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Role for IKK2- and NEMO-Kinase Mediated Nuclear Factor kappa B (NF-κB) Activation in CD4+ T Lymphocytes in Nephrotoxic Serum Nephritis (NTN) Induced Glomerulonephritis Mice

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Abstract

Accumulative evidences indicated the critical role for CD4+ T cells in the pathogenesis of glomerulonephritis, the molecule(s) involving in regulation of the development and functions of CD4+ T cells to mediate glomerulonephritis is poorly understood. NF-kB transcription family is known as a major regulator of T cells development and functions and participates into multiple inflammation based diseases. However, whether NF-κB functions in CD4+ T cells are critical for glomerulonephritis or not remains unclear. NF-KB is activated by an IkB kinase (IKK) complex comprised of two distinct kinase subunits, IKK1 (IKK α) and IKK2 (IKK β), plus a regulatory protein, NEMO (IKKy), which is essential for activation of NF-kB. Here, we specifically deleted IKK2 and/or NEMO in CD4+ T cells to investigate the role of canonical NF-κB pathway in glomerulonephritis. In general, our data showed that knockout of IKK2 and/or NEMO in CD4+ T cells decreased the number of T cells as previously reported under physiological conditions. However, mice with knockout of IKK2 and/or NEMO in CD4+ T cells did not alter the progression of glomerulonephritis showing similar renal functions by examination of albumin-to-creatinine ratio and blood urea nitrogen levels (BUN), and comparable morphology of kidney quantifying the by glomerular/tubulointerstitium damage, and renal crescent levels compared to control mice at 10th day in a well-established nephrotoxic serum nephritis (NTN) induced glomerulonephritis model. We did find an increased infiltration

ability of CD3+ and CD4+ T cells into kidney after NTN induction in all types of knockout mice but eventually exhibited similar number of CD3+ T cells and comparable percentage of CD4+ T cells residual in overall renal and glomerular tissues by immunohistochemical and FACS analysis. Interestingly, the infiltration levels for different subtypes of CD4+ T cells were distinct from each other in the injury kidney: more Th1 and Th17 cells and less Treg cells were observed in IKK2 and/or NEMO knockout mice comparing with control mice after NTN induction. However, similar expression levels of proinflammatoy chemokines, including IL-1b, TNF-α, CCL2, CCL5 and CCL20 in all types of knockout mice and control mice, were detected. Consistently, the activation of inflammatory related regulator NF-KB in renal cells was also unaltered by western blotting analysis. Thus, our observations implied that inactivation of NF-kB in CD4+ T cells is not involved in alteration of the severity of NTN induced glomerulonephritis.

In addition, to uncover which molecules in CD4+ T cells participate into NTN induced glomerulonephritis, microarray based genome profiling was performed by comparing genes expression in CD4+ T cells from kidney spleen that with or without NTN induction. Thus, to identify novel molecule(s) which are essential/critical for T cells mediated glomerulonephritis greatly benefits to clinic treatment of inflammatory renal diseases.

Key words: T cells, NF-kB, glomerulonephritis, microarray

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1 Introduction

1.1 Kidney anatomy

Kidney is an extremely important organ to maintain homeostasis. A normal and matured human kidney contains around one million of mutually independent neuphrons. As the basic structural and functional unit of kidney, nephron functions mainly to finely control and maintain the concentration of water and soluble substances at certain ranges. A nephron is made up by two main structures: the glomerulus and the double hairpin-shaped tubule (Kurts et al., 2013). The glomerulus is a very small and ball-like shape structure composed of capillary blood vessel and is enclosed in a cup-shaped structure called the Bowman's capsule. The "gap" between glomerulus and Bowman's capsule is Bowman's space where small molecules such as water and sodium salt can move freely, but large proteins and cells are not allowed to move in or outside. The tubule is the other very tiny tube where pass through the waste and other recyclable substances filtered out from the glomerulus. The space between the tubules is called the tubulointerstitium where habors the intrarenal immune system containing of dendritic cells, macrophages and fibroblasts (Fig. 1).



Fig. 1 Kidney anatomy Schematically showing general structure of nephron and its cellular components. A normal and matured human kidney contains around one million of mutually independent neuphrons. A nephron is made up by two main structures: the glomerulus and the double hairpin-shaped tubule. The glomerulus is a very small and ball-like shape structure composed of capillary blood vessel and is enclosed in a cup-shaped structure called the Bowman's capsule. The "gap" between glomerulus and Bowman's capsule is Bowman's space where small molecules such as water and sodium salt can move freely, but large proteins and cells are not allowed to move in or outside. The space between the tubules is called the tubulointerstitium where habors the intrarenal immune system containing of dendritic cells, macrophages and fibroblasts (adapted from(Kurts et al., 2013)).

1.2 Glomerulonephritis (GN)

Glomerulonephritis (GN), a common inflammatory kidney disease, is the main cause for the end stage of renal failure (<u>Kim et al., 2009</u>). Although GN could be triggered by genetic and/or environmental factors, it is believed that GN eventually developed by immune responses against self-antigens or foreign antigens (<u>Ikezumi et al., 2004</u>; <u>Kim et al., 2009</u>; <u>Tipping and Holdsworth</u>, <u>2006b</u>) and observed as inflammation in glomeruli and small blood vessels of the kidneys (<u>Hricik et al., 1998</u>). However, the exactly pathological mechanism about the GN is still unknown.

The detailed molecular mechanisms of proinflammatory response in GN have been intensively investigated. The pioneering research on the pathogenesis of glomerulonephritis have been devoted to the characterization of humoral immune responses in the initiation of glomerular injury, highlighting the critical role of antibody and the complement responses (Glassock, 1978; Rovin and Schreiner, 1991; van Alderwegen et al., 1997). Nevertheless, following studies questioned the exclusive player of humoral immune response in GN because the participation of only antibody and complement cannot fully explain multiple events occurring in both experimental and human glomerulonephritis (Rovin and Schreiner, 1991). Using mouse model suggested that the pathogenesis of GN is more complicated (Cunningham et al., 1999; Rovin and Schreiner, 1991) and mice deficient in various complement proteins still could generate inflammation (Sylvestre et al., 1996).

Recently, evidence has been slowly accumulated and the involvement of T cells has been recognized in progress of GN. More and more studies provided evidence that the T cells play an important role in the pathophysiology of GN, not only as helpers for antibody producing B cells, but also as immune effector cells (Heymann et al., 2009; Kalluri et al., 1997; Panzer and Kurts, 2010; Reynolds et al., 1993; Salama et al., 2001; Wu et al., 2002). The direct evidence for T cells in the pathogenesis of GN is supported by the findings that inhibiting T cells increases the life span of nephritic mice (Schiffer et al., 2003) and depletion of CD4+ T cells decreased autoantibody production and attenuated GN pathology (Reynolds and Pusey, 1994). In addition, injection of activated OVA-specific CD4+ T cells with the naïve CD8+ T cells in transgenic mice expressing the model antigen OVA in kidney podocytes caused accumulation of both CD4+ and CD8+ T cells in the kidney and lead to GN (Kim et al., 2009). Although the exact role of T cells in glomerular immunopathology is unclear, two possible mechanisms refer to macrophage recruitment to the kidney mediated by Th1 cells (Li et al., 1997) and epitope spreading caused by T cell-mediated damage and antigen release (Bolton et al., 2005) probably elucidate the underlying mechanisms at certain level. However the detailed mechanisms on the role of T cells in glomerulonephritis remains to be determined since it is still no clear answers on the basic but critical questions like how T cells induces/participates the progress GN and what the potential autoantigens are.

1.3 T cells development

T cells are the dominant cell types that participate in cell-mediated immune responses. They have functions as both regulators and effectors of the immune response. Naive conventional T cells seed and differentiate into matured T cells in the thymus. T-cell development in the thymus starts with CD4-CD8- double negative (DN) thymocytes, then progresses to CD4+CD8+ double positive (DP) thymocytes and finally becomes CD4+ or CD8+ single positive (SP) thymocytes and enter the circulation (Germain, 2002) (Fig. 2). A small part population of thymocytes differentiates into natural regulatory T cells (nTreg) in the thymus. Once they enter into peripheral via circulation system, they will encounter specific antigens or particular environmental conditions leading to the differentiation into effector subsets (Fig. 2). Naive CD8+ T cells can differentiate into CD8+ effector cells or CD8+ memory cells when migrate to the periphery. Naive CD4+ T cells can differentiate into various subsets of effector cells upon encountering specific antigens consisting of type 1 T helper cells (Th1), Th2, Th17, Th9, follicular T helper cells (Tfh), and Th22. In addition, some naive CD4+ T cells also differentiate into induced regulatory T cells (iTreg) in specific microenvironments (Oh and Ghosh, 2013). Importantly, Th1, Th2, Th17, and Treg cells are generally accepted playing a central role in the pathogenesis of proliferative and crescentic glomerulonephritis (Panzer and Kurts, 2010).



Fig. 2 T cells development Naive conventional T cells seed and differentiate into matured T cells in the thymus. T-cells development in the thymus start with CD4-CD8- double negative (DN) thymocytes, then progress to CD4+CD8+ double positive (DP) thymocytes and finally become CD4+ or CD8+ single positive (SP) thymocytes and enter the circulation. Naive CD8+ T cells can differentiate into CD8+ effector cells or CD8+ memory cells when migrating to the periphery. Naive CD4+ T cells can differentiate into various subsets of effector cells upon encountering specific antigens consisting of type 1 T helper cells (Th1), Th2, Th17, Th9, follicular T helper cells (Tfh), and Th22. In addition, some naive CD4+ T cells also differentiate into induced regulatory T cells (iTreg) in specific microenvironments. A small part population of thymocytes differentiates into natural regulatory T cells (nTreg) in the thymus.

1.4 T cells in GN

Accumulated evidences suggest that T cells play an important role in initiation and progression of GN. Although intensively studies are focusing on CD4+ T cells, the roles of CD8+ T cells and Treg on development of GN have also been gradually explored (Tipping and Holdsworth, 2006b).

1.4.1 CD4+ T cells and GN

The deposit of CD4+ T cells in glomerular from patients suggests a potential role of helper T cells in directing GN (<u>Neale et al., 1988</u>; <u>Stachura et al., 1984</u>). It was observed that blocking the functions of Th1 by deficiencies or specific antibodies of cytokines (for example, IL-12 or IFN-Gamma, etc.) attenuates GN injury (<u>Kitching et al., 1999a</u>; <u>Kitching et al., 2005a</u>). Conversely, administration of IL-12, the key Th1 cytokine to enhance Th1 functions, exacerbates GN (<u>Kitching et al., 1999c</u>). In addition, mice with deficiencies of Th2 cytokines like IL-4 and IL-10 show more susceptibility to GN (<u>Kitching et al., 1998</u>; <u>Kitching et al., 1998</u>; <u>Kitching et al., 1997</u>) or after glomerular injury is established (<u>Kitching et al., 1997</u>) provides protective roles from development of GN. Overall, these data suggest heir critical role in initiation of immune responses in GN.

In addition, CD4+ T cells also take key effector roles by recruiting macrophages. In a mice GN model, depletion of CD4+ T cell in the effector phase of the disease (after the nephritogenic immune response is established) can effectively prevent glomerular macrophage recruitment and GN injury (<u>Huang et al., 1997</u>). Thus, proinflammatory cytokines including IFN-Gamma, IL-12, IL-1b, and TNF from T cells, macrophages, and intrinsic

renal cells during the effector phase of GN have been partially explored in experimental models (<u>Timoshanko et al., 2002</u>; <u>Timoshanko et al., 2001</u>; <u>Timoshanko et al., 2004</u>; <u>Timoshanko et al., 2003</u>).

1.4.2 CD8+ T cells and GN

Cytotoxic effect from CD8+ T cells are also possible effector mechanism of GN since CD8+ T cells are observed in human and some experimental models of GN. Blocking CD8+ T cells using antibody prevent the development of GN without obvious alteration of circulating levels of nephritogenic antibodies in experimental Wky rats models (Kawasaki et al., 1992; Reynolds et al., 2002). In addition, the expression level of cytotoxic factor granzyme B was found to be significantly reduced in glomerular when depletion of CD8+ T cells, providing further evidence for involvement of T cell-mediated cytotoxicity in development of GN (Reynolds et al., 2002). However, in a mice model of GN with CD8-deficient showed more sever injury, excluding the role of CD8+ T cells in development of GN in mice but arguing their protective effects (Tipping et al., 1998). Although the observations of functional roles of CD8+ T cells in development of GN from rats and mice model are controversy, more elegant and intensive studies should be performed to examine the role of CD8+ T cells in development of GN.

1.4.3 Treg and GN

Dysfunction of Treg contributes to the development of GN by suppressing

autoreactive T cells (<u>Tipping and Holdsworth, 2006b</u>). Depletion of CD25+CD4+ Treg by neonatal thymectomy promoted development of GN in a mice model (<u>Bagavant and Tung, 2005</u>). Interestingly, transfer of Treg cells from naive mice attenuated the accumulation of T cells and macrophage in glomerulus and suppressed development of GN, while the transferred Treg cells from nephritic mice made the injury worse (<u>Wolf et al., 2005</u>), suggesting a potential treatment strategy for GN by transferring intact Treg cells.

Overall, T cell mediated immune response is critical for the development and progression of GN although some data are still not conclusive or controversial. However, the molecules that regulate T cell mediated immune response in GN are still in puzzled and need to be further determined.

1.5 Nuclear factor kappa B (NF-κB)

NF-κB is a family of dimeric transcription factors regulating the gene expression of several adhesion molecules, cytokines and chemotactic proteins involved in inflammation, immune response and cell proliferation (Baeuerle and Henkel, 1994; Danilewicz and Wagrowska-Danilewicz, 2013; Guijarro and Egido, 2001; Hayden and Ghosh, 2011).

1.5.1 The members of NF-κB family

The NF-kB family is comprised of five members: RelA (p65), RelB, c-Rel, NF-

 κ B1 (p105/p50), and NF- κ B2 (p100/p52) (<u>Hayden and Ghosh, 2011</u>) (Fig.3) that exhibit as homodimers or heterodimers with distinct gene regulatory functions. Different members share an N-terminal Rel homology domain, which is responsible for DNA-binding. The members of p65, c-Rel, and RelB contain a transcription activation domain and are hence capable of regulating transcription (<u>Baldwin, 1996</u>; <u>Barnes and Karin, 1997</u>; <u>Oh and Ghosh, 2013</u>). Because the most abundant dimer in majority of cell types and well-studied is the p50-p65 dimer, "NF- κ B" is usually refers to this dimer (<u>Guijarro and Egido, 2001</u>). It should be noted that knockout mice for all of the NF- κ B genes are available except the p65 knockout is lethal, implying functional redundancy among other members of the family (Fig. 3).



Fig. 3 The members of NF-κB family and its canonical pathway. "NF-κB" is usually refers to the most abundant dimer in majority of cell types and well-studied one: p50-p65 dimer. NF-

 κ B activation relies on IKK activity. In normal conditions, NF- κ B activation is inhibited by binding with I κ B. Once stimulations given, IKK will be activated and phosphorylates I κ B which promotes the dissociation of I κ B and NF- κ B. The phosphorylated I κ B will be degraded by ubiquitase. However, NF- κ B dimer will enter into nuclear and bind to specific DNA sequence(s) to promote transcription.

1.5.2 I κ B (inhibitor for κ B)

I κ B, the inhibitor of κ B, physically associates with NF- κ B dimers to retain them in the cytoplasm and maintain their inactive form (Kanarek and Ben-Neriah, 2012). The IkB is a family includes IkB α , IkB β , IkB γ , IkB ϵ , Bcl-3, the precursors of NF-kB1 (p105), and NF-kB2 (p100) (Ghosh et al., 1998; Whiteside and Israel, 1997). Of these, the most important and bestcharacterized regulator of mammalian NF-κB is IκBα (Karin and Ben-Neriah, 2000). Thus, IkBa contains a regulatory region at N-terminal that is required for phosphorylation/stimulation-induced degradation-the key step for NF-kB activation. It should be reminded that $I\kappa B\alpha$ also terminates NF- κB activation. Newly synthesized IkBa enters into the nucleus and binds with NF-kB to promote its dissociation from target DNA (Karin and Ben-Neriah, 2000). Then, the inactive form of IkBa-NF-kB complex re-exports to the cytoplasm (Arenzana-Seisdedos et al., 1997). However, the potential functions of the other members of IkB family are not well known (Guijarro and Egido, 2001).

1.5.3 IKK complex

Although NF-KB activation is preserved by directly interact with IKB, its status

is finely regulated by the IκB kinase (IKK). With the inflammatory stimulation, the IκB kinase (IKK), which is comprise of two catalytically active kinase (IKKα and IKKβ) and a regulatory subunit NEMO (NF-κB essential modulator, also known as IKKγ) (Fig 1.3), phosphorylates IκB protein and leads to the ubiquitination and degradation, resulting in NF-κB dimers released from IκB and translocate to the nucleus where they bind to κB binding site and modulate the target gene expression (<u>Hayden and Ghosh, 2008; Oh and</u> <u>Ghosh, 2013; Perkins, 2007</u>). NEMO is essential for IKK activity. However, although highly sequence identity and similarity is shared by IKKα and IKKβ, their functions are not always the same, which refers to the canonical and non-canonical pathways for activation of NF-κB.

1.5.4 The canonical and non-canonical pathways of NF-κB

The functional characterization of IKK α and IKK β in knockout mice split two distinct signaling transduction pathways to NF-kB activation according to the different stimuli, the IKK subunits activated, and the NF-kB/lkB genes targeted (Karin and Ben-Neriah, 2000). The canonical NF-kB pathway mainly targets to p65:p50 heterodimers although other combinations of dimers are also involved in (Oh and Ghosh, 2013; Perkins, 2007; Sun, 2011). The canonical NF-KB activation usually happens rapidly and transiently and is normally induced by inflammatory cytokines, pathogen-associated molecules, and antigen receptors. Once stimulation induced, IKK β is necessary and sufficient

to phosphorylate IkB α or IkB β in an IKK γ /NEMO-dependent manner (Karin and Ben-Neriah, 2000), which in turns liberate NF- κ B from I κ B. Thus, cells with IKK β were shown to be able to activate NF- κ B upon stimulation with proinflammatory cytokines such as TNF α or interleukin-1 (IL-1) (Li et al., 1999a; Li et al., 1999b) (Fig. 4). The role of IKK β in canonical NF- κ B signaling, however, remains unclear.

While in the non-canonical pathway, NF- κ B is activated by more specific members of the TNF cytokine family, such as BAFF, lymphotoxin- β , or CD40 ligand that dominantly relies on IKK α , but not IKK β or IKK γ /NEMO. IKK α is believed to selectively phosphorylate p100 associated with RelB (<u>Scheidereit</u>, <u>2006</u>; <u>Senftleben et al., 2001</u>). Together with its upstream effector NIK (NF- κ B-inducing kinase) acting, IKK α functions as both an IKK α -activating kinase as well as a scaffold linking IKK α and p100 (<u>Xiao et al., 2004</u>) to induce a phosphorylation-dependent proteosomal processing of p100 to p52, which eventually mediates the persistent activation of RelB/p52 complex to target specific kB elements (<u>Chen and Greene, 2004</u>; <u>Lawrence, 2009</u>; <u>Sun, 2011</u>), (Fig. 4).

Thus, diseases involved in the genetic mutations of IKK have been clinically identified (<u>Senegas et al., 2015</u>). The mutations in NEMO gene cause hypohidrotic ectodermal dysplasia with immune deficiency (HED-ID) in males (<u>Shifera, 2010</u>). IKKα mutation causes 'Cocoon syndrome' which shows severe multiple organs developmental defects (<u>Lahtela et al., 2010</u>). The

deficiency of IKK β , however, is not as detrimental in the canonical NF- κ B activation as the loss of NEMO, which is mainly due to the compensation roles taking by IKK α to some extent to retain the IKK function to activate canonical NF- κ B pathway when lacking of IKK β (<u>Makris et al., 2000a;</u> <u>Schmidt-Supprian et al., 2003</u>). Although IKK β is not essential for fully activate canonical NF- κ B pathway, a severe combined immunodeficiency (SCID) were recently identified in several individuals (<u>Burns et al., 2014</u>; <u>Mousallem et al., 2014</u>; <u>Nielsen et al., 2014</u>; <u>Pannicke et al., 2013</u>). However, the underlying mechanisms on how loss of functions of IKK causes distinct diseases remain to be intensively studied.



Fig. 4 Canonical and non-canonical pathways of NF-κB activation (adapted from(<u>Sun,</u> <u>2011</u>)).

1.5.5 Role of NF-kB in T cells development and functions

T cell differentiation, proliferation, and activation are controlled by gene programs of NF-kB (Paul and Schaefer, 2013; Siebenlist et al., 2005). The T cells development undergoes four distinct developmental stages that are from immature CD4-CD8- double negative 1 (DN1) to DN4 to matured CD4+CD8+ double positive T cells within the thymus. During the transition from DN3 to DN4 stage, a T cell receptor (TCR) β –NF- κ B–dependent survival step permits TCRa gene rearrangement and expression, resulting in the formation of TCRαβ CD4+CD8+ T cells (Fig. 5) (Gerondakis et al., 2014; Oh and Ghosh, 2013; Schmidt-Supprian et al., 2004; Siebenlist et al., 2005). The survival signals at the DN3-DN4 stages most likely depend on the activation of NF-KB with p50-p65 heterodimers and the absence of which at the stage DN3 results in high frequencies of apoptosis (Aifantis et al., 2001; Voll et al., 2000). Furthermore, the proliferation of immature T cells during the transition from DN3 to DN4 is also obviously diminished when inhibition of NF-KB activity (Aifantis et al., 2001). However, it is still unclear whether this is a direct consequence of a need for NF-kB during proliferation or a secondary outcome of increased apoptosis (Gerondakis et al., 2014).



Fig. 5 Role of NF-KB in T cells development (adapted from (Gerondakis et al., 2014)).

Once CD4+ T cells matured in the thymus, they will migrate to the periphery and encounter antigens. Upon stimulation, naive CD4+ T cells differentiate into distinct subsets of effector cells allowing for triggering immune responses against specific antigens as introduced above (Section 1.3 and 1.4). Thus, NF-kB participates in directly or indirectly regulating differentiation of different Th cell types following activation of naive CD4+ T cells, supporting their proliferation, survival and functions as well (Gerondakis et al., 2014). For example, the major role of Th1 cells is against intracellular viral or bacterial pathogens, predominantly through the production of IFN-y. NF-kB members, including p65, p50 and c-Rel (Corn et al., 2005; Hilliard et al., 2002), are essential for the development and function of Th1 cells, especially in the production of IFN-y by interacting with other transcriptional factors involving in T-bet, signal transducer and activator of transcription 4 (STAT4) and STAT1 (Balasubramani et al., 2010). Th2 cells are involved in the response to

extracellular pathogens and in allergic reactions by secretion of IL-4, as well as IL-5, IL-6, IL-9, IL-13, and IL-25. Th2 differentiation relies on a master regulator of transcriptional factor GATA3 which is tightly regulated by NF-kB (Das et al., 2001). CD4+ T cells with p50-deficiency were unable to induce GATA3 expression under Th2-differentiating conditions in vivo and in vitro. Hence, impairment of Th2 development in p50-deficient mice caused a defective response to allergic airway inflammation (Das et al., 2001). In addition, NF-KB can bind the enhancer sites of IL-4 locus and induce IL-4 expression in cooperation with nuclear factor of activated T cells (NFAT) to affect Th2 functions (Li-Weber et al., 2004). Th17 CD4+ T cells produces proinflammatory cytokines such as IL-17A, IL-17F, and IL-22, and participates in the immune response against extracellular bacteria. Recently, a possible role of NF-kB in the differentiation and function of Th17 cells was indicated because a defective IL-17 gene expression and Th17 cell differentiation was observed in c-Rel- or p65-deficient T cells (Ruan et al., 2011). However, the other report suggests that c-Rel is not required for Th17 cell differentiation (Visekruna et al., 2010). Thus, the role of the different NF-kB subunits in differentiation and function of Th17 is still controversy. Th9 is a subset of Thelper cells producing IL-9. Although their development and function remain poorly understood, NF-KB pathway is likely critical for Th9 differentiation (Xiao et al., 2012) and regulates IL-9 expression in T cells (Early et al., 2009), indicating NF-κB is involved in development and functions of Th9 cells. Tfh

cells express the chemokine receptor CXCR5 and produces, which are important for B-cell differentiation and maturation (Ma et al., 2012). NF-кB regulates Tfh cells development (Hu et al., 2011) and is critical for both CXCR5 (Serre et al., 2011) and IL-21 expression (Chen et al., 2010). Treg cells generally suppress or downregulate induction and proliferation of effector T cells (Sakaguchi et al., 2008). The role of NF-kB on Treg development and function is not well known. An indirect evidence of NF-kB regulates Treg development is from the study focusing on the role of PDK1 on T cell development (Park et al., 2010). It is shown that PDK1-deficient mice exhibit dramatically increased numbers of $\gamma\delta T$ cells which is normally inhibited by Treg cells, suggesting loss of PDK1 decreases the number of functional Treg cells. As PDK1 could activate NF-KB, which implies that NF-KB probably take effects on Treg cells development. A more direct evidence showing NF-κB is also critical for Treg cells functions. Constitutively activation of IKKB rescued the suppressive function of Ubc13 (an E2 ubiquitin-conjugating enzyme that is known to activate IKK and NF- κ B)-deficient Treg cells (Chang et al., 2012). The role of NF-kB on Th22 development and functions has not been elucidated (Jia and Wu, 2014).

Besides, NF- κ B is also required for CD8+ and nTreg cells differentiation, maturation and functions (<u>Oh and Ghosh, 2013</u>). Thus, NF- κ B plays a pivotal role of regulation of T cells development and functions.

1.5.6 NF-kB and Glomerulonephritis

The inactivated NF-kB was also found in renal cells and can be activated with stimulation, both in vivo and in vitro. Thus, accumulative evidence suggested that NF-kB plays an important role in many glomerulonephritis, especially by immune-mediated ones (Sanz et al., 2010). However, most of the studies only showed the descriptive data link NF-kB activation to human and experimental kidney disease, the comprehensive mechanisms on how NF-kB functions to kidney disease are not clear (Sakurai et al., 1996b; Sanz et al., 2010). In classic opinions of immune-mediated glomerulonephritis, immune complex formation and deposition in the kidney result in glomerular inflammation with recruitment of leukocytes, and the activation and proliferation of resident renal cells. It should be noted that proinflammatory role of NF-kB in inflammation is phase-dependent: NF-kB down-regulates inflammatory genes, up-regulates anti-inflammatory genes during resolution phase. Although it is still uncertain whether it is also the case in GN, the transition of distinct roles in different phases of inflammation is guite interesting. Thus, the cellular and molecular mechanisms underlying glomerular cell activation of NF- κB are not yet fully understood in human and experimental glomerulonephritis (Danilewicz and Wagrowska-Danilewicz, 2013; Zheng et al., 2006).

1.6 Nephrotoxic serum nephritis (NTN) model of GN

To study the pathophysiology of immune complex-induced GN in mice, a wellestablished model is nephrotoxic serum nephritis (NTN). NTN is a kind of model in which animals are injected with antibodies (typically generated from rabbits or sheep) into glomerular basement membrane (GBM) and cause subsequent acute glomerular injury (Quigg et al., 1998).

In this model, heterologous antibodies were administrated in the host's GMB. The pathogenesis of this disease is divided into two phases: the primary phase and the secondary phase. In primary phase (the immediate injury), the heterologous antibody binds to GBM and causes immediate injury. The secondary phase (autologous) begins 6-8 days after the injection and depends on the immunological response of the host to the injected heterologous anti-GBM antibodies. In addition, the primary and secondary phases approximately occur at the same time usually resulting in a transitory period of polymorphonuclear leucocyte (PMN) infiltration and T lymphocytes, monocytes/macrophages, proteinuria, and the formation of crescents and markedly persistent alterations (Chen et al., 2002; Dixon et al., 1961; Lehmann et al., 1969). The proteinuria, proliferative and inflammatory glomerular changes appear in NTN animals including crescent formation, leukocyte infiltrates and capillary aneurysms, intraglomerular deposition, which are mainly located in the periglomerular and interstitial region. Further, the tubulointerstitial compartment showed tubular dilation, necrosis and atrophy, and protein casts and tubular protein reuptake due to proteinuria.

The serial studies showed that the different immune cells take effects at different stages (Kurts et al., 2013). In the first days following induced NTN,

innate immune response mainly mediates renal damage, including neutrophils, mast cells and interleukin-17 (IL-17)-producing γδ T cells (Kurts et al., 2013). Simultaneously, T cells specific for the heterologous antibodies are primed in the lymphatic tissues and attractive to infiltrate into the kidneys. Th17 cells are firstly shown up in the kidney after 4 days NTN induction (Summers et al., 2009). If inflammation resistant, Th1 cells are recruited, which in turns recruit more proinflammatory cells (Summers et al., 2009). Treg cells are also present at this stage and attempt to control inflammation (Wolf et al., 2005b). 14 days later, host antibodies against the heterologous antibodies increasingly contribute to kidney injury. Thus, more and more studies have indicated that the immunity mediated by T lymphocytes, especially Th1, Th17 and Treg cells, play an important role in the NTN nephritis model (Kuroda et al., 1994; Okada et al., 2009). In addition, the balance between pro-inflammatory and anti-inflammatory factors derived from distinct T cells determines the severity of GN.

1.7 Aims of this project

GN is a T cells mediated inflammatory kidney disease (<u>Azadegan-Dehkordi et</u> <u>al., 2015</u>; <u>van Alderwegen et al., 1997</u>), however, the underlying mechanisms remain uncertain. NF-κB participates into regulating T cells development and

functions and dysfunction/abnormal activation of NF-κB is observed in human and experimental GN (<u>Auwardt et al., 2000</u>; <u>Sanz et al., 2010</u>; <u>Tak and</u> <u>Firestein, 2001</u>), suggesting a possible role of NF-kB on initiation and/or progression of GN by regulation of T cells development and functions. Thus, in this study, I mainly examined the effects of NF-kB in CD4+ T cells on GN by using CD4+ T cells type specific targeted knockout mice with NTN model.

1.7.1 To functional analysis of the roles of NF-kB in CD4+ T cells on GN with NTN model

Previously intensive studies showed a role of CD4+ T cells on GN. Although NF-kB takes effects in development and functions of CD4+ T cells and is implicated involving in GN (Panzer et al., 2009), the roles of NF-kB specifically in CD4+ T cells on initiation and progression of GN is still unknown. By using Cre-Loxp knockout system, I specifically deleted two genes encoding IKK2 and NEMO individually or together, two activators of NF- kB pathway, in CD4+ T cells and examine their roles in NTN mice. I mainly focused on the 10 days after induction since CD4+ T cells dominant immune response at this time point.

1.7.2 To detect the underlying mechanisms on how NF-kB deficient CD4+ T cells affects the progression of GN in NTN model

The mechanisms involving in T cells mediated GN could be as a result of abnormal T cells proliferation, infiltration and activation which in turn releases

proinflammatory factors to induce kidney injury.

The recruitment of CD4+ T cells into kidney is an important sign for kidney inflammation (<u>van Alderwegen et al., 1997</u>). Fluorescence-activated cell sorting (FACS) was used to count the distribution of different types of CD4+ T cells, including Th1, Th17 and Treg cells, in blood, spleen and kidney.

Proinflammatory factors are the main mediators for the GN. Multiple cytokines were measured by quantative reverse transcription polymerase chain reaction (qRT-PCR).

The damage of kidney could be also from the inflammatory response in kidney cells. NF- kB activation is also observed in kidney inflammation, hence, activity of NF- kB from kidney tissue was also examined.

1.7.3 Genome profile of NF-kB deficient CD4+ T cells affects in NTN model

To intensively understand how behaviorally difference between wild type and NF-kB deficient CD4+ T cells in NTN model, micro-array based genome profile was performed. Thus, by systemically analyzing the up- or down-regulated molecules may interpret the possible role of NF-kB in development and functions CD4+ T cells in NTN model.

2 Materials and Methods

2.1 Materials

2.1.1 Animals

8-10 weeks-old male mice (20-25 g b.w.) were breed in UKE-Eigenzucht. All animals were raised under specific pathogen-free conditions. Animal experiments were performed according to national and institutional animal care and ethical guidelines and were approved by local committees (FI 16/05 and 17/10).

2.1.2 Animal model

The nephrotoxic serum nephritis (NTN) animal model was induced in 8-10 weeks-old male transgenic mice by intraperitoneal injection of sheep-antimouse antiserum as show in following table.

Weight of mice	Serum Volume Inject
>25g	700ul
23.1-25g	665ul
21.1-23g	630ul
19.1-21g	595ul
17.1-19g	560ul
<17g	525ul

2.1.3 Primers

All primers were synthesized by Invitrogen. Primers were designed using Stepone Software v2.0 from Applied Biosystem.

a) Primers for qPCR

Primer	Sequences
TNEa	Fw: AAA TGG CCT CCC TCT CAT CAG T
	Rev: GCT TGT CAC TCG AAT TTT GAG AAG
II -1B	Fw: CCT TCC AGG ATG AGG ACA TGA
	Rev: TCA TCC CAT GAG TCA CAG AGG AT
	Fw: CCT TCC AGG ATG AGG ACA TGA
	Rev: TCA TCC CAT GAG TCA CAG AGG AT
CCI 5/ Pantos	Fw: GCA AGT GCT CCA ATC TTG CA
CCE5/ Names	Rev: CTT CTC TGG GTT GGC ACA CA
	Fw: CCA CCT CTG CGG CGA AT
	Rev: CGG TCT GTG TAT CCA AGA CA
185	Fw: CAC GGC CGG TAC AGT GAA AC
	Rev: AGA GGA GCG AGC GAC CAA A

b) Primers for genotyping

Primer	Sequences
CD4Cre	FW: CGA GTG ATG AGG TTC GCA AG
	RW: TGA GTG AAC GAA CCT GGT CG
IKK2 flox	FW: CCT TGT CCT ATA GAA GCA CAA C
	RW: GTC ATT TCC ACA GCC CTG TGA
NEMO	209: CGT GGA CCT GCT AAA TTG TCT
	210: ATC ACC TCT GCA AAT CAC CAG
	211: ATG TGC CCA AGA ACC ATC CAG

c) Primers for the knock out mice genotyping

mu IKK2	FW: CAC AAT CAG GCG ACA GGT GAA
	RW: TGC CGA AGC TCC AGT AGT GAA
mu NEMO	FW: AGC GCT GCC TGG AAG AGA ATC AAG
	RW: ACC TGG ACG CTG TGC TGC TGC TGT

2.1.4 Antibodies

a) Antibodies for western blotting

1st antibody	Species	Dilution	Company	Use
ß-Actin	mouse	1:3000	Cell signal	WB
ΙΚΚβ	rabbit	1:1000	Cell signal	WB
Phospho-IKKα/IKKβ	rabbit	1:1000	Cell signal	WB
ΙκΒα	rabbit	1:1000	Cell signal	WB
Phospho-ΙκΒα	rabbit	1:1000	Cell signal	WB
2st antibody	Species	Dilution	Company	Use
Anti-mouse-HRP	goat	1:40000	Cell signal	WB
Anti-rabbit-HRP	goa	1:10000	Cell signal	WB

b) Antibodies for FACS staining

CD45	PerCP	
CD4	APCeFluor780 or FITC	
CD3	AF700	
CD25	APC	BD Horizon
CD8	V500	
Foxp3	PE, FITC	

2.1.5 Devices

Apparatus	GmbH
Autoanalyzer	Hitachi 717; Roche
AbiPrism Sequence Detection System 7000	Applied Biosystems
ELISA- Reader	Severin
II. MATERIALS AND METHODS

Heating block (Neoblock 1)	Bosch	
Fridge 4°C	Nanodrop Technologies	
Fridge -20°C	LG	
Fridge -80°C	WTW	
Magnetic stirrer (Variomag Maxi)	Sarstedt	
Microwave (Microwave 800)	Brand	
Microwave	Agfa	
Nanodrop spectrophotometer (ND 1000)	Eppendorf	
PC	Fröbel Labortechnik	
pH- Meter (Inolab)	Biometra	
Pipette	Eppendorf	
Pipette (Accu- jet)	Eppendorf	
Cell Strainer 40µm	BD Biosciences, Germany	
Cell Strainer 70µm	BD Biosciences, Germany	
Falcon FACS tubes	BD Biosciences, Germany	
Falcon tubes 15 ml	BD Biosciences, Germany	
Falcon tubes 50 ml	BD Biosciences, Germany	
X-ray film developer (CP 1000)	Intas	
Shaker (Thermo Mixer comfort)	Janke & Kunkel	
Shaker (Rocky)	Scaltec	
Thermocycler	Köttermann	
Table centrifuge (Typ 5415 R)	Heraeus	
Table centrifuge (Mini Spin)	Heraeus	
UV-table and documentation system	Heraeus	
Vortex	Severin	
Libra (SBA 41)	Bosch	
Heat bath	Nanodrop Technologies	
Workbench (Hera Safe)	LG	
Centrifuge (Biofuge primo R)	WTW	
Centrifuge (Megafuge 1.0 R)	Sarstedt	

Lichtmicroscop (Axioscop)	Zeiss
Lichtmicroscop (Axioobserver)	Zeiss
Lichtmicroscop (Axiovert 25)	Zeiss
Lichtmicroscop (IM 35)	Zeiss
Lichtmicroscop (LSM 510 beta)	Zeiss
Software	
Adobe Illustrator	Adobe
Adobe Photoshop	Adobe
Excel	Microsoft
Stepone Software v2.0	Applied Biosystems USA
Windows 7	Microsoft
GraphPad Prism 5	GraphPad Software, USA
FACS Diva	BD Biosciences, Germany
Axiovision	Zeiss, Germany
T-Base	4D SAS, France
Powerpoint	Microsoft
Image J	

2.1.6 Buffer

1) Hypotonic Buffer A:

10 mM HEPES pH 7.9

10 mM KCl

0.1 mM EDTA pH 8.0

0.1 mM EGTA pH 8.0

Add freshly: 1 mM DTT and protease inhibitor mix, 1 mM Na-Vanadate, dilute 1:100

2) Hypertonic Buffer B:

20 mM HEPES pH 7.9

400 mN NaCl

1 mM EDTA

1 mM EGTA

Add freshly: 1 mM DTT and protease inhibitor mix, 1 mM Na-Vanadate,

diluted 1:100

3) Formalin- solution (4%)

4% Formalin

0.4 % Eosin

in Sorensen's Buffer

4) Protein lysis Buffer

50 mM HEPES pH 7.9

140 mM Nacl

2 mM EDTA Ph 8.0

1% NP40

10%Glycerol

Add freshly: protease inhibitor mix, diluted 1:100 and optional, 1 mM Na-Vanadate

5) 10x TBS: (1 Liter)

80g Nacl

24.2g Tris

Use 25% Hcl to adjust Ph 7.6

6) Blotting (Transfer) Buffer: (1 Liter)

3.0g Tris

15.0g Glycin

Add 200 ml Methonal

7) Blocking Buffer

TBS-T + 5% dry milk

8) 10x Mops Buffer: (1 Liter)

10.0g 0.1%SDS

3.0g 1mM EDTA

60.6g 50mM Tris

104.8g 50mM MOPS

9) MACS Buffer: (500ml)

20ml PBS

100mg BSA

80ul EDTA (0.5M, pH 8.0)

10) Coating Buffer: (1 Liter)

0.05M Carbonate-Bicarbonate, pH 9.6

11) Postcoat Buffer: (1 Liter)

50mM Tris

0.14M Nacl

0.05% Tween 20, pH 8.0

12) Sample/Conjugate Buffer: (1 Liter)

50mM Tris 0.14M Nacl 1% BSA

13) Washing solution: (1 Liter)

50mM Tris

0.14M Nacl

0.05% Tween 20, pH 8.0

14) Basic medium for T-cell cultures

RPMI 1640

5% Penicillin-Streptomycin

5% Hepes

10% FCS

0.1% Mercaptoethanol

2.1.7 Chemicals

β Mercaptoethanol (βME)	Invitrogen, Canada
Aqua ad iniectabilia	Baxter, Switzerland
Bovine Serum Albumin (BSA)	Sigma-Aldrich, USA
Cellwash	BD Biosciences, Germany
Collagenase D	Roche, Germany
DNAse I	Roche, Germany
DTT	Sigma-Aldrich, USA
EDTA	Sigma-Aldrich, USA
Ethanol abs	JT Baker, Netherlands
Ethanol danatured	Walter, Germany
Ethidiumbromid	Bio-Rad, Germany
FACS Clean	BD Biosciences, Germany
FACS Flow	BD Biosciences, Germany

FACS Rinse	BD Biosciences, Germany	
FCS	Invitrogen, Canada	
Filmentwickler Roentoroll 25	Tetenal, Germany	
Filmfixierer Superfix 25	Tetenal, Germany	
HEPES	Invitrogen, Canada	
Isofluran	Abbot, USA	
Protease 24	Sigma-Aldrich, USA	
RPMI 1640	Invitrogen, Canada	
Hydrochloric acid	Merck, Germany	
Sterile HBSS	Invitrogen, Canada	
Sterile PBS	Lonza, USA	
Trypanblau 0,5%	Biochrom, Germany	
Trypsin	Sigma-Aldrich, USA	
4% to 12% Bis-Tris NuPage gel	Invitrogen	

2.1.8 Kits

CD4+ T Cell Isolation Kit II mouse	Miltenyi Biotech USA	
CD4+CD25+ Regulatory T Cell Isolation	Miltenyi Biotech USA	
Kit mouse		
NucleoSpin® RNA II Mini Kit	Macherey-Nagel, Germany	
ELISA Kit, E101	Bethyl Laboratories, USA	
RNeasy micro kit 50	QIAGEN, Germany	
CytoChemPlus (AP) Polymer Bulk Kit	Zytomed, Germany	
Cytofix/Cytoperm Kit	BD Biosciences, Germany	
Foxp3 FITC Intracellular Staining Kit	BD Biosciences, Germany	
Agilent RNA 6000 Nano Kit	Agilent Tech., Germany	

2.2 Methods

2.2.1 Genotyping

The DNA of transgenic mouse lines was isolated from tail biopsies. The tails were immersed in a mixture liquid of 100µl extraction buffer (Sigma Aldrich, Germany) and 25µl lyse tissueprep (Sigma Aldrich, Germany) for 10 min at 65 $^{\circ}$ C. Then 100µl neutralization buffer (Sigma Aldrich, Germany) was used to recover the DNA. After that the normal PCR was done with the isolated DNA. The PCR product was separated on 1.5% agarose gel and by means of Ethidium bromide visualizes.

2.2.2 Animals

Mice expressing Cre recombinase under the CD4 promoter from the Jackson Laboratory (Bar Harbor, ME, strain B6.129P2(C)-Cd19tm1(cre)Cgn/J) were crossbred with mice containing a floxed IKK2 or NEMO gene. The mice were generously provided by: Dr. M. Karin (San Diego) for IKK2f/f mice, Dr. C. Wilson (Seattle) for CD4Cre mice, and Dr. Manolis Pasparakis, Cologne, for NEMO-f/f mice. Efficiency and specificity of the IKK2 or NEMO deletion were assessed by PCR from FACS-sorted CD4+ and CD4- splenocytes (BD ARIAIII Cytometer, Becton Dickinson, Germany). All animals were raised under specific pathogen-free conditions.

2.2.3 Preparation of sheep anti-mouse GBM serum

Sheep anti-mouse antiserum was prepared as described previously(<u>Panzer et al., 2007</u>). In brief, glomeruli of C57BL/6 mice were isolated from the renal cortex through a series of sieves of decreasing pore size (250-, 150- and 75- mm mesh), and disrupted by sonication. The GBM fractions were then collected by centrifugation. For immunization, 1 mg GBM protein was emulsified with 1 ml Freund's complete adjuvant and was administered to the sheep by subcutaneous (s.c.) injection. Anti-mouse GBM serum was raised in goat by repeated immunization.

2.2.4 NTN model injection

NTN was induced in 8-10-wk-old male Cre/ IKK2/ NEMO/ IKK2NEMOdeficient and wild-type mice (22 to 26 g body weight) by intraperitoneal injection of nephrotoxic sheep serum as described previously. The dose of serum injected into mice is following:

Weight of mice	Serum Volume Inject
>25g	700ul
23.1-25g	665ul
21.1-23g	630ul
19.1-21g	595ul
17.1-19g	560ul
<17g	525ul

2.2.5 Organ extract

The organ harvest was performed on the 10th day after the NTN induction. 35

The animals were anesthetized by isoflurane for the whole procedure. The abdominal cavity was opened with an abdominal incision and blood was collected from the aorta by 1 ml syringe (23G, 0.6mm x 25mm) with heparin inside. The kidneys were exposed and dissected. The animals died during blood collection, but for safety neck dislocation was performed in the end. The kidney and spleen were extracted and put into 2ml 10% FCS/ RPMI 1640 buffer and 5ml HBSS buffer, respectively, in 4°C. There are two kidneys for each mice, one is for the FACS analysis; the other was divided into three parts, one for the extraction of nuclear proteins, one for RNA isolation and the last for immunohistochemical studies.

2.2.6 Functional Studies

Mice were housed in metabolic cages 6 hours for urine collection at the third day and 12 h before the organ extract the 10th day after NTN induction. The 3rd day proteinuria was assessed semiquantitatively scoring the albumin band as negative (0), little (+), moderate (++), or severe (+++) and the severe mice were chosen. The albuminuria of collected urine was determined by standard ELISA analysis (Mice-Albumin Kit; Bethyl, Montgomery, TX). Further, the urinary creatinine was calculated by standard laboratory methods. When the mice were killed, blood was collected for blood urea nitrogen (BUN) measurement by standard laboratory methods.

For the microarray RNA analysis experiment, the urine was collected only

at the third day after NTN injection and proteinuria was assessed semiquantitatively by the albumin band: negative (0), little (+), moderate (++), or severe (+++). The severe (+++) mice were selected.

2.2.7 CD4+ cells RNA extracted from spleen for microarray RNA analysis

Spleens were extracted from mice at 10th day after NTN induction and CD4+ splenocyte was isolated with CD4+ T cell isolation kit (Miltenyi Biotec, USA, CA) in accordance with the instruction of the manufacturer. CD4+ cell was sorted with flow cytometry by FACS sorting core unit of UKE. RNA of CD4+ cell ($\leq 5 \times 106$ /ml) was extracted by NucleoSpin® RNA kit (MACHEREY-NAGEL, Düren, Germany), or ($\leq 5 \times 105$ /ml) by RNeasy micro kit 50 (QIAGEN, Germany). The RNA quality of the collected CD4+ T cell was assessed by Agilent RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany) as the instruction of the manufacturer. The total RNA should be \geq 200ng and the RNA integrity number (RIN) should be \geq 8.0.

2.2.8 DNA Microarray Hybridization and Analysis

100ng of total RNA were applied for Cy3-labelling reaction using the one color Quick Amp Labeling protocol (Agilent Technologies; Waldbronn, Germany). Labeled cRNA was hybridized to Agilent's SurePrint G3 Mouse GE 8x60K microarray (Design ID: 074809) for 16h at 68°C and scanned using the Agilent DNA Microarray Scanner. Raw expression values were calculated by

the software package Feature Extraction 10.5.1.1(Agilent).

Raw data were further analysed using R package "Limma". Raw data were log2 transformed and quantile normalized. For testing differential gene expression normalized data sets were filtered for informative genes (showing at least expression values > log2(50) in more than two samples). For statistical analysis and assessing differential expression, limma uses an empirical Bayes method to moderate the standard errors of the estimated log-fold changes(<u>Smyth, 2004</u>).

2.2.9 Isolation of splenocyte for flow cytometry analysis

Spleens were aseptically removed from mice at 10 d after NTN induction. Cells were isolated, and red blood cells were removed by lysis. The frequency of IFN—producing cells was assessed by mouse-specific IFN- ELISPOT kit (BD Biosciences, San Diego, CA) in accordance with the instruction of the manufacturer. Briefly, splenic cells were cultured at a density of 4 × 10⁶ cells / ml in serum-free X-Vivo 20 medium (BioWhittaker, Walkersville, MD) in the presence or absence of 20 g/ml sheep IgG (Sigma) in a 96-well nitrocellulose microplate coated with an IFN—specific mAb. After 72 h of incubation at 37°C, plates were washed three times and incubated for 2 h at room temperature with a biotinylated anti–IFN- antibody. IFN- spot-forming cells were revealed with a streptavidin– horseradish peroxidase and 3-amino- 9-ethyl-carbazole chromogen. The number of IFN- spot-forming cells in each well was quantified

manually with an inverted microscope, and the results were expressed as number of IFN- spot-forming cells per 10⁶ cells.

2.2.10 Isolation of renal cells

Previously described methods for renal cell isolation from murine kidneys were used (Panzer et al., 2007). In brief, kidneys were finely crushed and digested for 45 min at 37°C with 0.4 mg/ml collagenase D (Roche, Mannheim, Germany) and 0.01 mg/ml DNase I in DMEM (Roche) supplemented with 10% heat-inactivated FCS. Cell suspensions were filtered through 40-µm nylon meshes and washed with HBSS without Ca2+ and Mg2+ (Invitrogen, Karlsruhe, Germany). Renal cell suspension was used for flow cytometric analysis.

2.2.11 Isolation of renal RNA

The RNA was isolated with <u>NucleoSpin® RNA</u> kit (MACHEREY-NAGEL, Düren, Germany) as the instruction of the manufacturer. In briefly, 1/3 kidney was homogenized disrupted and lysis with 100:1 mixture of RA1 buffer and ßmercaptoethanol and the lysate was transferred to the NucleoSpin column in the collected tube, centrifuged 1min, 11,000 g. Then adjusted the RNA binding condition by 70% ethanol and transfer the mixture supernatant into the NucleoSpin column and centrifuged 1min, 11,000g to bind the RNA. Desalting the silica membrane with Membrane Desalting Buffer, centrifuged 1min, 11,000g. Add rDNase mixture to digesting the DNA, incubated 10 min at room temperature. Last, washed the column 3 times with 200 ul RA2 buffer, 600 ul RA3 buffer and finally 250 ul buffer RA3 respectively. The RNA was then eluted with 50 ul RNase-free water. For the subsequent work, the RNA was frozen at -80° C.

2.2.12 Isolation of renal proteins

Kidneys were harvested from mice and put into 2 ml 10 % RPMI / FBS media, and all the procedures were performed on ice. 1/3 kidney was homogenized crushed in hypotonic buffer A. The suspension was transferred to a 1.5 ml Eppi tube and incubated for 15 min in ice. 100 ul of 10% NP40 were added, mixed and centrifuged 13,000 rpm for 30 s, 4°C. After centrifuge, the supernatant was collected in Eppi tube, which contained cytoplasmic proteins. Then, the pellet was resuspended in hypertonic buffer B and incubated for 20 min on a rotor in the refrigerator and centrifuged 13,000 rpm for 5 min, the supernatant were the nuclear proteins.

2.2.13 cDNA

The RNA concentration was determined photometrically at 320 nm and 400 ng RNA were used for transcription into cDNA with the following reaction mixture.

mixture I:

2 µl Hex Primer (100 ng/µl, Invitrogen)

2 µl dNTPs (10 mmol)

16 µl H2O

Heated at 65 $^\circ\!\mathrm{C}$, for 5 min and added mixture II.

mixture II:

8 µl 5xPuffer + DTT (Invitrogen)

6 µl RNAse out (Invitrogen)

2 µl MMLV-Reverse Transkriptase (Invitrogen)

Then the samples were put into PCR machine used the protocol as follow:

10 min bei 25℃

 $60 \text{ min bei } 42^\circ\!\mathrm{C}$

10 min bei 70° C

2.2.14 Real-Time reverse transcription–PCR analysis

Total renal RNA was prepared according to standard laboratory methods as described previously (Panzer et al., 2007). Real-time PCR was performed for 40 cycles (initial denaturation 95°C for 10 min; denaturation 15 s at 95°C; primer annealing and elongation 1 min at 60°C) with 1.5l of cDNA samples in the presence of 2.5µl (0.9µM) of specific murine primers (primer sequences are available upon request) and 12.5µl of 2× Platinum SYBR Green qPCR Supermix (Invitrogen, Paisley, UK) in an AbiPrism Sequence Detection System 7000 (Applied Biosystems, Stadt,CA). To account for small RNA and

cDNA variability, we ran an 18S rRNA PCR in parallel. All samples were run in duplicate and normalized to 18S rRNA as described (Panzer et al., 2007). Relative quantification of gene expression was calculated using the $\triangle \triangle$ CT method (Panzer et al., 2006).

2.2.15 Flow cytometry

Before the incubation with antibody, nonspecific staining was blocked with normal mouse serum (SigmaAldrich). For fluorescence-activated cell sorter (FACS) analysis of renal cells and splenocytes, the isolated cells were stained for 25 min at 4°C with the following fluorochrome-conjugated antibodies: CD3 (AF700 17A2; R&D Systems, Wiesbaden, Germany), CD45 (PerCP 30-F11; Pharmingen, Heidelberg, Germany) CD4 (APC H7 GK1.5; Miltenyi, Bergisch Gladbach, Germany), CD25 (APC 30-F11; Pharmingen, Heidelberg, Germany), CD8 (V500) and FoxP3 (PE220803; R&D Systems). Dead cells were always excluded with propidium iodide. Experiments were performed on a Becton Dickinson (Heidelberg, Germany) FACScalibur using the software CellQuest (Becton Dickinson).

The evaluation of the FACS measurement was performed according to the following Schematic diagram: after all non-leukocytes and duplicates were excluded (Fig. 6A), the leukocytes were extracted by the expression of antibody of CD45 (Fig. 6B), from which the CD3+ T-cells can be isolated (Fig. 6C); which were then divided into CD4 + helper T cells (Fig. 6D). The CD4+

population was further divided into IFN-γ-producing Th1 cells and IL-17producing Th17 cells (Fig. 6E). Regulatory T cells (Tregs) were separated by the expression of the transcription factor Foxp3 (Fig. 6F).



Fig. 6 The schedule of the flow cytometry A) All non-leukocytes and duplicates were excluded; B) the leukocytes were extracted by the expression of antibody of CD45; C) the

CD3+ T-cells was isolated from CD45; D) CD3+ T cells were then divided into CD4 + helper T cells; E) the CD4+ population was further divided into IFN- γ -producing Th1 cells and IL-17-producing Th17 cells; F) Regulatory T cells (Tregs) were separated by the expression of the transcription factor Foxp3 from the CD4+ T cells.

2.2.16 Histology and Immunohistochemistry

Tissues were fixed in 4% neutral buffered formalin and routinely processed and embedded in paraffin. Crescent formation, glomerular fibrin, and glomerular sclerosis were counted in a blinded fashion in 100 consecutive glomeruli per mice using periodic acid-Schiff (PAS) stained tissue sections.

A semi-quantitative score for tubular injury and for acute tubular necrosis was calculated for each animal by a double-blinded observer as described before (Hunemorder et al., 2015). The percentage of tubules that displayed cellular necrosis, loss of brush border, interstitial edema, vacuolization, and tubule dilation were scored as follows: 0 = none, 1 = 25%, 2 = 26-50%, 3 = 51%-75%, $4 \ge 76\%$. For each animal, at least 10 high-power fields (hpf) were examined (x200).

Immunohistochemistry was performed as described in detail previously (Meyer-Schwesinger et al., 2009). The pan-T cell marker CD3 antibodies were used. For immunohistochemistry, 1-3µm thin paraffin sections were baked overnight at 40°C, deparaffinized, and rehydrated. Antigen retrieval was performed by microwave boiling and protease digestion. Unspecific binding was blocked with 5% normal horse or goat serum (Vector,

Burlingame, VT) for 30 min at room temperature. Primary antibody incubations were performed in 5% horse or goat serum overnight at 4°C, followed by incubation with biotinylated affinity purified donkey secondary antibodies. Color development was performed with the ABC-AP kit (Vector) according to the manufacturer's instructions. Neufuchsin was used as substrate for alkaline phosphatase, and nuclei were counter stained with hematoxylin for light microscopic evaluation. Stainings were evaluated under an Axioskop and photographed with an Axiocam HRc using the Axiostar software.

2.2.17 Western blotting

Immunoblots were performed as described previously. Gel electrophoresis and protein transfer to polyvinylidene difluoride membranes were performed according to standard protocols. In brief, samples were lysed in tissue protein extraction reagent (T-PER; Pierce Biotechnology, Inc., Rockford, IL) containing phosphatase inhibitors, and denatured with 4X lithium dodecyl sulfate. Samples were separated on a 4% to 12% Bis-Tris NuPage gel (Invitrogen Corp.) in NuPage running buffer. Polyvinylidine fluoride membranes (Millipore Corp., Bedford, MA) were blocked (5% nonfat milk) before incubation with primary antibodies diluted in Superblock blocking reagent (Pierce Biotechnology, Inc.). Binding was detected via incubation with horseradish peroxidase– coupled secondary antibodies (1:10,000; 3% nonfat

milk). Protein expression was visualized using ECL SuperSignal (Pierce Biotechnology, Inc.) according to the manufacturers' instructions, using Amersham imager 600 machine (<u>GE Healthcare Life Sciences</u>). Immunoblots were analyzed using ImageJ software (<u>Meyer-Schwesinger et al., 2009</u>).

2.2.18 Mouse albumin quantitative ELISA

Mouse anti-sheep IgG antibody titers were measured by ELISA using urine that were collected 3rd day after induction of NTN and 12 h before mice were killed. In brief, ELISA microtiter plates were coated with 100µl of 100µg/ml sheep IgG (Sigma, Taufkirchen, Germany) in carbonate-bicarbonate buffer overnight at 4°C. After blocking with 1% BSA in TBS, the plates were incubated with serial dilutions of mouse urine (starting at 1:100 to 1: 12,500) for 1 h at room temperature. Bound mouse IgG was detected using peroxidase-conjugated goat anti-mouse IgG (Biozol, Eching, Germany) at 1:1000, TMB peroxidase substrate, and absorbance readings (at 450 nm) on a spectrophotometer. Lack of cross-reactivity of the secondary antibody with sheep IgG was demonstrated by omitting the primary antibody. Ig isotypes were measured using the ELISA technique already described. Mouse urine was diluted 1:100 for IgG1, IgG2a, and IgG2b measurement. The bound mouse Ig isotypes were detected using peroxidase-conjugated rabbit antimouse IgG1, IgG2a, and IgG2b antibodies (Zymed-Invitrogen, Karlsruhe, Germany) at a dilution of 1:1000.

The urine samples were diluted with Sample Diluent according to the outcome of Multistix according to the following Scheme:

Classification	Dilution
Spur	1:100
+	1:1000
++	1:10000
+++	1:50000
++++	1:200000

The sample and a standard series with mouse albumin concentrations were in the following consolidated pipetted into the plate and incubated for 1 h at RT. From each sample double values were made and the mean value of the concentrations was used.

Standard	Consentration		Sample	Conjugate
			Diluent	
S0	10000 ng/ml	2µl Stock solution	198µl	
S1	1000 ng/ml	100µl S0	900µl	
S2	500 ng/ml	300µl S1	300µl	
S3	250 ng/ml	300µl S2	300µl	
S4	125 ng/ml	300µl S3	300µl	
S5	62,5 ng/ml	300µl S4	300µl	
S6	31,25 ng/ml	300µl S5	300µl	
S7	15,625 ng/ml	300µl S6	300µl	
S8	7,8 ng/ml	300µl S7	300µl	

2.2.19 Statistical Analysis

Results in the text are expressed as means \pm SD. Differences between individual experimental groups were compared by one-way ANOVA test otherwise statement. Statistical significance was defined as p<0.05.

3 Results

3.1 PCR based genotyping of transgenic knockout mice

By crossing the IKK2^{#/#}, NEMO^{#/#} and IKK2^{#/#}NEMO^{#/#} mouse with the CD4-*Cre* mouse, a cell-specific knockout of IKK2, NEMO and IKK2NEMO were achieved under control of the Cre promoter. The Cre promoter is specific for CD4+ T cells. We did the genotyping with the animal biopsies to check the related gene(s) was (were) deleted (Fig. 7).



Fig. 7 The genotyping of knockout animals The PCR products indicating the Cre and flox(fl) were expressed under different conditions. Each panel represents an individual mouse. For each genotyping, five independent mice samples were shown.

3.2 Functional studies

Our previous study showed NF-κB was activated around 3, 10, 21 days after 49

NTN induction, suggesting inflammatory responses were induced at those time points. In present study, we attenuated the NF-kB activity in the CD4 positive (CD4+) T cells specifically. In addition, since the T lymphocytes kinetics is slow and takes major effects in the mid-to-late stages (around 7-10 days after inflammation induced) in NTN induced inflammatory responses. Therefore, to investigate the role of the NF-kB mediated CD4+ T cells function in the NTN animals, we always focused on the functional activation of NF-KB at the 10th day after NTN induction. Firstly, the blood was collected at 10th day when sacrificed and urine was collected at 3rd day (to check whether NTN induction was successful or not) and 12 hours before the mice were sacrificed at 10th day after NTN induction. The albumin-to-creatinine ratio and blood urea nitrogen (BUN) was studied. It showed that the albumin-to-creatinine ratio was significantly elevated in all mice at 3 days after the disease induction, but it was obviously higher in the NEMO knockout mice and IKK2 knockout mice when compared with the CD4Cre mice (CD4Cre-T3: 175.8 ± 10.70; CD4CreNEMO-T3: 384.4 ± 77.92; CD4CrelKK2-T3: 480.4 ± 83.49; CD4CreIKK2NEMO-T3: 239.3 ± 30.43; CD4Cre-T3 vs CD4CreNEMO-T3, p* = 0.0108; CD4Cre-T3 vs CD4CreIKK2-T3, p*** = 0.0004; CD4Cre-T3 vs CD4CrelKK2NEMO-T3, p = 0.8885), (Fig. 8A). However, at the 9th day of NTN there was no much difference between the CD4Cre mice and all types of knockout mice (12 hours before the mice sacrificed) (CD4Cre-T9: 58.68 ± 7.182; CD4CreNEMO-T9: 59.40 ± 4.881; CD4CrelKK2-T9: 60.10 ± 18.39; 50

CD4CrelKK2NEMO-T9: 75.23 ± 4.693; CD4Cre-T9 vs CD4CreNEMO-T9, p > 0.9999; CD4Cre-T9 vs CD4CrelKK2-T9, p > 0.9999; CD4Cre-T9 vs CD4CrelKK2NEMO-T9, p = 0.456) (Fig. 8B). In addition, there was also no difference between the CD4Cre mice and all types of knockout mice in the blood urea nitrogen levels (BUN; mg/dL) at the 10th day after disease induction though (CD4Cre-T10: 33.75 ± 1.851; CD4CreNEMO-T10: 30.00 ± 1.348; CD4CrelKK2NEMO-T10: 30.00 ± 1.348; CD4CrelKK2NEMO-T10: 32.50 ± 2.146; CD4Cre-T10 vs CD4CreNEMO-T10, p = 0.3167; CD4Cre-T10 vs CD4CrelKK2-T10, p = 0.7114; CD4Cre-T10 vs CD4CrelKK2NEMO-T10, p > 0.9999) (Fig. 8C), indicating a more server kidney damage in NEMO and IKK2 knockout mice at the 3rd day after NTN induction but with a similar renal function between CD4Cre (control) and all the knockout mice at the 10th day after NTN induction.



Fig. 8 The renal function study of NTN mice

A) Albumin-to-creatinine ratio of 3 days (T3) after NTN. The knockout mice (NEMO or IKK2 knockout mice) had a higher albumin-to-creatinine ratio, suggesting a worse renal function; B) albumin-to-creatinine ratio of 9th day (T9) after NTN. There was no significant difference between the all types of knockout mice and Cre mice; C) The blood urea nitrogen level at 10 days after NTN was measured. There was no difference between the all knockout groups and the Cre group; D) Schematically summary image showed the kidney function (albumin-to-creatinine ratio) of the knockout mice and Cre mice at different time points. The trend lines were estimated automatically based on the data of T3 and T9 after NTN induction.

Although a more server kidney damage was observed in NEMO and IKK2 knockout mice comparing to CD4Cre mice at early stage (3rd after NTN induction), similar functional results were observed between control and knockout mice (Fig. 8D). These data suggested that our NTN induced GN model was successful. In addition, it seems the roles of NF-κB in CD4+ T 52

cells to mediate NTN induced GN at early (3rd) and mid-to-late (10th) stages were different. However, the server impaired renal function in NEMO or IKK2 knockout mice at the 3rd day after NTN induction was unlikely due to the abnormal CD4+ T cell functions because the innate immune response was dominant at this stage which mainly involved in neutrophils, mast cells and interleukin-17 (IL-17)-producing γδ T cells (Kurts et al., 2013). It should be noted that it can not exclude the possibility that inhibition of NF-kB in CD4+ T cells altered the other immunity components such as eosinophils, basophils, neutrophils, monocytes, macrophages and B lymphoid cells during the innate immune response stage. Surprisingly, we did not observe any difference of kidney damage at the 10th day after NTN induction between CD4Cre (Control) and all types of knockout mice (Fig. 8D) although it was suggested that (CD4+) T cells take a majority role to mediate inflammation in kidney at this stage (Kurts et al., 2013). Thus, our observations implied that although NF-κB is critical for CD4+ T cells development and functions, it seems that NF-kB is not an essential factor that affects CD4+ T cells development and functions to eventually modulate kidney injury at late stage of NTN.

3.3 Quantification of renal tissue damage in NTN mice

To further assess whether the difference of kidney tissue injury exists between the knockout mice and Cre (control) mice at 10th NTN induction, Periodic acid–Schiff (PAS, a method used to detect polysaccharides (e.g. glycogen)) stained kidney sections were evaluated, and mucosubstances (e.g. glycoproteins, glycolipids and mucins) in tissues, was performed to evaluate the morphology of kidney tissues by light microscopy. The score of glomerular damage and the present of glomerular crescents were valued as previously described (<u>Turner et al., 2008</u>). In addition, the tubuleinterstitium was also analyzed. A semi-quantitative score for the presence of glomerular crescents, glomerular sclerosis and tubule-interstitial injury was calculated for each genotype in a double-blinded observation procedure.

3.3.1 Glomerular morphology damage

Glomerular morphology damage changes included hyper-cellularity, formation of cellular crescents, intra-glomerular deposition of PAS-positive material, moderate mesangial proliferation and prominent fibrocellular proliferation. The score of the glomerular was quantitated as 0 (normal glomerular), 1 (slight formation of crescents or slight morphology change), 2 (deposited in the glomerular or slight mesangial proliferation, a lot of lymphocytes infiltrated in glomeruli and the structure of glomerular looked a little disorder), 3 (mesangial proliferation looked severe, the structure of glomerular was destroyed and more lymphocytes infiltrated) and 4 (the structure of glomerular was almost gone and badly destroyed, fibrotic, more cells infiltrated intra- or periglomerular) (Fig.9A).



Fig. 9 Characterization of NTN model of glomerulonephritis by PAS staining

A) Representative images of the glomerular morphology. The score was quantitated as 0 (normal glomerular), 1 (slight formation of crescents or slight morphology change), 2 (deposited in the glomerular or slight mesangial proliferation, a lot of lymphocytes infiltrated in glomeruli and the structure of glomerular looked a little disorder), 3 (mesangial proliferation looked severe, the structure of glomerular was destroyed and more lymphocytes infiltrated), and 4 (the structure of glomerular was almost gone and badly destroyed, fibrotic, more cells infiltrated intra- or peri-glomerular), respectively. B) The statistical graph of the glomerular morphology damage between knockout mice and control mice at 10th day after NTN induction.

The morphology damage score analysis showed that there was no difference between the control mice (Cre mice) and all types of knockout mice at 10^{th} days after NTN induction (CD4Cre: 0.8050 ± 0.06659 N=9;

CD4CreNEMO: 0.8107 \pm 0.06941 N=7; CD4CreIKK2: 0.7875 \pm 0.04270 N=10; CD4CreIKK2NEMO: 0.9278 \pm 0.07551 N=9; CD4Cre vs CD4CreNEMO, p > 0.9999; CD4Cre vs CD4CreIKK2, p > 0.9999; CD4Cre vs CD4CreIKK2NEMO, p = 0.5278) (Fig. 9B), which was according with the findings observed in the renal function study.

3.3.2 Renal crescent scores study

Crescentic glomerulonephritis is a morphologic expression of severe glomerular injury which can be caused by a lot of aetiologies and pathogenic mechanisms (Couser, 1988). The cause of the crescent formation is because of the rupture of the glomerular capillaries, which allows cellular and humoral inflammatory mediators to spill into Bowman's space. This structural phenomenon indicates severe glomerular injury which usually correlates with severe active glomerulonephritis and rapid loss of renal function (Tang et al., 2001) (Fig. 10A). Therefore, the crescent score analysis is also a useful method to evaluate the function of kidney. In our study, we did not find any difference between the knockout mice and Cre mice at 10th day of the NTN (CD4Cre: 42.86 ± 2.072 N=7; CD4CreNEMO: 44.38 ± 3.332 N=8; CD4CrelKK2: 42.92 ± 2.181 N=6; CD4CrelKK2NEMO: 43.75 ± 1.357 N=10; CD4Cre vs CD4CreNEMO, p > 0.9999; CD4Cre vs CD4CreIKK2, p > 0.9999; CD4Cre vs CD4CreIKK2NEMO, p > 0.9999) (Fig. 10B), suggesting specific

inhibition of NF-κB in CD4+ T cells did not alter the renal function at the 10th day after NTN induction. Thus, this data further confirmed that there was no obvious difference between Cre (control) and all types of knockout mice in mediating kidney injury in NTN induced GN animals.



Fig. 10 Renal crescent score analysis A) Representative image of crescent structure of glomerular, B) The statistical graph of the crescent scores analysis. There was no difference observed between the Cre mice and all types of knockout mice after NTN induction.

3.3.3 Renal tubulin interstitium study

Structural abnormalities of tubules and interstitium are common phenotypes in renal diseases and often associated with dysfunctional kidney (Hooke et al., 1987). At the 10th day of NTN induction, we did find the tubulin interstitium damage from all genotypes of knockout mice was significantly attenuated compared to the CD4Cre mice (Fig. 11B) (CD4Cre: 34.61 ± 2.369 N=9; CD4CreNEMO: 24.46 ± 1.185 N=7; CD4CrelKK2: 27.08 ± 1.467 N=6; CD4CreIKK2NEMO: 26.00 ± 1.776 N=10; CD4Cre vs CD4CreNEMO, p** = 0.0025; CD4Cre CD4CrelKK2, 0.0389; CD4Cre vs vs CD4CrelKK2NEMO, p** = 0.0050), suggesting inhibition of NF-kB in CD4+ T 57

cells protects the tubule interstitium from NTN induced inflammatory injury.



Fig. 11 Renal tubulin interstitium study A) Representative images showing renal tubulin interstitium in different groups. B) The statistical graph of tubule interstitium measurement. All types of knockout mice showed a mild but significant decreased scores comparing with CD4Cre (control) mice, suggesting the renal tubulin interstitium damage was slightly mitigated from CD4+ T cells NEMO and/or IKK2 knockout mice.

3.4 T lymphocyte recruitment in control mice and NTN mice

Based on the above analysis on renal functions and renal tissue morphology, inhibition of NF-kB activation by knockout IKK2 and/or NEMO specifically in CD4+ T cells caused server damage of renal functions at early stage (3rd day) after NTN induction, but did not aggravate or mitigate the damage of renal functions and renal tissue morphology at mid-to-late stage (10th day) after NTN induction. These observations promoted us to explore whether the T lymphocytes infiltration at this mid-to-late stage (10th day of NTN induction) of the disease is altered or not. Thus, we analyzed the infiltration of the total T (CD3+) lymphocytes.

3.4.1 Renal T lymphocyte recruitment

CD3 is initially expressed in the cytoplasm of pro-thymocytes, the stem cells from which T-cells arise in the thymus. The pro-thymocytes differentiate into common thymocytes, and then into medullary thymocytes. It is at this later stage that CD3 antigen begins to be present on the cell membrane. The antigen is found to exist on the membranes of all mature T-cells but not other cell types in immune system. This high specificity, combined with the presence of CD3 at all stages of T-cell development, makes it a useful immunohistochemical marker for T-cells in tissue sections.

Here, first, the number of CD3+ T cells in kidney at indicated time points was counted by immunohistochemical staining. In all types of knockout mice

before the NTN induction (Non NTN mice), the CD3+ lymphocyte infiltration was significant reduced compared to Cre mice (CD4Cre: 36.33 ± 6.394 N=4; CD4CreNEMO: 8.770 ± 1.422 N=5; CD4CreIKK2: 14.95 ± 3.771 N=5; CD4CreIKK2NEMO: 6.838 ± 2.500 N=4; CD4Cre vs CD4CreNEMO, p*** = 0.0004; CD4Cre vs CD4CreIKK2, p** = 0.0040; CD4Cre vs CD4CreIKK2NEMO, p*** = 0.0004) (Fig. 12A and B). This reduced infiltration into intact kidney could be due to either the ability of infiltration was decreased or less CD3+ T lymphocytes were developed when inhibition of NF- κ B activation in CD4+ T lymphocytes, which was indistinguishable in our present study.

However, at the 10th day after the NTN induction, there were numerous CD3+ T lymphocyte infiltrated into the kidney not only the Cre mice but also the knockout mice. However, there was no significant difference of infiltrated CD3+ T lymphocytes between Cre mice and knockout mice at 10th day after NTN induction (CD4Cre: 125.5 ± 11.88 N=3; CD4CreNEMO: 179.7 ± 21.61 N=4; CD4CreIKK2: 141.2 ± 16.34 N=3; CD4CreIKK2NEMO: 160.8 ± 1.469 N=4; CD4Cre vs CD4CreNEMO, p > 0.9999; CD4Cre vs CD4CreIKK2, p > 0.9999; CD4Cre vs CD4CreIKK2NEMO, p > 0.9999; CD4Cre vs CD4CreIKK2NEMO, p > 0.9999) (Fig. 12C and D). However, it was shown that the fold change of CD3+ T lymphocytes infiltration was obviously elevated in all types of knockout mice after NTN induction when comparing the relative change of CD3+ T cells after and before NTN induction (the ratio of mean from corresponding groups after and before NTN 60

induction: CD4Cre-NTN/con = 4.319632; CD4CreNEMO-NTN/con = 19.179510; CD4CrelKK2-NTN/con = 10.356740; CD4CrelKK2NEMO-NTN/con = 23,869290) (Fig. 12E). These data indicated that knockout of NEMO and/or IKK2 in CD4+ T cells can recruit more CD3+ T lymphocytes trafficking into the inflammatory kidney at the 10th day of NTN induction although they exhibited fewer CD3+ T cells infiltration in kidney before the NTN induction and similar number of CD3+ T cells after NTN induction comparing with CD4Cre (control) mice. These observations suggested that the inhibition of NF-κB by knockout IKK2 and/or NEMO did affect the T cells' trafficking into kidney under physiological condition although the possibility of less CD3+ T cells were differentiated and developed could not be excluded. However, the ability to traffic into kidney was elevated when encountering with kidney inflammation. Although it is unlikely the case, however, the possibility can not be excluded that the elevated proliferation of CD3+ T cells in suit in all types of knockout mice which eventually resulted in similar number of CD3+ T cells were present in kidney from Cre and all types of knockout mice after NTN induction.



Fig.12 The renal CD3+ T lymphocyte recruitment analysis A) Representative images of CD3+ T cell staining in the non-NTN mice; B) The statistical graph of CD3+ T cell counting. All types of knockout mice exhibited less CD3+ cell infiltration into kidney compared with the Cre mice before the NTN induction; C) Representative images of CD3+ T cell staining in NTN mice; D) The statistical graph of CD3+ T cell counting in NTN mice. Both the Cre mice and knockout mice had a large number of T lymphocytes infiltration and had not difference between each other at 10th days after NTN induction; E) Fold change analysis about the recruitment of CD3+ T lymphocytes after the NTN induction. The knockout mice showed an elevated ability to recruit more CD3+ T lymphocytes infiltration comparing with the Cre mice after NTN induction.
3.4.2 Glomerular T lymphocyte recruitment

To further examine the ability of T lymphocyte recruitment, we counted the CD3+ T lymphocytes infiltration not only in the renal tissue but also in the single glomerular before NTN and after NTN induction. The similar phenotypes to the general renal tissues were observed: there were fewer CD3+ T lymphocytes infiltration into the glomerular in all types of knockout mice before NTN induction (CD4Cre: 5.619 ± 0.3056 N=4; CD4CreNEMO: 3.955 ± 0.1562 N=5; CD4CrelKK2: 3.865 ± 0.4135 N=5; CD4CrelKK2NEMO: 3.050 ± 0.1440 N=4; CD4Cre vs CD4CreNEMO, $p^{**} = 0.0037$; CD4Cre vs CD4CrelKK2, $p^{**} = 0.0024$; CD4Cre vs CD4CrelKK2NEMO, $p^{***} = 0.0001$) (Fig. 13A and B). 10 days after NTN induction, a huge number of CD3+ T cell rushed to the peri-glomerular in all the animals. However, there was no significant difference between all types of knockout mice and Cre mice (CD4Cre: 16.64 ± 0.4140 N=9; CD4CreNEMO: 18.07 ± 0.6203 N=7; CD4CrelKK2: 17.27 ± 0.7075 N=6; CD4CrelKK2NEMO: 17.91 ± 0.9427 N=6; CD4Cre vs CD4CreNEMO, p = 0.3289; CD4Cre vs CD4CreIKK2, p > 0.9999; CD4Cre vs CD4CrelKK2NEMO, p = 0.5086) (Fig.13C). In fold change analysis, it showed all types of knockout mice recruited more CD3+ T lymphocyte peri-glomerular than the Cre mice (CD4Cre-NTN/con = 2,960821; CD4CreNEMO-NTN/con = 4,568358; CD4CreIKK2-NTN/con = 4,468521; CD4CrelKK2NEMO-NTN/con = 5,872951) (Fig. 13D). Thus, the phenotypes

of CD3+ T lymphocytes recruitment in glomerular structure and general renal tissues were consistent.

Still, before NTN induction, the trafficking of CD3+ T cells in all types of knockout mice was reduced but it is not clear such reduction was due to less number of total CD3+ T cells or the decrease of the trafficking ability. In addition, with less CD3+ T cells infiltration into glomerular before NTN induction in knockout mice but nearly same high amount of CD3+ T lymphocytes at 10th day of NTN as Cre mice, indicating more CD3+ T lymphocytes recruitment into kidney which could be due to either one of following or combined possibilities: NTN induction may increase the total number of CD3+ T lymphocytes because of new development of CD3+ T lymphocytes in lymphatic organs although the ability of recruiting CD3+ T lymphocytes into kidney is not changing. Conversely, the total number of CD3+ T lymphocytes is not changed but the ability to recruit CD3+ T cells into kidney is elevated. In addition, knockout of NEMO and/or IKK2 in CD4+ T lymphocytes may promote proliferation in suit after NTN induction. It should be noted that, all knockout mice were specifically targeted to CD4+ T lymphocytes, a subset of CD3+ T lymphocytes. Thus, the changes of CD3+ T cells we observed were dominant by the alteration of CD4+ T cells or the inhibition of NF-κB in CD4+ T cells could affect CD3+ T cells development and/or functions remains unclear.



Fig. 13 Glomerular T lymphocyte recruitment

A) Representative images of CD3+ T cell staining in glomerular. Left: the representative image of CD3+ T lymphocytes staining in Non NTN of CD4Cre mice; right: the representative image of CD3+ T lymphocytes infiltrate peri-glomerular in NTN of CD4Cre mice; B) The statistical graph of CD3+ T lymphocytes counting in non NTN mice. All types of knockout mice exhibited fewer CD3+ T lymphocytes infiltration in glomerular; C) The statistical graph of CD3+ T cell counting in NTN mice. Cre mice and all types of knockout mice showed more CD3+ lymphocytes infiltration in peri-glomerular comparing with non NTN mice, and there was no difference between each other group at 10th day after NTN; D) Fold change analysis of CD3+ T lymphocytes after and before the NTN induction.

3.5 FACS analysis

To further explore what exactly kind of subtype of T lymphocyte infiltrated in the immune system in our transgenic mice, we did the FACS analysis about the trafficking of CD3+, CD4+, Th1, Th17 and Treg T lymphocytes in different conditions.

3.5.1 CD3+ T lymphocytes analysis

CD3+ is an antigen appearing in all stage of T lymphocytes and is used to distinguish the T lymphocytes from others. Our FACS results showed that in all the knockout mice the percentage of T cells was significantly decrease in blood (circulatory system), spleen (peripheral immune system) and kidney (local inflammation) no matter before the NTN or after the NTN. Before the NTN induction: (blood: CD4Cre: 16.43 ± 1.417 N=4; CD4CreNEMO: 2.280 ± 0.3455 N=5; CD4CrelKK2: 9.740 ± 1.336 N=5; CD4CrelKK2NEMO: 2.900 ± 0.1000 N=3; CD4Cre vs CD4CreNEMO, p**** < 0.0001; CD4Cre vs CD4CrelKK2, $p^{**} = 0.0016$; CD4Cre vs CD4CrelKK2NEMO, $p^{****} < 0.0001$) (Fig. 14A); (spleen: CD4Cre: 12.93 ± 0.2955 N=4; CD4CreNEMO: 2.220 ± 0.3397 N=5; CD4CreIKK2: 8.075 ± 1.183 N=4; CD4CreIKK2NEMO: 1.933 ± 0.2963 N=3; CD4Cre vs CD4CreNEMO, p**** < 0.0001; CD4Cre vs CD4CreIKK2, *p**** = 0.0006; CD4Cre vs CD4CreIKK2NEMO, *p***** < 0.0001) (Fig. 14B); (kidney: CD4Cre: 17.60 ± 3.210 N=4; CD4CreNEMO: 4.280 ± 0.7677 N=5; CD4CrelKK2: 8.160 ± 0.5573 N=5; CD4CrelKK2NEMO: 6.533 ± 1.244 N=3; CD4Cre vs CD4CreNEMO, p*** = 0.0002; CD4Cre vs CD4CrelKK2, $p^{**} = 0.0036$; CD4Cre vs CD4CrelKK2NEMO, $p^{**} = 0.0028$) (Fig. 14C). After NTN: (blood: CD4Cre: 18.07 ± 0.6386 N=3; CD4CreNEMO:

3.650 ± 0.3594 N=4; CD4CreIKK2: 8.567 ± 2.217 N=3; CD4CreIKK2NEMO: 3.180 ± 0.4363 N=5; CD4Cre vs CD4CreNEMO, $p^{****} < 0.0001$; CD4Cre vs CD4CreIKK2, $p^{***} = 0.0002$; CD4Cre vs CD4CreIKK2NEMO, $p^{****} < 0.0001$) (Fig. 14A); (spleen: CD4Cre: 14.43 ± 0.1764 N=3; CD4CreNEMO: 4.250 ± 0.6702 N=4; CD4CreIKK2: 6.733 ± 1.490 N=3; CD4CreIKK2NEMO: 4.880 ± 0.8564 N=5; CD4Cre vs CD4CreNEMO, $p^{****} < 0.0001$; CD4Cre vs CD4CreIKK2, $p^{***} = 0.0007$; CD4Cre vs CD4CreIKK2NEMO, $p^{****} = 0.0002$) (Fig. 14B); (kidney: CD4Cre: 38.97 ± 1.099 N=3; CD4CreNEMO: 29.10 ± 2.710 N=4; CD4CreIKK2: 14.23 ± 1.027 N=3; CD4CreIKK2NEMO: 21.40 ± 2.424 N=5; CD4Cre vs CD4CreNEMO, $p^{***} = 0.0007$) (Fig. 14C).

In addition, in each group, if compare the Non NTN and NTN mice, no obvious change was found about the CD3+ T cells infiltration in the blood and spleen after NTN induction (blood: CD4Cre/Non NTN vs. CD4Cre/NTN, p > 0.9999; CD4CreNEMO/ Non NTN vs. CD4CreNEMO/NTN, p > 0.9999; CD4CrelKK2/ Non NTN VS. CD4CrelKK2/NTN, p 0.9999: > CD4CrelKK2NEMO/ Non NTN vs. CD4CrelKK2NEMO/NTN, p > 0.9999, Fig. 14A); (spleen: CD4Cre/ Non NTN vs. CD4Cre/NTN, p = 0.8453; CD4CreNEMO/ Non NTN vs. CD4CreNEMO/NTN, p = 0.2439; CD4CreIKK2/ Non NTN vs. CD4CrelKK2/NTN, p > 0.9999; CD4CrelKK2NEMO/ Non NTN vs. CD4CrelKK2NEMO/NTN, p = 0.0603, Fig. 14B). In kidney (the local inflammation), however, the percentage of CD3+ T lymphocytes was elevated 67

significantly both in the Cre mice and all the knockout mice except for the IKK2 knockout mice after NTN induction. In IKK2 knockout mice, no obviously elevation about the T cells infiltration was found after the NTN induction (kidney: CD4Cre/ Non NTN vs. CD4Cre/NTN, $p^{****} < 0.0001$; CD4CreNEMO/ Non NTN vs. CD4CreNEMO/NTN, $p^{****} < 0.0001$; CD4CreIKK2/ Non NTN vs. CD4CreIKK2/NTN, p = 0.1791; CD4CreIKK2NEMO/ Non NTN vs. CD4CreIKK2NEMO/NTN, $p^{****} = 0.0001$) (Fig. 14C). Furthermore, we found in NEMO knockout mice the T cells infiltration was significantly higher than others. In the fold change analysis of CD3+ T cells infiltration between after NTN and before NTN induction, we found that the most striking elevated CD3+ T cells infiltration in kidney was observed in NEMO knockout mice.



Fig.14 FACS analyze about the CD3+ T lymphocyte

A) CD3+ T lymphocytes analysis from blood. The percentage of CD3+ T lymphocytes was less in all the knockout mice before or after NTN induction and no obvious elevation of CD3+ T lymphocytes in blood after NTN induction. B) CD3+ T lymphocytes analysis from spleen. The percentage of CD3+ T lymphocytes was less in all the knockout mice before or after NTN induction compared with the Cre mice. A slight but not significant increment of CD3+ T lymphocytes infiltration in spleen after NTN induction was observed in both NEMO and NEMO/IKK2 knockout mice. C) The percentage of CD3+ T lymphocytes was less in all the

knockout mice before or after NTN induction comparing with CD4Cre mice. However, NTN induction significantly elevated CD3+ T cells infiltration in all genotypes. D) Fold change analysis of the T CD3+ T lymphocytes recruitment from organs of blood, spleen and kidney.

Overall, these data showed that the percentage of CD3+ T cells were significantly decreased in all knockout mice we examined in the Non NTN mice, which was the same to the result found in the immunochemical histology study. At the 10th day of NTN induction, the immunochemical histology study showed that the CD3+ T cells were highly trafficking in the renal while there were no differences between the Cre mice and knockout mice but the FACS study here showed that the CD3+ T cells' percentage was lower in knockout mice. The reasons could be that in the immunochemical histology staining the CD3+ T cells were studied in the total amount but in FACS analysis the CD3+ T cells were isolated from the leukocytes and the CD3+ T cells were explored in the percentage of leukocytes. The total number of CD3+ T cells at 10th day of NTN was the same in all mice while percentage of CD3+ T cells was decreased in FACS suggesting the leukocytes were significantly elevated in the knockout mice at the 10th day of NTN. However, the part of T cells is small part of leukocytes and we didn't sure what kinds of the leukocytes were increased in the knockout mice at 10th day of NTN. Therefore, deletion of NF-KB activity specifically in CD4+ T cells probably not only affected the total T cells' differentiation, development and functions but also may affect other parts of leukocytes' function. Secondly, the most 70

strikingly elevated CD3+ T cells were detected in kidney but not in blood or in spleen in all types of knockout mice, indicating the kidney (the local inflammation) is the main target for immune response when NTN induction, and their ability of CD3+ T lymphocytes infiltration into kidney was not impaired. Thirdly, in kidney, at the 10th day of the NTN the CD3+ T cells infiltration was still less in all knockout mice although the CD3+ T cells were significantly elevated in all mice, which is different from what we find in the immunohistochemistry study. In the immunohistochemistry study, the total number of CD3+ T cells at the 10th day of NTN was similar between all types of knockout mice and Cre (control) mice. In the FACS analysis, however, the percentage of the CD3+ T lymphocytes was lower than the Cre mice. Thus, the altered percentage of CD3+ T cells did not represent of the change number of CD3+ T cells since the total leukocytes number could be affected when deletion of the NF-KB activation in the CD4+ T lymphocytes. Lastly, the most striking CD3+ T lymphocytes infiltration into kidney was observed in NEMO knockout mice comparing with IKK2 or NEMO/IKK2 knockout mice. However, the underlying mechanism is still missing.

3.5.2 CD4+ T lymphocytes analyze

Since the knockout mice are specifically targeted to CD4+ T cells, the infiltration of the subtype of CD4+ T lymphocytes in the NTN animals was

examined. CD4+ T cells play a central role in modulating cellular immunity by releasing T cell cytokines. Mature T helper (Th) cells express the surface protein CD4 and are referred to as CD4+ T cells. Such CD4+ T cells are generally treated as having a pre-defined role as helper T cells within the immune system, particularly in the adaptive immune system. These cells help suppress or regulate immune responses. They are essential in B cell antibody class switching, in the activation and growth of cytotoxic T cells, and in maximizing bactericidal activity of phagocytes such as macrophages (Zhu and Paul, 2008).

Our FACS studies showed that before the NTN induction (in non NTN mice), the percentage of CD4+ T lymphocytes infiltration was less in the NEMO and IKK2/NEMO knockout mice but higher in IKK2 knockout mice when comparing with the Cre mice in both blood and spleen organs (blood: CD4Cre: 53.48 ± 0.3119 N=4; CD4CreNEMO: 35.30 ± 3.078 N=5; CD4CreIKK2: 65.22 ± 2.344 N=5; CD4CreIKK2NEMO: 32.13 ± 4.237 N=3; CD4Cre vs CD4CreNEMO, $p^{***} = 0.0010$; CD4Cre vs CD4CreIKK2, $p^* = 0.0242$; CD4Cre vs CD4CreIKK2NEMO; 20.010; CD4Cre vs CD4CreIKK2; 57.10 ± 1.028 N=4; CD4CreIKK2NEMO: 20.03 ± 2.660 N=3; CD4Cre vs CD4CreIKK2NEMO, $p^{***} = 0.0001$; CD4Cre vs CD4CreIKK2; 57.10 ± 1.028 N=4; CD4CreIKK2NEMO: 20.03 ± 2.660 N=3; CD4Cre vs CD4CreIKK2NEMO, $p^{****} < 0.0001$; CD4Cre vs CD4CreIKK2NEMO in kidney but no significant change in NEMO T

knockout mice (kidney: CD4Cre: 44.38 ± 1.266 N=4; CD4CreNEMO: 41.76 ± 3.056 N=5; CD4CreIKK2: 64.92 ± 1.380 N=5; CD4CreIKK2NEMO: 32.53 ± 2.019 N=3; CD4Cre vs CD4CreNEMO, p > 0.9999; CD4Cre vs CD4CreIKK2, $p^{****} < 0.0001$; CD4Cre vs CD4CreIKK2NEMO, $p^* = 0.0139$).

After NTN induction, the percentage of CD4+ T cells was still less in the NEMO and IKK2/NEMO knockout mice and more in IKK2 knockout mice compared with the Cre mice in blood (blood: CD4Cre: 43.43 ± 0.3180 N=3; CD4CreNEMO: 23.48 ± 0.9277 N=4; CD4CrelKK2: 62.23 ± 1.790 N=3; CD4CrelKK2NEMO: 25.90 ± 4.028 N=5; CD4Cre vs CD4CreNEMO, p** = 0.0023; CD4Cre vs CD4CrelKK2, $p^{**} = 0.0057;$ CD4Cre vs CD4CrelKK2NEMO, $p^{**} = 0.0042$, Fig. 15A). However, there was no much change in all types of knockout mice when comparing with CD4Cre mice in the spleen and kidney (spleen: CD4Cre: 29.47 ± 1.391 N=3; CD4CreNEMO: 24.25 ± 5.903 N=4; CD4CrelKK2: 46.50 ± 3.288 N=3; CD4CrelKK2NEMO: 19.74 ± 2.502 N=5; CD4Cre vs CD4CreNEMO, p > 0.9999; CD4Cre vs CD4CrelKK2, p = 0.0538; CD4Cre vs CD4CrelKK2NEMO, p = 0.3110); (kidney: CD4Cre: 37.33 ± 1.619 N=3; CD4CreNEMO: 36.73 ± 4.114 N=4; CD4CrelKK2: 28.23 ± 2.684 N=3; CD4CrelKK2NEMO: 36.04 ± 4.558 N=5; CD4Cre vs CD4CreNEMO, p > 0.9999; CD4Cre vs CD4CrelKK2, p = 0.5500; CD4Cre vs CD4CreIKK2NEMO, p > 0.9999) (Fig. 15B and C).

Interestingly, NTN induction did not elevate the percentage of CD4+ T lymphocytes in all genotypes (comparing the change after and before NTN 73 induction for each group). Interestingly, CD4+ T lymphocytes with IKK2 knockout showed elevated percentage of CD4+ T lymphocytes infiltration before NTN induction was dramatically decreased to the comparable level of all the other genotypes after NTN induction in kidney (Blood: CD4Cre/ Non NTN vs. CD4Cre/NTN, p**** < 0.0001; CD4CreNEMO/ Non NTN vs. CD4CreNEMO/NTN, ρ < 0.0997; CD4CrelKK2/ Non NTN vs. CD4CrelKK2/NTN, p > 0.9999; CD4CrelKK2NEMO/ Non NTN vs. CD4CrelKK2NEMO/NTN, p = 0.2572)(Fig.15 A); (spleen: CD4Cre/ Non NTN CD4Cre/NTN, $\rho = 0.1150$; CD4CreNEMO/Non NTN VS. VS. CD4CreNEMO/NTN, p >0.9999; CD4CrelKK2/ Non NTN vs. CD4CrelKK2/NTN, p = 0.0887; CD4CrelKK2NEMO/ Non NTN VS. CD4CrelKK2NEMO/NTN, p > 0.9999)(Fig. 15B); (kidney: CD4Cre/ Non NTN VS. CD4Cre/NTN, p = 0.6199; CD4CreNEMO/Non NTN VS. CD4CreNEMO/NTN, p = 0.9744; CD4CreIKK2/ Non NTN VS. CD4CrelKK2/NTN, p**** < 0.0001; CD4CrelKK2NEMO/ Non NTN vs. CD4CrelKK2NEMO/NTN, p > 0.9999)(Fig. 15C).



Fig. 15 FACS analysis of CD4+ T lymphocytes

A) CD4+ T lymphocytes analysis from blood. The percentage of CD4+ T lymphocytes was less in NEMO and NEMO/IKK2 knockout mice and more in IKK2 knockout mice before or after NTN induction. NTN induction did not alter the percentage of CD4+ T lymphocytes in all genotypes except in NEMO knockout mice, which showed a little bit reduction. B) CD4+ T lymphocytes analysis from spleen. The percentage of CD4+ T lymphocytes was less in NEMO and NEMO/IKK2 knockout mice and more in IKK2 knockout mice before or after NTN induction. C) The percentage of CD4+ T lymphocytes was less in NEMO AND NEMO/IKK2 knockout mice and more in IKK2 knockout mice and slightly decreased in NEMO/IKK2 knockout mice before NTN induction. NTN induction did not alter the percentage of CD4+ T lymphocytes in all genotypes except in IKK2 knockout mice before NTN induction.

mice, which showed a dramatic reduction of the percentage of CD4+ T lymphocytes. It should be noted that the percentage of CD4+ T lymphocytes in all genotypes was similar after NTN induction.

Overall, specific knockout of NEMO or NEMO/IKK2 in CD4+ T lymphocytes decreased the percentage of CD4+ T lymphocytes in blood and spleen under physiological condition. Knockout of IKK2 in CD4+ T lymphocytes increased the percentage of CD4+ T lymphocytes in blood and spleen before NTN induction. There was no obvious change after NTN induction in all genotypes. The distinct change in IKK2 knockout mice and NEMO or NEMO/IKK2 knockout mice may reflect the different role of NEMO and IKK2 in regulation of CD4+ T lymphocytes development and/or function. In kidney, however, only knockout of IKK2 showed obvious increment of percentage of CD4+ T lymphocytes but knockout of NEMO and NEMO/IKK2 did not alter the percentage of CD4+ T lymphocytes before NTN induction. After NTN induction, we did not observe obvious change of the percentage of CD4+ T lymphocytes in each individual group/genotype except in IKK2 knockout mice showing dramatically decreased the percentage of CD4+ T lymphocytes, which resulted in a similar percentage of CD4+ T lymphocytes in all genotypes after NTN induction. It should be noted that although NTN induction did not increase the percentage of CD4+ T lymphocytes in both CD4Cre (control) mice and all types of knockout mice, the absolute number of CD4+ T lymphocytes was significant elevated since the number of CD3+ T 76

lymphocytes was dramatically increased in kidney after NTN induction.

3.5.3 Treg, Th17 and Th1 T lymphocytes analyze

Although the percentage of the overall CD4+ T cells infiltration in kidney from all genotypes was similar after NTN induction, the infiltration of distinct subtypes of CD4+ T lymphocytes was investigated since they behavior not always identically. It is known that Treg, Th17 and Th1 CD4+ T cells are involved in GN, thus, we analyzed how these distinct subtypes of CD4+ T cells were distributed.

The regulatory T cells (Tregs), known as suppressor T cells, are a subpopulation of CD4+ T cells that modulate the immune system. These cells generally suppress or downregulate induction and proliferation of effector T cells (Shevach, 2000). In our study, Treg infiltration was less in all types of knockout mice after NTN induction in blood. While it was showed there was no much difference of the Treg cells infiltration in spleen from all genotypes. In kidney, however, both the NEMO knockout mice and NEMO/IKK2 double knockout mice exhibited less Treg cells infiltration compared to Cre mice while there was no difference in the IKK2 knockout mice (Fig. 16A).

T helper 17 cells (Th17) are a subset of T helper cells producing proinflammatory cytokine interleukin 17 (IL-17). Th17 cells play a role in adaptive immunity protecting the body against pathogens and play an

important role in maintaining mucosal barriers and contributing to pathogen clearance at mucosal surfaces, but they have also been implicated in autoimmune and inflammatory disorders. The loss of Th17 cell populations at mucosal surfaces has been linked to chronic inflammation and microbial translocation (Zambrano-Zaragoza et al., 2014). In this study, the percentage of Th17 T cell after NTN was significantly elevated in all knockout mice in spleen; but in kidney, the Th17 T cell was significantly elevated only in NEMO and double knockout mice (Fig. 16B).

Type 1 helper (Th1) cells are a lineage of CD4+ T cell producing interleukin (IL)-2, gamma-interferon (IFN-gamma) and tumour necrosis factor-beta and are involved in cellular immunity. Th1 cells induce delayed-type hypersensitivity reactions promoting cell-mediated immune responses and are required for host defense against intracellular viral and bacterial pathogens (Zhang et al., 2014). In spleen, the percentage of Th1 T cells was significantly higher only in the NEMO/IKK2 knockout mice. In kidney, the percentage of Th1 T cells were significantly higher in the NEMO and IKK2 knockout mice but not for NEMO/IKK2 knockout mice when comparing with the Cre mice (Fig. 16C).

Thus, these data suggested that inhibition of NF-κB in CD4+ T cells does affect the infiltration of Th1, Th17 and Treg cells in multiple organs, including kidney, after NTN induction. However, it seems that these alterations have no obvious effects on kidney functions since the similar impaired kidney functions 78 were observed in all genotypes.



Fig.16 FACS analyze about the Treg, Th17 and Th1

A) The FACS analysis of Treg cells: (a) The percentage of Treg cells in blood. All types knockout mice showed less Treg cells infiltration after NTN induction; (b) The percentage of Treg cells in spleen. There were not difference in all mice; (c) The percentage of Treg cells in kidney. Both NEMO knockout mice and NEMO/IKK2 knockout mice showed less Treg T cells than the Cre mice after NTN induction. B) The FACS analysis of Th17 cells: (a) The percentage of Th17 cells was significantly elevated in NEMO and NEMO/IKK2 knockout mice in spleen after NTN induction; (b) In all knockout mice, the percentage of Th17 cells was

significantly elevated after NTN induction comparing with the Cre mice; C) The FACS analysis of the Th1 T cell: (a) The percentage of Th1 cells in spleen. After NTN induction, only the NEMO/IKK2 knockout mice had a significantly higher Th1 cells infiltration compared to the CD4Cre mice; (b) In kidney, the NEMO and IKK2 knockout but not NEMO/IKK2 knockout mice had a significantly higher Th1 cells infiltration compared to CD4Cre mice.

3.6 Western Blotting analysis

It was reported the NF-kB activation in renal is critical for kidney disease, especially mediated by immune responses (Sanz et al., 2010). Since there was no obvious difference observed in T cells infiltration, we would like to know whether NF-kB activity was changed in NTN induced kidney. We did western blotting to further check how about the NF-kB activation related proteins levels from kidney tissues at the 10th day of NTN. First, we checked the nucleoprotein of IKK β , it was shown that IKK β protein was down-regulated in NEMO knockout mice but not IKK2 and NEMO/IKK2 double knockout mice (CD4Cre: 1.204 ± 0.1387 N=3; CD4CreNEMO: 0.3826 ± 0.1151 N=7; CD4CrelKK2: 0.6517 ± 0.1620 N=7; CD4CrelKK2NEMO: 1.000 ± 0.1327 N=11; CD4Cre vs CD4CreNEMO, $p^* = 0.0174$; CD4Cre vs CD4CreIKK2, p = 0.1585; CD4Cre vs CD4CrelKK2NEMO, p > 0.9999) (Fig. 17A and B). As for the IkB of nucleoprotein, it was declined only in IKK2 knockout (CD4Cre: 1.841 ± 0.3291 N=3; CD4CreNEMO: 1.112 ± 0.3258 N=7; CD4CreIKK2: 0.7331 ± 0.2008 N=7; CD4CrelKK2NEMO: 1.000 ± 0.1414 N=11; CD4Cre vs CD4CreNEMO, p = 0.2927; CD4Cre vs CD4CreIKK2, $p^* = 0.0449$; CD4Cre vs

CD4CrelKK2NEMO, p = 0.1370) (Fig. 17A and B). However, the active form of IKK $\alpha\beta$ of nucleoprotein, phosphorylated IKK $\alpha\beta$ (pIKK $\alpha\beta$), there was not change in all the mice (CD4Cre: 1.020 ± 0.3411 N=3; CD4CreNEMO: 0.5562 ± 0.03514 N=3; CD4CrelKK2: 0.7587 ± 0.2053 N=7; CD4CrelKK2NEMO: 1.000 ± 0.2844 N=7; CD4Cre vs CD4CreNEMO, p > 0.9999; CD4Cre vs CD4CrelKK2, p > 0.9999; CD4Cre vs CD4CrelKK2NEMO, p > 0.9999) (Fig. 17A and B). Thus, these was no change in the ration between pIKK $\alpha\beta$ and IKK β (CD4Cre: 0.8659 ± 0.1885 N=3; CD4CreNEMO: 0.8339 ± 0.1168 N=3; CD4CrelKK2: 1.014 ± 0.2557 N=8; CD4CrelKK2NEMO: 1.079 ± 0.3186 N=9; CD4Cre vs CD4CreNEMO, p > 0.9999; CD4Cre vs CD4CrelKK2, p > 0.9999; CD4Cre vs CD4CrelKK2NEMO, p > 0.9999; CD4Cre vs CD4CrelKK2, p > 0.9999; CD4Cre vs CD4CrelKK2NEMO, p > 0.9999; CD4Cre vs CD4CrelKK2, p > 0.9999; CD4Cre vs CD4CrelKK2NEMO, p > 0.9999; CD4Cre vs CD4CrelKK2, p > 0.9999; CD4Cre vs CD4CrelKK2NEMO, p > 0.9999) (Fig. 17A and B), suggesting there was no NF- κ B activity changing between the CD4Cre mice and all types of knockout mice.



Fig. 17 Western Blotting of nucleoprotein(NP)

A) Representative images of western blotting of nucleoprotein from NTN induced mice. B) The statistical graph of IKK β , I κ B, pIKK $\alpha\beta$ and pIKK $\alpha\beta$ / IKK β protein.

Furthermore, we also check about the protein level in cytoplasmic protein (CP). We found there was no difference between all types of knockout mice and CD4Cre mice after NTN induction for both the IKK β and I κ B protein (IKK β : CD4Cre: 0.9040 ± 0.1662 N=3; CD4CreNEMO: 1.170 ± 0.2782 N=7;

CD4CrelKK2: 1.212 ± 0.08853 N=7; CD4CrelKK2NEMO: 1.000 ± 0.1004 N=11; CD4Cre vs. CD4CreNEMO, p > 0.9999; CD4Cre vs. CD4CrelKK2, p = 0.9943; CD4Cre vs. CD4CrelKK2NEMO, p > 0.9999) (Fig. 18A and B); (I κ B: CD4Cre: 1.257 ± 0.3446 N=3; CD4CreNEMO: 1.001 ± 0.1630 N=7; CD4CrelKK2: 0.8784 ± 0.2458 N=5; CD4CrelKK2NEMO: 1.000 ± 0.1164 N=11; CD4Cre vs. CD4CreNEMO, p > 0.9999; CD4Cre vs. CD4CrelKK2, p = 0.7956; CD4Cre vs. CD4CrelKK2NEMO, p > 0.9999) (Fig. 18A and B).

Α



Fig. 18 Western Blotting of cytoplasmic protein (CP) A) Representative images of western blotting of cytoplasmic protein from NTN induced mice. B) The statistical graph of IKK β , I κ B, protein.

Overall, there were no differences found on $I\kappa B$ kinases activity between the knockout mice and CD4Cre (control) mice at 10th day after NTN induction, suggesting a comparable activation of NF-κB presented in all genotypes at 10th day after NTN induction. Thus, this finding further indicated that deletion of NF-κB activity in CD4+ T cells did not attenuate or aggravate the injury of the renal tissue.

3.7 Kidney chemokine expression

Chemokines are important mediators for immune mediated kidney injury. We finally detected some chemokines which were implicated to involve in immune mediated response in kidney.

IL-1 β is a member of the interleukin 1 family of cytokines. This cytokine is produced by activated macrophages as a proprotein. This cytokine is an important mediator of the inflammatory, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis (Smirnova et al., 2002). In our study, we didn't find different between the knockout mice and CD4Cre (control) mice at 10th day of NTN (CD4Cre: 1.883 ± 0.06489 N=3; CD4CreNEMO: 3.158 ± 0.5223 N=4; CD4CreIKK2: 2.253 ± 0.6365 N=3; CD4CrelKK2NEMO: 2.250 \pm 0.4722 N=5; CD4Cre vs CD4CreNEMO, p =0.3328; CD4Cre CD4CrelKK2, 0.9999; CD4Cre vs > р VS CD4CrelKK2NEMO, p > 0.9999) (Fig. 19A).

Tumor necrosis factor (TNF), the primary role is to regulate the immune cells, is a cell signaling protein (cytokine) involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction. It is mainly produced by activated macrophages, and also came from many other cell types such as CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons (Locksley et al., 2001). It was shown there was no obvious change between knockout mice and CD4Cre (control) mice (CD4Cre: $9.303 \pm 2.596 \text{ N=3}$; CD4CreNEMO: $17.07 \pm 1.293 \text{ N=4}$; CD4CreIKK2: $11.12 \pm 1.933 \text{ N=3}$; CD4CreIKK2NEMO: $13.85 \pm 1.871 \text{ N=5}$; CD4Cre vs CD4CreIKK2NEMO, *p* = 0.0592; CD4Cre vs CD4CreIKK2, *p* > 0.9999; CD4Cre vs CD4CreIKK2NEMO, *p* = 0.3686) (Fig. 19B).

The chemokine (C-C motif) ligand 2 (CCL2) who belongs to the CC chemokine family, is a small cytokine and is also referred to as monocyte chemotactic protein 1 (MCP1) and small inducible cytokine A2. CCL2 recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation produced by either tissue injury or infection (Carr et al., 1994; Xu et al., 1996). The CCL2 was decreased only in NEMO knockout mice at 10th day after NTN but no alteration in other types of knockout mice (CD4Cre: 34.47 ± 2.912 N=3; CD4CreNEMO: 15.61 ± 2.313 N=4; CD4CrelKK2: 24.66 ± 4.021 N=3; CD4CrelKK2NEMO: 31.12 \pm 3.284 N=5; CD4Cre vs CD4CreNEMO, p^{**} = 0.0067; CD4Cre vs CD4CrelKK2, p = 0.2408; CD4Cre vs CD4CreIKK2NEMO, *p* > 0.9999) (Fig. 19C).

Chemokine (C-C motif) ligand 5 (CCL5), known as RANTES (regulated on activation, normal T cell expressed and secreted), is chemotactic for T cells, eosinophils, and basophils, and plays an active role in recruiting leukocytes into inflammatory sites (Donlon et al., 1990). CCL5 is also a target gene of NF- κ B activity that expressed by T lymphocytes, macrophages, platelets, synovial fibroblasts, tubular epithelium, and certaintypes of tumor cells (Soria and Ben-Baruch, 2008). NF- κ B activation by different stimuli such as CD40L or IL-15 induces CCL5 production (Aldinucci et al., 2012; Chenoweth et al., 2012). At 10th day after NTN induction, there was no difference between all genotypes (CD4Cre: 3.483 ± 1.059 N=3; CD4CreNEMO: 4.825 ± 0.5033 N=4; CD4CreIKK2: 3.367 ± 0.4018 N=3; CD4CreIKK2NEMO: 5.798 ± 0.8773 N=5; CD4Cre vs CD4CreNEMO, p = 0.8358; CD4Cre vs CD4CreIKK2, p > 0.9999; CD4Cre vs CD4CreIKK2NEMO, p = 0.1928) (Fig. 19D).

Chemokine (C-C motif) ligand 20 (CCL20), also named liver activation regulated chemokine (LARC) or Macrophage Inflammatory Protein-3 (MIP3A), is a small cytokine belonging to the CC chemokine family. It is strongly chemotactic for lymphocytes and weakly attracts neutrophils (Hieshima et al., 1997). CCL20 is implicated in the formation and function of mucosal lymphoid tissues via chemoattraction of lymphocytes and dendritic cells towards the epithelial cells surrounding these tissues. It was showed that the CCL 20 expression was elevated only in the NEMO knockout mice at 10^{th} day after NTN induction (CD4Cre: 4.617 ± 2.127 N=3; CD4CreNEMO: 88.18 ± 15.17 86

N=4; CD4CreIKK2: 9.670 ± 1.853 N=3; CD4CreIKK2NEMO: 25.33 ± 6.607 N=5; CD4Cre vs CD4CreNEMO, p^{***} = 0.0003; CD4Cre vs CD4CreIKK2, p > 0.9999; CD4Cre vs CD4CreIKK2NEMO, p = 0.4483) (Fig. 19E).



Fig. 19 Kidney chemokine analysis There was no obvious difference of the chemokine of IL 1b(A), TNFa(B) CCL2(C), CCL5(D) and CCL20(E) from kidney in all genotypes at 10th day after NTN induction except a decreased expression of CCL2(C) in NEMO knockout mice, and an increment of CCL20(E) in NEMO knockout mice.

3.8 Microarray based genome-wide gene expression profiling

Although inhibition of NF-κB activation in CD4+ T cells did not alter the severity of kidney injury in NTN model, the role of CD4+ T cells on immune mediated kidney diseases could not be excluded. Thus, we expected other

possible molecules but not IKK2 and/or NEMO are critical for CD4+ T cells mediated NTN induced kidney injury. To detect which molecules may involve in the disease, microarray based genome-wide gene expression profiling in CD4+ T cells before and after NTN induction was performed.

Gene clusters analysis was shown in Fig. 3.9. In the condition of logFC \geq 1 and adjust p value \leq 0.05, the changed genes were listed in the Table 1. Interestingly, there were only few genes were selected under such criterion. There was no down-regulated gene in the NEMO ablation mice but two genes, the Cnn3 (calponin 3, acidic) and Amigo 2 (adhesion molecule with Ig like domain), were found up-regulated. In IKK2 deletion mice, 3 genes were found up-regulated and 14 genes were found down-regulated. In Cre (Control) mice 14 genes were found up-regulated and 17 genes were down-regulated. In addition, in the wild type mice 8 genes were up-regulated but 97 genes were down-regulated when comparing the T0 mice and the NTN T10 mice (Table 1).



Fig. 20 Microarray RNA analysis

Gene clusters of different genotypes before or at 10 days after NTN induction.

Down-regulated				Up-regulated			
wild		CD4Cre	CD4Cre	wild		CD4Cre	CD4Cre
type	CD4Cre	IKK2	NEMO	type	CD4Cre	IKK2	NEMO
Мро	lglc2	Tsc22d3		Hsph1	lqsec3	Hsph1	Cnn3
Car2	Mzb1	Ddit4		Zmynd10	Gm7867	Zbtb32	Amigo2
Tspo2	Blnk	Txnip		3010001F 23Rik	Tenm4	Zmynd10	
Ccr3	lgkv6-15	Zfp36l2		Rbm3	Gm4877		
Mt1	Mt1	Gm4956		Saysd1	Gm8292		
Drc1	Emilin2	Bbc3		Gm6335	Gfra4		
lqsec3	Lmo2	Arrdc3		Kif1b	Halr1		
Pon3	Csf2rb	Gnal		Pde6g	Glis1		
Car1	Cbfa2t3	Cirbp			Rs1		
Tuba8	Pparg	114			Olfr1500		
Gm6970	ll1rl1	Mettl20			Gm5316		
Hist1h1b	Ascl2	Cxcr4			Mir3091		
Ccna2	Smtn	2410022M 11Rik			Ltbp3		
Fignl1	Popdc2	Pik3ip1			Baiap2l2		
AnIn	Fam20a						
Ube2c	Fut7						
Ascl2	Cacna1s						
Mcm10							
Nusap1							
Dtl							
Birc5							
Cenpk							
114							
Cdk1							
Blvrb							
Ccnb1							
Shcbp1							
Ska1							
E2f8							
Cd63							
Kif20a							
Icam4							
Elane							
Pbk							
Aspm							

Cdca3				
Cdca5				
lglc2				
Mt2				
Rad51ap1				
Ticrr				
Prkar2b				
Cdc6				
ll1rl1				
Sapcd2				
Slc43a3				
Ankle1				
Myo1d				
Cenpe				
Padi4				
Sgol1				
Fam109b				
Cks1brt				
Selenbp1				
Ncapg2				
Spc25				
Cela1				
Uhrf1				
Egfl7				
Prc1				
Cdkn3				
Top2a				
Cdca8				
Ect2				
Csprs				
Melk				
Gm32460				
Ctse				
Popdc2		 	 	
Ncaph				
Aunip				
Tk1				
Fam20a				
Cks1b				
Neil3				
Eomes				

Cenpi				
Kif22				
0				
Cenpp				
Bub1				
Nuf2				
Cdc20				
Csrp2				
Cpne2				
Sccpdh				
Syce2				
Gins1				
Amica1				
E2f1				
Trip13				
Ctla4				
Tmbim1				
Gins2				
Zwilch				
Matn2				
Gabarapl1				
Chaf1a				

4 Discussion

Glomerulonephritis is a range of immune-mediated disorders that result in inflammation within the glomerulus and other compartments of the kidney. In the last decades despite many studies devoted to explore the molecular signaling pathways of glomerulonephritis and made great advances, the exactly pathological mechanism of glomerulonephritis is still unknown and the treatments for glomerulonephritis remain non-specific, hazardous, and only partly successful. Glomerulonephritis therefore remains a common cause of end-stage kidney failure worldwide.

The pathogenesis mechanisms of glomerulonephritis are numerous, complex and remains incompletely understood, and are mainly focus on antibody, complement, coagulation, intrinsic kidney cells and leucocytes (Chadban and Atkins, 2005). The common feature of glomerulonephritis is the appearance of inflammatory leucocytes in both the glomerular and the interstitial compartments of the kidney and the dominant cell types are macrophages and T lymphocytes. More and more studies have shed light on T lymphocyte cell-mediated immune responses and the involvement of T cells has been recognized to play an import role in the pathophysiology of glomerulonephritis (Panzer and Kurts, 2010; Salama et al., 2001; Wu et al., 2002). It is generally accepted that, in particular, CD4+ T helper cells play a central role in the pathogenesis of proliferative and crescent glomerulonephritis (Wu et al., 2002). But the exactly mechanism still remain 93

unknown.

NF- κ B has been found to be important for the development of T cell. The activation, differentiation, proliferation, and effector function of T cells are controlled by the gene programs of NF-kB (Gerondakis et al., 2014; Siebenlist et al., 2005). The complete blocking of NF-kB activity by ablation of NEMO caused an arrest in single-positive thymocytes. Partially impairment of IKK function by specific deletion of IKK2 impairs the development of the Treg and CD4+ effector/memory cells (Oh and Ghosh, 2013). Therefore, the NF-κB has an important role in the development of T cells. In this project, the IKK2 and NEMO were selectively deleted in CD4+ T lymphocyte to investigate the role for IKK2- and NEMO-kinase mediated nuclear factor kappa B activation in an experimental model of immune mediated injury (NTN) mice. The aim of this project is to explore the role of NF-kB-mediated CD4+ T cells in NTN mice and to understand the mechanisms of IKK2- and NEMO- driven systemic selective activation in renal infiltrating CD4+ T cells after NTN injury, which might have therapeutic implication to develop future therapeutic strategies for glomerulonephritis.

In our previous works, it was found that NF-κB played an important role in experimental glomerulonephritis (<u>Panzer et al., 2009</u>). *In vivo* systemic therapy with a specific IKK2 inhibitor reduced NF-kappa B activation in the kidney and inhibited renal inflammatory responses when mice were pretreated with the inhibitor, not however, when IKK2 inhibitor therapy were 94 delayed until day 4 after NTN induction (in preparation). Systemic application of IKK2 inhibitor reduced NF-kappa B activation both in renal and the trafficking of the inflammatory cells. Further, we found in the NF-κB was high activity at the 3rd, 10th and 21st day after NTN induction. These data suggested a possible role of NF-κB in CD4+ T cells on mediating NTN induced kidney injury.

In present study, we found that the renal function was more seriously impaired at 3rd day after NTN induction in the CD4+ T lymphocytes specific NEMO and IKK2 knockout mice in which the NF-kB activation was inhibited by measuring albumin/creatinine ratio. However, at 10th day after NTN induction, there was no obvious difference in all genotypes and no worse or attenuated renal function was observed in knockout mice, although the T cells dominant kidney inflammatory responses at this time point (Kurts et al., 2013). We further confirmed this finding by examining multiple other parameters including BUN test, PAS staining based glomerular morphology damage scores and renal crescent scores study (renal tissue damage), from which the same findings were observed at 10th day of NTN: no worse or attenuated renal tissue and functional damage found in knockout mice when compared with the CD4Cre (control) mice. Although a slightly reduction of the injury in tubule-interstitium test in all types of knockout mice, the overall structures and functions of kidney did not show any improvement or worse at 10 days after NTN induction when inhibit the NF-kB function in CD4+ T cells by specifically 95

knockout of NEMO and/or IKK2.

To characterize the how T cells behavior at 10th day after NTN induction in detail, we did the immunohistochemical staining about the CD3+ T cells and the FACS study about the different types of T cells in the blood, spleen and kidney. In immunohistochemical staining, the trafficking of CD3+ T cells were significantly decreased in the knockout mice in the non NTN mice (before NTN induction) and the same finding was found in the FACS study. These data suggested that inhibition of the NF-κB function in the CD4+ T cells did affect the T cells' differentiation and development and also further confirmed by previous reports that NF-KB is critical for different types of CD4+ T cells development (Oh and Ghosh, 2013). At the 10th day after NTN induction in immunohistochemical study, it showed a high trafficking number of CD3+ T cells into kidney comparing before NTN induction but without difference between all types of knockout mice and CD4Cre(control) mice. In the FACS study, we did observe very highly trafficking of CD3+ T cells into kidney after 10 days NTN induction, however, it was less CD3+ T cells trafficking in the knockout mice than the CD4Cre(control) mice. This results was a little bit difference from the finding in the immunohistochemical study, which probably is due to that the CD3+ T cells were counted in total amount in the immunohistochemical study while in the percentage of leukocytes in FACS study.

CD4+ T lymphocytes were measured by FACS study only. In kidney, there 96

was no change observed in all genotypes before and after NTN induction except in the IKK2 knockout mice: the CD4+ T cells were highly traffic into before the NTN induction but obviously reduced at 10th day of NTN induction comparing with the CD4Cre(control) mice. In a similar study by using CD4-Cre to specifically knockout IKK2 and NEMO in CD4+ T cells, deficient of NEMO in CD4+ T cells decreased the population of CD8 single-positive cells while did not significantly reduce CD4-SP in thymus. Importantly, only few of CD4+ and CD8+ T cells could be detected in the periphery (Schmidt-Supprian et al., 2003). All the observations are consistent with what we examined in the NEMO and NEMO/IKK2 knockout mice. However, they also observed the deficient of IKK2 under CD4-Cre condition caused reduced number of CD4+ and CD8+ T cells in the periphery but did not affect thymocyte development (Schmidt-Supprian et al., 2003), which partially counteracts with our observation-an elevated percentage of CD4+ T cells (account to CD3+ T cells) in IKK2 knockout mice without NTN induction. Thus, unlike an essential role of NEMO on canonical NF-κB activation, residual IKK activation can still occur due to IKK1 being able to compensate to some extent the lack of IKK2 activity, which is an already known phenomenon (Makris et al., 2000b; Schmidt-Supprian et al., 2003). In addition, it should be noted that IKK1 not only mediates non-canonical NF-kB pathways but also regulates biological functions independently with NF-KB by its nuclear localization and ability to phosphorylate Histone H3 (Anest et al., 2003; Yamamoto et al., 2003).

Consistently, although reduced in the number of CD3+ T cells showed in all types of knockout mice, more CD3+ T cells were detected in IKK2 knockout mice compared to NEMO or NEMO/IKK2 knockout mice. Overall, knockout of NEMO or NEMO/IKK2 in CD4+ T cells resulted in reduced T cells in the periphery reflects a critical role of canonical NF-kB on T cells development. However, knockout of IKK2 alone in CD4+ T cells showed an increased CD4+ T cells implies a more complex network on NF-KB activity dependently or independently regulates T cells development. In addition, the mechanisms on why inhibition of NF-κB activation in CD4+ T cells resulted in reduced T cells in the periphery are not well-known. However, it is implied that NF-kB is required for T cells survival after leaving thymus because an increased number of apoptotic cells were observed in the populations (Schmidt-Supprian et al., 2003), and probably by regulating anti-apoptotic Bcl-2 family member expression (Zheng et al., 2003).

Although there were fewer CD3+ and CD4+ T cells present in the knockout mice except for an increment of CD4+ T cells in IKK2 knockout mice in kidney, the ability to recruit T cells into kidney was increased in those knockout mice. Although accumulative studies suggest a positive relationship between glomerular dysfunction and T cells infiltration (van Alderwegen et al., 1997) and the activation of NF-kB (Sanz et al., 2010) in multiple glomerulonephritis diseases and experimental animal kidney inflammatory models, the direct evidence for NF-kB mediated T cells recruitment in injury renal is still missing.
Our data did provide a negative relationship between the activity of NF-kB and ability of T cells recruitment in injury renal since in the fold change study the T cells recruitment ability is higher in knockout mice though there was no obvious difference of T cell trafficking at 10th day of NTN by counting the number of CD3+ T cells in different compartments of kidney. Interestingly, CD3+ T cells in blood, spleen and kidney were obviously lower in all the knockout mice not only before the NTN but also after the NTN induction by FACS but for the CD4+ T cells, one of subtypes of T cells, no significant change was observed except for the IKK2 knockout mice. The percentage of CD3+ T cells was less in the knockout mice after NTN but the percentage of CD4+ subtype T cells were not reduced at 10th day of NTN in the knockout mice, suggesting a CD4+ T cells dominant the NTN induced renal injury, which is consistent with previous observations (Huang et al., 1997; Tipping and Holdsworth, 2006a; Tipping et al., 1998). In addition, the similar renal dysfunctions were detected in all genotypes further confirmed the previous studies that number of T cells infiltration is critical for kidney inflammatory responses (Hooke et al., 1987; Markovic-Lipkovski et al., 1990, 1991).

The different subtypes of CD4+ T cells play different roles on mediating NTN induced kidney inflammation (Azadegan-Dehkordi et al., 2015; Tipping and Holdsworth, 2006a; Turner et al., 2010). Th1 cytokine deficiencies attenuate renal injury (Kitching et al., 1999b; Kitching et al., 2005b) and administration of IL-12 to activate Th1 functions exacerbates renal disease 99

(Kitching et al., 1999d). In our FACS study the Th1 percentage was higher in knockout mice, the higher percentage of Th1 didn't mean the the total number of Th1 was higher. What's more, even the total number of Th1 was higher in the knockout mice at 10th day of NTN we can't sure those Th1 cells have function for the NF-kB active was delete in the CD4+ T cells. Th17 promotes inflammation by the secretion of IL-17 and tumor necrosis factor α (TNF- α) leading to up regulate expression of numerous pro-inflammatory cytokines and chemokines such as CCL2 by local tissue and infiltrating inflammatory cells. IL-17 knockout mice showed attenuated renal pathology and preserved renal function despite an intact Th1 response (Paust et al., 2009). Th17 cells also can induce the expression of CXCL1 for early renal injury and Th1 cellrecipients showed more CCL2 and CCL5 and most likely for the renal injury at later time points (Summers et al., 2009). Previous study show the Th17 is a kind of proinflammatory cytokines but our FACS analysis showed a significant higher percentage of Th17 in the knockout mice at 10th day of NTN. The reason is the same as what we explain about the Th1. However, a potential role for Treg cells to attenuate renal injury has been demonstrated in NTN mice: transfer of naïve Treg cells decreased glomerular T cells and macrophage accumulation and suppressed development of GN, but Treg cells from nephritic mice aggravated disease (Wolf et al., 2005a). In this study the Treg was reduced in knockout mice. Although there was no change of total CD4+ T cells infiltration between different genotypes at 10th day of NTN, the 100

infiltration of different subtypes of CD4+ T cells were distinct: knockout mice exhibited more percentage of Th1 and Th17 infiltration but less percentage of Tregs after NTN induction when comparing with Cre mice. However, knockout mice did not develop more server renal dysfunctions after NTN induction. These could be due to that the infiltration Th cells lacking of NF-KB that modulates their abilities to induce or attenuate inflammatory responses since NF-KB is critical for Th cells functions (Oh and Ghosh, 2013). This hypothesis was supported by examination of multiple proinflammatory chemokines expression, like IL-1b, TNF-α, CCL2, CCL5 and CCL20, all of which did not show significant change after NTN induction in all genotypes. Although an increment of CCL20 was detected in NEMO knockout mice, the decreased CCL2 may complement to their proinflammatory effects. Furthermore, the distinct properties of Th cells infiltration also probably did not contribute to inflammation of renal cells since NF-kB activation in renal cells did not show obvious change by western blotting analysis. Thus, NF-kB in renal cells is critical to mediate inflammatory induced renal injury (Brahler et al., 2012; Sakurai et al., 1996a; Sanz et al., 2010). In our western blotting studies to detect different IkB kinase activity, there was no difference observed between the knockout mice and CD4Cre(control) mice, which supplied the other evidence to explain that no difference was found about the renal function between the knockout mice and the control mice (Cre mice) at 10th day after NTN induction.

Overall, our data suggested that specific deletion of NMEO and/or IKK2 in CD4+ T cells did increase the T cells recruitment into NTN induced inflammatory kidney. However, it is still unclear whether those elevated recruited T cells were functional or not since those T cells did not change the proinflammatory chemokines expression and inflammatory states of renal cells, and eventually had no obvious effects on modulating injury renal functions. Actually, these somewhat incongruent observations are consistent with other reports. For example, IKK2-deficient CD4+ T cells still can activate NF-kB to a reduced but significant extent in response to various stimuli in vitro and show a comparable humoral immune in vivo (Schmidt-Supprian et al., 2003), which is probably due to complementary role of IKK1. However, NEMO knockout CD4+ T cells probably upregulates non-canonical NF-κB pathway to control immune responses (van Delft et al., 2015). Alternatively, NF-KB activation, in some cases, is independent with NEMO functions (Saitoh et al., 2002).

Thus, the molecules mechanisms on T cells, especially CD4+ T cells mediated kidney diseases are not well understood. Microarray based genome profiling was performed by comparing genes expression in CD4+ T cells from spleen of mice with or without NTN induction which would greatly benefit to identify novel candidates that are essential and/or critical for T cells mediated renal inflammatory diseases, providing new targets for clinic treatment of glomerulonephritis.

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