Differential Roles of Interleukin 6 on Immune Responses in Experimental Glomerulonephritis

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Differential Roles of Interleukin 6 on Immune Responses in Experimental Glomerulonephritis

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ADAM17	a disintegrin and metalloproteinase metallopeptidase domain 17
AiaMPOGN	autoimmune anti-myeloperoxidase glomerulonephritis
ANCA	anti-neutrophil cytoplasmic antibody
AP-1	activating protein 1
Aqua dest.	distilled water
ATP	adenosintriphosphat
BATF	basic leucine zipper transcription factor, ATF-like
BSA	bovine serum albumin
BUN	blood urea nitrogen
CD	cluster of differentiation
cDNA	complementary desoxyribonucleic acid
cfu	colony forming unit
cGN	crescentic glomerulonephritis
CO_2	carbon dioxide
cpm	counts per minute
Cre	“causes recombination” recombinase
CT	cycle threshold
d	day(s)
ddH_2O	double-distilled water
DMEM	Dulbecco’s modified Eagle’s medium
DNA	desoxyribonucleic acid
dNTP	desoxiribonucleosid triphosphate
DTT	dithiothreitol
EAG	experimental auoimmune glomerulonephritis
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
fl / flox	flanked by loxP site

FoxP3	forkhead-box-protein P3
Fw /fw	forward
°C	degree Celsius
g	gram(s)
GBM	glomerular basement membrane
gcs	glomerular cross section
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GN	glomerulonephritis
gp130	glycoprotein 130
h	hour(s)
H_2O	water
H_2SO_4	sulfuric acid
HCL	hydrochloric acid
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
hpf	high power field
HRP	horseradish peroxidase
i.p.	intraperitoneal
IFNγ	interferon gamma
IL	interleukin
Ig	immunoglobuline
JAK	Janus kinase
KO	knockout
l	liter(s)
LAG-3	lymphocyte-activation gene 3
LCM	L-929 conditioned medium
LysM	lysozyme M
MACS	magnetic-activated cell sorting
M-CSF	macrophage colony-stimulating factor
MAP	mitogen activated protein
mg	milligram(s)
min	minute(s)
ml	milliliter(s) (liter x 10^{-3})

mM	milimole (mole x 10^{-3})
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
n.s.	not significant
NaCl	sodium chloride
NaH_2PO_4 x H_2O	monosodium phosphate
Na_2HPO_4 x $2H_2O$	disodium hydrogen phosphate
$NaNO_2$	sodium nitrite
NF-κB	nuclear factor'kappa-light-chain-enhancer' of activated B-cells
ng	nanogram(s) (gram x 10^{-9})
NH_4Cl	ammonium chloride
nmol	nanomole (mole x 10^{-9})
NTN	nephrotoxic nephritis
o/n	over night
OD_{450}	optical density / absorbance at 450nm wavelength
OVA	ovalbumin
p	p(robability)-value
P/S	peniciln-streptomycin
PAS	periodic acid-Schiff stain
PBS	phosphate buffered saline
PMA	phorbol 12-myristate 13-acetate
pg	pictogram (gram x 10^{-12})
PCR	polymerase chain reaction
qRT-PCR	quantitative real-time PCR
RNA	ribonucleic acid
ROR	RAR (retinoic acid related)-related orphan receptor
rpm	rounds per minute
RPMI 1640	Roswell Park Memorial Institute medium
RPGN	rapid progressive glomerulonephritis
RT	room temperature
Rv /rev	reverse
s	second(s)
s.c.	subcutaneous

SD	standard deviation
SEM	standard error of the mean
SLE	systemic lupus erythematosus
STAT3	signal transducer and activator of transcription 3
TAE	tris-acetate-EDTA
TCR	T-cell receptor
TGFβ	transforming growth factor beta
Th/ T_H	T-helper (cell)
TMB	3,3',5,5'-Tetramethylbenzidine
TNFα	tumor necrosis factor-alpha
Tr1	type 1 regulatory T-cell
T_{reg}	regulatory T-cell ($CD4^+CD25^+FoxP3^+$)
Tris	Tris(hydroxymethyl)-aminomethane
U	units
µg	microgram (gram x 10^{-6})
µl	microliter(s) (liter x 10^{-6})
µM	micromole (mole x 10^{+6})
V	Volt
WT	wildtype
x g	multiplied gravitation
YFP	yellow fluorescent protein

1 Introduction

1.1 Glomerulonephritis

Glomerulonephritides (GN) are a heterogeneous group of immune-mediated renal diseases, commonly characterized by inflammation of the glomeruli and surrounding area. GN is the third most prevalent cause of terminal renal failure (after diabetic and hypertensive/ ischemic nephropathy) in Europe and the USA. Glomerulonephritis accounts for 10-15% of all patients with a terminal renal failure [1,2]. GN is hallmarked by acute or chronically developing renal failure with hematuria and/or proteinuria.

Different forms of glomerulonephritis are known. They are classified into nephritic or nephrotic forms, which in turn are caused by different disease entities. The most aggressive form of glomerulonephritis is the rapid progressive glomerulonephritis (RPGN or crescentic GN) with a poor prognosis and a rapid progression to failure within days to several weeks. RPGN is classified into three pathophysiologically different subtypes: Type I RPGN, also known as anti-GBM nephritis, is caused by auto-antibodies directed against the glomerular basement membrane (GBM). Deposition of immune complexes is representative for type II RPGN. It is predominantly associated with systemic lupus erythematosus (SLE). The most frequent form of RPGN is type III RPGN. It is hallmarked by damage of the glomeruli in a so called “pauci immune”, neither immune-complex deposition-related nor anti-GBM antibody-related manner. It is thought to be caused by activation of neutrophils in response to anti-neutrophil cytoplasmic antibodies (ANCA). The most common form of type III RPGN is an ANCA-associated vasculitis such as Wegener granulomatosis. Although the above mentioned forms of RPGN are induced by different stimuli, they are collectively characterized by interaction of infiltrating immune cells with glomerular tissue and formation of glomerular crescents [1].

The pathology of most forms of glomerulonephritis remains widely unknown. As a consequence, currently used therapeutic strategies are unspecific and show a

high frequency of severe side-effects. Therefore, the search for new therapeutic targets is a priority for nephrologists and scientists worldwide.

1.2 Role of immune cells in glomerulonephritis

A characteristic hallmark of glomerulonephritis is the immigration of inflammatory cells into renal tissue. Infiltrating T-helper cells (T_H-cells or Th-cells) play a central pathophysiologic role in initiation and progression of the disease [3,4]. Mainly, it can be differentiated between Th1, Th2 and Th17 immune response. Differentiation of naïve T-helper cells into these subgroups is driven by subset specific cytokine environment [5–8].

The Th1 immune response is marked by production of the cytokine interferon-γ (IFN-γ) [9]. In contrast, the Th2 immune response is characterized by generation of immunoglobulin E (IgE) and production of IL-4, IL-5 and IL-13 [10]. The third and less characterized subgroup of Th17 cells is identified by production of the eponymous cytokines IL-17A and IL-17F, as well as the production of the cytokines IL-22 and granulocyte-macrophage colony-stimulating factor (GM-CSF) and recruitment of neutrophil granulocytes [11,12].

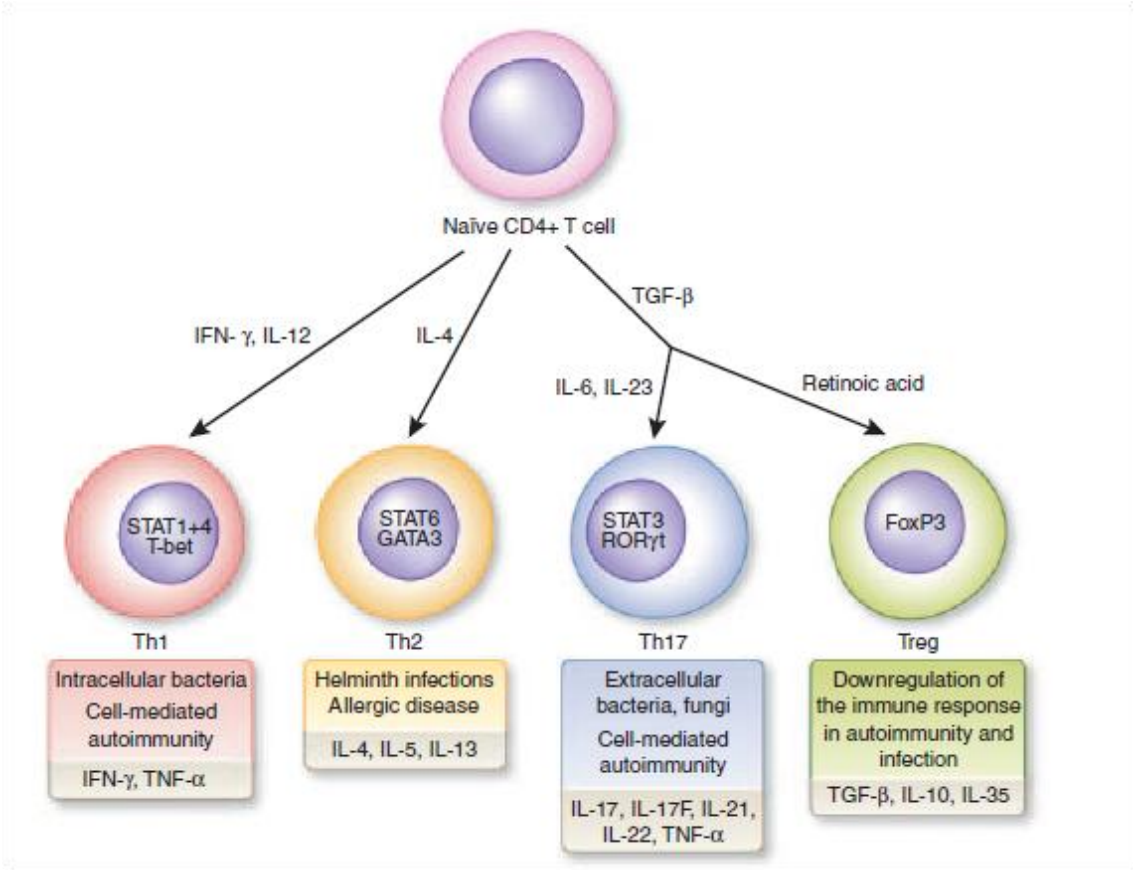

Figure 1.1: Specific cytokine secretion and environment for differentiation of Th1, Th2, Th17 and Treg cells (figure from [13]).
Naïve T cells differentiate into Th1, Th2, Th17 and T_{reg} cells depending on the predominant cytokine environment. Different signaling pathways are activated, which lead to induction of a subset of specific transcription factors. Once activated, these transcription factors in turn mediate the secretion of linage specific cytokines.

Regulatory T-cells (T_{regs}) oppose these pro-inflammatory subtypes of T-helper cells. T_{regs} secrete a bulk of anti-inflammatory cytokines, such as IL-10 and IL-35, which counteract the convalescence of the autoimmune processes [13]. In former studies, the most significant role in pathogenesis of proliferative glomerulonephritis was thought to be played by Th1 cells [14,15]. However, current studies also assume the recently described Th17 immune response to contribute to renal pathogenesis and damage [16–19].

1.3 Th-17 cells

In 2007, a new subset of T helper cells was discovered, distinct from Th1 and Th2 cells, that appears to play a key role in autoimmune diseases [20–22]. These cells are characterized by recruiting neutrophil granulocytes and, basically, the production of interleukin 17A, IL-17F, IL-22 and GM-CSF. Accordingly they were named Th17 cells [11,12].

A couple of key molecules are responsible for differentiation and proliferation of Th17 cells. It has been implicated that interleukin 6 (IL-6) and transforming growth factor beta (TGF-β) induce Th17 cell differentiation from naïve T cells [7,23–25]. These intermediate cells then secrete interleukin 21 (IL-21) which leads to expansion of Th17 cells in an autocrine positive feedback loop. Subsequently, interleukin 23 (IL-23) is essential for stabilization, activation, and terminal differentiation with induction of effector cytokines and trafficking molecules of Th17 cells [26–28]. Induction of the central transcription factors signal transducer and activator of transcription 3 (STAT3), the retinoic-acid-receptor-related orphan receptors alpha (RORα) and gamma (RORγ) and, referring to recent studies, possibly also basic leucine zipper transcription factor ATF-like (BATF) occur already at the beginning of Th17 cell differentiation [29–31]. They are crucial for Th17 formation and mediate expression of characteristic Th17 gene products as well as effector cytokines IL-17A, IL-17F, IL-9, IL-21 and IL-22 as shown in figure 1.2.

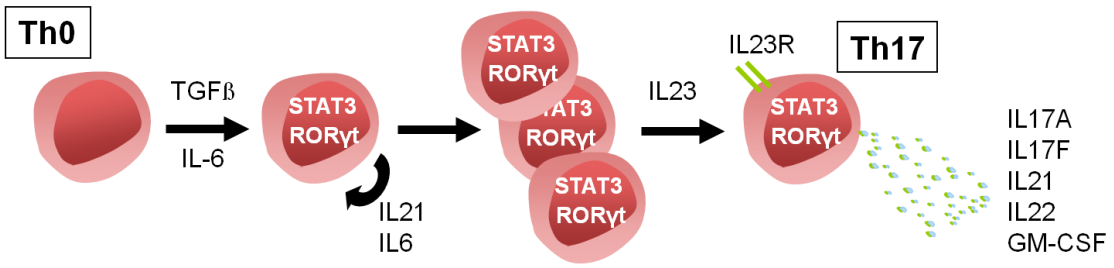

Figure 1.2: Th17 cell differentiation.
Interleukin 6 and transforming growth factor β induce activation of transcription factors STAT3 and RORγt in naïve T-helper cells. Secretion of interleukin 21 leads to expansion in an autocrine positive feedback loop. Interleukin 23 causes stabilization and activation of Th17 cells. Mature Th17 cells are hallmarked by secretion of IL-17A, IL-17F, IL-21, IL-22 and GM-CSF (figure from [32]).

1.4 Relevance of Th17 cells in glomerulonephritis

Recent studies show a functional relevance of Th17 immune response in experimental models of autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, uveitis and colitis [33–35]. However, the role of Th17 immune responses differs in these studies depending on the used model and disease and show partially contrary effects [36,37]. The influence of Th17 immune response on glomerulonephritis has been confirmed by multiple studies including our own. Initial studies by our group could unveil a significant role of IL-23 and IL-17 in the course of glomerulonephritis in a murine model for the first time [16]. The role of IL-23 in the course of glomerulonephritis was independently described by another group in a model of experimental autoimmune glomerulonephritis (EAG) [18]. In the myeloperoxidase specific model of anti-neutrophil cytoplasmic antibodies (ANCA) associated nephritis, the pathogenic character of IL-17A could be underlined [17]. Moreover, antigen specific activated Th17 cells were shown to induce proliferative glomerulonephritis [19]. Subsequent studies emphasize the pathogenic role of IL17 in murine models [38,39] as well as in the course of glomerulonephritis in human patients [40].

1.5 Regulatory T-cells

A pivotal subtype of immune system modulating T cells is a subpopulation of so called regulatory T cells (T_{reg}), formerly known as suppressor T cells (T_S). A hallmark of regulatory T cells is secretion of anti-inflammatory cytokines, such as IL-10, IL-35 and TGF-β, which counteract the convalescence of autoimmune processes [13]. Release of these anti-inflammatory cytokines suppresses development and differentiation of other immune cells, such as T helper cells [41–43]. Thus, T_{regs} are a decisive mediator of immune responses. Due to their suppressing effects on pro-inflammatory immune cells, T_{regs} keep the immune responses in balance and prevent excessive reaction [41,44]. Furthermore, selective manipulation of regulatory T cells in mouse models has suggested that

modulation of T_{regs} can be a helpful tool for treatment of autoimmune diseases, cancer and for the prevention of transplant rejection [45–47]. To date, different forms of regulatory T cells are known. The best characterized ones are those, that express CD4, CD25 and, FoxP3 (FoxP3$^+$ regulatory T cells, or T_{regs}) [48,49]. Nevertheless, the molecular mechanisms that underlie the suppressing or regulatory activity of T_{regs} are not fully known yet.

1.5.1 FoxP3$^+$ Regulatory T-cells

The best studied subtype of regulatory T cells are the FoxP3 (forkhead box protein 3) positive T_{regs} [48,50]. They are essential key players for self-tolerance and homeostasis of the immune system by suppressing harmful excessive or aberrant immune responses [51,52]. Recent studies suggest that there are different populations of T_{regs} that are specialized for neutralization of Th1, Th2 or Th17 effector cell responses [41,53–55]. Generation of these specific regulatory T cells seems to underlie mechanisms, quite similar to those that lead to generation of T effector cells [54,56–59]. Recently, it has been shown that deletion of the Th17 specific transcription factor STAT3 in regulatory T cells leads to an excess of Th17 immune response and generation of a severe colitis [41]. Figure 1.3 shows a possible model of generation and differentiation of different regulatory T cell subtypes.

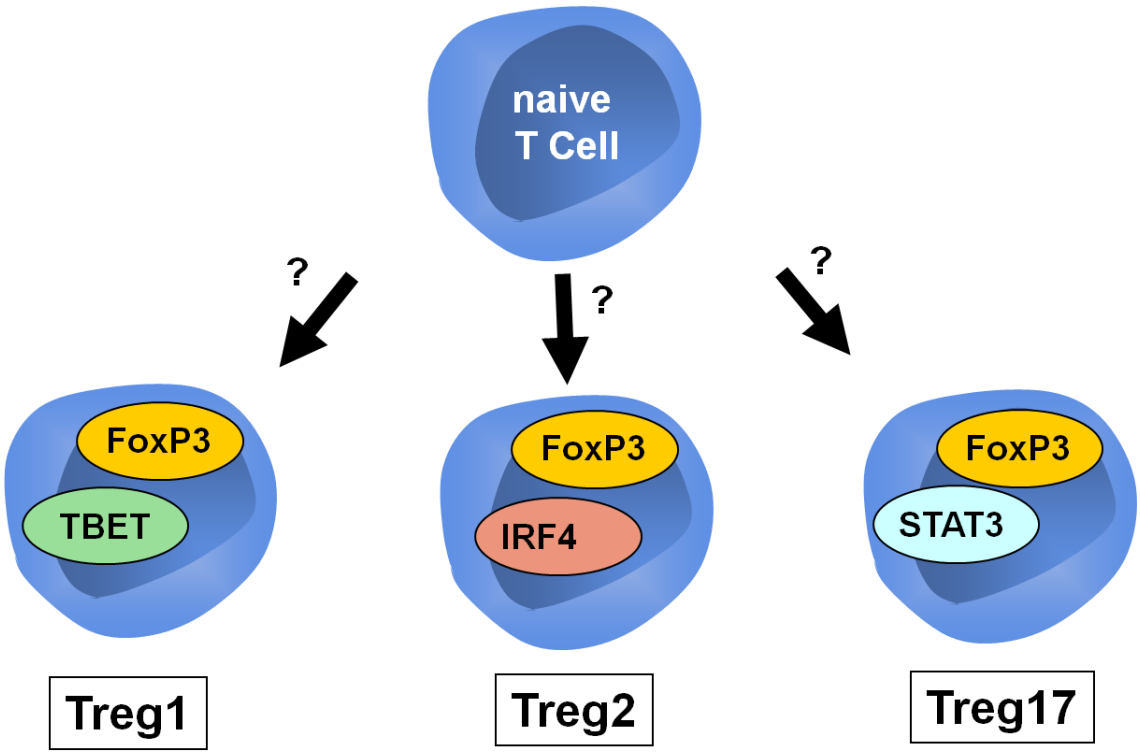

Figure 1.3: Model for generation of different T_{reg} subsets.
The transcription factor FoxP3 is crucial for the differentiation of naïve T cells into different subsets of regulatory T cells. The fate into which subpopulations of T_{regs} each cell differentiates is dependent on other transcription factors, which are specific for related effector cells. STAT3 e.g. which is in charge of differentiation into T_H17 cells, is also responsible for differentiation into $T_{reg}17$ cells. The mechanism for induction of FoxP3, and hence the control of differentiation into regulatory T cells is not fully understood (figure from [32]).

1.5.2 Tr1 cells

Tr1 cells are another subtype of regulatory T cells. To date, these cells are not well characterized and very difficult to detect. Just like $FoxP3^+$ regulatory T-cells, Tr1 cells seem to have a major impact on regulation of immune responses [60]. They are hallmarked by secretion of IL-10, IFN-γ, TGF-β, and IL-5, while secretion levels of IL-2 and IL-4 are low [61–65]. By this representative cytokine pattern, Tr1 cells can be differentiated from polarized and unpolarized T cells. Following activation, proliferation of Tr1 is limited due to the autocrine secretion of the suppressively acting IL-10, which is a major reason for the hitherto poor characterization of Tr1 cells [66]. Recent studies suggest that Tr1 cells are characterized by their coexpression of CD49b and the lymphocyte activation gene 3 (LAG-3) [67].

However, Tr1 cells have been shown to suppress both type1 and type2 immune responses by secretion of TGF-β and/or IL-10. In addition, Tr1 cells inhibit antibody production of B cells and antigen presentation of monocytes and dendritic cells [62,68–73]. Recently, it has been shown that IL-6 induces the differentiation of Tr1 cells [74].

1.6 Interleukin 6

Interleukin 6 (IL-6), formerly known as interferon-β2 (IFNB2) is a pleiotropic cytokine which is involved in the regulation of cell growth, proliferation, differentiation, survival, and gene activation [75–84]. Thereby, it plays an important role in a broad range of cellular and physiological processes like immune response, inflammation, hematopoiesis, and oncogenesis [83].

IL-6 was originally described as B-cell differentiation factor (BSF-2), that is responsible for the final maturation of B-cells into antibody producing cells [85,86]. However, studies using recombinant IL-6 or anti-IL-6 antibodies have also revealed effects on T-cells, hepatocytes, hematopoietic progenitor cells and even neuronal cells [75,77,84]. IL-6 has also been shown to act as a potent growth factor for human myeloma and plasmacytoma cells [87,88]

IL-6 is secreted by a variety of cells, among others by T-cells and macrophages [22,76,77,82,89,90]. IL-6 is involved in the induction of acute phase proteins and in the control of neutrophil and monocyte responses [91,92]. It also plays an important role in the control of monocytes and neutrophils. Furthermore, IL-6 is involved in lymphocyte survival and in the support of B cell growths and antibody production [90,93,94]. As previously described, IL-6 is also crucial for differentiation of naïve T cells into Th17 cells [23,24].

In 1993 and 1997 IL-6 was shown to act anti-inflammatory in two different rat models of glomerulonephritis [95,96]. However, the multifunctional role of IL-6 and its impact on the course of the disease in glomerulonephritis is poorly understood.

1.6.1 IL-6 Signaling

Signaling of IL-6 can be proceeded via two variable pathways. In the classical IL-6 signaling pathway, IL-6 binds to the interleukin 6 receptor (IL-6R). IL-6R is a membrane protein that is expressed on hepatocytes and some leukocytes subsets only. It consists of two different subunits: the IL-6Rα chain (also called CD126) to which IL-6 binds directly, and the signal-transducing glycoprotein gp130 (also called CD130) [97].

The alternative IL-6 signaling pathway is the so called "IL-6-*trans*-signaling". Here, IL-6 binds to a soluble IL-6 receptor (sIL-6R). After binding of IL6 to sIL-6R, the resulting IL-6/sIL-6R complex binds to gp130, which is expressed on the surface of most cell types. To counteract an unspecific IL-6-*trans*-signaling, a soluble gp130 form (sgp130) binds to and thereby inactivates the IL-6/sIL-6R complex.

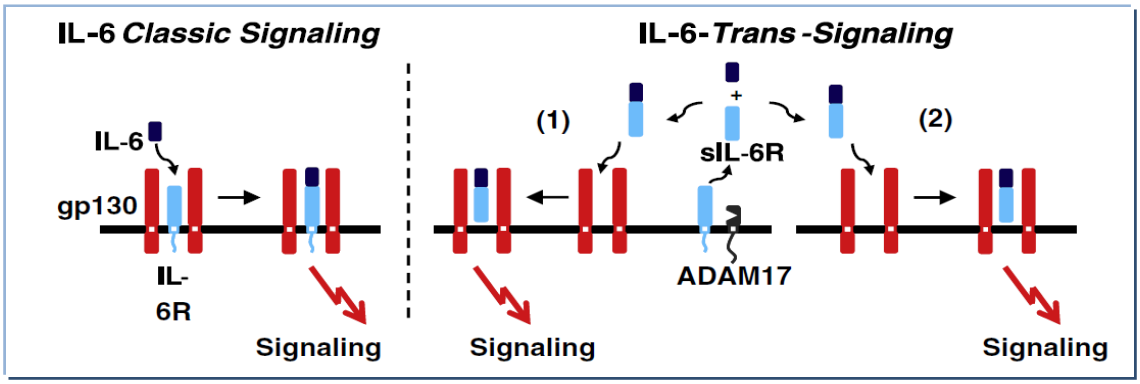

Figure 1.4: Classical and trans-signaling pathway of IL-6.
Cells that express both gp130 and the IL-6R are responsive to the IL-6 *classical-signaling*. However, cells that express only gp130 can be activated by the IL-6/sIL-6R complex. Activation of cells by the IL-6/sIL-6R complex is termed *trans-signaling*. The sIL-6R is generated by proteolytic cleavage of the membrane bound precursor. The sIL-6R binds IL-6 with comparable affinity as the membrane bound form and mediates gp130 activation in an (1) autocrine or (2) paracrine manner [94].

Different cellular events are initiated following IL-6 binding to its receptor, including activation of Janus Kinase (JAK) kinases and Ras-mediated signaling. Activation and phosphorylation of JAK kinases lead to activation of transcription factors, such as STAT3 (Signal Transducers and Activators of Transcription-3) and to the activation of the tyrosine-specific protein phosphatase SHP2 (SH2

(Src Homology-2) Domain-containing Tyrosine Phosphatase) [98,99]. Phosphorylation of STAT3 results in its dimerization and subsequent translocation into the nucleus and activation of STAT3 dependent gene transcription. SHP2 links cytokine receptor activation to Ras/MAP (Mitogen-Activated Protein) kinase pathways. [100].

The Ras-mediated pathway activates MAP kinases resulting in activation of the transcription factors Elk1 and NF-IL-6 (C/EBP-Beta). These and other transcription factors, such as Activating Protein-1 (AP-1) and Serum Response Factor (SRF), regulate different promoters and enhancers that respond to IL-6 and other signaling factors [101].

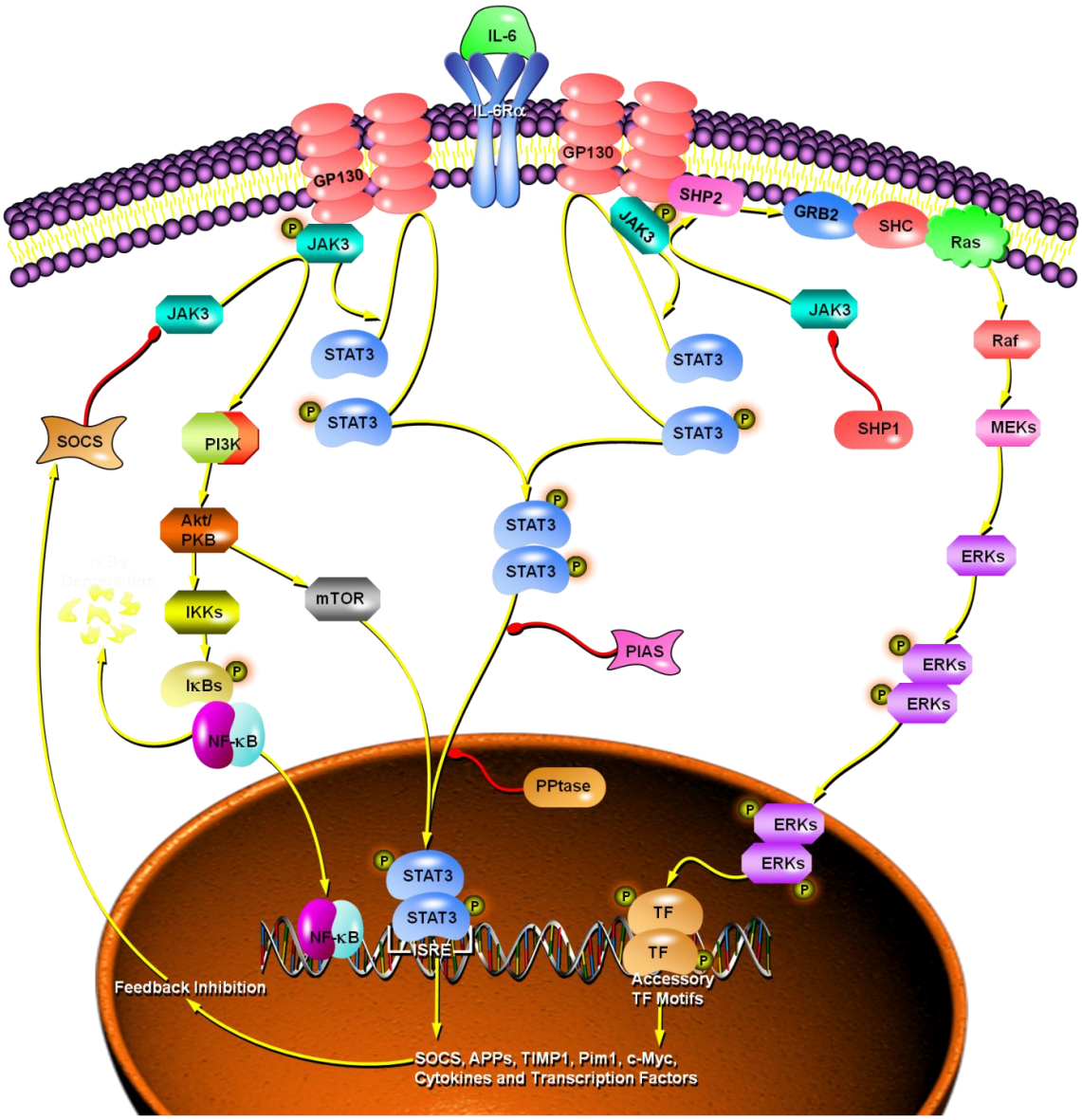

Figure 1.5: Intracellular signaling of IL-6.
Signaling of IL-6 is predominantly mediated by activation of JAK3, which in turn leads to phosphorylation of transcription factors, such as NFκB and STAT3. Phosphorylation and dimerization of STAT3 results in translocation of STAT3 into the nucleus and transcription of IL-6 dependent genes. [source: SABioscience[102]]

1.6.2 Pro-inflammatory functions of IL-6

IL-6 is a multifunctional cytokine which signals not only via two different pathways but also shows variable effects on different cell types. Therefore, it can either act in a pro-inflammatory or in an anti-inflammatory way.

IL-6 together with TGFβ is crucial for differentiation of CD4 T-cell precursors into pro-inflammatory Th17 cells [8,22,23,103,104]. Thus, IL-6 has been shown

to act both protective and exacerbating in different autoimmune diseases such as diabetes, atherosclerosis, systemic lupus erythematosus, behcet's disease, and rheumatoid arthritis [105–109]. In addition, levels of IL-6 expression are increased in many patients with mesangial proliferative glomerulonephritis [110], and also in rodents with experimental analogues [111]. Therefore, expression of IL-6 is also suggested to be a good marker for glomerular cell proliferation and it is assumed that IL-6 is involved in crescent formation and tubulointerstitial injury [89].

1.6.3 Anti-inflammatory functions of IL-6

It is known, that IL-6 has inhibitory effects on macrophages, by inducing the expression of anti-inflammatory cytokines such as sTNFR and IL-1ra [112–114]. These cytokines in turn down-regulate the production of pro-inflammatory cytokines like TNFα and IL-1 [113,115–117], which finally results in the inhibition of macrophages. In addition, Riedy and Stewart showed that IL-6 can also directly inhibit the proliferation of macrophages *in vitro* [118]. Due to the inhibitory effects on tumor necrosis factor alpha (TNFα) and interleukin 1 (IL-1) and the capacity to promote the secretion of interleukin 1 receptor antagonist (IL-1ra) and IL-10, IL-6 also functions as an anti-inflammatory cytokine [112,114,119]. In a rat model of nephrotoxic nephritis (NTN), IL-6 has been shown to have significant anti-inflammatory properties [96]. In addition, IL-6 has anti-inflammatory effects in rat anti GBM-nephritis [120]. Recent studies also describe IL-6 as a crucial inducer of anti-inflammatory Tr1-cell generation [74].

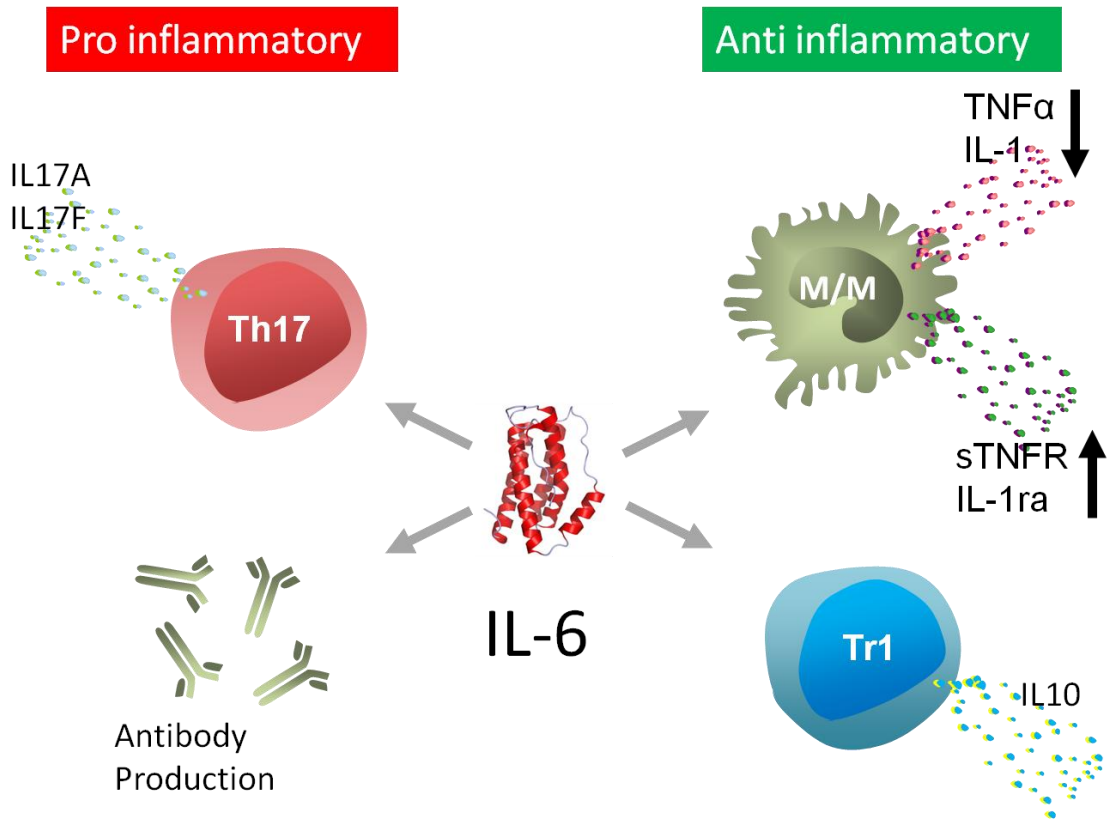

Figure 1.6: Differential roles of IL-6.
IL-6 functions in a pro-inflammatory manner by its importance in differentiation and proliferation of pro-inflammatory Th17 cells and their secretion of pro-inflammatory cytokines IL-17A and IL-17F. In addition, IL-6 supports the growth of B-cells and their antibody production. IL-6 also affects the decrease of cytokine secretion of TNFα and IL-1 and the increase of anti-inflammatory proteins sTNFR and IL-1ra by monocytes / macrophages. In addition, it also induces the generation of Tr1 cells.

1.6.4 IL-6 in renal inflammatory disease

As IL-6 is known to be a mediator for different immune responses, there have always been suggestions, that IL-6 might play a role in different forms of glomerulonephritis. The first investigation concerning the role of IL-6 in RPGN was the inhibition of IL-6R by a monoclonal antibody in a murine NZW x NZB lupus nephritis model, which revealed the amelioration of the disease [121]. These findings were confirmed by antibody mediated inhibition of IL-6 in a murine model of MRL-lpr SLE [122]. In addition, studies administrating recombinant human IL-6 in lupus mice reveal an aggravating effect on the course of disease [123], which verifies the therapeutic effect of IL-6 inhibition once again. Recent investigations in IL-6 deficient lupus mice also showed significant ameliorating effects on the course of SLE [124]. In contrast,

administration of IL-6 in a murine model of NTN has been shown to act protectively [95,96]. Despite the well characterized functions of IL-6 in SLE, its role in cGN is only poorly understood.

1.6.5 Clinical implications of IL-6 – tocilizumab

Since IL-6 has been shown to mediate immune response in different diseases in pro- or anti-inflammatory way, respectively, there is an interest in developing anti-IL-6 agents [125,126]. The first anti-IL-6 agent, that has been approved for rheumatoid arthritis so far is tocilizumab (RoActemra®/Actemra®, Roche), a monoclonal antibody which specifically binds to IL-6R, thereby blocking IL-6 signaling [127]. Treatment with tocilizumab has been shown to ameliorate clinical symptoms and histological damage in rheumatoid arthritis [128,129]; however, its effects on renal disease have not been studied so far.
Despite the well described properties of IL-6, its impact on the Th-17 immune responses in glomerulonephritis is only partially understood.

1.7 Aim of the study

Glomerulonephritides are a group of important autoimmune diseases which often result in terminal renal insufficiency. Th17 responses were shown to exacerbate the progression of glomerulonephritis. However, the role of IL-6 driven maturation of Th17 cells on the progression of glomerulonephritis is still elusive to date.
The pleiotropic cytokine IL-6 is a rewarding subject to study as IL-6 directed therapies have recently been introduced into clinical practice. To this end, tocilizumab, a monoclonal antibody directed against the IL-6 receptor, has been approved for rheumatoid arthritis and shows good progress in curing symptoms and disorders. The role of tocilizumab in renal disease has not been addressed so far.

In this study, it was attempted to unveil the influence of IL-6 signaling on Th17 immune responses in experimental glomerulonephritis. To elucidate the role of IL-6, two different experimental mouse-models for rapid progressive glomerulonephritis were used. In addition it was investigated whether the effect of IL-6 in experimental glomerulonephritis is mediated by the classical or the *trans*-signaling pathway.

2 Materials and Methods

2.1 Materials

2.1.1 Equipment

CO_2-Incubators	Haereus, Germany
TissueLyser	Qiagen, Germany
Balance	Ohaus, Germany
LSR II Flow Cytometer	BD Biosciences, Germany
Centrifuges	Haereus, Germany
Thermo Cycler	BioMetra, Germany
Real Time Cycler	Applied Biosystems, USA
Power Supply	Pharmacia Biotech, USA
Gel Electrophoresis System	PeqLab Biotechnologies, Germany
ELISA Reader MRX	Dynatech Laboratories, USA
NanoDrop Spectrophotometer	PeqLab Biotechnologies, Germany

2.1.2 Chemicals

2-propanol	Carl Roth, Germany
β-Mercaptoethanol	Life Technologies, USA
Acetic acid	Carl Roth, Germany
Agarose	Life Technologies, USA
Aqua ad iniectabilia	Braun Melsungen, Germany
Brefeldin A	Sigma Aldrich, USA
Bovine Serum Albumin (BSA)	Sigma Aldrich, USA
Carbonate/Bicarbonate	Merck, Germany
Cell Wash	BD Biosciences, Germany
Cell Cytofix/Cytoperm	BD Biosciences, Germany
Collagenase	Roche, Switzerland
DMEM	Life Technologies, USA
DNase I	Roche, Switzerland

Dithiothreitol (DTT)	Sigma Aldrich, USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, USA
Ethanol absolute	JT Baker, The Netherlands
Ethanol denatured	Walter, Germany
Ethidiumbromide	BioRad, Germany
FACS Clean	BD Biosciences, Germany
FACS Flow	BD Biosciences, Germany
FACS Rinse	BD Biosciences, Germany
Fetal Bovine Serum (FBS)	Life Technologies, USA
Formalin	Merck, Germany
Hank's Balanced Salt Solution (HBSS)	Life Technologies, USA
HEPES	Life Technologies, USA
Hydrochloric acid	Merck, Germany
Ionomycin	Merck, Germany
Isoflurane	Abbott, USA
L-Glutamine	PAA, Germany
Penicillin/Streptomycin	Life Technologies, USA
Periodic acid	Merck, Germany
Perm Wash	BD Biosciences, Germany
Phorbol 12-myristate 13-acetate (PMA)	Sigma Aldrich, USA
Phosphate Buffered Saline (PBS)	Lonza, Switzerland
Protease 24	Sigma Aldrich, USA
RPMI 1640	Life Technologies, USA
Sodium chloride	J.T. Baker, The Netherlands
Sodium phosphate, dibasic	Merck, Germany
Sodium phosphate, monobasic	Merck, Germany
Tris	Sigma Aldrich, USA
Trypan blue 0.5%	Biochrom, Germany
Trypsin	Sigma Aldrich, USA
Tween-20	Carl Roth, Germany
X-VIVO™ 10	Lonza, Switzerland
Xylene	Merck, Germany

2.1.3 Kits

Name	Supplier
XNAT2 Extract-N-Amp Tissue PCR Kit	Sigma Aldrich, USA
Griess Reagent System	Promega, USA
Mouse IL-2 ELISA MAX™ Standard	BioLegend, USA
Mouse IL-4 ELISA MAX™ Standard	BioLegend, USA
Mouse IL-6 ELISA MAX™ Standard	BioLegend, USA
Mouse IL-10 ELISA MAX™ Standard	BioLegend, USA
Mouse IL-17A ELISA MAX™ Standard	BioLegend, USA
Mouse GM-CSF ELISA MAX™ Standard	BioLegend, USA
Mouse IFNγ ELISA MAX™ Standard	BioLegend, USA
Mouse TNFα ELISA MAX™ Standard	BioLegend, USA
Mouse IL-1ra/IL-1F3 Quantikine ELISA Kit	R&D Systems, USA
Mouse IL-17F DuoSet	R&D Systems, USA
CD4 (L3T4) MicroBeads, mouse	Miltenyi Biotech, Germany
$CD4^+$ T Cell Isolation Kit II, mouse	Miltenyi Biotech, Germany
$CD4^+CD25^+$ Regulatory T Cell Isolation Kit, mouse	Miltenyi Biotech, Germany

Table 2.1: Used kits

2.1.4 Solutions and buffers

2.1.4.1 Buffers for FACS analysis

<u>erythrocyte lysis buffer</u>

Solution A: 170 mM Tris/ HCl, pH 7.6

Solution B: 160 mM Ammonium chloride

Mix Solution A and B at the ration of 1:9

<u>MACS buffer</u>

PBS

0.5% BSA

2 mmol EDTA

2.1.4.2 Buffers for electrophoresis of nucleic acids

1x TAE buffer

40 mM Tris

20 mM Acetic acid

1 mM EDTA

A.dest.

2.1.4.3 Buffers for Immunohistochemistry

Sorensen's Phosphate Buffer

0.2 M $Na_2HPO_4xH_2O$

0.2 M $NaH_2PO_4xH_2O$

Ad 1000 ml A.dest.

pH 7.2-7.4

2.1.4.4 Cell Culture Medium

Medium for cultivation of primary splenocytes

RPMI-1640

1% HEPES

1% penicillin/ streptomycin

10% ml FBS

2.1.5 Primers

2.1.5.1 Primers for genotyping

Name	Sequence	Annealing temperature
FoxP 3 wt forward	*TGGACCGTAGATGAATTTGAGTT*	64°C
FoxP 3 wt reverse	*CCAGATGTTGTGGGTGAGTG*	64°C
FoxP 3 Cre forward	*AGGATGTGAGGGACTACCTCCTGTA*	60°C
FoxP 3 Cre reverse	*TCCTTCACTCTGATTCTGGCAATTT*	60°C
CD4 Cre forward	*CCTGGAAAATGCTTCTGTCCGTTTG*	56°C
CD4 Cre reverse	*ACGAACCTGGTCGAAATCAGTGCG*	56°C

IL-6R flox forward	*GAAGGAGGAGCTTGACCTTGG*	64°C
IL-6R flox reverse	*AACCATGCCTATCATCCTTTGG*	64°C
LysM Cre wt	*CCCAGAAATGCCAGATTACG*	62°C
LysM wt	*CTTGGGCTGCCAGAATTTCTC*	62°C
LysM common	*TTACAGTCGGCCAGGCTGAC*	62°C
IL-10 flox forward	*CCAGCATAGAGAGCTTGCATTACA*	60°C
IL-10 flox reverse	*GAGTCGGTTAGCAGTATGTTGTCCAG*	60°C

Table 2.2: Primers for genotyping

2.1.6 Antibodies

2.1.6.1 Antibodies for FACS analysis

Name	clone	dilution	source
rabbit anti mouse CD3e	500A2	1:100	BD Horizon, USA
rat anti mouse CD 4	GK1.5	1:100	BD Pharmingen, USA
rat anti mouse CD8a	53-6.7	1:100	eBioscience, USA
rat anti mouse CD11b	M1/70	1:100	BD Pharmingen, USA
hamster anti mouse CD11c	HL3	1:100	BD Horizon, USA
rat anti mouse CD19	1D3	1:100	BD Pharmingen, USA
rat anti mouse CD44	IM7	1:100	BD Pharmingen, USA
rat anti mouse CD45	30-F11	1:100	BD Pharmingen, USA
mouse anti mouse CD45.1	A20	1:100	eBioscience, USA
mouse anti mouse CD45.2	104	1:100	eBioscience, USA
hamster anti mouse CD49b	HMa2	1:100	BioLegend, USA
rat anti mouse CD62L	MEL-14	1:100	BioLegend, USA
hamster anti mouse CD69	H1.2F3	1:100	BioLegend, USA
rat anti mouse CD206	C068C2	1:100	BioLegend, USA
rat anti mouse F4/80	BM8	1:100	BioLegend, USA
rat anti mouse/rat FoxP3	FJK-16s	1:125	eBioscience, USA
rat anti mouse GM-CSF	Mp1-22E9	1:100	eBioscience, USA
rat anti mouse Gr1(Ly6C/Ly6G)	RB6-8C5	1:100	BioLegend, USA
rat anti mouse IFNγ	XMG1.2	1:100	eBioscience, USA
rat anti mouse IL-10	JES5-16E3	1:100	eBioscience, USA
rat anti mouse IL-17A	TC11-18H10	1:100	BD Horizon, USA

mouse anti human Ki67	B56	1:100	BD Horizon, USA
rat anti mouse LAG-3	C9B7W	1:100	BioLegend, USA
rat anti mouse Ly6C	AL-21	1:100	BD Pharmingen, USA
rat anti mouse Ly6G	1A8	1:100	BD Pharmingen, USA
rat anti mouse MHC II	I-A/I-E	1:100	eBioscience, USA
mouse anti mouse NK1.1	PK36	1:100	eBioscience, USA
mouse anti mouse RORγt	Q31-378	1:300	BD Pharmingen, USA
rat anti mouse TNFα	MP6-XT22	1:100	BD Pharmingen, USA
hamster anti mouse γδ	GL3	1:100	BioLegend, USA

Table 2.3: Antibodies for FACS analysis

2.1.6.2 Antibodies for immunohistochemistry

name	clone	source
rabbit Anti-human CD3	A0452	Dako, Germany
rat anti-mouse F4/80	BM8	BMA Biomedicals, Germany
anti-mouse/human MAC2	M3/38	Cedarlane-Laboratories, Canada
anti-mouse/rat FoxP3	FJK-16s	eBioscience, Germany
rabbit anti-mouse/rat IgG	E0468	Dako, Germany
rat anti-mouse Gr1	NIMP-R14	Hycult, The Netherlands

Table 2.4: Antibodies for immunohistochemistry

2.1.7 Mouse strains

name	source
C57BL/6_2010	*UKE VTH*
IL-6R$^{flox/flox}$	Jackson Laboratories, Maine, USA
IL-6$^{(-/-)}$	G. Tiegs, Hamburg, Germany
LysM^{Cre}	Jackson Laboratories, Maine, USA
FoxP3^{Cre} YFP	A. Y. Rudensky, New York, USA
CD4^{Cre}	C. Wilson, Washington, USA

Table 2.5: Mouse strains

2.2 Methods

2.2.1 Animal experiments

loxP-site flanked IL6R$^{flox/flox}$ mice (initially derived from Jackson Laboratories, Maine, USA) were a kind gift of Prof. Jürgen Scheller, University Hospital, Düsseldorf, Germany. Macrophage specific deletion of IL-6R was achieved by crossbreeding with LysM^{Cre} mice (initially derived from Jackson Laboratories, Maine, USA), which were a generous gift from Prof. Giesa Tiegs, UKE, Hamburg, Germany. Th-cell specific deletion of IL-6R was achieved by crossbreeding with mice expressing the Cre recombinase under the control of the CD4 locus, which were a kind gift from Dr. Christopher B. Wilson, University of Washington, Seattle, USA. Efficiency and specificity of the IL-6R deletion was assessed by PCR (see Table 2.3 for detailed list).
All experiments were performed according to national and institutional animal care and ethical guidelines and were approved by local ethical committees (approval numbers 63/10, 37/11 and Org441). Mice were raised under specific pathogen free conditions at the Central Animal Facility of the University Hospital Eppendorf, Hamburg, Germany.

2.2.1.1 NTN model

2.2.1.1.1 Generation of NTN serum

Generation of NTN serum was done as described previously by Assmann et al. [130] and shown schematically on figure 2.1. In brief, glomerular basement membranes from mice were dissected and purified. Sheep (Eurogentec, Belgium) were immunized with this antigen. Blood from sheep was collected and sheep anti-mouse GBM antibodies were precipitated and dialyzed.

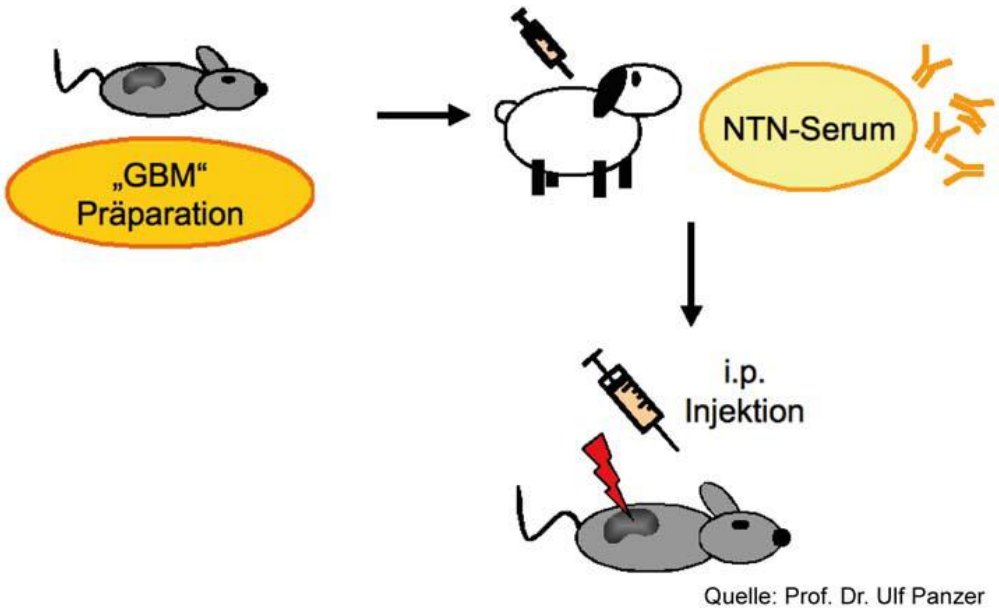

Figure 2.1: Generation of NTN serum.
GBM from mice were dissected and purified. Sheep immunized with this antigen produce anti-mouse GBM antibodies, which are purified from blood.

2.2.1.1.2 Induction of NTN

NTN was induced by intraperitoneal (i.p.) application of 0.25 – 0.7 ml (depending on serum specificity) NTN-serum at day 0 and mice were sacrificed at day 10. For the accelerated NTN model, sheep IgG (dissolved in PBS) was diluted 1:1 with complete Freund's adjuvant and homogenized via sonication. Mice were pre-immunized subcutaneously (s.c.) with 0.5 mg sheep IgG per 20 g bodyweight at day -4. NTN was induced as described previously.

2.2.1.2 MPO model

Recombinant mouse Myeloperoxidase (MPO) and mouse MPO (from differentiated 32Dcl3 cells) were generated as described previously by Apostolopoulos et al. [131]. Ovalbumin (OVA) was purchased from Sigma-Aldrich. 8-10 week old male C57BL/6 mice (obtained from the Jackson Laboratory) were injected i.p. with 10 µg MPO dissolved in PBS and mixed 1:1 with Freud's complete adjuvance on day -14 and Freud's incomplete adjuvant

on day -7, respectively. 200 µl of PBS containing 500 µg of α-IL-6R (Chugai Pharma) or rat IgG (Sigma-Aldrich) as control were administrated intraperitoneally at day 0 or at day -15 and -7, respectively. Induction of glomerulonephritis was achieved by intravenously application of a subnephritogenic dose of NTN on day 0. Mice were sacrificed on day 4.

2.2.1.3 Metabolism cages

Mice were placed into metabolic cages on day 2 and 9 after induction of glomerulonephritis or day 3 after induction of AiaMPOGN. After 4-6 hours or 24 hours in AiaMPOGN, respectively, urine was collected and stored at -20 °C until further analysis (see 2.2.6).

2.2.2 Cell Culture Techniques

2.2.2.1 Preparation of L929-conditioned medium

L929 cells (ATCC CCL-1) were plated at a density of 1.2 x 10^6 cells on a 15-cm cell culture dish containing 20 ml of DMEM supplemented with 10% FBS, L-Glutamine and P/S. Cells were cultivated in a humidified incubator with 5% CO_2 at 37 °C for 14 days. Supernatants were collected, centrifuged at 1500 rpm for 10 min and filtered through a 0.45 µm filter to remove cell debris. L-929 conditioned medium (LCM) was stored at -20 °C.

2.2.2.2 Isolation of macrophages from mice

Mice were injected i.p. with 1 ml thioglycolate. To isolate macrophages peritoneal cavity was washed with 5 ml ice cold PBS at day 4. Peritoneal macrophages were washed twice with ice cold PBS and resuspended in RPMI 1640 with L-glutamine containing 1% penicillin-streptomycin, 1% HEPES, 20% FCS and 20% LCM.

2.2.2.3 Macrophage proliferation assay

Peritoneal macrophages were isolated as described above. 3 ml of macrophage culture were plated at a concentration of 3.3 x 10^2 cells/ml on a 6-well plate. Cells were cultivated in a humidified incubator with 5% CO_2 at 37 °C. Colony formation in presence or absence of 100 U/ml IL-6 was monitored for 14 days. Analysis was performed by counting colony forming units (cfu). Cfus were determined as a group of cells with cell-cell contacts or at least a distance of less than 1x cell width and a total number of cells of n>3.

2.2.2.4 Splenocyte stimulation assay

96-well flat-bottomed plates (BD Falcon) were coated with anti-CD3 (1 µg/ml) and incubated over night (o/n) at 4 °C. Splenocytes from FoxP3 Cre^+ YFP mice were prepared as described in 2.2.4.3. $CD4^+$ T-cells were separated with "$CD4^+$ T cell isolation kit II" (Miltenyi Biotec, Germany) by magnetic-activated cell sorting (MACS) according to the manufacturer's guidelines. Cells were stained with mice specific anti-CD45 and anti-CD4 antibodies, conjugated with PerCP or APC-H7, respectively, and sorted by fluorescence activated cell sorting (FACS) for YFP-positive or YFP-negative $CD4^+$ cells. Sorted cells were resuspended in RPMI-1640 with L-glutamine, supplemented with 1% penicillin-streptomycin, 1% HEPES, 10% FCS. Cells were plated at a density of 5 x 10^5 cells/ml on a 96-well flat-bottomed plate and stimulated with soluble anti-CD3 (1 µg/ml) and anti-CD28 (1 µg/ml). Cells were cultured in the presence or absence of IL-6 (20 ng/ml) for 3 days. Supernatants were pooled and stored at -20°C until further analysis. Cells were harvested and stained as described in 2.2.4.4 for FACS analysis.

2.2.3 Methods in molecular biology

2.2.3.1 Isolation of genomic DNA from mouse tail

Genomic DNA from mouse tails was isolated using the XNAT2 Extract-N-Amp Tissue PCR Kit (Sigma Aldrich, USA) according to the manufacturer's instructions. DNA was stored at 4 °C for short-term storage.

2.2.3.2 Genotyping of mouse by PCR

2 µl 10 x Dream Taq buffer (Fermentas, USA)
0.4 µl dNTPs (Fermentas, USA)
1 µl Primer forward
1 µl Primer reverse
0.2 µl Dream Taq Polymerase (Fermentas, USA)
12.4 µl ddH2O

PCR reaction mix was prepared as described above. Primers for genotyping are listed in table 2.3. Three µg of template genomic mouse DNA was added and gently mixed. The reaction was placed in a thermal cycler and PCR was performed using the cycling condition described below.

Cycling conditions

95 °C	3 min	
95 °C	45 s	35 x
50-60 °C	45 s	
72 °C	45 s	
72 °C	2 min	
4 °C	∞	

2.2.3.3 Electrophoresis of DNA

1-2 % agarose gels were cast using the BioRad SubCell® gel casting system. Samples were loaded onto the gel and electrophoresis was usually performed at 80-120 V for 45-60 min. DNA was visualized by staining the gel with ethidium bromide solution and analyzed under UV light using alphamanager® mini gel documentation (ProteinSimple, San Jose, USA).

2.2.4 Methods in immunology

2.2.4.1 Preparation of single cell cultures from mouse kidney

Kidneys were extracted from mice and cut in small pieces. 5 ml of digestion medium was added before treatment with 10 µl DNase I and 20 µl Collagenase. After an incubation time of 40 min at 37 °C, kidneys were broke up using a 70 µM cell strainer (BD Biosciences, Germany). Cell strainers were rinsed extensively with HBSS. Cell suspension was then centrifuged at 300x g for 10 min at 4 °C. Supernatant was discarded and cells were resuspended in 2 ml erythrocyte lysis buffer. After 5-7 minutes, reaction was stopped by adding HBSS. Cells were centrifuged at 300x g for 10 min at 4 °C and cell pellet was resuspended in 10 ml HBSS on ice. To prepare a single cell suspension, cells were then separated by using a 40 µM cell strainer (BD Biosciences, Germany). Again, cell strainers were extensively washed with HBSS and cells were again centrifuged at 300x g for 10 min at 4 °C. Pellets were resuspended in 2 ml HBSS and transferred to BD FACS tubes.

2.2.4.2 Stimulation of kidney cells with PMA/Ionomycin

Mouse kidney cells were prepared as described in 2.2.4.1. Cells were centrifuged at 300x g for 10 min at 4 °C and resuspended in 1 ml X-VIVO™ 10 supplemented with ß-Mercaptoethanol with or without PMA (0.05 µg/ml)/ Ionomycin (1 µg/ml). After 30 minutes of incubation cells were vortexed and 2

µl/ml of Brefeldin A solution (10 µg/ml) was added. Cells were then incubated for 4 h in a CO_2 incubator.

2.2.4.3 Preparation of single cell cultures from mouse spleen

Spleens were extracted from mice and put on ice. HBSS was added and spleens were homogenized by using a 70 µM cell strainer (BD Biosciences, Germany). Cells were centrifuged at 300x g for 10 min at 4 °C and supernatant was discarded. Erythrocytes were lysed by incubation of the pellet with 1 ml erythrocytes lysis buffer for 10 min at room temperature. Lysis was stopped by adding HBSS. After centrifugation, cell pellet was resuspended in 10 ml HBSS and filtered using a 40 µM cell strainer (BD Biosciences, Germany). Cells were centrifuged again and cell pellet was resuspended in 5 ml RPMI-1640 for primary splenocytes. Cell number was counted and $4x10^6$ cells were transferred to a new falcon tube. After centrifugation, cells were resuspended in 1 ml RPMI-1640 for primary splenocytes and plated on 24-well plates. Cells were incubated at 37 °C in a CO_2 incubator. On day 3, supernatants were collected in 1.5 ml reaction tubes. Samples were centrifuged at 5000 rpm for 10 min at 4 °C and stored at -20 °C until determination of cytokine levels by ELISA.

2.2.4.4 Staining for FACS analysis

Cells for FACS analysis were prepared and stimulated as described in chapter 2.2.4.1 to 2.2.4.3. All centrifugation steps were done at 300x g for 10 min at 4 °C and cells were kept on ice during staining procedure.

After stimulation cells were blocked using mouse albumin (50 µg/µl) for 15-30 min at 4°C. 2 to 3 ml PBS were added and cells were centrifuged. LIVE/DEAD dye was then incubated with the cells for 30 min (light-protected). PBS was added again and cells were centrifuged. For surface staining cells were incubated with different antibodies raised against surface proteins (see table 2.1.5.3) for 20-30 min (light-protected). Cells were then washed twice with 1-2 ml of Cell Wash (BD Biosciences, Germany) and centrifuged. 250 µl of FixPerm (BD Biosciences, Germany) was added and cells were incubated for 20 min. Next, cells were washed twice with PermWash (BD Biosciences, Germany) and

incubated with antibodies against intracellular proteins (see table 2.1.5.3) for 30 min. Cells were washed twice with PermWash and resuspended in 200 µl PBS. Prior to FACS analysis, cells were filtered twice with a 30 µM cell strainer and centrifuged at 100x g for 1 min at 4°C.
Staining for intranuclear proteins was performed with FoxP3 Staining Kit (eBioscience, Germany) according to manufacturer's guideline. Therefore, cells were treated as described previously, fixation/ permeabilization as well as washing steps with BD reagents were replaced by eBioscience reagents.

2.2.4.5 Detection of albumin in mouse urine by ELISA

Albumin ELISA was performed with reagents purchased from Bethyl Laboratories, USA and prepared according to manufacturer's instructions. On day one, ELISA plates (Nunc immunoplates MaxiSorp C) were coated with anti-mouse-albumin antibody and incubated over night (o/n) at 4 °C. Next day, coated ELISA plates were washed three times with washing buffer and blocked with postcoat buffer for 1 hour at RT. Dilutions of urine samples were prepared (1:1,000 – 1:200,000 according to Stix-levels (see 2.2.6.1)) with sample diluent. ELISA plates were washed three times and samples were loaded onto the plate. Serial dilutions of mouse albumin standard (1,000 ng/ml – 7.8 ng/ml) were prepared and also loaded onto the plate. After 1 hour of incubation at RT, plates were extensively washed (5x). HRP-conjugated second antibody diluted 1:50,000 in sample diluent was then added and incubated for 1 h at RT. Again, ELISA plates were washed five times followed by addition of TMB peroxidase substrate and peroxidase solution B. Reaction was stopped by H_2SO_4 and extinction was measured in an ELISA plate reader at OD_{450nm}.

2.2.4.6 Immunoglobulin ELISA

All solutions were purchased from Bethyl Laboratories, USA and prepared according to manufacturer's instructions. On day one, ELISA plates (Nunc immunoplates MaxiSorp C) were coated with sheep IgG and incubated over night (o/n) at 4 °C. Next day, coated ELISA plates were washed three times with washing buffer and blocked with postcoat buffer for 1 hour at RT. Serial

dilutions of serum samples were prepared (1:100 – 1:12500) with Sample diluent. ELISA plates were washed three times and samples were loaded onto the plate. After 1 hour of incubation at RT, plates were extensively washed (5x). HRP-conjugated second antibody (IgG, IgG1, IgG2a, IgG2b, IgG2c, IgG3, or IgA) diluted 1:1000 in sample diluent was then added and incubated for 1 h at RT. Again, ELISA plates were washed five times followed by addition of TMB (peroxidase substrate and peroxidase solution B mixed 1:1). Reaction was stopped by H_2SO_4 and extinction was measured in an ELISA plate reader at OD_{450nm}.

2.2.4.7 Cytokine ELISA

For the detection of cytokines in splenocyte culture supernatants different ELISA Kits were used (see 2.1.3.1 for detailed list). All kits were used according to manufacturer's instructions.

2.2.4.8 MPO ELISA

Detection of serum mouse anti-mouse MPO antibodies by ELISA was performed as describes previously [131].

2.2.4.9 [^{3}H]Thymidine Assay

[^{3}H]Thymidine assay was performed as described previously [18].

2.2.5 Methods in histology

Light microscopy and immunohistochemistry were performed by routine procedures. Crescent formation and severe glomerular necrosis (two or more layers of cells visible in Bowman's space or >50% of the glomerular tuft affected) were determined in 50 glomeruli per mouse in PAS-stained paraffin embedded kidney-sections in a blinded manner as described previously [132]. Paraffin-embedded sections (2 µm) were either stained with an antibody

directed against the pan-T cell marker CD3 (A0452; Dako, Hamburg, Germany), the monocyte/macrophage cell markers F4/80 (BM8, BMA Biomedicals, Hiddenhausen, Germany) and MAC2 (M3/38; Cedarlane-Laboratories, Burlington, Ontario, Canada) or either sheep or mouse IgG (Jackson Immuno Research Laboratories), and developed with a polymer-based secondary antibody–alkaline phosphatase kit (POLAP; Zytomed, Berlin, Germany), as published previously [133,134]. Glomerular CD3 and MAC2-positive cells were quantified in 50 glomerular cross-sections (gcs), F4/80 and interstitial CD3 positive cells were counted in 30 tubulointerstitial high power fields (hpf, magnification x 400) per kidney-section in a blinded fashion.

2.2.6 Protein biochemistry

2.2.6.1 Measuring of protein concentration in urine

To determine the concentrations of proteins in urine of mice, 15 µl of mouse urine was load onto multistix® 10 SG (Siemens, Germany) according to manufacturer's instructions. Urine was incubated for 60 seconds and protein concentration was visually determined by comparison with manufacturer's scale. The generated data served as a basis for the dilutions that were used in Albumin ELISA.

2.2.6.2 Determination of creatinine and blood urea nitrogen (BUN) in urine

Detection of creatinine and BUN was performed at the Zentrallabor, University Hospital Eppendorf, Germany.

3 RESULTS

3.1 Role of IL-6 signaling in experimental glomerulonephritis

Interleukin 6 (IL-6) is a pleiotropic cytokine acting as a central regulator of both pro-inflammatory and anti-inflammatory processes [93,94]. It is a major inducer of Th17 responses, for stimulation of acute phase protein synthesis and plays a role in the control of activation and regulation of monocytes and neutrophils. In addition, IL-6 is involved in lymphocyte survival and supports the growth of B cells and their antibody production [90,93,94,135]. In several autoimmune diseases, IL-6 has been shown to act in either pro- or anti-inflammatory manner [95,96,105–110,136–139]. Glomerulonephritides are important autoimmune disease resulting in terminal renal insufficiency. Most cell populations regulated by IL-6 have been shown to be involved in GN pathogenesis e.g. Th17 cells, neutrophils, macrophages and pathogenic antibodies [17,135,136,138,140]. To date, the role of IL-6 in the progression of glomerulonephritis is still elusive.
In this study two different mouse-models for glomerulonephritis were used to address the role of IL-6, the NTN-model (see 2.2.1.1) and the MPO-model (see 2.2.1.2).

3.2 Anti IL-6R treatment (m-tocilizumab) exacerbates crescentic NTN glomerulonephritis

IL-6 is known to be crucial for pro-inflammatory Th17 immune response development [22]. At first, we questioned whether an inhibition of IL-6 signaling ameliorates glomerulonephritis by repressing Th17 cell development. To unveil the role of the IL-6 signaling on the experimental crescentic glomerulonephritis, C57BL/6 mice were injected intraperitoneally with the monoclonal murine antibody anti-IL6R (m-tocilizumab, Chugai) or IgG, respectively. For extensive blocking of IL-6R, injection was performed on day -1, one day prior to NTN administration, and on day 5 (figure 3.1 A).

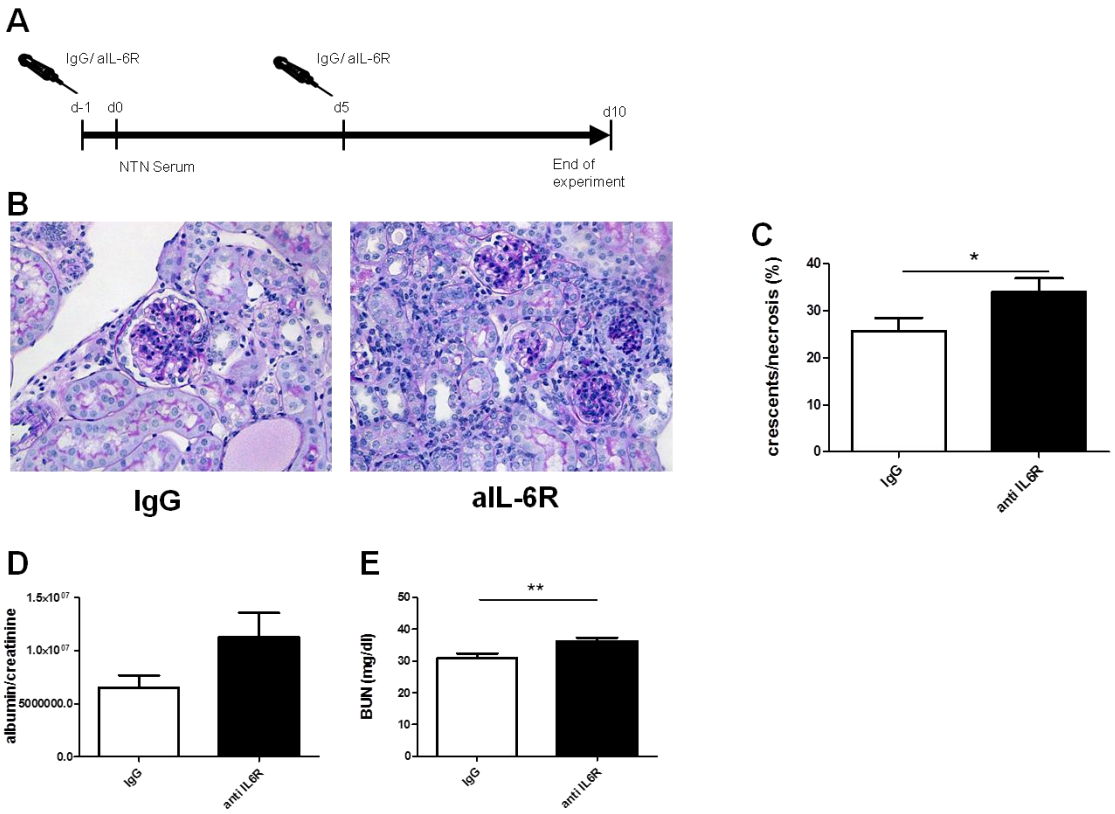

Figure 3.1: Renal is aggravated after m-tocilizumab treatment.
(A) Experimental setup: for blocking of IL-6R mice were injected with m-tocilizumab on day 1 and day 5. NTN was induced on d0. Mice were sacrificed on d10. **(B)** Representative photographs of PAS stained kidney sections from IgG and aIL-6R treated C57BL/6J mice at day 10 after induction of NTN (magnification x 400) show exacerbated renal damage in m-tocilizumab treated mice. **(C)** Quantification of glomerular crescents, interstitial T-cells **(D)** and infiltrating macrophages **(E)** reveals higher renal damage in m-tocilizumab treated mice. Renal function was assessed by determination of albumin-creatinine ratio **(F)** and serum BUN levels **(G)** on day 10. Bars represent means ± SD. *P<0.05; **P<0.01; n.s.= not significant .

PAS stained kidney sections of IgG and aIL-6R treated mice at day 10 after induction of NTN show aggravated renal damage in m-tocilizumab treated mice (figure 3.1 B, C). In addition, higher numbers of interstitial $CD3^+$ T-cells (figure 3.1 D) and infiltrating macrophages (figure 3.1 E) are detectable after treatment with m-tocilizumab. Albumin-creatinine ratio (figure 3.1 F) and serum BUN levels (figure 3.1 G) on day 10 were also significantly increased in aIL-6R treated mice.

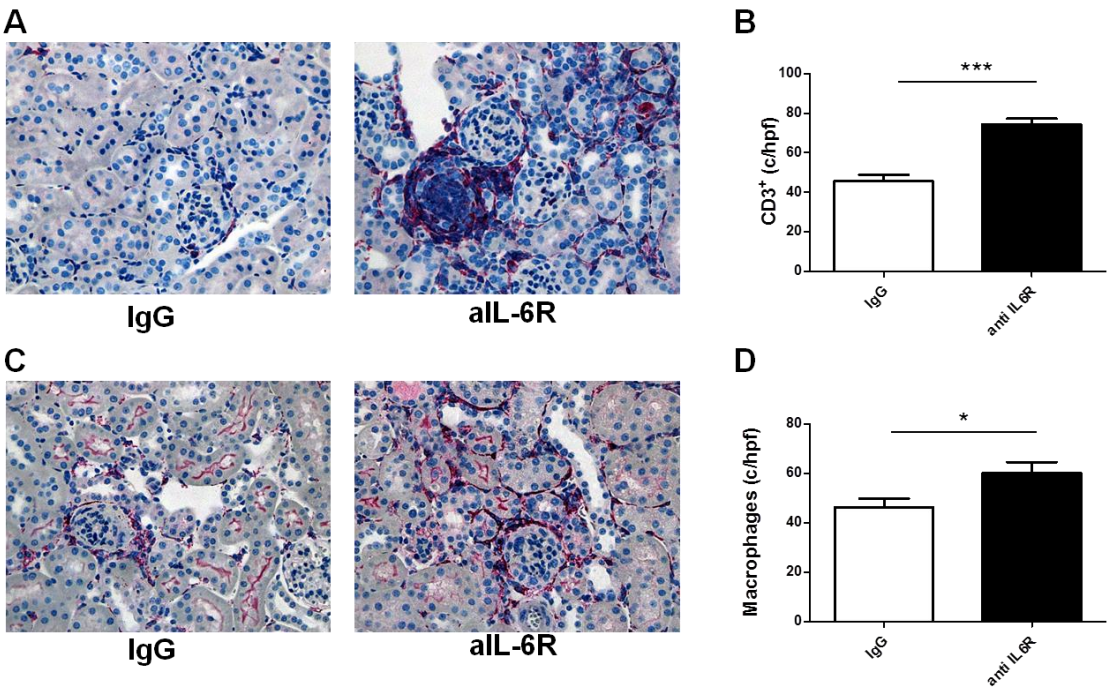

Figure 3.2: Analysis of renal leukocytes infiltration after m-tocilizumab treatment in NTN. **(A)** $CD3^+$ IHC staining of kidney sections from IgG and m-tocilizumab treated mice at day 10 after induction of NTN (magnification x 400). **(B)** Quantification of infiltrating $CD3^+$ T-cells in renal interstitium. **(C)** F4/80 IHC staining of kidney sections from IgG and m-tocilizumab treated mice at day 10 after induction of NTN (magnification x 400). **(D)** Quantification of infiltrating macrophages in renal interstitium. Bars represent means ± SD. *P<0.05; *** P<0.001.

Immunohistochemical staining for T-cell specific CD3 antibody in kidney sections at day 10 after induction of NTN revealed a high appearance of infiltrating $CD3^+$ T-cells in inflamed kidney tissue (figure 3.2 A). In addition, staining for macrophage specific F4/80 antibody in kidney sections also showed increased macrophage infiltration (figure 3.2.C). Treatment with m-tocilizumab significantly enhanced renal T-cell (figure 3.2 B) and renal $F4/80^+$ macrophage infiltration (figure 3.2 D).

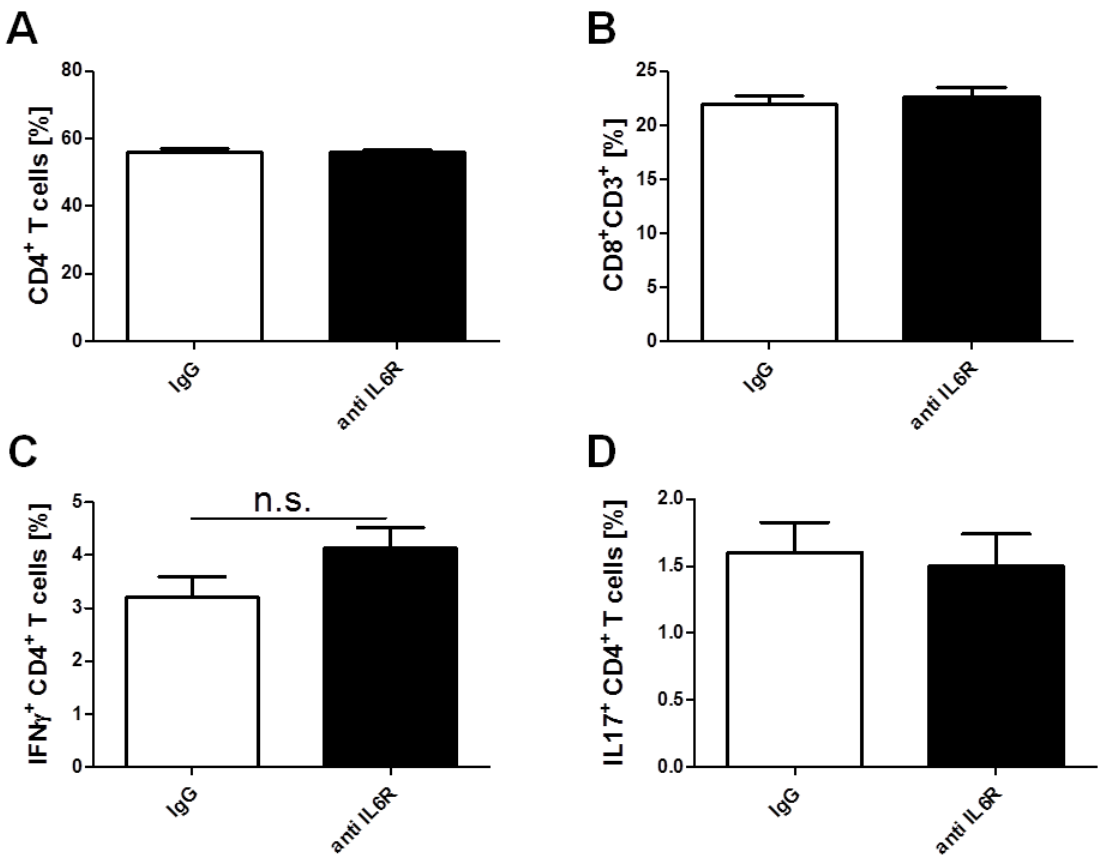

Figure 3.3: Renal leukocyte subtype infiltration after m-tocilizumab treatment of NTN nephritis.
Percentages of $CD4^+$ **(A)** and $CD8^+$ **(B)** T-cells remained unaltered in the kidney. **(C)** Renal Th1 immune response was determined by flow cytometry measurement of IFNγ producing $CD4^+$ T-cells. **(D)** Renal Th17 immune response was determined by flow cytometry measurement of IL-17 producing $CD4^+$ T-cells. Error bars represent means ± SD. n.s.= not significant .

While histological and clinical parameters reveal exacerbation of disease in the kidney (cf. 3.1), cellular distribution of $CD4^+$ (figure 3.3 A) and $CD8^+$ (figure 3.3 B) T-cells remained unaltered. Surprisingly, flow cytometric analysis of Th1 immune responses by IFNγ producing Th-cells (figure 3.3 C) and Th17 immune responses by IL17 producing Th cells (figure 3.3 D) did not show significant differences in the kidney, either.

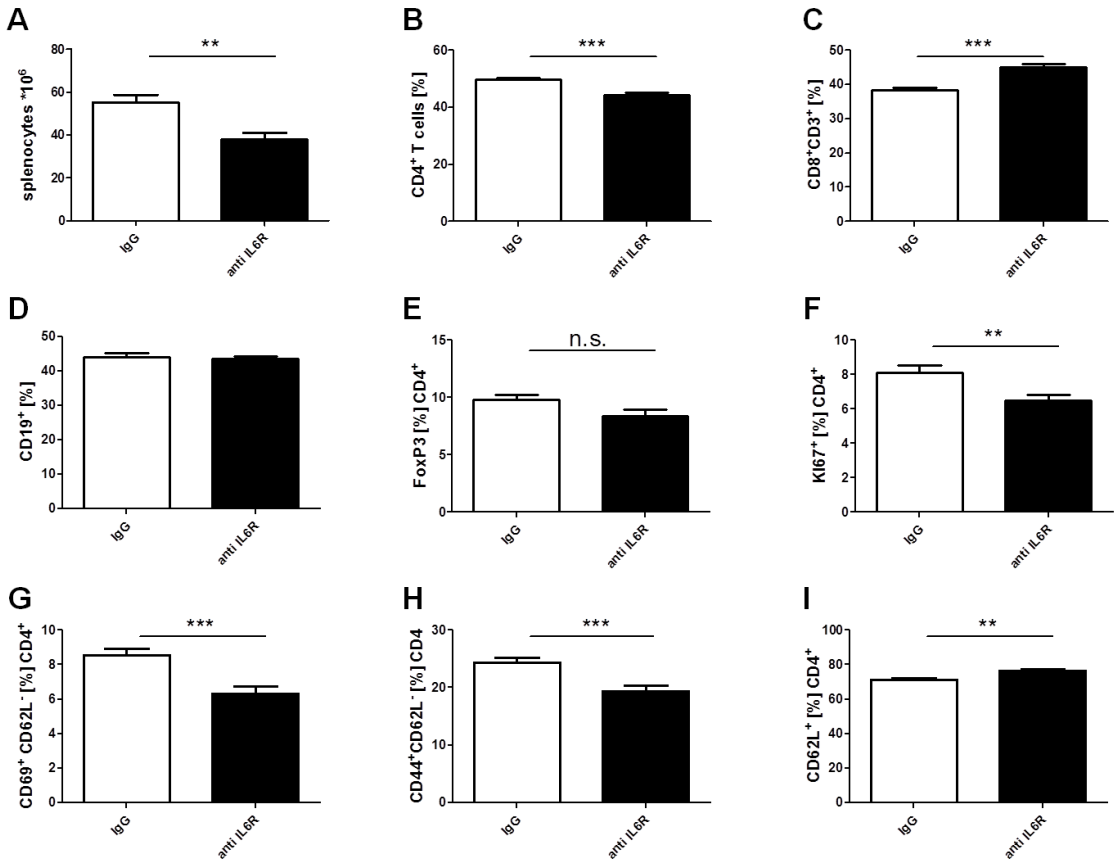

Figure 3.4: Cellular subtype composition of spleens after m-tocilizumab treatment in NTN.
(A) Whole numbers of splenocytes are reduced in m-tocilizumab treated mice, as well as splenic $CD4^+$ Th-cells **(B)**. Percentages of $CD8^+$ T-cells **(C)** are significantly increased in antibody treated mice, whereas percentage of B-cells **(D)** and $FoxP3^+$ T_{regs} **(D)** in spleen remain unaltered. **(E)** Proliferation of Th-cells was determined by expression of KI67. Measurement of early developing Th-cells showed significant reduction in m-tocilizumab treated mice **(G, H)**, whereas late activation marker CD62L is distinctively higher expressed. Error bars represent means ± SD. **P<0.01; *** P<0.001; n.s.= not significant.

Analysis of systemic immunity showed decreased splenocyte numbers (figure 3.4 A), as well as a lower percentage of $CD4^+$ T-cells (figure 3.4 B). On the other hand, the proportion of $CD8^+$ T-cells was significantly elevated (figure 3.4 C), whereas percentages of CD19 positive B-cells (figure 3.4 D) and importantly also FoxP3 positive regulatory T-cells (figure 3.4 E) remained unaltered. Proliferation of Th-cells in the spleen was determined by expression of Ki67 and showed lower Ki67 expressing Th-cells in aIL-6R treated animals (figure 3.4.F). Measurement of activation status of $CD4^+$ T-cells in the spleen indicated lower

numbers of early and late activated Th-cells (figure 3.4 G, H), but higher numbers of naïve T-cells (figure 3.4 I).

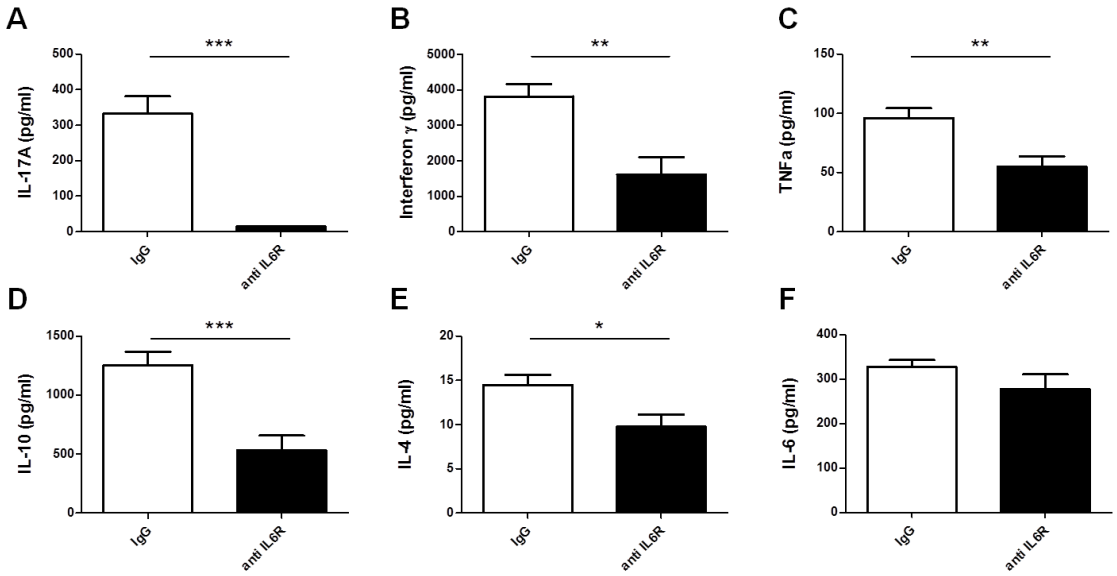

Figure 3.5: Systemic immune response after m-tocilizumab treatment.
Splenocytes were cultured and re-stimulated with IgG for 72 h. Cytokine ELISAs were performed from supernatants, revealing decreased production of pro-inflammatory cytokines IL-17A **(A)**, IFNγ **(B)** and TNFα **(C)** in m-tocilizumab treated mice. Production of anti-inflammatory cytokines IL-10 **(D)** and IL-4 **(E)** was also reduced in m-tocilizumab treated animals, whereas secretion of IL-6 **(F)** remained unaltered. Error bars represent means ± SD. *P<0.05; **P<0.01; *** P<0.001.

Monitoring of cytokine production of sheep IgG re-stimulated splenocyte cultures also demonstrated significant differences between m-tocilizumab treated animals and controls. Secretion of the pro-inflammatory cytokine IL-17A was markedly reduced (figure 3.5 A) when m-tocilizumab was applied. In addition, secretion of further pro-inflammatory cytokines like IFNγ (figure 3.5 B) and TNFα (figure 3.5 C) was also significantly reduced. Interestingly, the production of the anti-inflammatory cytokines IL-10 (figure 3.5 D) and IL-4 (figure 3.5 E) was also diminished in the culture supernatants of aIL-6R treated mice. In contrast, levels of IL-6 secretion remained unaltered (figure 3.5 F)

3.3 Aggravation of crescentic nephritis is mediated by inhibition of classical but not alternative IL-6 signaling

IL-6 mediates signal transduction via two different pathways, the classical IL-6 signaling pathway and the IL-6-*trans*-signaling. While the classical pathway is mediated by binding to interleukin 6 receptor (IL-6R), which expression is limited to hepatocytes, and leukocytes, *trans*-signaling is mediated by binding of IL-6 to the signal-transducing glycoprotein gp130, which is ubiquitously expressed. To unveil which signaling pathway is involved, C57BL/6J mice were treated with either aIL-6 antibody, which inhibits both signaling pathways, or sgp130Fc which inhibits the *trans*-signaling only. As control, C57BL/6J mice were injected i.p. with IgG. For extensive inhibition of either classical or *trans*-signaling, mice were injected i.p. aIL-6, sgp130Fc, or IgG, respectively, one day prior to and on day 5 after NTN application (figure 3.6 A).

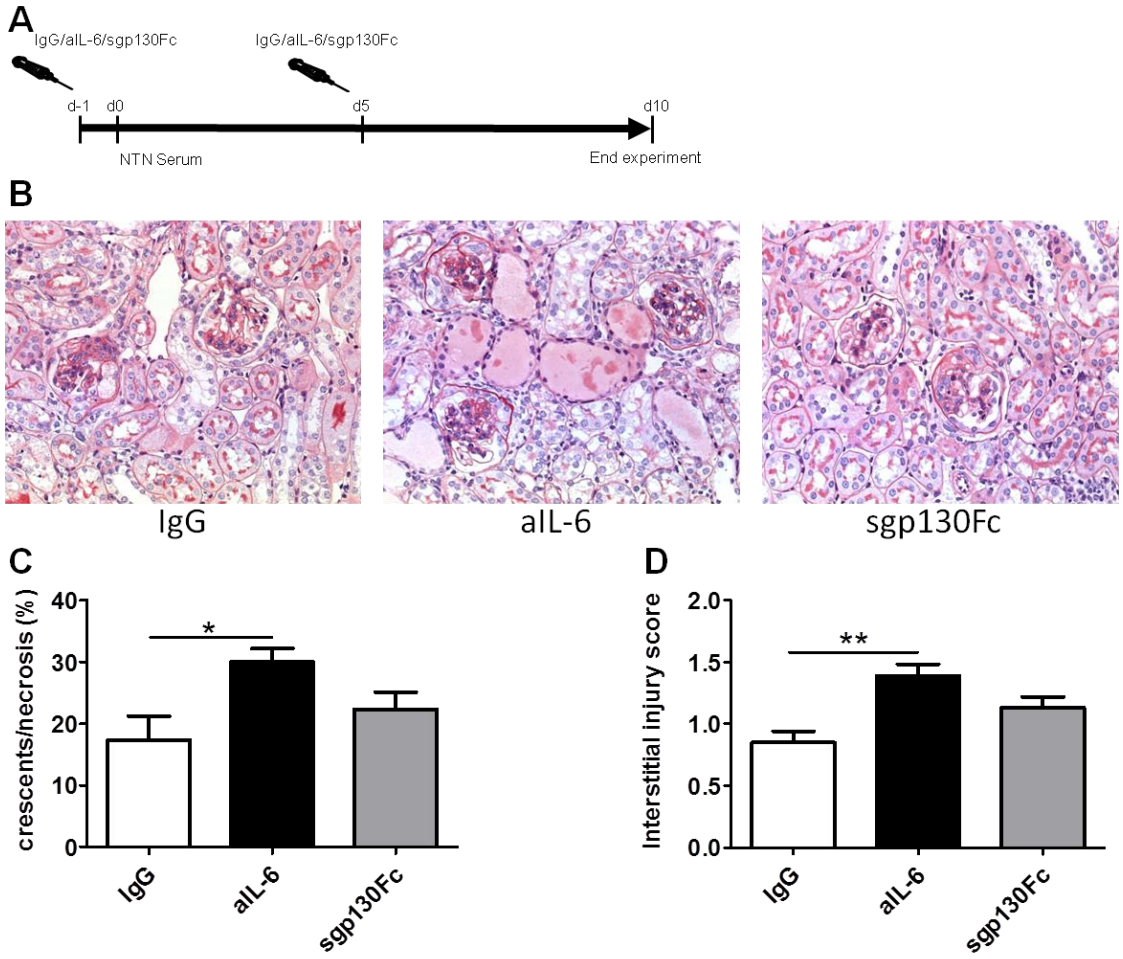

Figure 3.6: Renal injury after inhibition of classical or trans-signaling of IL-6 in NTN.
(A) Experimental setup: for blocking of IL-6 mice were injected with aIL-6 or spg130Fc on days d-1 and d5. NTN was induced on d0. Mice were sacrificed on d10. **(B)** Representative photographs of PAS stained kidney sections from IgG, aIL-6 and spg130Fc treated C57BL/6J mice at day 10 after induction of NTN (magnification x 200) show exacerbated renal damage in aIL-6 treated mice. sgp130Fc treated mice show renal damage comparable to IgG treated control mice. **(C)** Quantification of glomerular crescents reveals higher renal damage in aIL-6 treated mice. **(D)** Quantification of interstitial injury shows increased damage in aIL-6 treated mice. Error bars represent means ± SD. *P<0.05; **P<0.01.

PAS stained kidney sections of IgG, aIL-6, and sgp130Fc treated mice at day 10 after induction of NTN all show significant renal damage (figure 3.6 B). Inhibition of both IL-6 signaling pathways by aIL-6 resulted in significant aggravation of disease in terms of formation of glomerular crescents and interstitial injury, while specific blockade of IL-6 *trans*-signaling by the fusion protein sgp130Fc did not (figure 3.6. C, D).

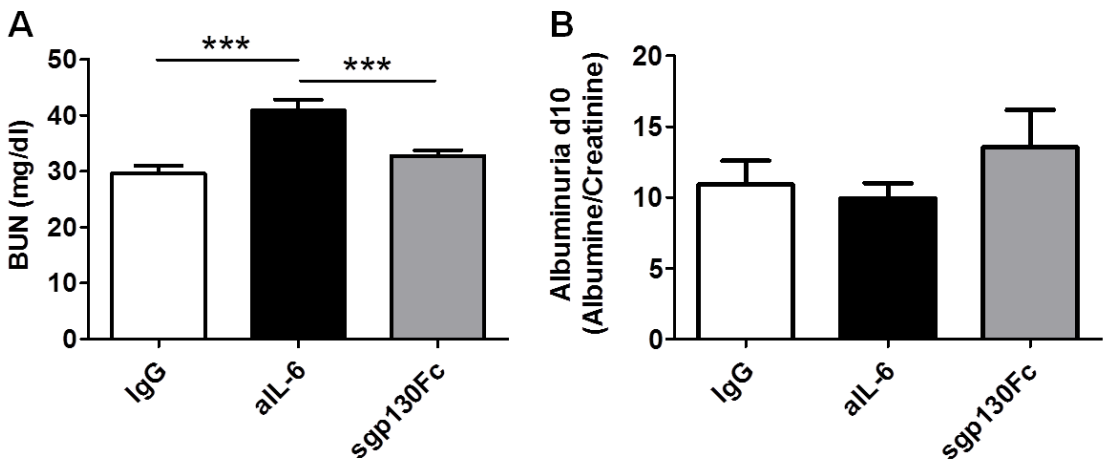

Figure 3.7: Renal functional injury after differential IL-6 inhibition.
Renal function was assessed by serum BUN levels **(A)** and urinary albumin-creatinine ratio **(B)** on day 10. Error bars represent means ± SD. *** P<0.001.

As a parameter of renal dysfunction, serum BUN levels were determined and showed a more severe course of disease in aIL-6 treated mice, whereas BUN levels under the blockade of *trans*-signaling via sgp130Fc remained unaltered (figure 3.7 A). The urinary albumin-creatinine ratio did not alter between differential treatment regimes (figure 3.7 B).

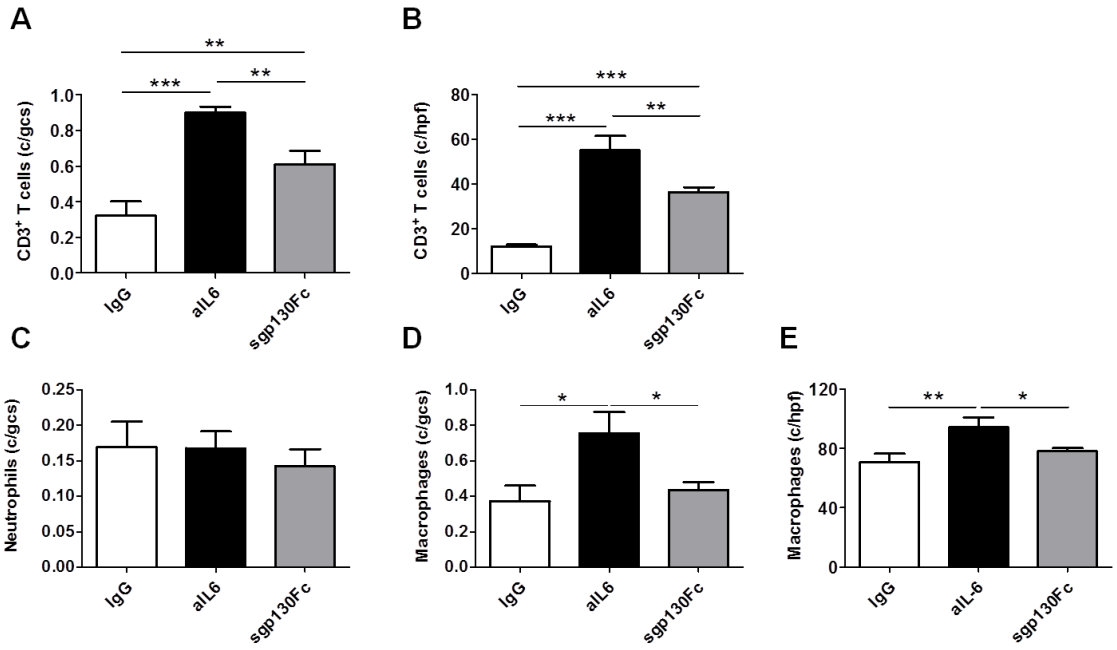

Figure 3.8: Analysis of renal leukocyte infiltration by immunohistochemistry.
Kidney sections from IgG, aIL-6 and spg130Fc treated C57BL/6J mice at day 10 after induction of NTN were stained by IHC. Quantification of $CD3^+$ T-cells per glomerular cross section **(A)** or high power field **(B)** reveals higher number of infiltrating T-cells in the kidney. **(C)** Numbers of neutrophils per glomerular cross section were unaffected by differential treatment. Quantification of Mac-2^+ macrophages per glomerular cross section **(D)** and F4/80^+ macrophages per high power field **(E)** feature increased infiltration of macrophages into the kidney in aIL-6 treated mice. Bars represent means ± SD. *P<0.05; **P<0.01; *** P<0.001.

Analysis of renal leukocyte infiltration by immunohistochemistry revealed higher cellular infiltration rates into the kidney of aIL-6 treated mice, displayed by elevation of $CD3^+$ T-cells in both glomeruli (figure 3.8 A) and interstitium (figure 3.8 B). While numbers of infiltrating neutrophils into the glomeruli remained unaffected by either treatment (figure 3.8 C), more infiltrating Mac-2^+ macrophages in the glomeruli (figure 3.8 D) and F4/80^+ macrophages in the interstitium (figure 3.8 E) were detectable in aIL-6 treated mice.

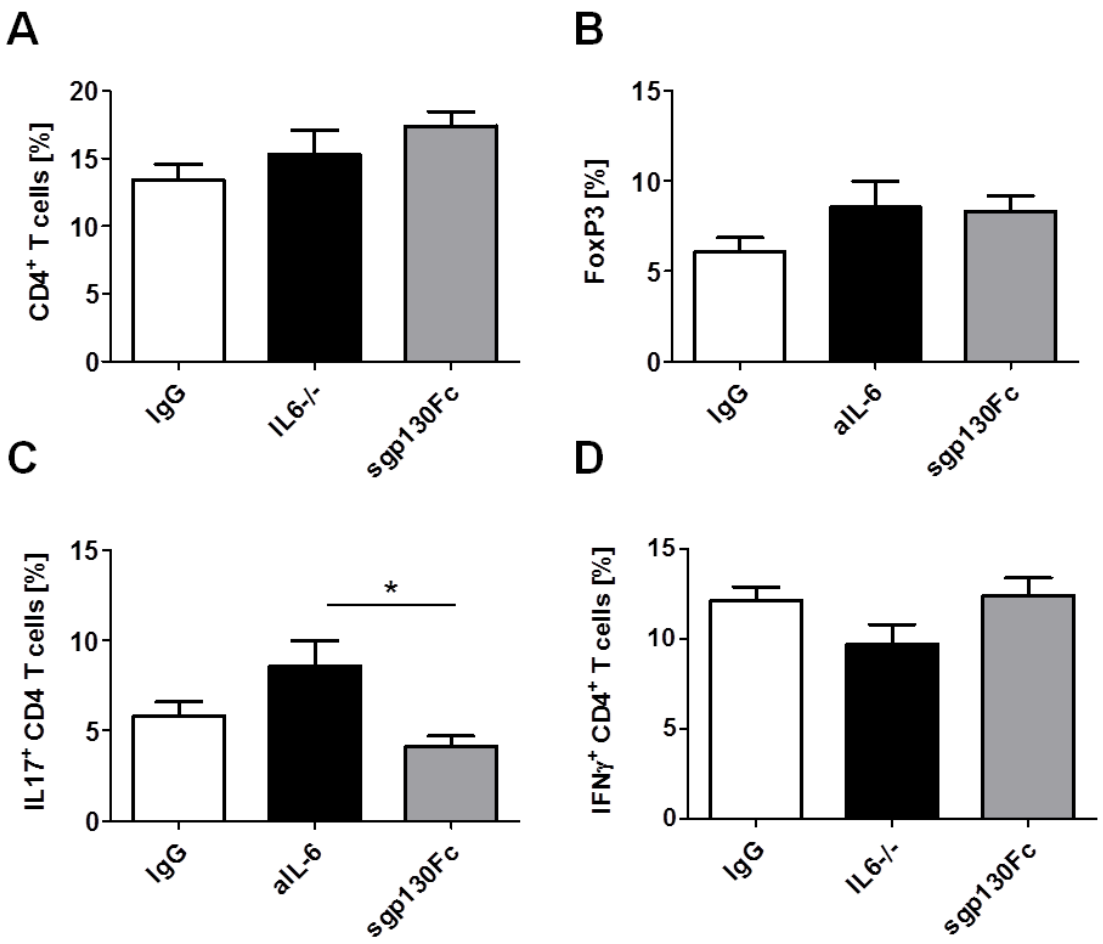

Figure 3.9: Renal T-cell subtype infiltration after differential IL-6 signaling suppression in the kidney.
Percentages of $CD4^+$ T-cells **(A)** and $FoxP3^+$ T_{regs} **(B)** remained unaltered in the kidney. **(C)** Renal Th17 immune response was enhanced in aIL-6 treated mice. **(D)** Renal Th1 immune response was unaltered. Bars represent means ± SD. *P<0.05.

Similar to m-tocilizumab treatment, usage of aIL-6 or sgp130Fc clearly demonstrated an aggravation of disease determined by (immuno-)histological and clinical parameters (cf. figure 3.6-3.8), but showed no difference in cellular subtype distribution of $CD4^+$ T-cells (figure 3.9 A) and $FoxP3^+$ T_{regs} (figure 3.9 B). Flow cytometric analysis of Th17 immune responses by IL17 producing Th cells (figure 3.9 C) and Th1 immune responses by IFNγ producing Th cells (figure 3.9 D) did not show any differences in the kidney in comparison with control mice either.

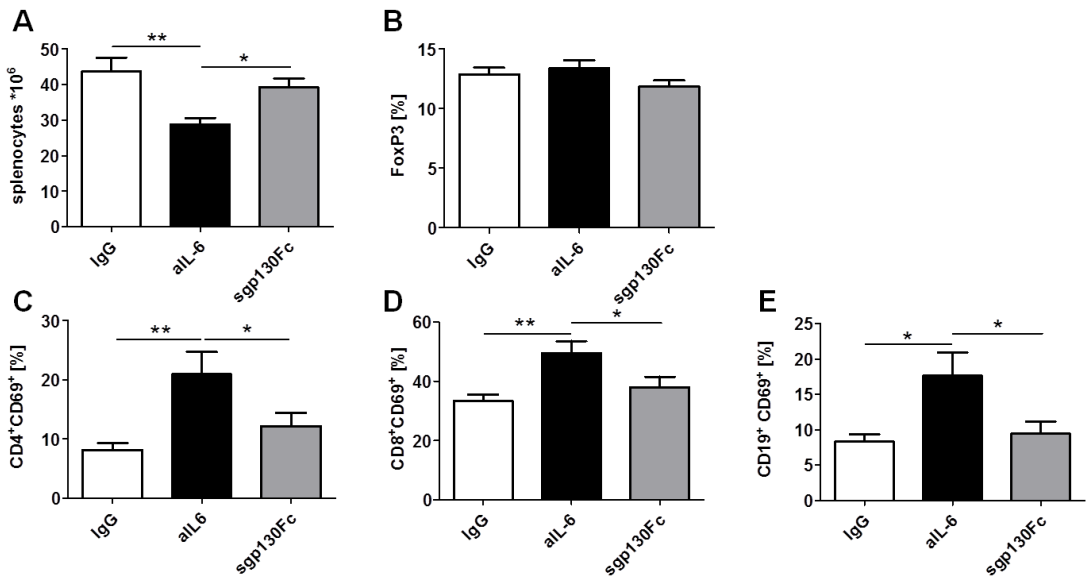

Figure 3.10: Splenocyte composition after differential IL-6 signaling suppression.
(A) Total numbers of splenocytes were reduced in aIL-6 treated mice. Percentages of $FoxP3^+$ T_{regs} **(B)** remained unaltered. Activation of $CD4^+$ T-cells **(C)**, $CD8^+$ T-cells **(D)** or activation status of B-cells **(E)** was enhanced in aIL-6 treated mice. Error bars represent means ± SD. *P<0.05; **P<0.01.

As shown in figure 3.10, the total numbers of splenocytes (A), as well as the percentage of regulatory T-cells (B), activated $CD4^+$ T-cells (C), $CD8^+$ T-cells (D), and B-cells (E) in sgp130Fc treated mice did not differ compared to the IgG treated control mice. In contrast, aIL-6 mice showed significant reduction of total number of splenocytes (figure 3.10 A), but exhibited higher percentages of activated T-helper (figure 3.10 C), $CD8^+$ T-cells (figure 3.10 D), and B-cells (figure 3.10 E), whereas FoxP3 positive regulatory T-cells were unaltered (figure 3.10 B).

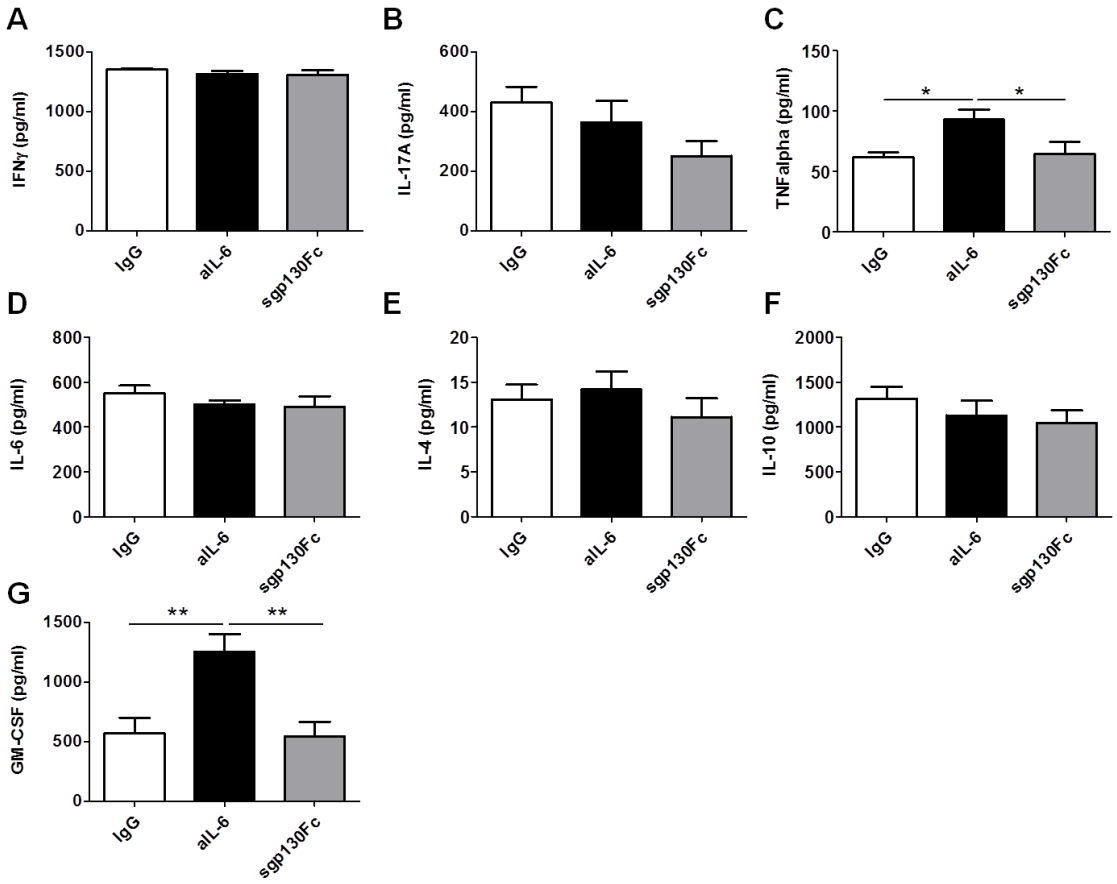

Figure 3.11: Systemic immune response after differential IL-6 signaling suppression in NTN.
Splenocytes were cultured and re-stimulated with IgG for 72 h. Cytokine ELISAs were performed from supernatants, revealing unaltered production of the pro-inflammatory cytokines IFNγ **(A)** and IL-17A **(B)**. Levels of TNFα **(C)** were increased in aIL-6 treated, but not in sgp130Fc treated mice. Secretion of IL-6 **(D)**, as well as secretion of the anti-inflammatory cytokines IL-4 **(E)** and IL-10 **(F)** also remained unaltered. Determination of GM-CSF from serum **(G)** revealed markedly increased secretion in aIL-6 treated mice. Error bars represent means ± SD. *P<0.05; **P<0.01 .

An increase of systemic cytokine expression in aIL-6 treated mice was observed for TNFα as highlighted in figure 3.11 C. Also cytokine levels of macrophage expanding GM-CSF (figure 3.11 G) taken from blood sera were significantly increased in aIL-6 treated mice compared to sgp130Fc treated mice and IgG controls. The levels of IFNγ (figure 3.11 A) and IL-17A (figure 3.11 B) in turn, remained unaltered. The same was true for the anti-inflammatory cytokines IL-4 (figure 3.11 E) and IL-10 (figure 3.11 F). The levels of IL-6 did not reveal differences in the tested groups either (figure 3.11 D).

3.4 IL-6 inhibition in the macrophage-dependent effector phase is sufficient to exacerbate nephritis

As shown above, inhibition of the blockade of both IL-6 signaling pathways by aIL-6 or m-tocilizumab resulted in significant aggravation of renal disease, whereas specific inhibition of IL-6 *trans*-signaling by the fusion protein sgp130Fc did not. Surprisingly, development of the nephritogenic Th17 response remained intact in antibody treated animals. Our findings raised the question, how suppression of IL-6 signaling resulted in aggravated course of glomerulonephritis. As mentioned above, IL-6 has an anti-inflammatory effect on macrophages by inducing the expression of anti-inflammatory cytokines sTNFR and IL-1ra [113–115,117]. In addition, IL-6 is known to suppress macrophage proliferation [118]. Since suppression of the IL-6 signaling resulted in an increase of infiltrating macrophages into the inflamed tissue, as demonstrated in figure 3.8, and we observed higher levels of GM-CSF, next the impact of IL-6 on macrophage activation in experimental glomerulonephritis was investigated.

C57BL/6J mice were pre-immunized subcutaneously with antigen for five days prior to induction of NTN to allow for nephritogenic Th17 responses to develop normally. NTN was induced on day 0; inhibition of differential IL-6 signaling pathways was performed by application of antibodies the same day (cf. figure 3.12 A).

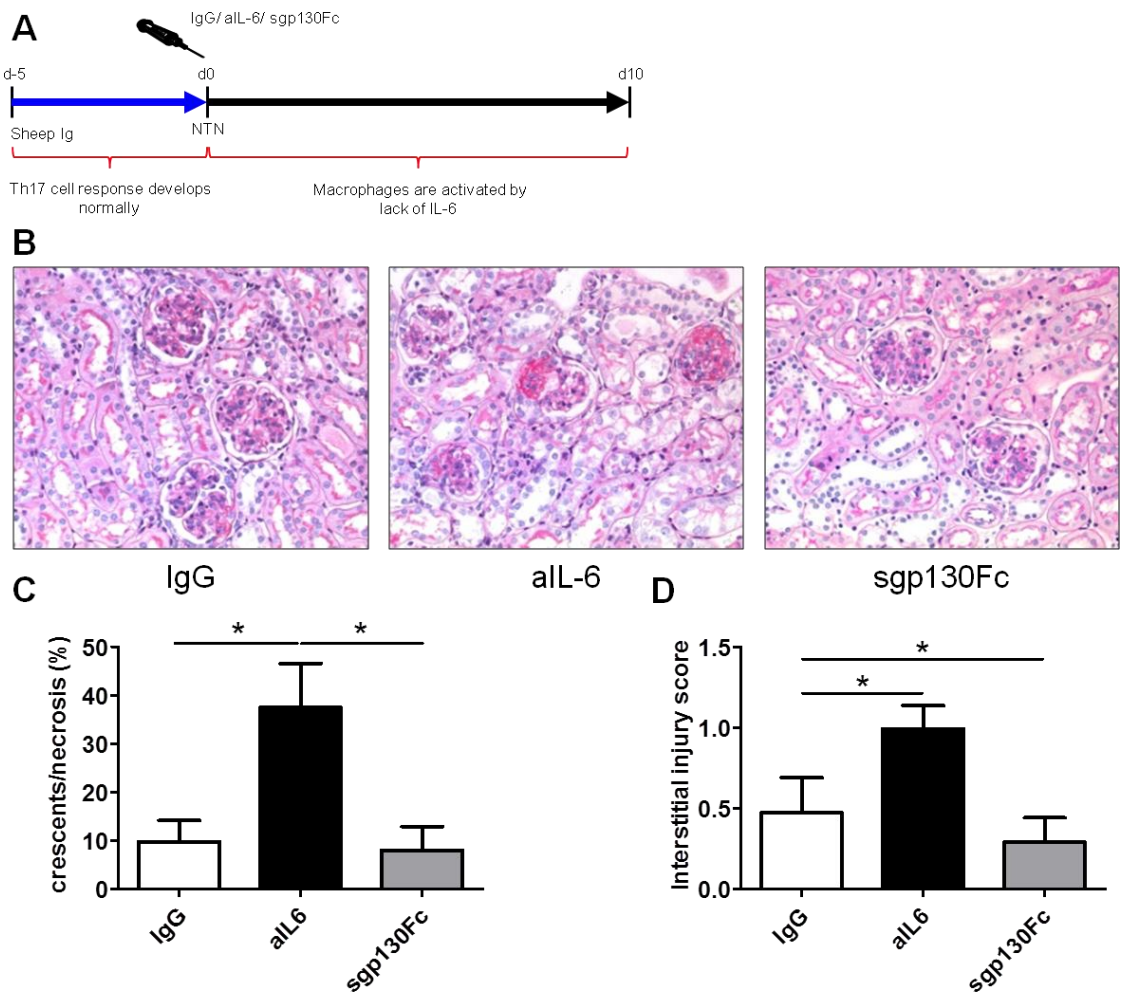

Figure 3.12: Renal injury is increased after inhibition of classical IL-6 signaling in macrophage dependent effector phase.
(A) Experimental setup. C57BL/6J mice were pre-immunized for 5 days with 0.5 mg sheep IgG in CFA. NTN was induced on d0. For blocking of IL-6 in the effector phase, mice were injected with aIL-6 or spg130Fc on d0. Mice were sacrificed on d10. **(B)** PAS staining of kidney sections from IgG, aIL-6 and spg130Fc treated C57BL/6J mice at day 10 after induction of NTN (magnification x 400). Quantification of glomerular crescents **(C)** and interstitial injury **(D)**. Bars represent means ± SD. *P<0.05.

PAS stained kidney sections at day 10 after induction of NTN of pre-immunized C57BL/6J mice treated with IgG, aIL-6, and sgp130Fc in the effector phase all showed significant renal damage (Figure 3.12 B). Inhibition of pan IL-6 signaling by aIL-6 in the macrophage dependent effector phase of NTN even aggravated the course of disease, whereas inhibition of *trans*-signaling had no effect (figure 3.12 C, cf. 3.6 C). Scoring of interstitial injury also demonstrated an aggravated course of disease when treated with aIL-6 antibody, but not when treated with sgp130Fc (figure 3.12 D).

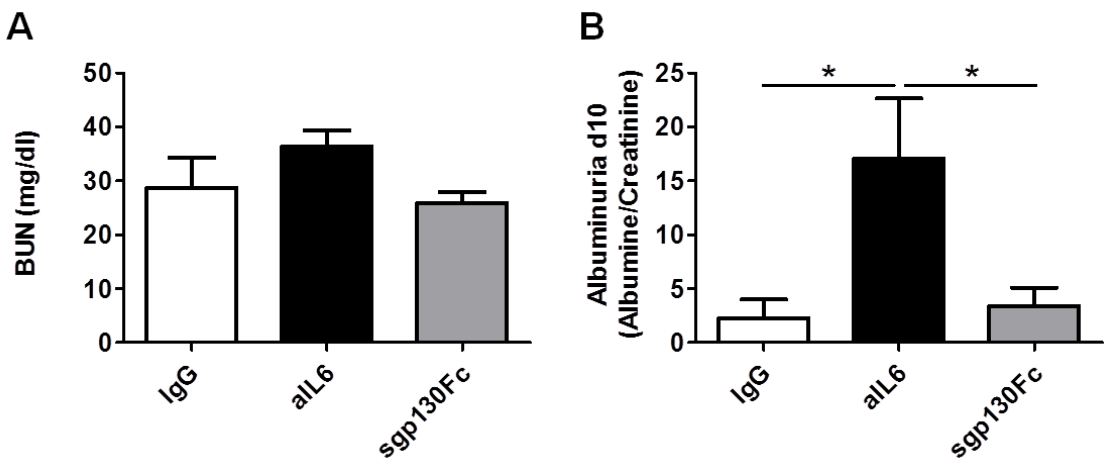

Figure 3.13: Renal functional injury after differential IL-6 inhibition in the effector phase of NTN.
Renal function was assessed by determination of serum BUN levels **(A)** and urinary albumin-creatinine ratio **(B)** on day 10. Error bars represent means ± SD. *P<0.05.

Inhibition of IL-6 signaling in the effector phase caused a non significant increase in blood urea nitrogen levels in all tested groups (figure 3.13 A), whereas the albumin-creatinine ratio was significantly increased in aIL-6 treated mice (figure 3.13 B). Specific blockade of IL-6 *trans*-signaling by the fusion protein sgp130Fc did not result in elevated albuminuria.

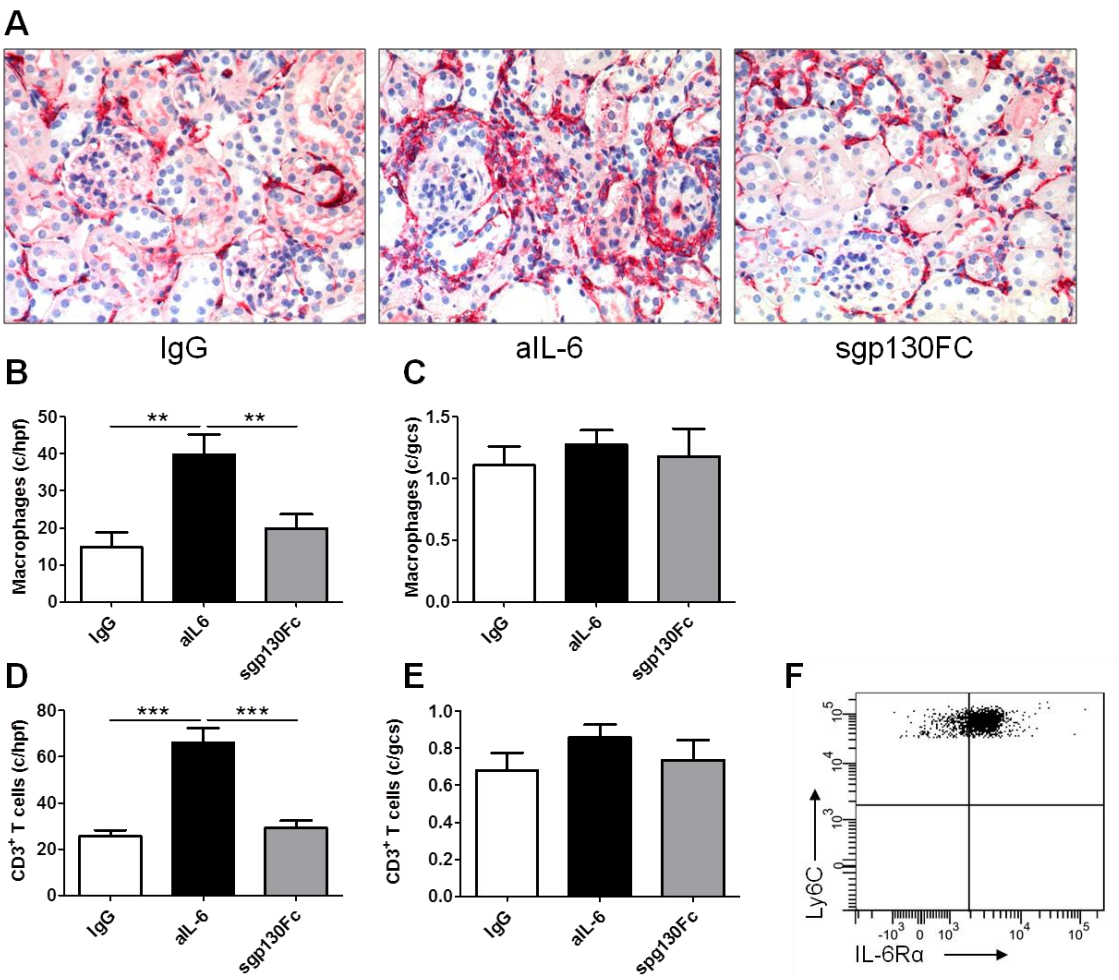

Figure 3.14: Analysis of renal leukocytes after differential IL-6 inhibition in the macrophage dependent effector phase of NTN.
(A) F4/80 IHC staining of kidney sections from IgG, aIL-6, and spg130Fc treated C57BL/6J mice at day 10 after induction of NTN (magnification x 400). **(B)** Quantification of infiltrating F4/80$^+$ macrophages in inflamed kidney tissue. **(C)** Quantification of infiltrating Mac-2$^+$ macrophages in glomerular cross section. Quantification of infiltrating CD3$^+$ T-cells in renal interstitium **(D)** or in glomerular cross section **(E)**. **(F)** FACS analysis of IL-6Rα expression on Ly6C positive macrophages. Bars represent means ± SD. *P<0.05; **P<0.01; *** P<0.001.

Immunohistochemical staining for macrophage specific F4/80 antibody in kidney sections at day 10 after induction of NTN of pre-immunized C57BL/6J mice revealed a high occurence of infiltrating macrophages in inflamed kidney tissue (figure 3.14 A). Treatment with aIL-6 but not sgp130Fc significantly enhanced renal F4/80$^+$ macrophage infiltration (figure 3.14 B) whereas renal Mac-2$^+$ macrophage infiltration remained unaltered (figure 3.14 C). In addition, aIL-6 treated animals showed an increased number of infiltrating CD3$^+$ T-cells in the kidney interstitium (figure 3.14 D) but not in the glomerulus (figure 3.14 E), whereas inhibition of *trans*-signaling had no effect. FACS analysis from spleens

clearly demonstrated the presence of IL-6Rα on the surface of macrophages (figure 3.14 F), which makes them susceptible for IL-6 treatment.

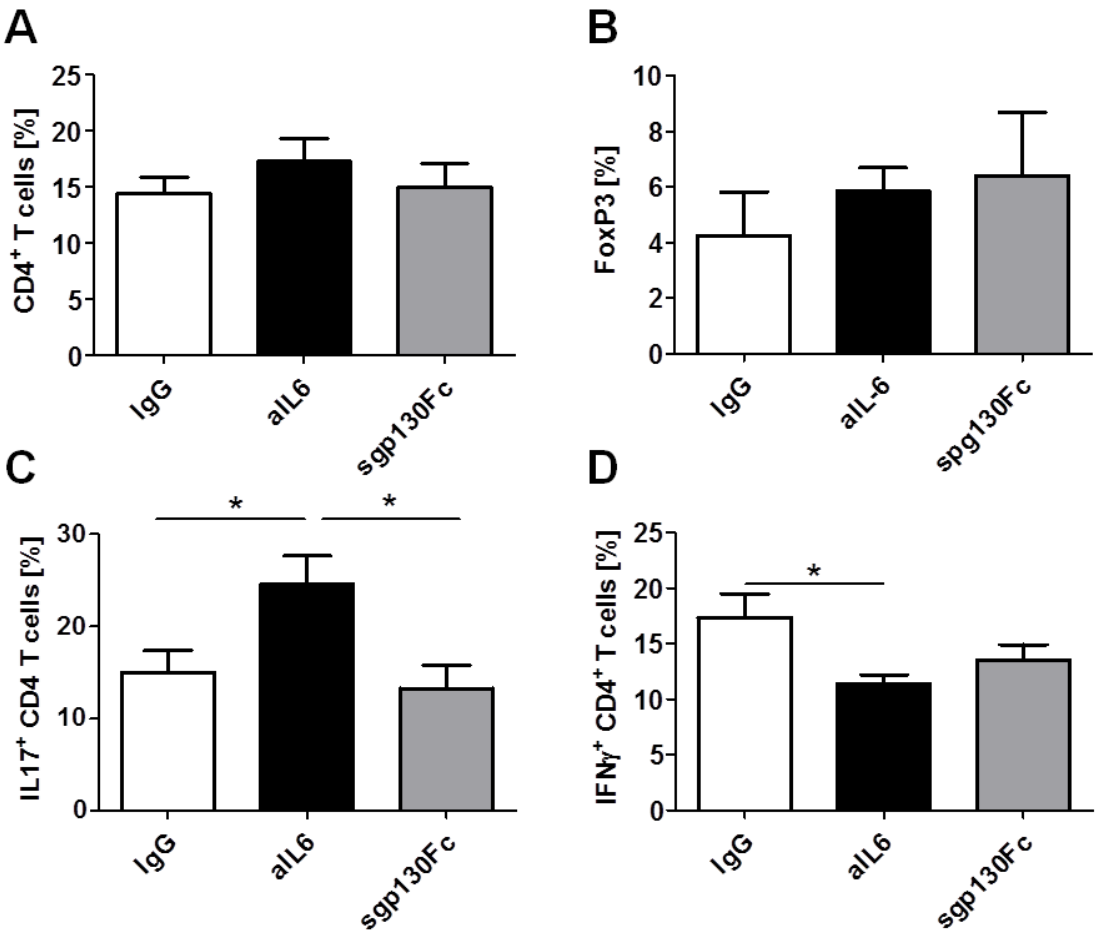

Figure 3.15: Renal T-cell subtype composition after differential IL-6 inhibition in the macrophage dependent effector phase.
Percentages of $CD4^+$ T-cells **(A)** and $FoxP3^+$ T_{regs} **(B)** remained unaltered in the kidney. **(C)** Renal Th17 immune response was determined by flow cytometry measurement of IL-17 producing $CD4^+$ T-cells. **(D)** Renal Th1 immune response was determined by flow cytometry measurement of IFNγ producing $CD4^+$ T-cells. Bars represent means ± SD. *P<0.05.

Renal distribution of Th-cells (figure 3.15 A) and $FoxP3^+$ T_{regs} (figure 3.15 B) were not affected by inhibition of either classic IL-6 signaling or *trans*-signaling in the effector phase. The renal Th17 immune response was even increased in aIL-6 treated mice, while specific blockade of IL-6 *trans*-signaling by sgp130Fc did not affect Th17 responses (figure 3.15 C). In contrast, Th1 immune response, defined by flow cytometry of IFNγ producing $CD4^+$ T-cells, was slightly decreased for aIL-6 treated animals compared to IgG controls (figure

3.15 D). Again, inhibition of *trans*-signaling revealed no differences in Th1 responses compared to the controls.

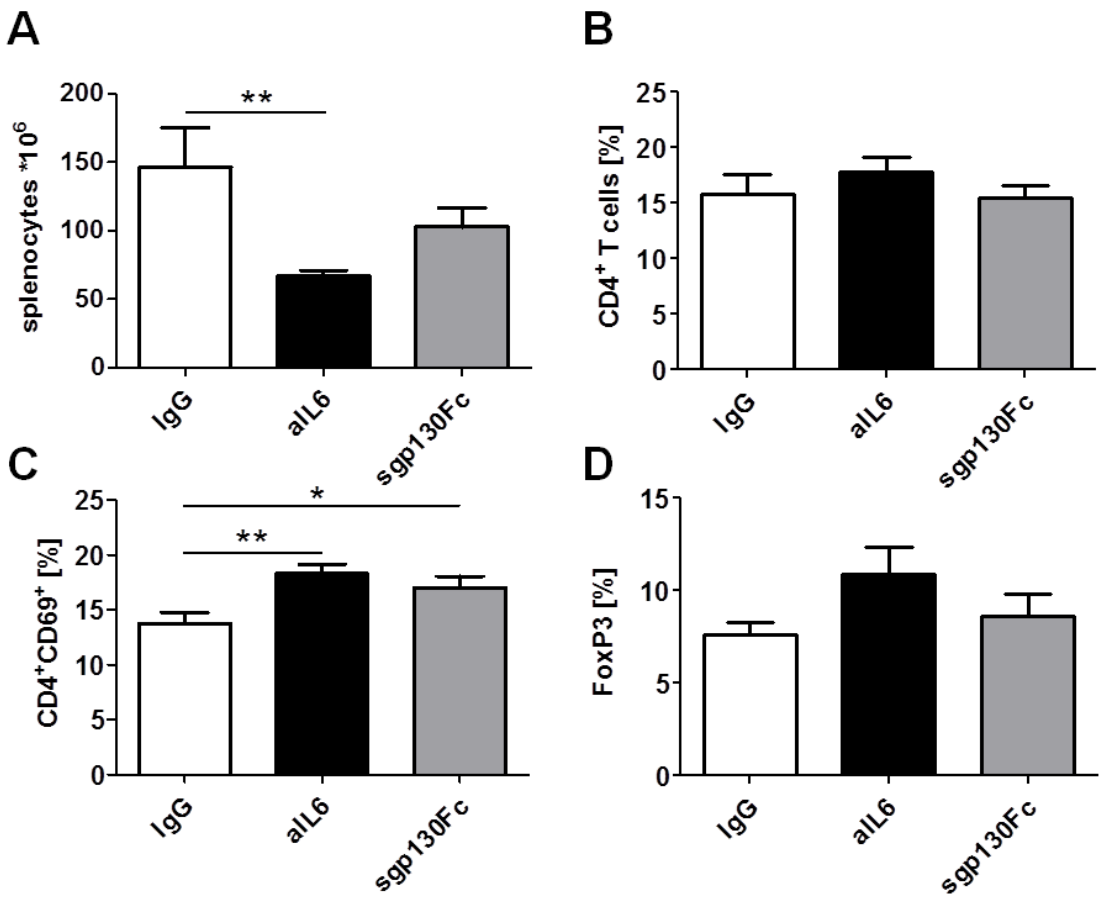

Figure 3.16: Splenocyte composition after differential inhibition of IL-6 in the macrophage dependent effector phase.
(A) Total numbers of splenocytes from IgG, aIL-6, and sgp130Fc treated C57Bl/6 mice were counted in single cell suspensions. Percentages of $CD4^+$ T-cells **(B)** in spleens of IgG, aIL-6, and sgp130Fc treated C57Bl/6 mice were analyzed by flow cytometry. Activation of $CD4^+$ T-cells **(C)** and percentage of $FoxP3^+$ T_{regs} **(D)** were analyzed by FACS staining of spleens from IgG, aIL-6 and sgp130Fc treated mice. Error bars represent means ± SD. *P<0.05; **P<0.01.

The total numbers of splenocytes were markedly decreased in aIL-6 but not in sgp130Fc treated mice (figure 3.16 A), whereas proportion of $CD4^+$ T-cells were unaltered in both antibody treated groups (figure 3.16 B). However, activation status of $CD4^+$ T-cells in both classic and *trans*-signaling affected groups was significantly elevated (figure 3.16 C). Percentage of T_{regs} did not differ in both antibody treated groups (figure 3.1 D).

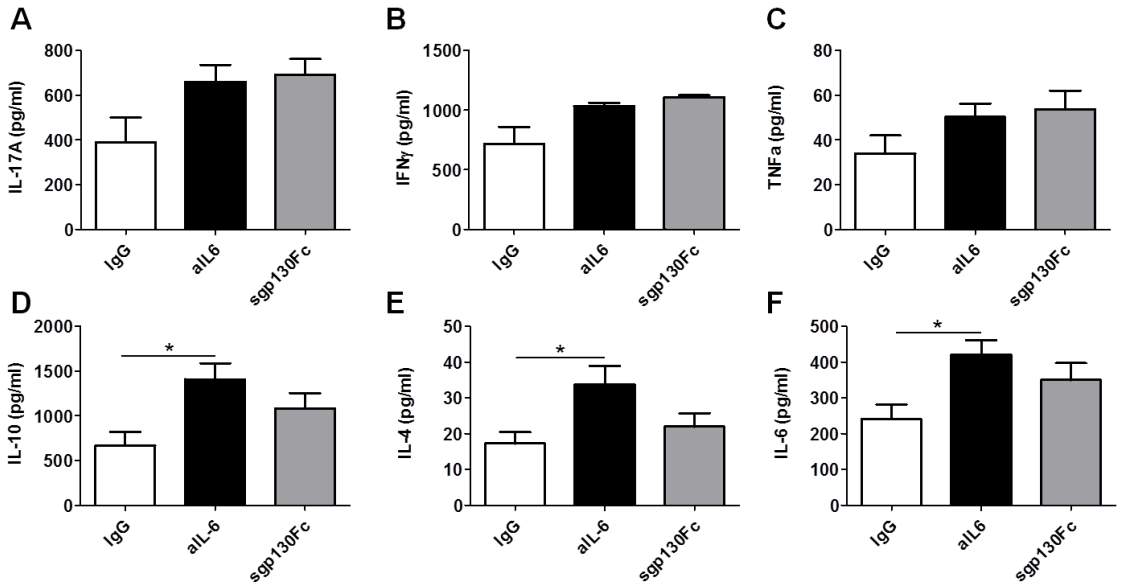

Figure 3.17: Systemic immune response after differential IL-6 signaling suppression in the effector phase.
Splenocytes were cultured and re-stimulated with IgG for 72 h. Supernatants were collected and levels of different cytokines were determined by ELISA The amounts of the pro-inflammatory cytokines IL-17A **(A)**, IFNγ **(B),** and TNFα **(C**) were consistent. The anti-inflammatory cytokines IL-10 **(D)** and IL-4 **(E)** were elevated after aIL-6 treatment. The levels of IL-6 were also increased in aIL-6 treated mice compared to controls **(F)**. Bars represent means ± SD. *P<0.05.

Analysis of the supernatants from splenocyte cultures demonstrated similar levels of the pro-inflammatory cytokines IL-17A (figure 3.17 A), IFNγ (figure 3.17 B), and TNFα (figure 3.17 C). Surprisingly, levels of the anti-inflammatory cytokines IL-10 (figure 3.17 D) and IL-4 (figure 3.17 E) were raised in aIL-6 treated animals. Secretion of IL-6 was also markedly elevated in aIL-6 treated mice figure 3.17 F).

In summary, late inhibition of pan IL-6 signaling by aIL-6 in the macrophage dependent effector phase of NTN was sufficient to aggravate the disease. Renal and systemic Th17 immune responses were even increased compared to IgG treated control mice. aIL-6 but not sgp130Fc treatment significantly enhanced F4/80^{+} renal macrophage infiltration. Specific inhibition of IL-6 *trans*-signaling by sgp130Fc did not affect the course of nephritis.

3.5 IL-6 acts in an anti-inflammatory manner by inhibiting macrophage proliferation

As shown in the previous chapters, inhibition of IL-6 signaling led to exacerbation of glomerulonephritis. So far, the results implicated differential roles of IL-6 in T-cell and macrophage differentiation, proliferation, immune response mediation, and cytokine expression. As inhibition of classical IL-6 signaling led to increased infiltration of macrophages and IL-6 is known to influence macrophage proliferation, we wanted to study this aspect. To determine the impact of IL-6 on macrophages, peritoneal macrophages from C57BL/6J mice were cultured *in vitro* in presence or absence of IL-6 for 14 days.

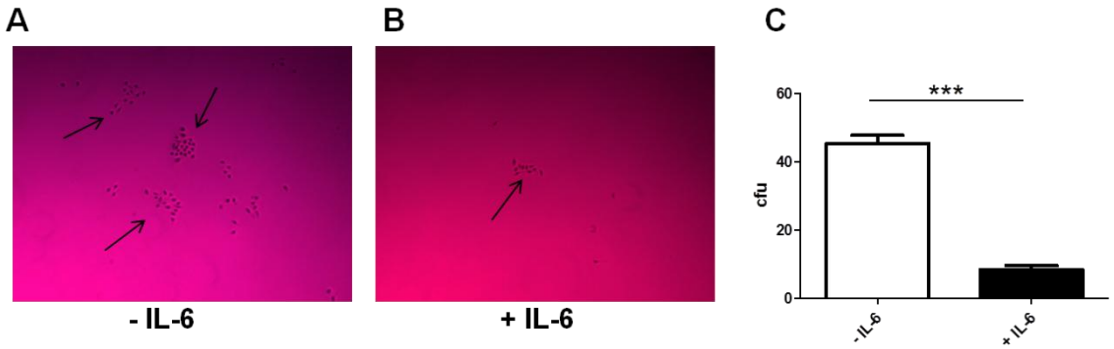

Figure 3.18: Analysis of IL-6 effects on macrophage proliferation *in vitro*
Peritoneal macrophages from C57BL/6J mice were cultured at 3.3 x 10^2 cells/ ml in absence **(A)** or presence **(B)** of recombinant IL-6 for 14 days. **(C)** Colony forming units >3 were counted. Cfus are marked with arrows. Bars represent means ± SD. *** P<0.001.

Proliferation of peritoneal macrophages *in vitro* was strongly inhibited by stimulation with recombinant IL-6 (figure 3.18 B), compared to unstimulated macrophages (figure 3.18 A). Diminished proliferation resulted in a significant reduction of total colony forming units (cfus) with lower numbers of cells (figure 3.18 C).

3.6 Macrophage specific abrogation of classical IL-6 signaling aggravates NTN

To address specific effects of IL-6 classical signaling on macrophages, we generated LysM^{Cre} x IL-6R$^{fl/fl}$ mice (cf. chapter 2.2.1), which specifically lack the IL-6R on macrophages while leaving IL-6 signaling in other cell types unaffected. NTN was induced as discusses above.

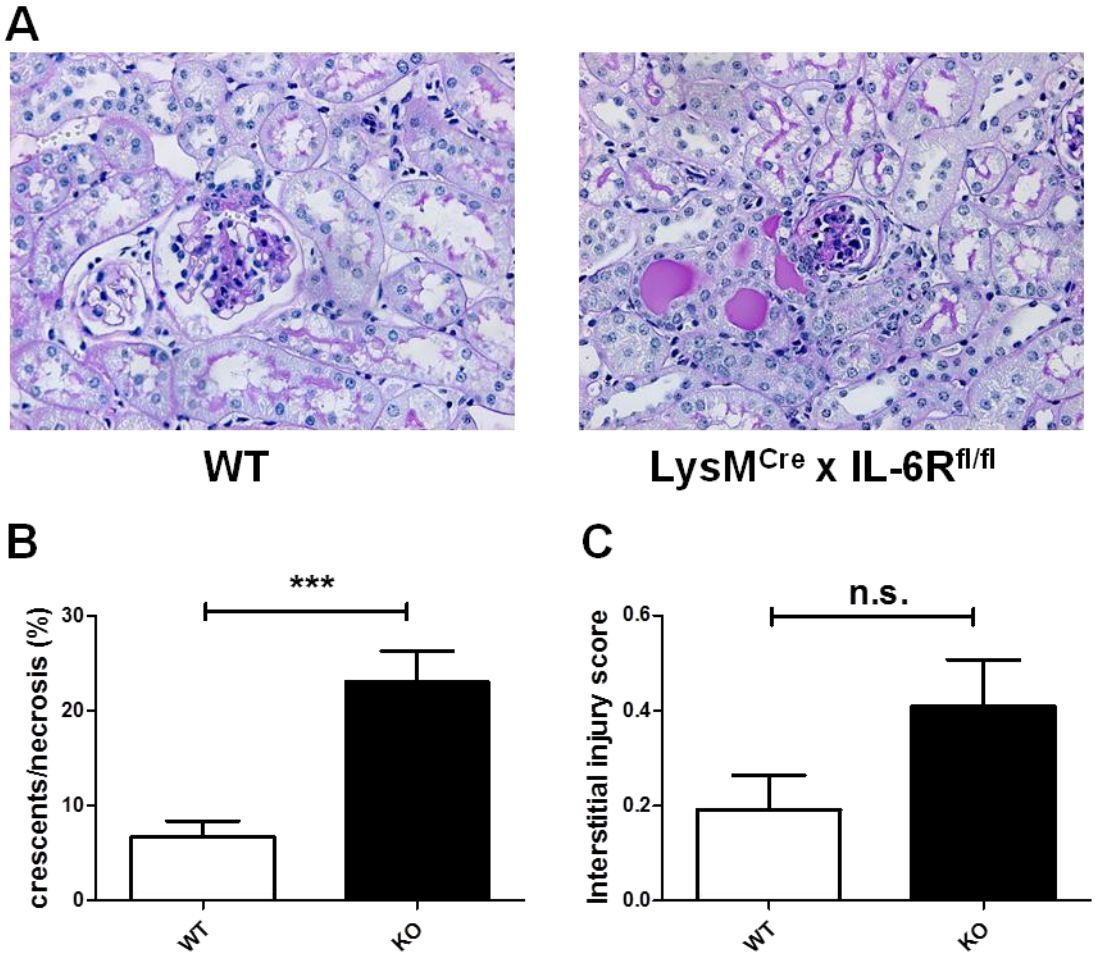

Figure 3.19: Renal injury is aggravated in LysM^{Cre} x IL-6R$^{fl/fl}$ mice in NTN nephritis.
(A) PAS staining of kidney sections from WT and LysM^{Cre} x IL-6R$^{fl/fl}$ mice at day 10 after induction of NTN (magnification x 400). **(B)** Quantification of glomerular crescents. **(C)** Quantification of interstitial injury. Error bars represent means ± SD. *** P<0.001; n.s.= not significant.

PAS stained kidney sections at day 10 after induction of NTN of mice lacking IL-6R in macrophages showed significantly aggravated renal damage (Figure 3.19

A). Formation of crescents was remarkably increased (figure 3.19 B) in cell specific knockout mice. Interstitial injury was also considerably elevated in LysM^{Cre} x IL-6R$^{fl/fl}$ mice, but did not reach statistically significant levels, whereas albuminuria (figure 3.20 A) and BUN levels (figure 3.20 B) remained unaltered.

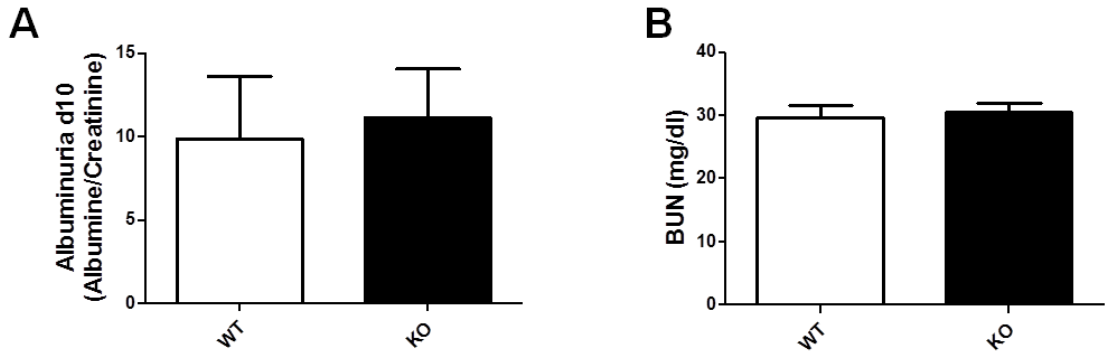

Figure 3.20: Renal functional parameters WT and LysM^{Cre} x IL-6R$^{fl/fl}$ in NTN nephritis. Renal function was assessed by determination of albumin-creatinine ratio **(A)** and serum BUN levels **(B)** on day 10. Bars represent means ± SD.

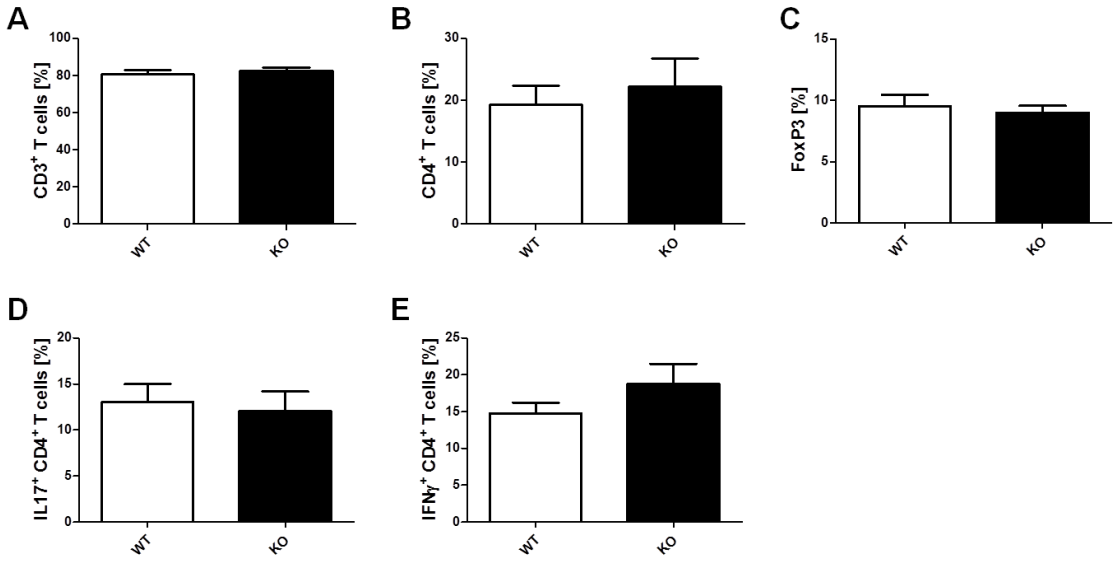

Figure 3.21: Renal T-cell subtypes of WT and LysM^{Cre} x IL-6R$^{fl/fl}$ mice in NTN nephritis. Percentages of CD3^{+} T-cells **(A)** CD4^{+} T-cells **(B),** and FoxP3^{+} T$_{regs}$ **(C)** remained unaltered in the kidney. **(C)** Renal Th17 immune response was determined by flow cytometry measurement of IL-17 producing CD4^{+} T-cells. **(D)** Renal Th1 immune response was determined by flow cytometry measurement of IFNγ producing CD4^{+} T-cells. Bars represent means ± SD.

FACS analysis indicated no difference in percentages of renal CD3^{+} (figure 3.21 A) and CD4^{+} (figure 3.21 B) T-cells. In addition, FoxP3^{+} T$_{regs}$ remained

unaltered in wildtype and knockout mice (figure 3.21 C). Renal Th17 (figure 3.21 D) and Th1 (figure 3.21 E) immune responses were also unaffected in LysM^{Cre} x IL-6R$^{fl/fl}$ mice.

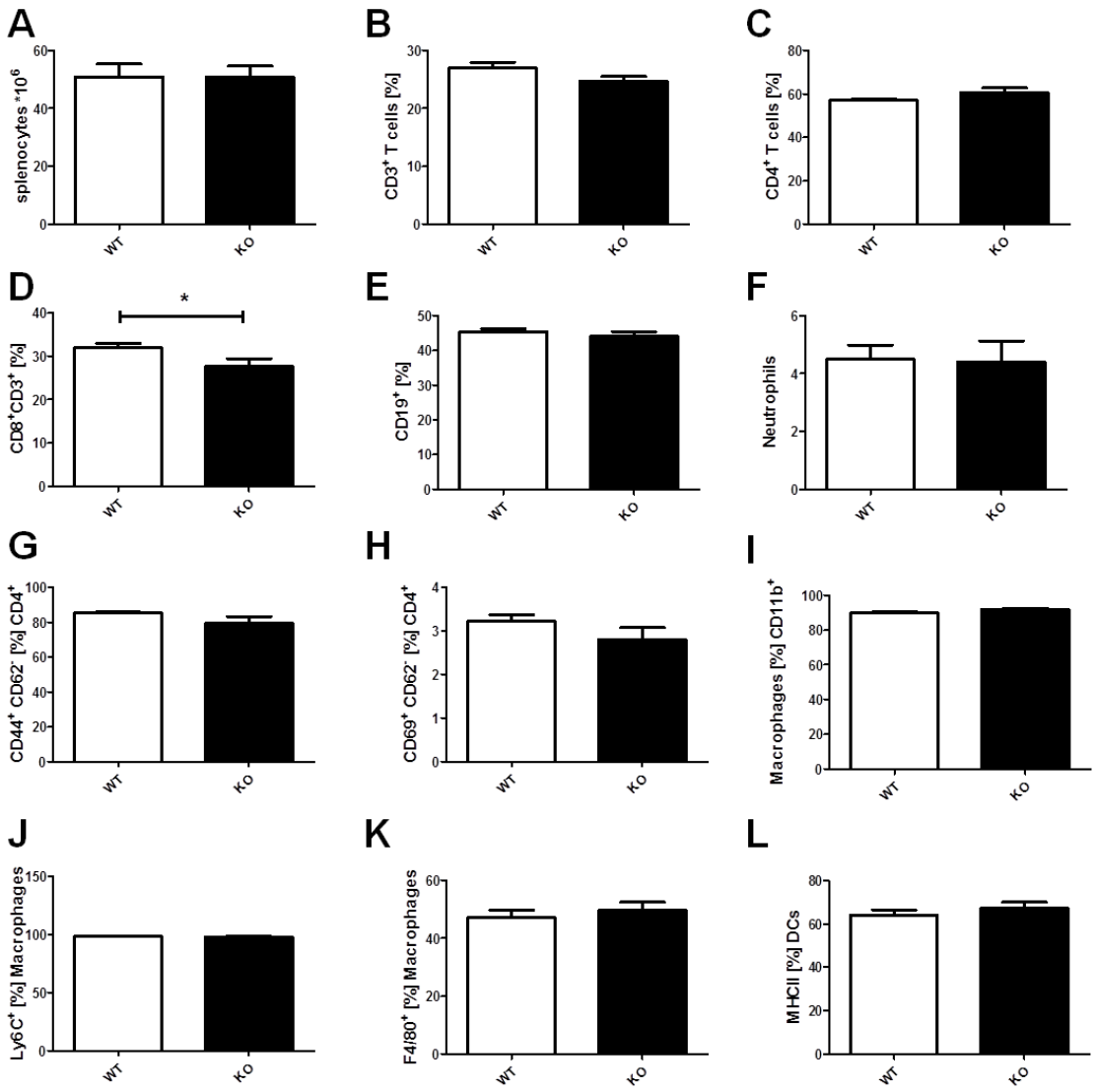

Figure 3.22: Splenocyte composition of WT and LysM^{Cre} x IL-6R$^{fl/fl}$ mice in NTN nephritis. **(A)** Total numbers of splenocytes from WT and LysM^{Cre} x IL-6R$^{fl/fl}$ mice were similar. Percentages of CD3^{+} T-cells **(B),** CD4^{+} T-cells **(C),** CD8^{+} **(D)**, B-cells **(E),** and neutrophils **(F)** in spleens of WT and LysM^{Cre} x IL-6R$^{fl/fl}$ mice were analyzed by flow cytometry. Percentages are shown. **(G)** Late and **(H)** early activation of Th-cells were similar. Percentages of total macrophages **(I)**, as well as Ly6C^{+} **(J)**, F4/80^{+} macrophages **(K)** and MHCII^{+} dendritic cells **(L)** were unaltered in WT and LysM^{Cre} x IL-6R$^{fl/fl}$ mice. Bars represent means ± SD. *P<0.05.

Specific knockout of IL-6R in LysM^{+} cells did not result in alteration of the cellular immune responses in the spleen, focusing on whole splenocyte numbers (figure 3.22 A), CD3^{+} T-cells (figure 3.22 B), CD4^{+} Th-cells (figure 3.22

C), B-cells (figure 3.22 E), or neutrophil granulocytes (figure 3.22 F). Late (figure 3.22 G) and early activation (figure 3.22 H) of $CD4^+$ T-cells also remained unaltered. Only the percentage of $CD8^+$ T-cells was slightly reduced in $LysM^{Cre}$ x $IL\text{-}6R^{fl/fl}$ mice (figure 3.22 D). In addition, splenocyte composition of macrophage subtypes also remained unaltered (figures 3.22 I-L).

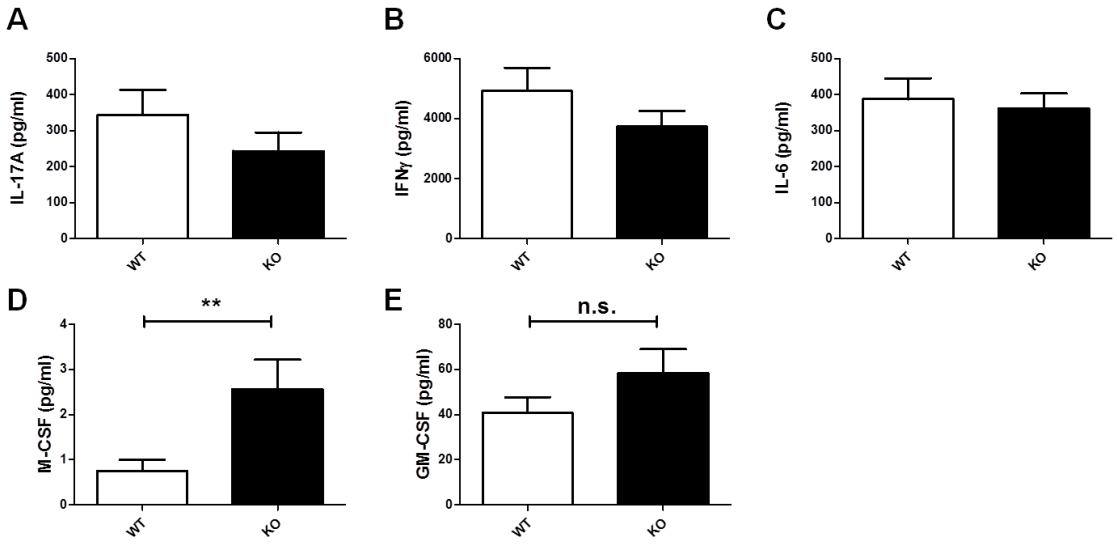

Figure 3.23: Systemic immune response of WT and $LysM^{Cre}$ x $IL\text{-}6R^{fl/fl}$ mice in NTN nephritis.
Splenocytes were cultured and re-stimulated with IgG for 72 h. Cytokine ELISAs were performed from supernatants, revealing similar production of IL-17A **(A)** and IFNγ **(B)**. Secretion levels of IL-6 **(C)** remained unaltered. Cytokine ELISAs from murine sera, however, show elevated levels of M-CSF **(D)** and GM-CSF **(E)** in $LysM^{Cre}$ x $IL\text{-}6R^{fl/fl}$ mice. Bars represent means ± SD. **P<0.01; n.s.= not significant.

Cytokine levels from splenocyte culture supernatants were measured by ELISA and revealed similar levels of the pro-inflammatory cytokines IL-17A (figure 3.23 A) and IFNγ (figure 3.23 B) in knockout mice. Likewise, secretion of IL-6 was unaffected (figure 3.23 C). Cytokine ELISA from murine sera, however, demonstrated significantly elevated levels of macrophage expanding and activating cytokine M-CSF in $LysM^{Cre}$ x $IL\text{-}6R^{fl/fl}$ mice (figure 3.23 D). Levels of GM-CSF were also increased in $LysM^{Cre}$ x $IL\text{-}6R^{fl/fl}$ mice, even though not statistically significant (figure 3.23 E).

In summary, induction of NTN in $LysM^{Cre}$ x $IL\text{-}6R^{fl/fl}$ mice again resulted in exacerbation of nephritis. In line, levels of M-CSF and GM-CSF were elevated in $LysM^{Cre}$ x $IL\text{-}6R^{fl/fl}$ mice, possibly indicating enhanced macrophage activation

as causative. However, aggravation of NTN in LysM^{Cre} x IL-6R$^{fl/fl}$ mice was not as severe as observed in aIL-6 treated mice.
This led to the assumption, that there might be another cell population, which is affected by IL-6 and mediates the course of glomerulonephritis in experimental NTN.

3.7 CD4^{+} T-cell specific abrogation of classical IL-6 signaling aggravates NTN

To assess IL-6 effects on CD4^{+} T-cells, we generated CD4^{Cre} x IL-6R$^{fl/fl}$ mice (cf. chapter 2.2.1). Mice bearing this genotype lack the IL-6 receptor in CD4 expressing cells, which should result in an impaired generation of a Th17 immune response. However, in addition, lack of IL-6 signaling in CD4^{+} T-cells could also result in reduction of anti-inflammatory Tr1 cell generation. To evaluate the importance of both possible pathways, NTN was induced and the outcome was studied.

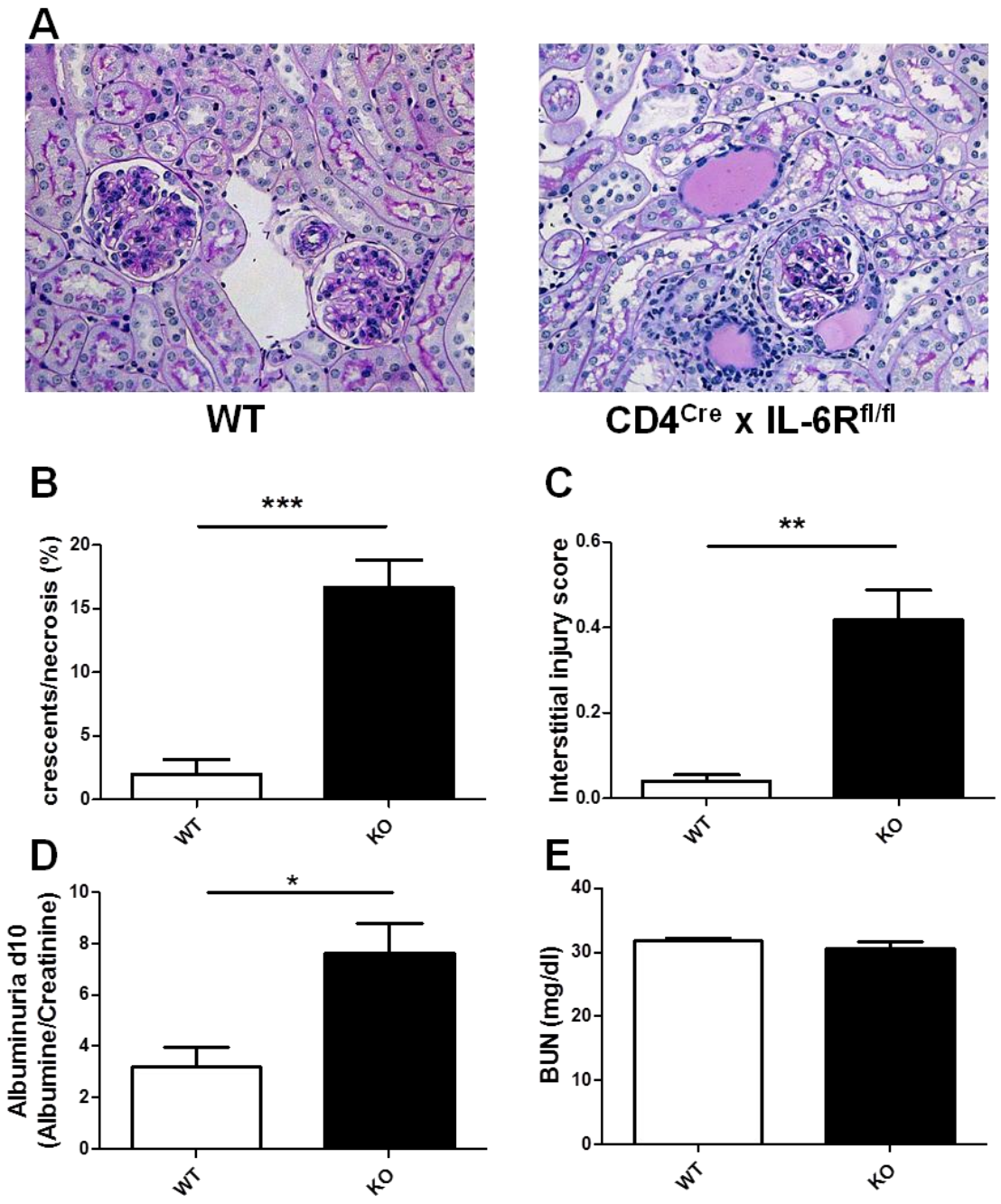

Figure 3.24: Renal injury of WT and CD4^{Cre} x IL-6R$^{fl/fl}$ in NTN model.
(A) PAS staining of kidney sections from WT and CD4^{Cre} x IL-6R$^{fl/fl}$ mice at day 10 after induction of NTN (magnification x 400). **(B)** Quantification of glomerular crescents. **(C)** Quantification of interstitial injury. Renal function was assessed by urinary albumin-creatinine ratio **(D)** and serum BUN levels **(E)** on day 10. Error bars represent means ± SD. *P<0.05; **P<0.01; *** P<0.001.

PAS stained kidney sections of mice lacking IL-6R in CD4^{+} T-cells at day 10 after induction of NTN displayed significantly elevated renal damage (figure 3.24 A) compared to WT mice. CD4^{Cre} x IL-6R$^{fl/fl}$ mice showed both, significantly

increased formation of crescents (figure 3.24 B) as well as interstitial injury (figure 3.24 C) in the kidney. In line, albuminuria was significantly increased in $CD4^{Cre}$ x $IL\text{-}6R^{fl/fl}$ mice (figure 3.24 D), whereas BUN levels remained unaltered (figure 3.24 E).

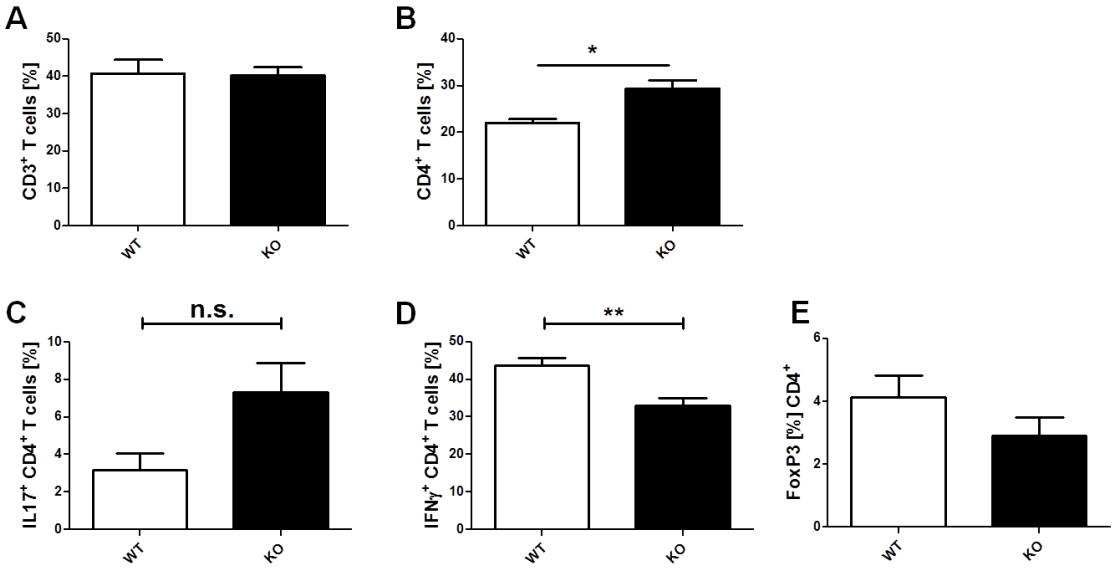

Figure 3.25: Renal T-cell subtypes of WT and $CD4^{Cre}$ x $IL\text{-}6R^{fl/fl}$ mice in NTN nephritis.
(A) Percentages of $CD3^+$ T-cells remained unaltered in the kidney. **(B)** Proportion of $CD4^+$ T-cells was found increased in $CD4^{Cre}$ x $IL\text{-}6R^{fl/fl}$ mice. **(C)** The renal Th17 immune response was increased, without reaching statistically significance. **(D)** The Th1 immune response was decreased in $CD4^{Cre}$ x $IL\text{-}6R^{fl/fl}$ mice. **(E)** Renal $FoxP3^+$ T_{regs} were unaltered. Bars represent means ± SD. *P<0.05; **P<0.01; n.s.= not significant.

Analysis of renal T-cell subsets by flow cytometry displayed no differences in the proportion of $CD3^+$ T-cells in the kidneys of WT and KO mice (figure 3.25 A). In contrast, $CD4^{Cre}$ x $IL\text{-}6R^{fl/fl}$ mice indicated increased percentages of renal $CD4^+$ T-cells (figure 3.25 B). Th17 immune response was slightly increased in, $CD4^{Cre}$ x $IL\text{-}6R^{fl/fl}$ mice (figure 3.25 C), but did not reach statistically significant levels, whereas the Th1 immune response was significantly decreased (figure 3.25 D). Percentages of T_{regs} were similar in WT and $CD4^{Cre}$ x $IL\text{-}6R^{fl/fl}$ mice (figure 3.25 E).

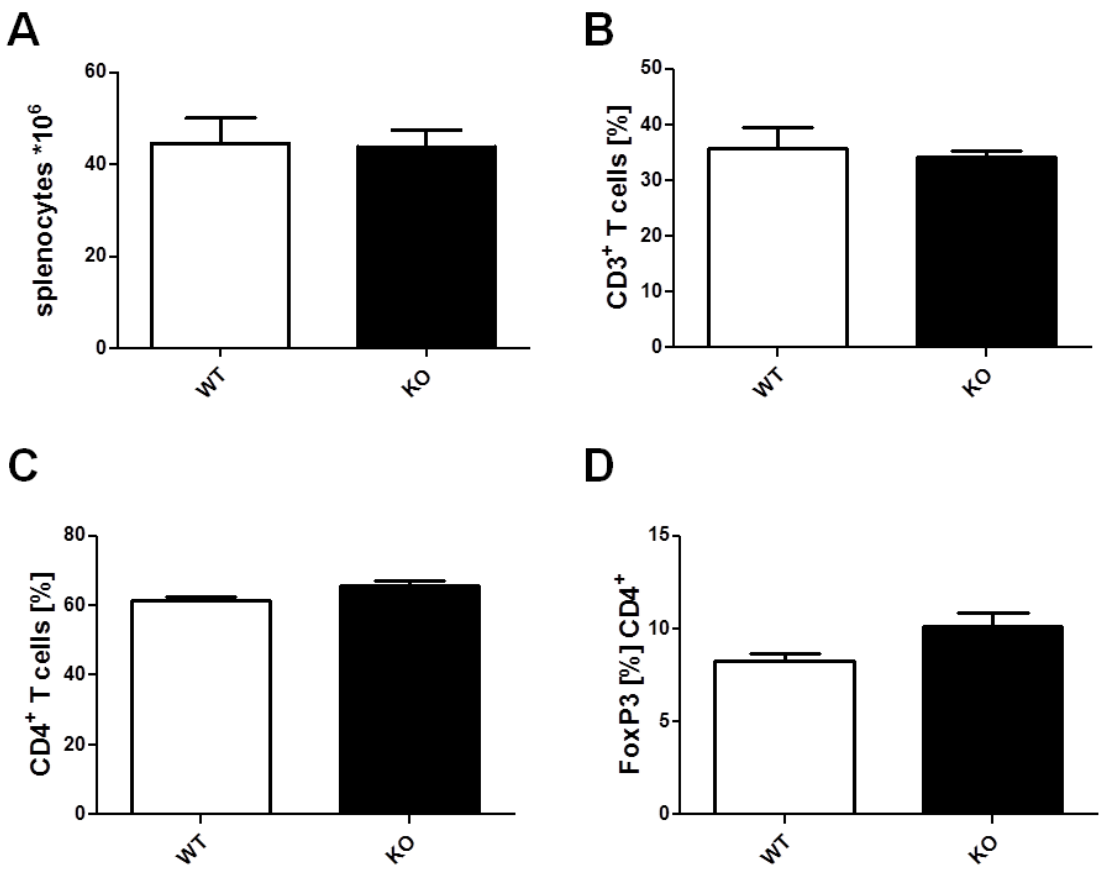

Figure 3.26: Splenocyte composition of WT and CD4^{Cre} x IL-6R$^{fl/fl}$ mice in NTN nephritis. **(A)** Total numbers of splenocytes from WT and CD4^{Cre} x IL-6R$^{fl/fl}$ mice were counted in single cell suspensions. Percentages of CD3^{+} T-cells **(B),** and CD4^{+} T-cells **(C)** were unaltered. **(D)** Percentages of FoxP3^{+} T_{regs} in the spleen were slightly reduced in ET mice. Bars represent means ± SD.

Mice lacking IL-6R in CD4^{+} cells were unaltered regarding to total splenocyte numbers (figure 3.26 A). Same is true for CD3^{+} (figure 3.26 B) and CD4^{+} T-cells (figure 3.26 C), as well as percentages of FoxP3^{+} T_{regs} (figure 3.26 D).

3.8 Anti-inflammatory Tr1 cells are dependent on IL-6

Data from the literature suggest an anti-inflammatory effect of IL-6 by induction of regulatory Tr1 cells [74,141]. We therefore wanted to assess the *in vitro* effect of IL-6 on this IL-10 secreting cell population. Whole murine splenocytes from transgenic mice with YFP expressing FoxP3^{+} regulatory T-cells were

sorted by FACS to obtain FoxP3$^+$ T_{regs} as well as FoxP3$^-$ CD4$^+$ T-cells (non-T_{regs}). Sorted non-T_{regs} and T_{regs} were stimulated with recombinant IL-6 for 72 h.

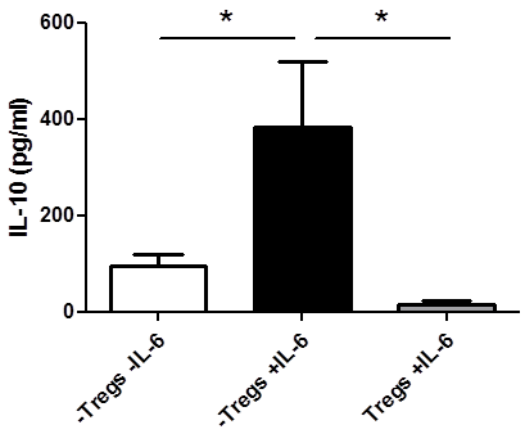

Figure 3.27: IL-10 production by T_{regs} and Tr1 cells after IL-6 stimulation *in vitro*.
Whole splenocytes were enriched for CD4$^+$ T-cells by MACS. CD4$^+$ T-cells were then sorted for expression of FoxP3 by FACS. Sorted T_{regs} and non-T_{regs} were cultured *in vitro* for 3 days in the presence or absence of IL-6. IL-10 production was assessed by ELISA. Bars represent means ± SD. *P<0.01.

Cytokine ELISA from supernatant revealed a pronounced increase in IL-10 secretion of FoxP3$^-$ CD4 T-cells, when stimulated with IL-6 (figure 3.27). Notably, FoxP3$^+$ T_{regs} showed no change of IL-10 production when stimulated with IL-6.

Based on these results, it was likely, even though not formally proven, that exacerbation of glomerulonephritis in CD4^{Cre} x IL-6R$^{fl/fl}$ mice might have resulted from impaired generation of anti-inflammatory Tr1 regulatory T-cells.

3.9 Complete absence of IL-6 impairs generation of Th17 immune responses and improves the course of NTN

Both, inhibition of IL-6 by antibodies as well as leukocyte subtype specific abrogation of IL-6R signaling resulted in aggravation of NTN. However, none of our IL-6 neutralization strategies resulted in significant impaired Th17 responses. We therefore wanted to assess effects of complete lack of IL-6 on Th17 responses and NTN.

Firstly, wild type and IL-6$^{-/-}$ mice were injected i.p. with NTN serum and sacrificed after 3 days to assess if induction of disease is equally efficient in WT and IL-6$^{-/-}$ mice (figure 3.28 A).

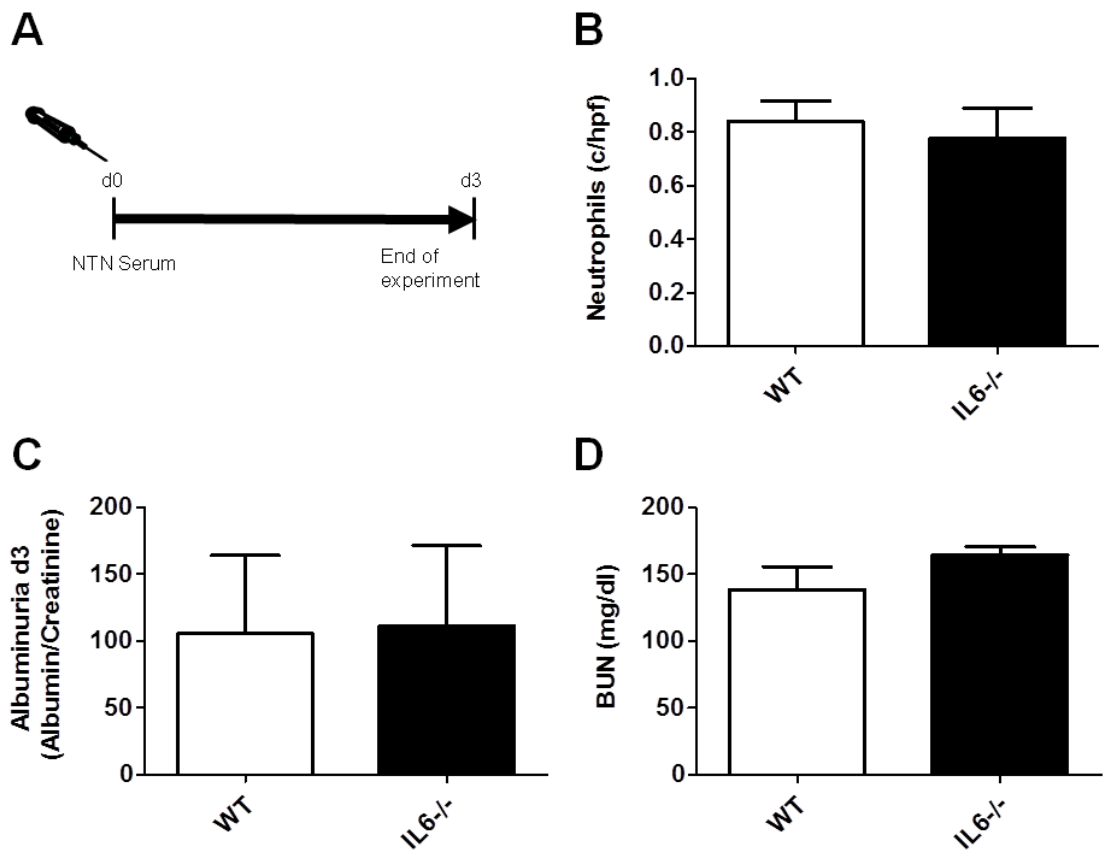

Figure 3.28: NTN in heterologous phase in IL-6$^{-/-}$.
(A) Experimental setup. **(B)** Neutrophil infiltration was quantified in GR-1 stained kidney sections at day 3. **(C)** Renal function was assessed by determination of albumin-creatinine ratio and serum BUN levels **(D)** on day 3. Bars represent means ± SD.

Induction of NTN in the heterologous phase in WT and IL-6 KO mice revealed no differences in infiltrating neutrophils into the kidney (figure 3.28 B). The same was true for albuminuria (figure 3.28) and BUN levels (figure 3.28 D). We therefore studied the autologous phase of disease (figure 3.29 A).

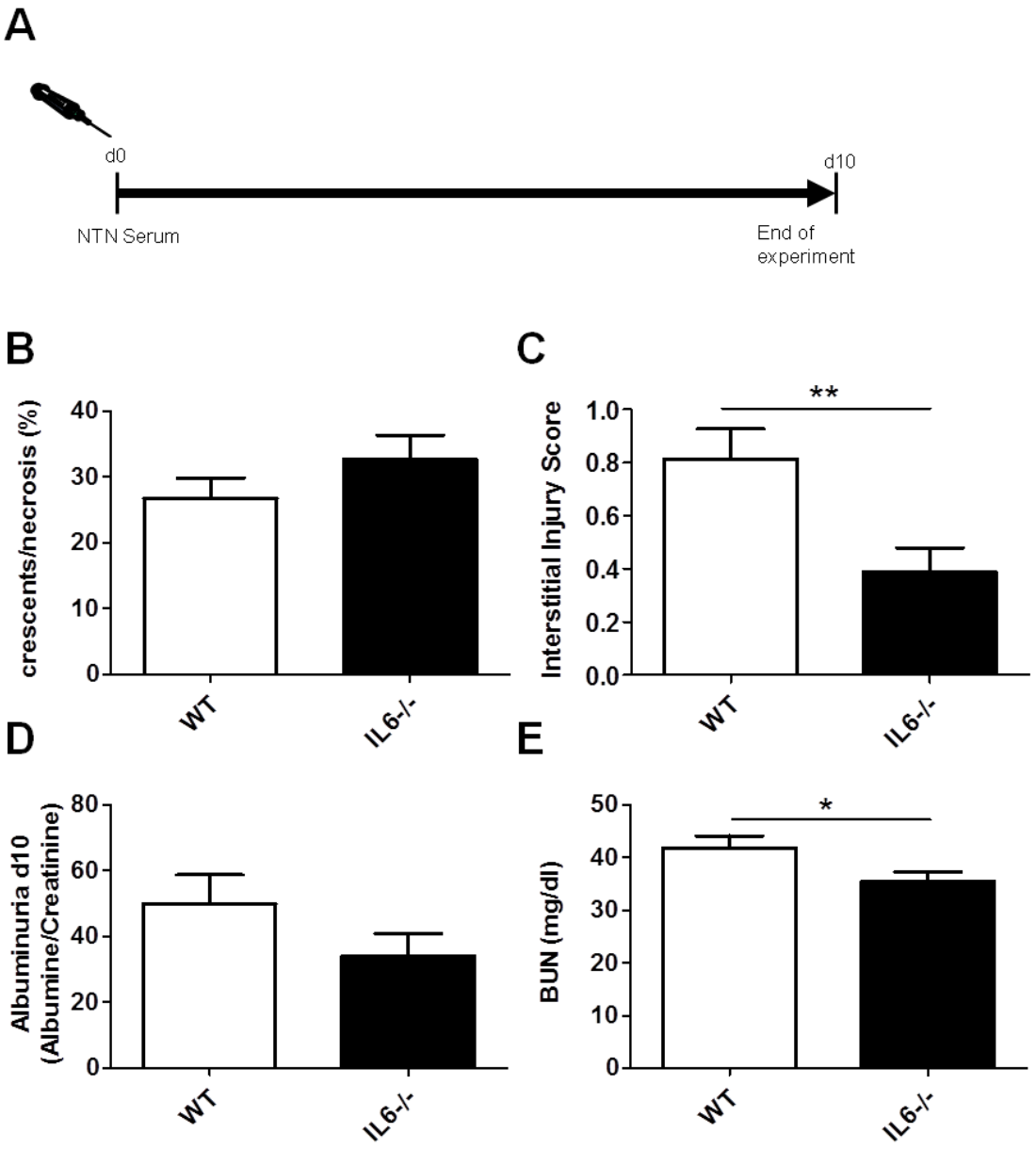

Figure 3.29: NTN in IL-6$^{-/-}$ mice.
(A) Experimental setup. **(B)** Quantification of glomerular crescents in WT and IL-6$^{-/-}$ mice. **(C)** Quantification of interstitial injury shows decreased damage in IL-6$^{-/-}$ mice. Renal dysfunction was assessed by urinary albumin-creatinine ratio **(D)** and serum BUN levels **(E)** on day 10. Bars represent means ± SD. *P<0.05; **P<0.01.

Formation of crescents was similar in both groups of mice (figure 3.29 B), but renal interstitial injury was clearly reduced compared to wildtype mice (figure 3.29 C). In addition, renal dysfunction was ameliorated in IL-6$^{-/-}$ mice with a trend to decreased albuminuria (figure 3.29 D) and significantly lower BUN levels (figure 3.29 E).

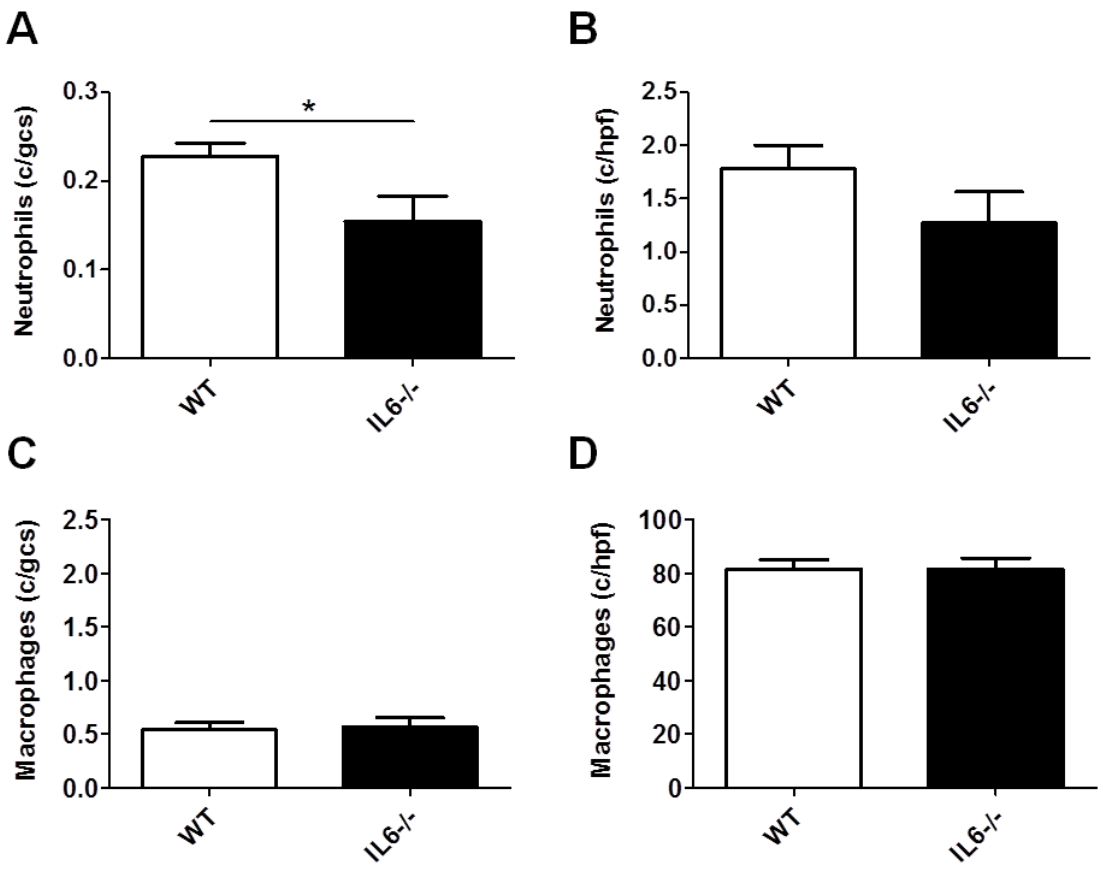

Figure 3.30: Renal leukocyte infiltration in WT and IL-6$^{-/-}$ mice after induction of NTN. Numbers of neutrophils per glomerular crescents **(A)** and high power field **(B)** were decreased in IL-6$^{-/-}$ mice. Quantification of Mac-2^{+} macrophages per glomerular crescent **(C)** and F4/80^{+} macrophages per high power field **(D)** were unaltered in WT and KO mice. Bars represent means ± SD. *P<0.01.

Immunohistochemically stained kidney sections at day 10 after induction of NTN of wildtype and IL-6$^{-/-}$ mice showed less infiltrating glomerular neutrophils (figure 3.30 A), while interstitial neutrophils remained unaltered (figure 3.30 B). Also, infiltrating macrophages remained unaltered in both glomeruli (figure 3.30 C) and interstitium (figure 3.30 D).

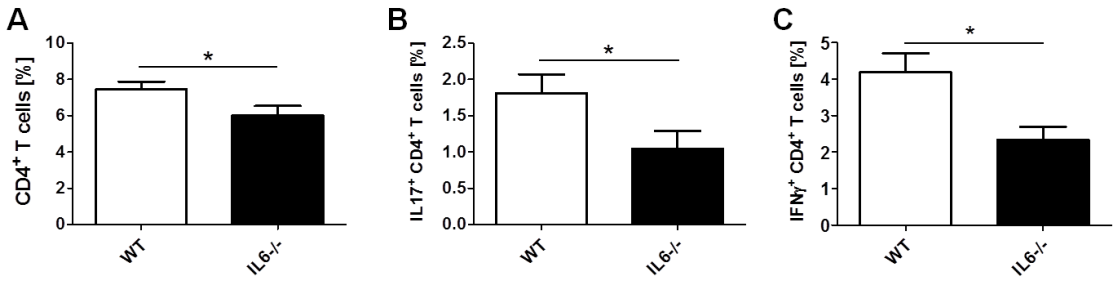

Figure 3.31: FACS analysis of renal T-cell cubtypes in WT and IL-6$^{-/-}$ mice after induction of NTN.
(A) Percentages of CD4^{+} T-cells were reduced in IL-6$^{-/-}$ mice in the kidney. **(B)** Renal Th17 immune response was measured by flow cytometry of IL-17 producing CD4^{+} T-cells. **(C)** Renal Th1 immune response was measured by flow cytometry of IFNγ producing CD4^{+} T-cells. Bars represent means ± SD. *P<0.01.

In addition, flow cytometric analysis revealed a lower percentage of renal CD4^{+} T-cells in IL-6$^{-/-}$ mice (figure 3.31 A). In contrast to our previous experiments, FACS analysis of IL-17A or IFNγ producing CD4^{+} T-cells in the kidney also unveiled reduced Th17 (figure 3.31 B) and Th1 (figure 3.31 C) immune response in IL-6$^{-/-}$ mice.

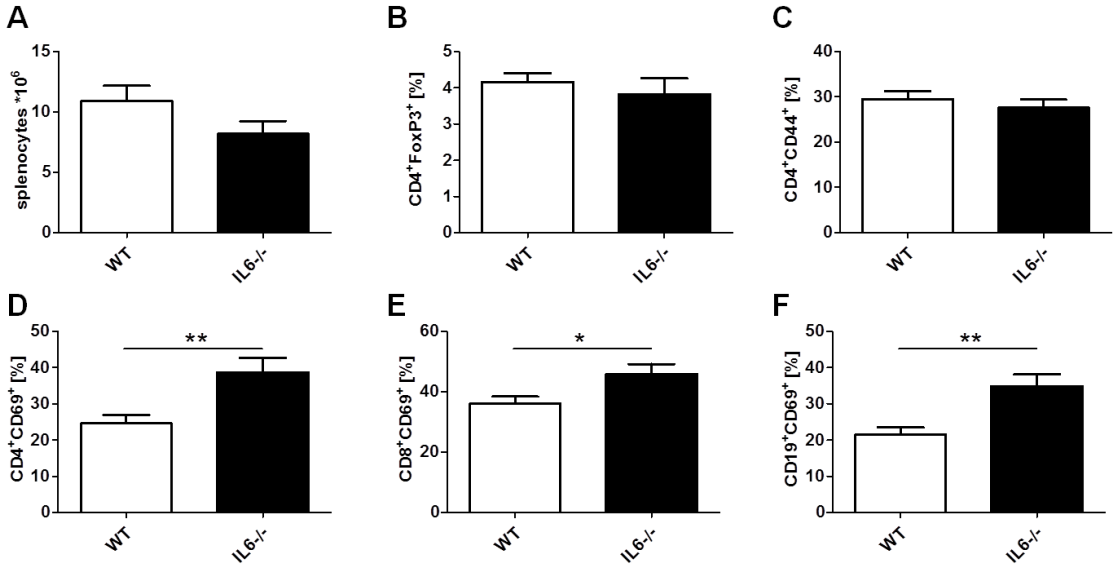

Figure 3.32: Splenocyte composition in WT and IL-6$^{-/-}$ mice after induction of NTN.
(A) Total numbers of splenocytes from WT and IL-6$^{-/-}$ mice were count in single cell suspensions. Numbers of FoxP3^{+} T$_{regs}$ **(B)** in spleens of WT and IL-6$^{-/-}$ mice were analyzed by FACS. Percentage is shown on the Y-axis. CD4^{+}CD44^{+} T-cells **(C)**, activation of CD4^{+} T-cells **(D)**, CD8^{+} T-cells **(E)** or activation status of B-cells **(F)** were analyzed by FACS staining of spleens from WT and IL-6$^{-/-}$ mice. Bars represent means ± SD. *P<0.05; **P<0.01.

Spleen sizes (figure 3.32 A), as well as percentages of T_{regs} (figure 3.32 B) remained similar. Assessment of $CD4^+$ T-cell activation unveiled unaltered expression of late activation marker CD44 (figure 3.32 C) whereas early activation of $CD4^+$ T-cells (figure 3.32 D), $CD8^+$ T-cells (figure 3.32 E) and B-cells (figure 3.32 F) was surprisingly upregulated. Induction of NTN in IL-6 deficient mice thus resulted in reduced nephritogenic Th17 immune response with subsequent amelioration of the disease. Importantly, also renal macrophage influx was not elevated as seen in the previous experiments using different IL-6 inhibition strategies. However, not all parameters of renal damage were improved and general leukocyte activation remained higher in the knockout group.

To better study effects mediated by the effects of IL-6 on Th17 responses, accelerated NTN was induced in wildtype and IL-6 deficient mice. In our hands this model leads to a much more prominent induction of Th17 immunity. Therefore, mice were pre-immunized with sheep IgG in complete Freund's adjuvant for 5 days to allow for Th17 immune responses to develop (figure 3.33 A). NTN was subsequently induced by i.p. injection of nephrotoxic serum.

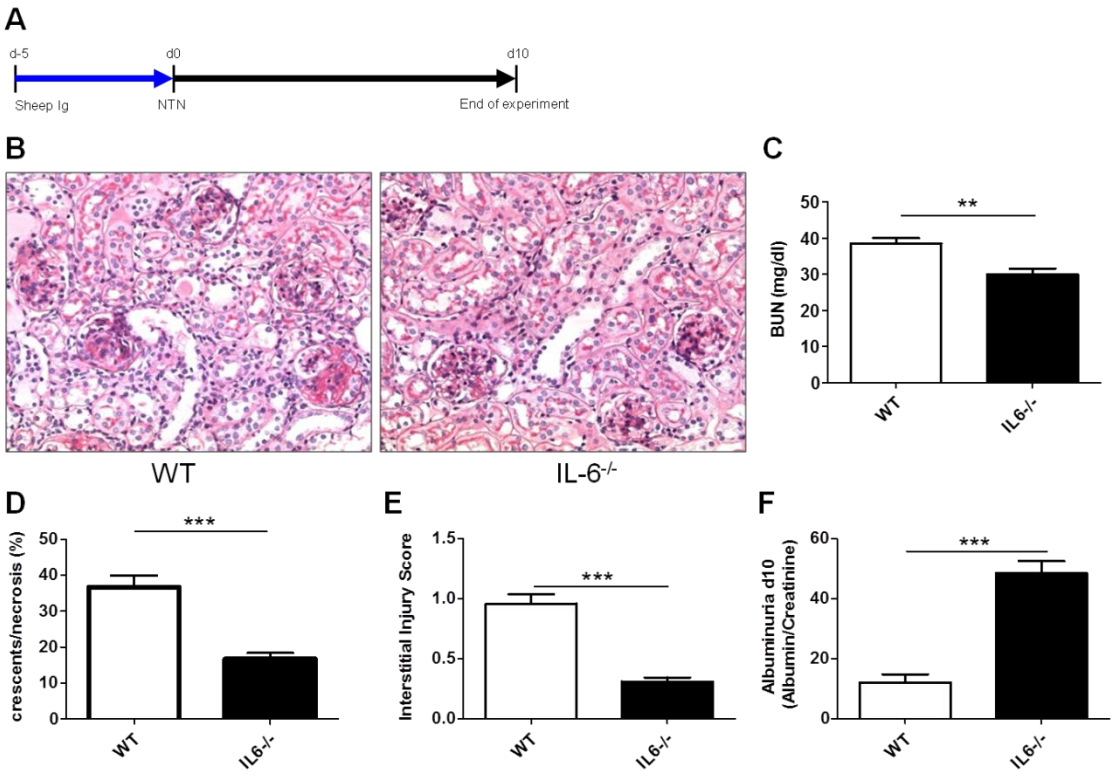

Figure 3.33: Accelerated NTN in IL-6$^{-/-}$.
(A) Experimental setup. C57BL/6J and IL-6$^{-/-}$ mice were pre-immunized with 0.5 mg sheep IgG in CFA for 5 days. NTN was induced on d 0 and mice were sacrificed on d 10. **(B)** PAS staining of kidney sections from WT and IL-6$^{-/-}$ mice at day 10 after induction of NTN (magnification x 400). Determination of renal injury was performed by quantification of glomerular crescents **(C)** and interstitial injury **(D)**. **(E)** Renal function was assessed by serum BUN levels at day 10. Bars represent means ± SD. **P<0.01; *** P<0.001.

PAS stained kidney sections at day 10 after induction of NTN of pre-immunized C57BL/6J and IL-6$^{-/-}$ mice showed significant amelioration of the disease (figure 3.33 B) in IL-6 deficient mice. Again, BUN levels at day 10 after induction of NTN were significantly reduced in IL-6$^{-/-}$ mice (figure 3.33 E) and revealed even more improvement of disease compared to previous results (cf. figure 3.29 E). In addition, in accelerated NTN crescent formation in glomeruli was also significantly reduced in IL-6$^{-/-}$ mice (figure 3.33 C), unlike our results in non-accelerated NTN (cf. figure 3.29 B). Again, renal interstitial injury was clearly reduced compared to wildtype mice (figure 3.33 D), as it was in non-accelerated NTN (cf. figure 3.29 C).

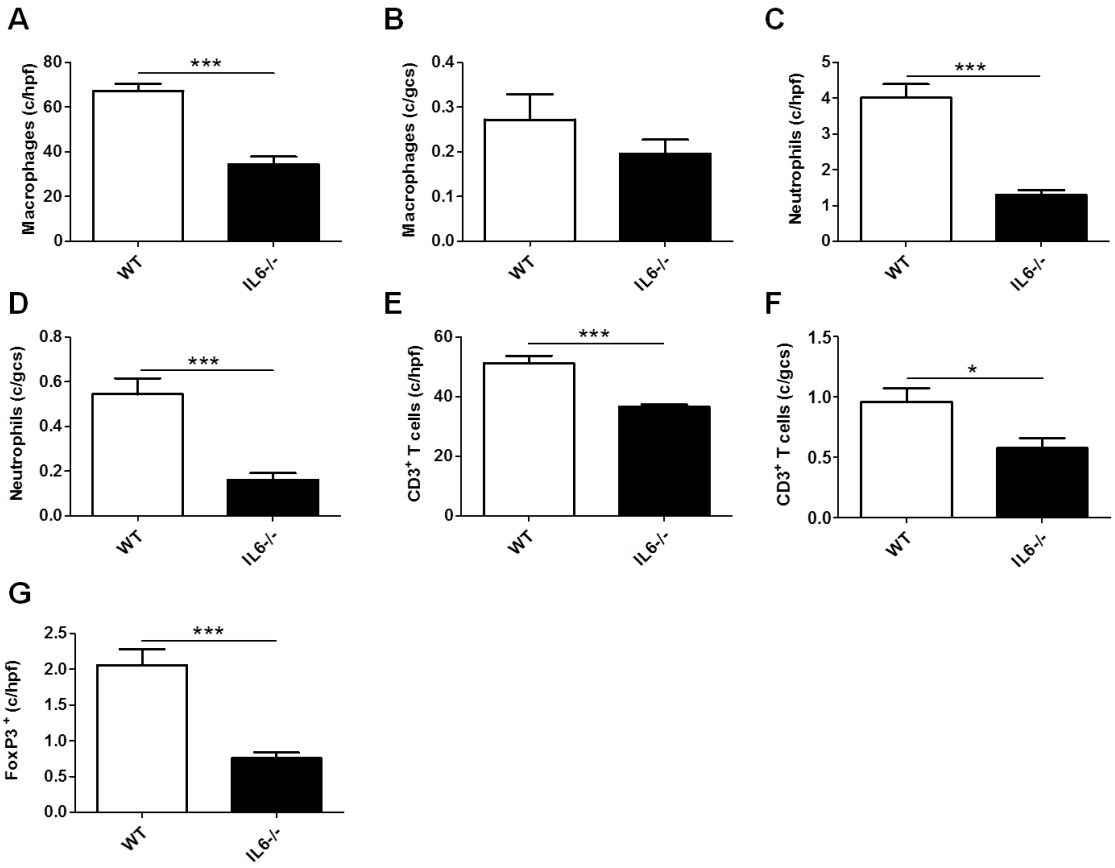

Figure 3.34: Renal leukocyte infiltration in WT and IL-6$^{-/-}$ mice after induction of accelerated NTN.
Kidney sections from WT and IL-6$^{-/-}$ mice at day 10 after induction of NTN were stained by IHC. Quantification of F4/80^{+} macrophages per high power field **(A)**, Mac-2^{+} macrophages per gcs **(B)**, neutrophils per high power field **(C)**, and neutrophils per gcs **(D)** were reduced in IL-6$^{-/-}$ mice. Quantification of CD3^{+} T-cells per high power filed **(E)** and gcs **(F)** revealed lower numbers of infiltrating T-cells in the kidneys of IL-6$^{-/-}$ mice. **(G)** Quantification of FoxP3^{+} T-cells showed diminished T_{regs} in the kidney of IL-6$^{-/-}$ mice. Bars represent means ± SD. *P<0.05; *** P<0.001.

The observed amelioration of disease in IL-6$^{-/-}$ mice was also matched by reduced leukocyte infiltration. Interstitial F4/80^{+} macrophages (figure 3.34 A) as well as glomerular Mac-2^{+} macrophages (figure 3.34 B) were reduced. Similarly, less infiltrating neutrophils were found in interstitial tissue (figure 3.34 C) and glomeruli (figure 3.34 D). Finally, numbers of CD3^{+} T-cells in interstitial tissue (figure 3.34 E) and glomeruli (figure 3.34 F) as well as the amounts ofFoxP3^{+} T_{regs} (figure 3.34 G) were significantly reduced in IL-6 deficient mice compared to WT animals.

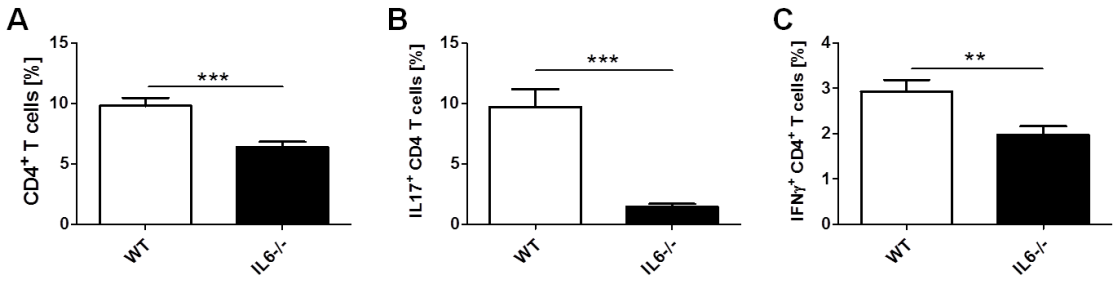

Figure 3.35: Renal T-cell subtype composition in WT and IL-6$^{-/-}$ mice after induction of accelerated NTN.
(A) Percentages of CD4^{+} T-cells were reduced in IL-6$^{-/-}$ mice in the kidney. **(B)** Renal Th17 immune response was measured by flow cytometry of IL-17 producing CD4^{+} T-cells. **(C)** Renal Th1 immune response was measurement by flow cytometry of IFNγ producing CD4^{+} T-cells. Bars represent means ± SD. **P<0.01; *** P<0.001 .

In line with protection from disease, both renal (figure 3.35 B) and systemic (figure 3.37 A) Th17 responses were almost absent in IL-6 deficient mice when compared to WT mice. In relation to non-accelerated NTN, reduction of renal CD4^{+} T-cells (figure 3.35 A) and renal Th1 immune response (figure 3.35 C) was even more pronounced.

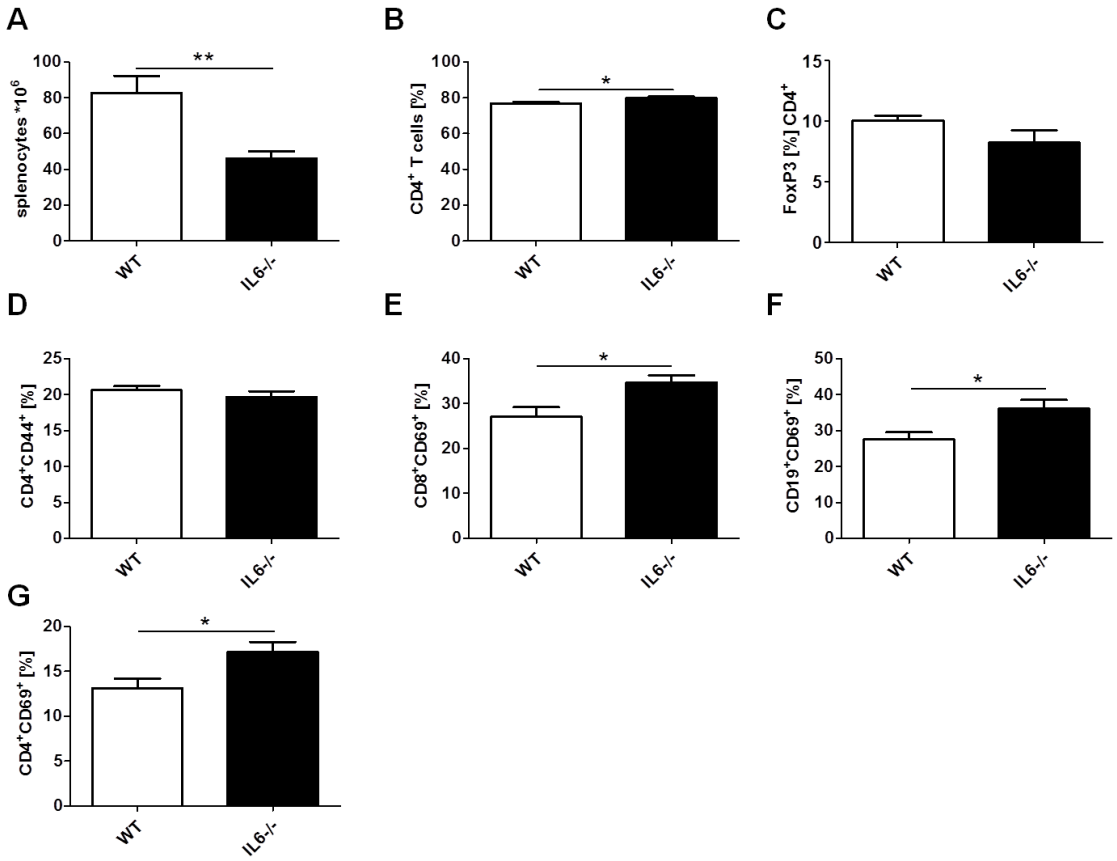

Figure 3.36: Splenocyte composition in WT and IL-6$^{-/-}$ mice after induction of accelerated NTN.
(A) Total numbers of splenocytes from WT and IL-6$^{-/-}$ mice were count in single cell suspensions. Percentages of CD4^{+} T-cells **(B)** and FoxP3^{+} T_{regs} **(C)** in spleens of WT and IL-6$^{-/-}$ mice were analyzed by FACS. CD4^{+}CD44^{+} T-cells **(D)**, early activation of CD8^{+} T-cells **(E)**, B-cells **(F),** and CD4^{+} T-cells **(D)**, were analyzed by FACS staining of spleens from WT and IL-6$^{-/-}$ mice. Error bars represent means ± SD. *P<0.05; **P<0.01.

IL-6 deficiency also resulted in reduced numbers of whole splenocytes (figure 3.36 A) in accelerated NTN, whereas distribution of T_{regs} (figure 3.36 C), as well as CD44^{+} CD4 T-cells (figure 3.36 D), remained unaltered. Interestingly, while total numbers of splenocytes were reduced in IL-6 deficient mice, percentages of CD4^{+} T-cells were increased (figure 3.36 B). Again, activation of CD4^{+} T-cells (figure 3.36 G), CD8^{+} T-cells (figure 3.36 E), and B-cells (figure 3.36 F) was upregulated, as had been observed in non-accelerated NTN (cf. figure 3.32).

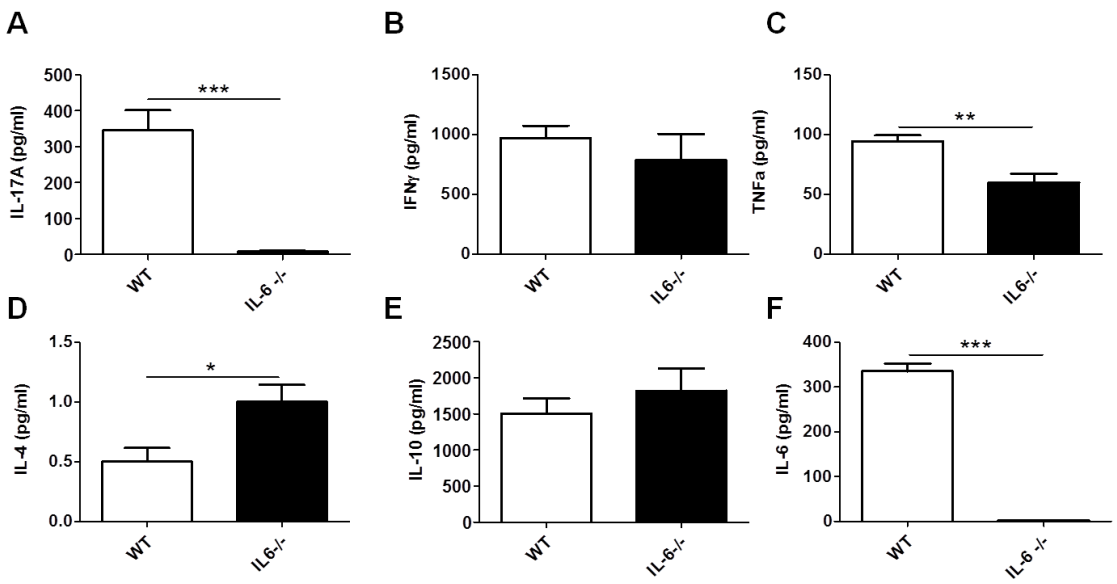

Figure 3.37: Systemic immune response in WT and IL-6$^{-/-}$ mice after induction of accelerated NTN.
Splenocytes were cultured and re-stimulated with IgG for 72 h. The supernatants were collected and analyzed for cytokine release by ELISA. **(A)** The production of IL-17A was almost absent. Systemic Th1 immune response remained unaltered **(B)**. Levels of TNFα **(C)** were reduced in IL-6$^{-/-}$ mice. Secretion of anti-inflammatory cytokines IL-4 **(D)** and IL-10 **(E)** was increased in IL-6$^{-/-}$ mice. Production of IL-6 was extinguished in IL-6$^{-/-}$ mice. Bars represent means ± SD. *P<0.05; **P<0.01; *** P<0.001.

Analysis of systemic immune responses by ELISA from splenocyte culture supernatants revealed a decreased secretion of the pro-inflammatory cytokines IL-17 A (figure 3.37 A), and TNFα (figure 3.37 C) in IL-6$^{-/-}$ mice. IFNγ levels remained the same (figure 3.37 B). On the other hand, secretion of the anti-inflammatory cytokine IL-4 (figure 3.37 D) was enhanced, while IL-10 (figure 3.37 E) was not different in IL-6$^{-/-}$ deficient mice. As expected, secretion of IL-6 was absent in the IL6$^{-/-}$ mice compared to the WT animals (figure 3.37 F).

In summary, complete absence of IL-6 impaired the development of nephritogenic Th17 immune responses and consequently ameliorated NTN. This improvement of diseases outcome was more prominent in accelerated NTN, which is a model that is much more dependent on IL-17.

3.10 Inhibition of IL-6 signaling in the induction but not the effector phase attenuates autoimmunity and glomerulonephritis in experimental ANCA vasculitis

Our studies documented both pro- and anti-inflammatory effects of IL-6. The pro-inflammatory functions of IL-6 involve development of Th17 responses and induction of antibody secretion. We therefore decided to study anti-IL-6 treatment in a different experimental mouse model, which strongly depends on antibodies and Th17 responses.

To define the role of IL-6 signaling in a model of anti-neutrophil cytoplasmic antibody (ANCA) vasculitis, autoimmune anti-myeloperoxidase (MPO) glomerulonephritis (AiaMPOGN) was induced in C57BL/6 mice.

In order to investigate whether inhibition of IL-6 signaling during the initiation phase of AiaMPOGN, when ANCA and nephritogenic Th17 responses develop, aIL-6R antibody or IgG control were applied on days -15 and -7 prior to induction of disease by NTN injection on day 0 (figure 3.38 A).

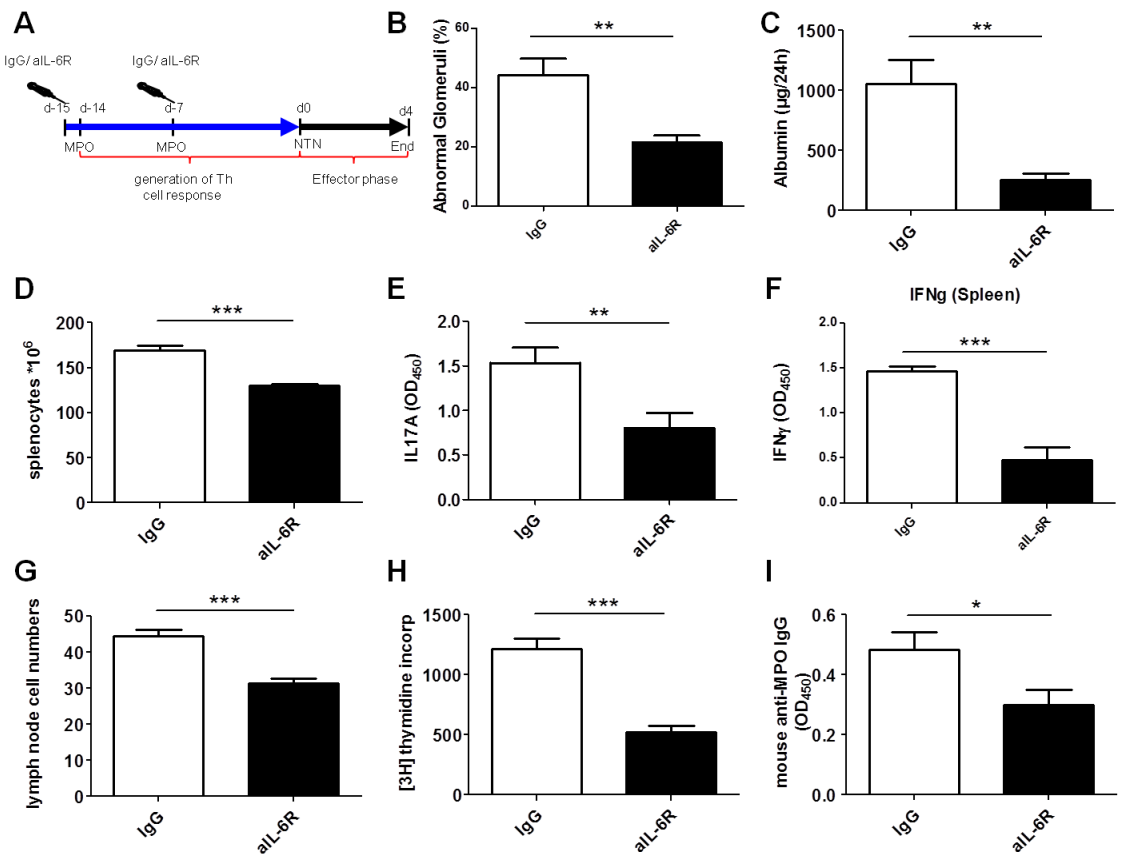

Figure 3.38: Renal and systemic effects of aIL-6R antibody blockade in the initiation phase of AiaMPOGN.
(A) Experimental setup. C57BL/6J mice were pre-immunized with murine MPO in Freund's adjuvant 14 and 7 day prior to NTN induction. Mice were injected with aIL-6R or control IgG on d-15 and d-7. NTN was administrated on d0 and mice were sacrificed on d4. **(B)** PAS staining of kidney sections were quantified for abnormal glomeruli. **(C)** Albuminuria was determined by ELISA. Total numbers of splenocytes **(D)** and lymph nodes **(G)** were counted. Secretion of the pro-inflammatory cytokines IL17A **(E)** and IFNγ **(F)** was quantified from splenocyte culture supernatants after 72h. **(H)** anti-MPO IgG was measured by ELISA. **(I)** Proliferation rate of splenocytes was determined by [^{3}H] thymidine assay. Bars represent means ± SD. *P<0.05; **P<0.01; *** P<0.001.

Early aIL-6R blockade diminished autoimmunity and attenuated glomerular injury in AiaMPOGN, as shown by lower percentages of abnormal glomeruli (figure 3.38 B) and lower amounts of albumin in the urine of m-tocilizumab treated animals (3.38 C). Total splenocyte numbers were markedly decreased in m-tocilizumab treated mice (figure 3.38 D), as were systemic Th17 (figure 3.38 E) and Th1 (figure 3.38 F) immune responses and total numbers of lymph node cells (figure 3.38 G). Total anti-MPO IgG levels were also reduced in mice treated with m-tocilizumab antibody (figure 3.38 H). Furthermore, splenocyte

proliferation was significantly diminished in the m-tocilizumab treated animals (figure 3.38 I).

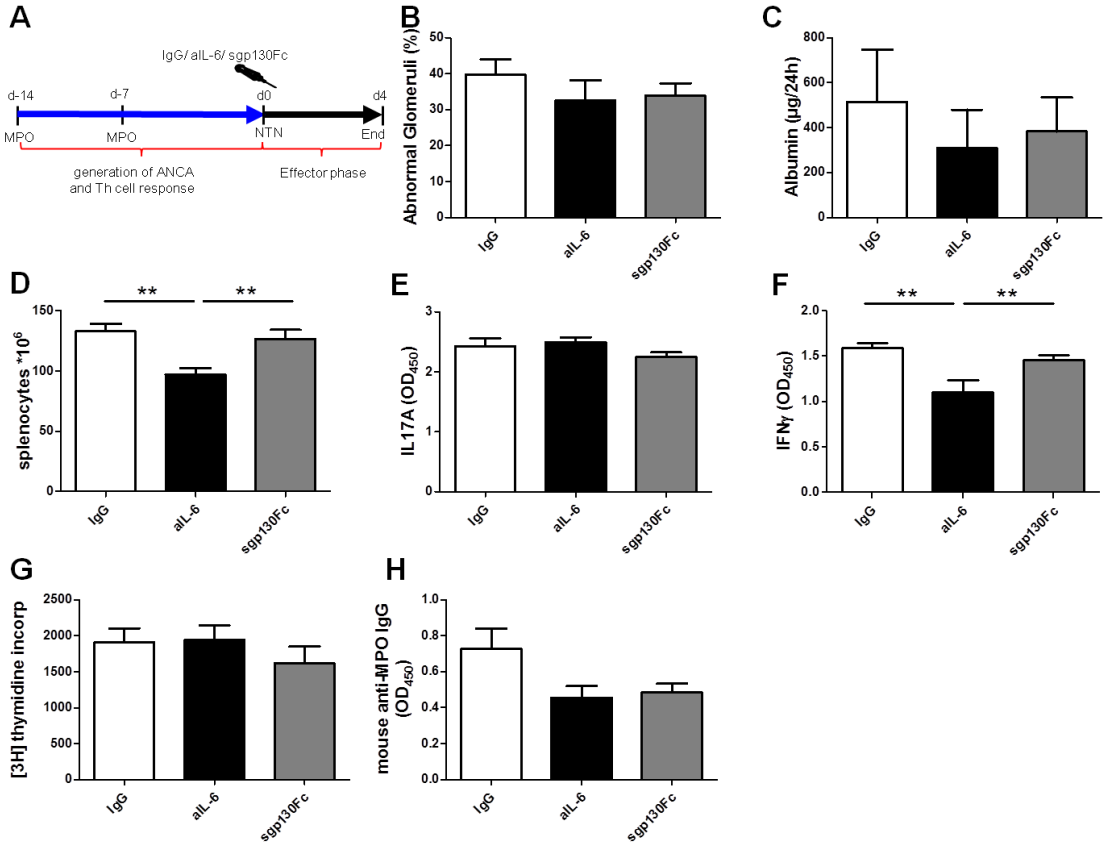

Figure 3.39: Renal injury of AiaMPOGN induced mice after blockade of classical or *trans*-signaling of IL-6 in effector phase.
(A) Experimental setup. C57BL/6J mice were pre-immunized with 0.5 mg murine MPO in Freund's adjuvant 14 and 7 day prior to NTN induction. Mice were injected with aIL-6, spg130Fc or IgG as control on d0. Mice were sacrificed on d 4. **(B)** PAS staining of kidney sections from IgG, aIL-6 and spg130Fc treated C57BL/6J mice were quantified for abnormal glomeruli. **(C)** Total numbers of splenocytes from IgG, aIL-6, and sgp130Fc treated C57Bl/6 mice were counted. Levels of IL17A **(D)** and IFNγ **(E)** were assessed from splenocyte culture supernatants after 72h. **(F)** Albuminuria was determined by ELISA. **(G)** anti-MPO IgG was measured by ELISA. **(H)** Proliferation rate of splenocytes was determined by [^{3}H] thymidine assay. Bars represent means ± SD. **P<0.01.

Next, we wanted to evaluate whether IL-6 directed treatment in the effector phase of the disease, after autoimmunity is already established, also has the potential to reduce renal injury. For this purpose, C57BL/6 mice were immunized with murine MPO on days -14 and -7. Antibodies for differential IL-6 signaling inhibition were applied at day 0. Nephritogenic immune responses were induced via application of subnephritogenic doses of NTN at day 0 and mice were sacrificed four days later (figure 3.39 A).

Appearance of abnormal glomeruli was similar in aIL-6 and sgp130Fc treated animals (figure 3.39 B). Total numbers of splenocytes were significantly reduced in mice treated with aIL-6 antibody, but not in animals treated with sgp130Fc (figure 3.39 C). Pro-inflammatory cytokines were assessed by ELISA. While Th17 immune responses remained unaltered independently of the inhibited signaling pathway (figure 3.39 D), blockade of pan IL-6 signaling by aIL-6 but not blockade of *trans*-signaling by the fusion protein sgp130Fc was capable of reducing Th1 immune responses (3.39 E). Albumin levels remained the same in all groups (figure 3.39 F). As expected, levels of anti-MPO IgG were also unaltered (figure 3.39 G). Proliferation of splenocytes, determined by [^{3}H]thymidine assay, was unaffected in both aIL-6 and sgp130Fc treated animals (figure 3.39 H).

In order to reveal, if aIL-6R antibody might be a more powerful tool for effector phase treatment of experimental ANCA vasculitis, AiaMPOGN was induced in C57BL/6 as described above, but mice were then treated with m-tocilizumab instead of aIL-6 or sgp130Fc (figure 3.40 A).

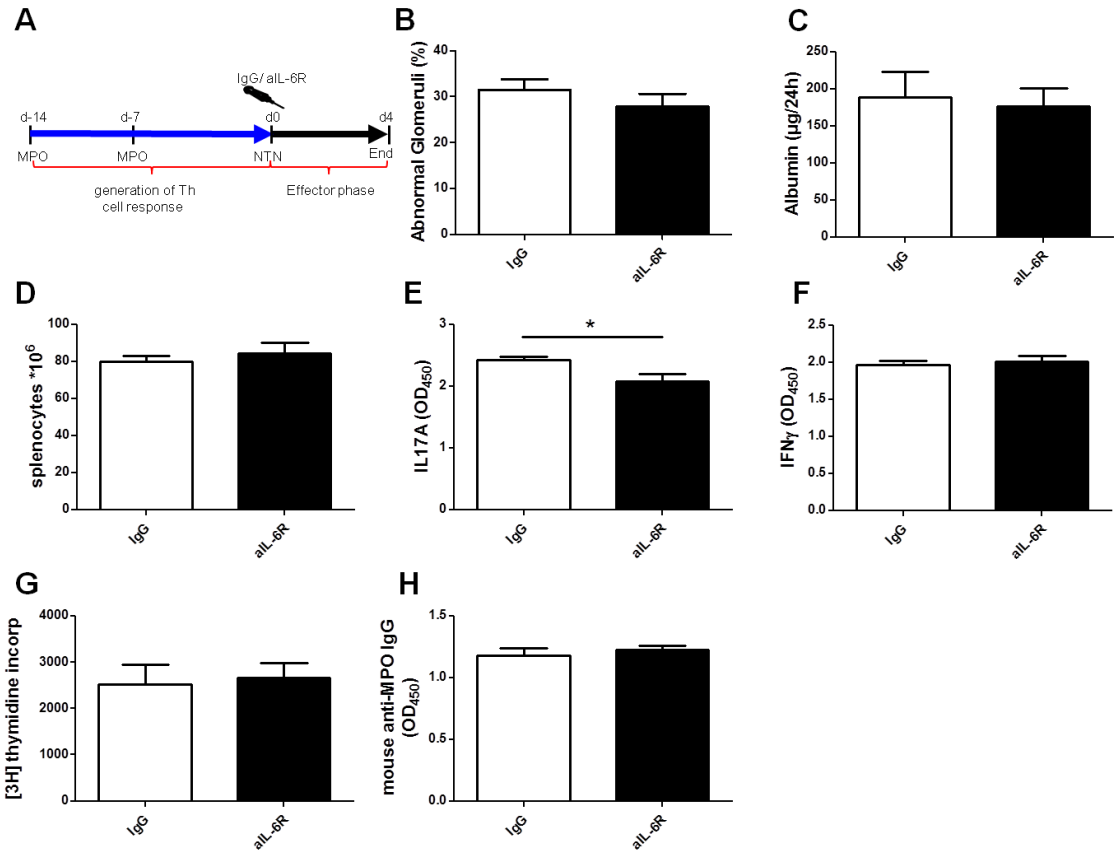

Figure 3.40: Renal and systemic effects of aIL6R antibody in effector phase of AiaMPOGN.
(A) Experimental setup. C57BL/6J mice were pre-immunized with 0.5 mg sheep IgG in Freund's adjuvant 14 and 7 day prior to NTN induction. Mice were injected with aIL-6R on d0 and sacrificed on d4. **(B)** PAS staining of kidney sections from aIL-6R and IgG treated C57BL/6J mice were quantified for abnormal glomeruli. **(C)** Total numbers of splenocytes from IgG or aIL-6R treated C57Bl/6 mice were counted. Secretion of the pro-inflammatory cytokines IL17A **(D)** and IFNγ **(E)** were performed using supernatants of splenocyte cultures after 72h. **(F)** Albuminuria was measured by ELISA. **(G)** anti-MPO IgG was measured by ELISA. **(H)** Proliferation rate of splenocytes was evaluated by [^{3}H] thymidine assay. Bars represent means ± SD. *P<0.05.

Formation of abnormal glomeruli again remained unaltered in aIL-6R treated mice (figure 3.40 B), as well as splenocyte numbers of treated and control mice (3.40 C). In contrast to aIL-6 treatment, which resulted in a reduction of Th1 immune response but revealed no variation in Th17 immune response, aIL-6R treatment induced weaker Th17 immune response (figure 3.40 D), but did not affect the Th1 immune response (figure 3.40 E). In addition, levels of albuminuria (figure 3.40 F), anti-MPO IgG (figure 3.40 G) as well as splenocyte proliferation rates did not show any variations between aIL-6R treated mice and controls (figure 3.40 H).

In conclusion, inhibition of IL-6 signaling in the effector phase by either sgp130Fc or aIL-6 or a-IL6R caused only slight effects on the course on AiaMPOGN and was insufficient to improve the disease. Inhibition of IL-6R signaling during initiation of autoimmune disease on the other hand was quite effective and significantly reduced renal injury.

4 Discussion

This study focuses on the differential roles of IL-6 in experimental glomerulonephritis. Glomerulonephritides are a heterogeneous group of autoimmune diseases which are mediated by different types of immune cells. Th1 and Th17 cells play an essential role in the development and progression of many forms of GN [15,17,39,132–134,142–144]. Previously, it has been shown that Th17 cells are strongly dependent on IL-6 during their differentiation [7,23,25]. In addition, macrophages have been shown to be crucial mediators of renal tissue damage [120]. Interestingly, macrophage differentiation, activation, and proliferation are also under control of IL-6 [118,120,145,146].

Therefore this study aimed to gain insights into the development of glomerulonephritis regarding the role of IL-6 in nephritogenic immune responses. In addition, the efficacy of IL-6 targeting therapeutics that have recently been introduced into clinical practice should be addressed.

To do so, the effects of IL-6 deficiency and inhibition in the Th-cell and macrophage dependent NTN model of crescentic glomerulonephritis and the role of IL-6 signaling in the murine model of ANCA antibody and Th17 dependent autoimmune anti-myeloperoxidase glomerulonephritis (AiaMPOGN) were analyzed.

Today's therapeutic approaches targeting glomerulonephritis are rather unspecific with a high incidence of serious adverse events. Recently, an anti-IL-6R directed therapy has been introduced into clinical practice for the treatment of human autoimmune diseases. This aIL-6R directed therapy showed clinical efficacy in treating rheumatoid arthritis (RA) and systemic juvenile idiopathic arthritis (sJIA) by improvement of clinical symptoms. The role in glomerulonephritis is unclear and has not been evaluated.

Infiltrating Th-cells seem to play a central role in initiation and progression of glomerulonephritis. Th1 and Th17 cell responses were described to be capable of inducing proliferative glomerulonephritis [3,4,14,17,132,134,142,144,147]. While Th1 cells and their role in glomerulonephritis have been established for years [3,4,14,15,142,147], the specific role of Th17 cells, which were first

described in 2006 [21,22], and the underlying mechanism of their regulation during disease are not well-characterized. Th17 cell maturation is crucially dependent on the presence of IL-6 [148,149]. In addition, levels of IL-6 were found to be elevated in patients with ANCA-associated vasculitis [150,151]. Also inhibition of IL-6 has been shown to act protective in different models of murine SLE [121–124]. Hence, anti IL-6 treatment could be expected to act protective in the therapy of glomerulonephritis.

On the other hand, studies in the 1990s by Karkar et al. have shown amelioration of the course of glomerulonephritis in a rat model of NTN by pre-treatment or continuous administration of recombinant IL-6 [95,96]. These data were established long before Th17 cells have been described and therefore had no focus on Th17 immune response. In addition, these data seem to be contradictory to the circumstance, that IL-6 induces Th17 cell differentiation, which leads to a pro-inflammatory immune response and therefore should aggravate the course of disease. The underlying mechanisms for these unexpected findings were, however, left unclear.

One explanation might derive from the fact that IL-6 is a multifunctional cytokine which mediates both pro- and anti-inflammatory processes [94]. It is not only crucially involved in the generation of pro-inflammatory Th17 responses [135,152] but also in the induction of anti-inflammatory IL-10 producing Tr1 cells [74,141,153] as well as in the down-regulation of macrophage activation [140,146]. Not surprisingly, the role of IL-6 in glomerulonephritis is not well defined to date.

In order to unveil the function and impact of IL-6 on glomerulonephritis, inhibition of IL-6 signaling was first studied in the murine model of induced experimental glomerulonephritis. NTN disease is induced by injection of polyclonal sheep antibodies raised against constituents of mouse glomeruli. The sheep immunoglobulin thus binds to mouse glomeruli and acts as a planted antigen. Initially, injection of NTN serum leads to acute non-specific renal injury. This so called heterologous phase is largely mediated by innate mediators (e.g. neutrophils and the complement system) during the first 3-4 days. In the subsequent autologous phase, when adaptive immune responses against sheep globulin have developed, injury is largely mediated by $CD4^+$ T cells.

These include especially Th17 and Th1 effectors but also regulatory T cells. In addition, macrophages have been shown to be pathogenic effectors.

4.1 Anti IL-6R treatment by m-tocilizumab exacerbates the clinical course of NTN nephritis

Tocilizumab is the first-in-class anti-IL-6 receptor agent that is approved for several autoimmune diseases. It has been shown to ameliorate the symptoms and disorders of RA and sJIA [128,129]. Tocilizumab is a monoclonal antibody that specifically binds to the soluble and membranous IL-6 receptor, thereby effectively blocking IL-6 signaling by interfering with both classical and *trans* pathways.

In theory, inhibition of IL-6 signaling by m-tocilizumab treatment was thought to result in a block of Th17 immune responses which in turn should lead to an amelioration of glomerulonephritis. Surprisingly, mice treated with m-tocilizumab showed a significant exacerbation of disease in contrast to controls as shown by immunohistochemistry (cf. figures 3.1, 3.2). All other tested clinical parameters, such as albuminuria (cf. 3.1 F) and BUN levels (cf. 3.1 G), also showed aggravation of disease in all m-tocilizumab treated animals as well. Despite the well described potency of m-tocilizumab to effectively block IL-6 signaling, there was no difference in renal infiltration of Th17 cells (cf. figure 3.3). Only systemic Th17 immune response was significantly reduced in m-tocilizumab treated animals (cf. figure 3.5). Nevertheless, progression of disease was aggravated, which might be due to different IL-6 related functions or to differential IL-6 signaling in combination with insufficient inhibition of the renal Th17 responses by inhibition of IL-6R via m-tocilizumab.

As m-tocilizumab is known to inhibit both classical and *trans*-signaling of IL-6, the mentioned results could refer to effects occurring by either classical or *trans*-signaling. Classical signaling occurs only in hepatocytes and some leukocyte subtypes because only theses cell types express the membranous IL-6R while *trans*-signaling can effect almost any cell of the body.

In order to unveil which distinct IL-6 signaling pathway is important, C57BL/6 mice were treated with antibodies directed against either classical and *trans*-signaling or *trans*-signaling only. Thereby both IL-6 signaling pathways were investigated separately.

4.2 Inhibition of classical IL-6 signaling is responsible for exacerbation of glomerulonephritis

As mentioned previously, IL-6 is a cytokine with a bulk of different functions and regulatory capacities. Variable signaling effects are directed via two differential pathways [84,94,98,99]. Classical IL-6 signaling is initiated by binding of IL-6 to the membranous IL-6 receptor. This membranous receptor is expressed on the surface of hepatocytes and leukocytes only. In addition, IL-6 can bind to the soluble IL-6 receptor, to form an IL-6/sIL-6R complex, which binds to the signal transducing glycoprotein 130 [93,97]. As gp130 is expressed in a wide variety of cells, the observed exacerbation of glomerulonephritis may be due to differential signaling effects.

m-tocilizumab inhibits the membranous IL-6R as well as the soluble IL-6R. Hence, a precise analysis of the cell types that are influenced by the inhibition of IL-6 is not possible when using m-tocilizumab. The same is true for the discrimination between the previously described IL-6 signaling pathways.

In order to elucidate, how treatment with m-tocilizumab caused aggravation of the disease, both IL-6 signaling pathways were blocked individually in a new setup. Inhibition of classical and *trans* IL-6 signaling via an aIL-6 antibody resulted in aggravation of the disease as detected by quantification of interstitial damage and formation of crescents, as well as higher levels of BUN (cf. figures 3.6-3.8). Therefore aIL-6 treatment showed the same effects as m-tocilizumab treatment. In contrast, treatment with sgp130Fc, selectively blocking the alternative signaling pathway, resulted in an unaltered course of the disease compared to IgG controls. Analysis of systemic immune responses of the Th1 and Th17 characteristic cytokines IFNγ and IL-17 showed no differences in either aIL-6 treated or sgp130Fc treated mice compared to IgG controls (cf.

figure 3.11). Interestingly, although the total number of splenocytes was reduced, activation status of T- and B-cells was significantly increased in aIL-6 treated mice.

This demonstrates that only classical IL-6 signaling results in an exacerbation of glomerulonephritis. A further hint for the aggravating role of classical IL-6 signaling in the course of anti-GBM nephritis, is derived from the significantly elevated expression of GM-CSF, an important cytokine for the proliferation of macrophages [154]. In addition, only inhibition of classical signaling led to an increase macrophages infiltration into the kidneys.

These results clearly demonstrate for the first time an aggravating effect of IL-6 classical signaling-inhibition in the development and progression of NTN, whereas inhibition of *trans*-signaling via sgp130Fc did not lead to any differences in the course of disease. In addition, the obtained results for aIL-6 treatment were comparable to those gained by treatment with m-tocilizumab (cf. chapters 3.2, 3.3).

4.3 Inhibition of classical IL-6 signaling in the macrophage dependent effector phase is sufficient to exacerbate nephritis.

So far, it was demonstrated, that inhibition of IL-6 signaling exacerbates the course of disease in the NTN model of crescentic glomerulonephritis. Analysis of both IL-6 signaling pathways clearly implied that only classical signaling is involved in these aggravating processes. This allows the conclusion that the effects are mediated by IL-6 action on leukocytes since only these cells express the membrane bound IL-6 receptor. Analyses of Th17 responses showed no significant reduction of renal Th17 infiltration in contrast to our expectations. This could be due to the following reasons: Inhibition of IL-6 by either antibody is probably not complete and might be sufficient to prime a small number of Th17 cells. Once differentiated, these remaining Th17 cells proceed to a positive IL-6 independent feedback loop and proliferation [155], which finally results in a robust Th17 population [135]. But why was the clinical outcome in both aIL6-R and aIL-6 treated mice significantly worsened? This leads to the

conclusion that there is another effect of IL-6 resulting in an anti-inflammatory immune response. Our results suggest a possible macrophage specific immune response by elevated levels of macrophage proliferation promoting cytokine GM-CSF (cf. figure 3.11) and increased numbers of infiltrating macrophages into the kidney (cf. figure 3.8).

It is known, that IL-6 induces an anti-inflammatory phenotype in macrophages by inducing the expression of anti-inflammatory cytokines such as sTNFR and IL-1ra [112,113]. These cytokines in turn counteract the effects of pro-inflammatory cytokines like TNFα and IL-1 [113,115–117], which finally results in the inhibition of macrophages. In addition, Riedy and Stewart showed that IL-6 can also directly inhibit the proliferation of macrophages *in vitro* [118].

In order to elucidate the role of IL-6 on macrophages in the NTN model of experimental glomerulonephritis, mice were pre-immunized against the nephritogenic antigen for 5 days to allow for Th17 immune responses to develop normally and to exclude effects of aIL-6 treatment on development of immune responses. Subsequent blockade of pan IL-6 signaling by aIL-6 in the macrophage dependent effector phase of NTN was sufficient to aggravate the disease (cf. chapter 3.4). Compared to the previous experiments, renal and systemic Th17 immune responses were even increased (cf. figures 3.1 - 3.16). Again, specific blockade of IL-6 trans-signaling by sgp130Fc did not significantly affect the course of nephritis (cf. chapter 3.4). In addition, aIL-6, but not sgp130Fc treatment, significantly enhanced F4/80^{+} renal macrophage infiltration (cf. figure 3.14), which results in a more severe progression of disease. Also, both renal and systemic levels of pro-inflammatory TNFα, which is a known target of IL-6 in macrophages, were significantly elevated.

Mechanistically, the absence of IL-6 might therefore lead to inefficient down regulation of macrophages. As a consequence, activated macrophages induce further pro-inflammatory immune responses and aggravate nephritis.

4.4 IL-6 acts in an anti-inflammatory manner by inhibiting macrophage proliferation

In order to assess the anti-inflammatory effect of IL-6 on macrophage proliferation, stimulation of peritoneal macrophages with recombinant IL-6 was performed *in vitro* according to Riedy and Stewart [118]. IL-6 stimulated cultures of peritoneal macrophages showed a significant reduction of proliferation, which demonstrates the anti-inflammatory effect of IL-6 on macrophages (cf. chapter 3.5). In reverse, this implies that inhibition of IL-6 leads to enhanced macrophage proliferation, which in turn might cause increased pro-inflammatory immune responses.

4.5 Inhibition of IL-6 signaling in macrophages aggravates glomerulonephritis

To further unveil the role IL-6 signaling in macrophages in experimental glomerulonephritis, LysM^{Cre} x IL-6R$^{fl/fl}$ mice were generated, lacking IL-6R specifically in macrophages. As mentioned in the previous chapters, inhibition of IL-6 leads to enhanced macrophage activation and could thereby mediate the observed aggravated course of the disease. Because macrophages in LysM^{Cre} x IL-6R$^{fl/fl}$ mice do not express the IL-6R, they are no longer affected by classical IL-6 signaling. Thus, if IL-6 signaling in macrophages is indeed anti-inflammatory, an exacerbated course of the disease was to be expected. Indeed, histological sections of LysM^{Cre} x IL-6R$^{fl/fl}$ mice showed much more crescents and interstitial damage than control mice (cf. figure 3.19). In line elevated systemic M-CSF and GM-CSF levels (cf. figure 3.23) were observed which suggest an increased activation of macrophages as causative for aggravation of NTN.

4.6 Abrogation of IL-6 signaling in CD4+ T cells aggravates NTN by impairing anti-inflammatory Tr1 cell development

Possible other anti-inflammatory cell types that are known to be influenced by IL-6 are $CD4^+$ Tr1 cells [60]. To unveil a potential role of Tr1 cells in regulating Th17 immune responses in the course of glomerulonephritis, we generated $CD4^{Cre}$ x IL-$6R^{fl/fl}$ mice, lacking the IL-6 receptor specifically on $CD4^+$ cells, including Th17 cells and Tr1 cells. We initially expected these mice to show an attenuated course of the disease, as differentiation of naïve $CD4^+$ Th-cells into pro-inflammatory Th17 cells should be impaired. In addition, the equilibrium of Th17 / T_{reg} differentiation should have been skewed in favor of T_{reg} differentiation, thereby suppressing autoimmune responses. Surprisingly, induction of NTN in $CD4^{Cre}$ x IL-$6R^{fl/fl}$ mice also led to an aggravation of disease (cf. figure 3.24). This might be due to differential effects: Firstly, abrogation of Th17 cell differentiation, like in the antibody blockade studies was rather incomplete and not significantly suppressed. This might be due to incomplete Cre mediated excision of IL-6R on $CD4^+$ T cells. The remaining functional Th17 cells might then have expanded even more efficiently. Secondly, absence of IL-6R signaling might have interfered with development of $CD4^+$ anti-inflammatory Tr1 cells. Since these cells have no characteristic and distinguishing surface marker, we decided to study IL-6 effects on Tr1 cells *in vitro.*

Thus, in order to unveil if anti-inflammatory Tr1 induction might be stimulated by IL-6, T_{reg} depleted $CD4^+$ T-cell cultures, stimulated with or without recombinant IL-6, were analyzed for IL-10 secretion (cf. figure 3.27). Levels of IL-10 were significantly elevated in IL-6 stimulated $CD4^+$ T_{reg} depleted cells, compared to unstimulated cells, suggesting Tr1 being responsible for balancing immune responses under the influence of IL-6.

These findings therefore suggested, that blockade of classical IL-6 signaling not only down modulates macrophage activation and proliferation but also impairs Tr1 cell function.

4.7 Complete absence of IL-6 impairs generation of Th17 immune responses and improves the course of NTN

Blockade of IL-6 signaling by several antibodies and lineage specific IL-6R knockout demonstrated exacerbation of the course of NTN glomerulonephritis. However, none of our interventions resulted in significant reduction of renal Th17 responses. This is most likely due to small remaining amounts of functional IL-6 or intact IL-6 receptors.

Thus, we were interested, whether complete deficiency of IL-6 would abrogate Th17 responses and also lead to an aggravation or rather amelioration of glomerulonephritis. NTN was therefore induced in IL-6$^{-/-}$ and wild type mice.

Contrary to the antibody blockade and conditional receptor knockout studies, the course of the disease was attenuated in IL-6$^{-/-}$ mice. IL-6$^{-/-}$ mice showed amelioration of interstitial damage and clinical parameters (cf. figure 3.29).

In contrast to the antibody blockade studies, Th17 immune responses were quite significantly down regulated in IL-6$^{-/-}$ mice (cf. figure 3.31). The observed reduced Th1 immune responses might result as a consequence of impaired Th17 immune responses, as Th17 and Th1 immune responses are suggested to interact on each other [143]. Interestingly, total numbers of splenocytes were reduced in IL-6$^{-/-}$ mice, but activation of B-cells and CD4^{+} and CD8^{+} T-cells in the spleen was markedly increased as assessed by CD69 expression (cf. figure 3.32). This supports our hypothesis, that even though lack of IL-6 leads to amelioration of disease, IL-6 also has anti-inflammatory functions on leukocyte activation.

To further evaluate the effects of IL-6 on Th17 responses we next induced the model of accelerated NTN which in our hands shows much higher levels of renal and systemic Th17 responses than non accelerated NTN.

As hypothesized, the attenuating effects were even increased in accelerated NTN (cf. figures 3.33 – 3.37). IL-6$^{-/-}$ mice showed quite significantly reduced histological damage (cf. figure 3.33) due to less infiltrating inflammatory cells into the kidney (cf. figure 3.34). In addition, both renal and systemic Th17 immune responses were almost absent in IL-6$^{-/-}$ mice (cf. figures 3.35, 3.37) emphasizing the crucial role of IL-6 in Th17 cell differentiation. However, still a

generalized up-regulation of the activation marker CD69 on most splenic leukocyte subsets was again detectable in IL-6$^{-/-}$ mice (cf. figure 3.36), highlighting the additional anti-inflammatory properties of IL-6.

4.8 Inhibition of IL-6 receptor attenuates autoimmunity and glomerulonephritis in experimental ANCA vasculitis.

Our data shown above demonstrate dual effects of IL-6, which acts pro-inflammatory via induction of a nephritogenic Th17 immune response and anti-inflammatory via down-regulation of macrophage activation and activation of Tr1-cell differentiation in the experimental murine model of NTN nephritis. Inhibition of IL-6 or IL-6R resulted in a more severe course of disease in the mouse. Thus, the possible therapeutic use of anti-IL-6 directed therapies for treatment of human patients in clinical practice has to be approached with care. However, as pointed out before, glomerulonephritides are a heterogeneous group of diseases. Thus findings from one model cannot necessarily be translated to another. In this respect, several studies have shown that ANCA associated vasculitis leads to high systemic levels of IL-17 and IL-6 in human patients [150]. In addition, it is known that IL-6 is required for antibody production and therefore lack of IL-6 might reduce pathogenic ANCA levels. Hence, a therapeutic use of anti-IL-6 agents might be promising in treatment of ANCA-associated vasculitis. To define the role of IL-6 signaling in ANCA-vasculitis, the murine experimental model of AiaMPOGN [18,131] was used.
First, mice were pre-emptively treated with m-tocilizumab during the initiation phase of disease in which anti MPO ANCA as well as Th17 responses develop.
Unlike in our studies in the NTN model, treatment with m-tocilizumab resulted in attenuation of the course of disease (cf. figure 3.38). Early IL-6 receptor inhibition via m-tocilizumab diminished autoimmunity and ANCA production and consequently attenuated glomerular injury in AiaMPOGN. In a second experiment, mice were again immunized with murine MPO to allow for ANCA and Th-cell responses to generate, this time however, without blockade of IL-6 signaling. Subsequent inhibition of pan IL-6 signaling by aIL-6 or *trans*-signaling

via the fusion protein sgp130Fc in the effector phase after development of autoimmunity did not result in any differences (cf. figure 3.39). Neither humoral immunity nor renal damage was affected by either treatment (cf. figure 3.39). The same results were shown after inhibition of IL-6 signaling via m-tocilizumab in the effector phase only (cf. figure 3.40). This is most likely due to the fact, that Th17 immune responses and ANCAs had already developed normally, when anti-IL-6 signaling reagents were administrated. Why did IL-6 blockade not lead to aggravation of disease in AiaMPOGN which is in contrast to our findings in the NTN model? This can be explained by the fact that tissue injury in AiaMPOGN is not only dependent on Th17 immune response (like NTN) but also on IL-6 dependent ANCA antibodies directed against MPO (unlike NTN). The role of macrophages and TR1 cells has not been studied so far but our results point to a much less important role than in NTN. In conclusion, blockade of IL-6 signaling in the initiation phase of ANCA vasculitis offers exciting therapeutic potential for the human disease, especially as tocilizumab is already in clinical use for other inflammatory disease entities.

5 Summary

Glomerulonephritis (GN) is one of the most prevalent causes of terminal renal failure. The most aggressive form is referred to as rapid progressive glomerulonephritis (RPGN), with a poor prognosis and a rapid progression to failure within several weeks. GN in general is characterized by infiltration of T-cells and macrophages into the kidney, resulting in inflammation of the glomeruli and surrounding area.

The pleiotropic cytokine IL-6 is crucially involved in generation of pro-inflammatory Th17 responses but also in generation of anti-inflammatory Tr1 cells and down-regulation of macrophage activation. Recently, IL-6 directed therapies have been introduced to treat human autoimmune diseases. As its role in GN remains controversial we studied IL-6 function in the Th17 and macrophage dependent NTN model of crescentic GN.

Inhibition of IL-6 by m-tocilizumab or anti-IL-6 antibodies resulted in an aggravated course of the disease. Aggravation was shown to be mediated via the classical IL-6 signaling pathway. Studies in leukocyte specific IL-6R deficient mice showed that aggravation of nephritis was due to increased macrophage activation and decreased Tr1 cell differentiation. Th17 responses were not affected by either IL-6 blockade or receptor knockout. This was most likely due to small remaining amounts of functional IL-6 or IL-6 receptors.

In contrast, IL-6 deficient mice were significantly protected from NTN disease. Protection was most likely due to almost absent renal and systemic Th17 responses in complete absence of IL-6.

In addition, early IL-6R inhibition by m-tocilizumab diminished autoimmunity and attenuated glomerular injury in the antibody and Th17 dependent model of AiaMPOGN. This protective effect was due to suppressed development of Th17 immune responses and an abolished ANCA autoantibody production, mediated by IL-6R inhibition.

In conclusion this study further underlines the multi functionality of IL-6. We clearly demonstrated opposing effects of IL-6 which is 1) pro-inflammatory via induction of nephritogenic Th17 responses and 2) anti-inflammatory via

induction of IL-10 producing Tr1 cells and down-regulation of macrophage activation. The latter effects were mediated by classical rather than by alternative IL-6 signaling.

In addition, this study revealed that IL-6 critically directs development of AiaMPOGN by 3) mediating development of MPO directed Th17 responses and 4) production of ANCA antibodies. Based on our results in two different murine models, the use of anti-IL-6 directed therapies for the treatment of GN in clinical practice has to be approached with care. Our data indicate that the right disease entity and the right timing of application need to be chosen if IL-6 blockade is considered.

6 Zusammenfassung

Glomerulonephritis ist eine der häufigsten Ursachen der terminalen Niereninsuffizienz. Schlechte Heilungschancen und ein rasches Voranschreiten des Nierenversagens sind die Hauptmerkmale der rapid progressive Glomerulonephritis (RPGN), die die aggressivste Form der GN darstellt. Ein charakteristisches Merkmal der GN ist die Einwanderung von T-Zellen und Makrophagen in die Niere, was eine Entzündung der Glomeruli und des umgebenen Gewebes zur Folge hat.

Das pleiotrope Zytokin IL-6 ist ein zentraler Mediator für die Generierung der pro-inflammatorischen Th17-Antwort, besitzt aber auch anti-inflammatorische Wirkung, in dem es unter anderem die Differenzierung von Tr1-Zellen initiiert und die Aktivierung von Makrophagen hemmt. Unlängst haben anti-IL-6 gerichtete Therapien Einzug in den klinischen Alltag bei der Behandlung von Autoimmunkrankheiten gefunden. Ziel der zugrunde liegenden Studie war es, die kontroverse Rolle von IL-6 im Modell der Th17- und Makrophagen-abhängigen RPGN zu untersuchen.

Die Inhibierung von IL-6 mit murinem Tocilizumab beziehungsweise anti-IL-6-Antikörpern zeigten eine Verschlimmerung des Krankheitsverlaufes. Diese Verschlechterung wurde durch die klassische IL-6-Signalkaskade herbeigeführt. Die Untersuchungen in den Leukozyten-spezifischen IL-6R-Knockout-Mäusen zeigten, dass das verschlechterte Krankheitsbild aus einer Zunahme aktivierter Makrophagen und gleichzeitig aus einer Abschwächung der Tr1-Zell-Differenzierung resultierte. Die Th17-Antwort blieb sowohl bei Antikörper-Behandlung, als auch in den spezifischen Knockout-Mäusen unbeeinflusst, was mit verbleibenden, geringen Mengen an funktionellem IL-6 bzw. des IL-6-Rezeptors zu begründen ist.

In IL-6-defizienten Tieren führte die NTN-Induktion jedoch zu einer fast vollständig fehlenden systemischen und renalen nephritogenen Th17-Antwort mit konsekutiv signifikant besserem Verlauf der Nephritis.

Desweitern konnte gezeigt werden, dass die Behandlung mit murinem Tocilizumab zu einer Verminderung der Autoimmunität und zur Abmilderung

des glomerulären Schadens in einem Antikörper- und Th17-abhängigen ANCA-Modell führte (AiaMPOGN).

Zusammenfassend unterstreicht die vorliegende Arbeit die multifunktionale Rolle von IL-6. Darüber hinaus konnte eine entscheidende Rolle sowohl für die Th17-Antwort, als auch für Makrophagen in der NTN-Nephritis in der vorliegenden Arbeit gezeigt werden: Die partielle Blockade von IL-6 ist nicht ausreichend um die Entstehung einer pathogenen Th17-Antwort zu unterdrücken und verstärkt die Nephritis durch gesteigerte Makrophagen-Aktivierung und verminderte Induktion von IL-10 produzierenden Tr1-Zellen. Dieser pro-inflammatorische Effekt wird durch die klassische, nicht aber durch die alternative IL-6-Signalkaskade vermittelt. Komplette IL-6-Defizienz hingegen verhindert die Ausbildung einer nephritogenen Th17-Antwort, welches die Aktivierung der Makrophagen außer Kraft setzt und deutlich protektive Effekte aufweist. Des Weiteren zeigt diese Arbeit, dass IL-6 entscheidend die Entwicklung der zellulären und humoralen Autoimmunität in der ANCA-assoziierten GN lenkt.

Auf Grund der dargestellten Ergebnisse in zwei unterschiedlichen Modellen in der Maus sollte daher der Gebrauch von anti-IL-6 gerichteten Therapie-maßnahmen bei der Behandlung der GN im klinischen Alltag mit Vorsicht verwendet werden. Die hier gezeigten Daten weisen darauf hin, dass sowohl die Form der Erkrankung, als auch der Zeitpunkt des Behandlungsbeginns bei einer anti-IL-6- bzw. anti-IL-6R-Therapie sorgsam geprüft werden müssen.

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8 Publications and Conferences

Publications

Malte A. Kluger, Anett Ostmann, Michael Luig, Matthias Meyer, Bören Görke, Hans-Joachim Paust, Rolf A.K. Stahl, Ulf Panzer, Gisa Tiegs, Oliver M. Steinmetz *B Cell Derived IL-10 Does Not Vitally Contribute to Immunity and Clinical Course of Glomerulonephritis. (in revision)*

Michael Luig, Malte A. Kluger, Bören Görke, Hans-Willi Mittrücker, Stefan Rose-John, Rolf A.K. Stahl, Ulf Panzer, Oliver M. Steinmetz *IL-6 Mediates Crescentic Glomerulonephritis via Differential Effects on Th17 Responses and Macrophage Activation (in preparation)*

Sharon L. Ford, Michael Luig, Steven R. Holdsworth, Shaun A. Summers, Oliver M. Steinmetz *Inhibition of IL-6 Receptor Attenuates Autoimmunity and Glomerulonephritis in Experimental ANCA Vasculitis (in preparation)*

Conferences

Michael Luig, Malte A. Kluger, Bören Görke, Hans-Willi Mittrücker, Stefan Rose-John, Rolf A.K. Stahl, Ulf Panzer, Oliver M. Steinmetz *IL-6 vermittelt die rasch progrediente Glomerulonephritis über differentielle Effekte auf die Th17-Antwort und Makrophagen-Aktivierung – Kongress für Nephrologie 2012 "4. Jahrestagung der Gesellschaft für Nephrologie", Hamburg, Germany*

Michael Luig, Malte A. Kluger, Bören Görke, Hans-Willi Mittrücker, Stefan Rose-John, Rolf A.K. Stahl, Ulf Panzer, Oliver M. Steinmetz *IL-6 Mediates Crescentic Glomerulonephritis via Differential Effects on Th17 Responses and Macrophage Activation – American Society of Nephrology "ASN Kidney Week 2012", San Diego, USA*

9 Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbständig und ohne fremde Hilfe verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hamburg, im Juli 2013

Michael Luig