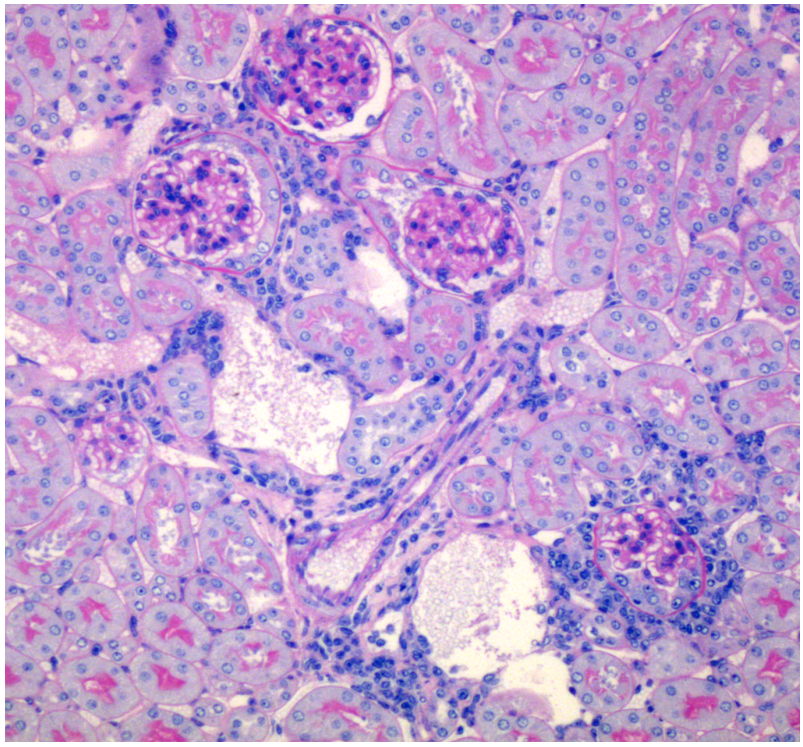
Mechanisms of T cell mediated kidney disease



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Prof. Dr. med. Christian Kurts
Prof. Dr. rer. nat. Norbert Koch

To my family

Nichts kann den
Menschen mehr stärken
als das Vertrauen, das
man ihm entgegenbringt.
(A. Harnack)

1. Index

2. Abbreviations

AdOVA	Adenovirus.OVA
AdeGFP	Adenovirus.eGFP-Luc
AICD	activation induced cell death
Alum	Aluminum hydroxide
aOTI	activated OT-I
aOTII	activated OT-II
APC	Antigen presenting cell
AP	Alkaline phosphatase
BSA	Bovine serum albumin
cDNA	complementary DNA
CLN	cervival lymph node
CLP	common lymphoid precursor
CloLip	Clodronate Liposome
CMP	common myeloid precursor
CTL	cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Desoxyribonucleic acid
DT	Diphteria Toxin
DTH	delayed type hypersensitivity
DTR	Diphteria Toxin Receptor
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
eGFP	enhanced GFP
ER	endoplasmatic reticulum
FACS	fluorescent associated cell sorting/screening
FCS	Fetal calf serum
GBM	Glomerular basement membrane
GFP	Green fluorescent protein
GN	Glomerulonephritis
Hel	Hen egg lysozyme
HEV	high endothelial venule
HPLC	high pressure liquid chromatography
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILN	inguinal lymph node
i.p.	intra-peritoneal
i.v.	intravenous
LPS	Lipopolysaccharide
Luc	Firefly Luciferase
mAb	monoclonal antibodies
MACS	magnetic associated cell sorting
MFI	mean fluorescence intensity
MHC	major histocompatibility
MLN	mesenteric lymph node
MMP	Matrix metalloproteinase
MW	Molecular weight
NOH	Nephrin OVA-Hel
nOTI	naïve OT-I
nOTII	naïve OT-II
OD	optical density
o.n.	over night
OVA	Ovalbumin
PAMP	pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
RLN	renal lymph node
RNA	Ribonucleic acid
SA	Streptavidin
s.c.	sub-cutaneous
Spl	Spleen
TCR	T cell receptor
TLR	Toll-like receptor
TGF	transforming growth factor
wks	weeks
wt	wild type

3. Abstract and Summary

Immune mediated glomerulonephritis (GN) is a major cause renal inflammation leading to terminal kidney failure. B cells and autoantibodies are well known to contribute to the progression of glomerular disease. The role of $CD8^+$ T cells, however, remains to be clarified, partially because of lack of suitable disease models.

To investigate this question, a transgenic murine model system was created by expressing a membrane-bound model antigen (OVA-HEL) under the control of the nephrin promoter specifically on glomerular podocytes (NOH mice). These cells would then be targets of adoptively transferred OVA- or HEL-specific lymphocytes. NOH mice could therefore be used as a model for antigen specific attacks of the cellular or humoral immune response. This approach has been used successfully in the past to elucidate mechanisms in other autoimmune diseases, e.g. Diabetes Type I and Autoimmune Encephalomyelitis.

$CD8^+$ OVA-specific T cells were activated exclusively in the renal draining lymph node in a DC dependent fashion, indicating cross-presentation of glomerular antigen whereas $CD4^+$ OVA-specific T cells remained inactive after adoptive transfer.

In contrast to the Diabetes models, even high numbers of OVA-specific $CD8^+$ T cells alone only inflicted minor glomerular damage. Co-injection of OVA-activated OVA-specific $CD4^+$ T helper cells induced influx of antigen specific T cells and inflammatory mononuclear cells and resulted in renal immunopathology. The infiltrate was predominantly periglomerular, similar to the histological changes seen in rapid progressive forms of glomerulonephritis. The infiltrate was dominated by myeloid dendritic cells (DC) expressing CD11c, CD11b and the costimulatory markers CD40 and CD86, proinflammatory DC of the Gr-1^{hi}CX3CR1^{lo} phenotype and also contained antigen-specific $CD8^+$ and $CD4^+$ T cells. DC activation and $CD8^+$ T cell effector function strongly depended on the availability of $CD4^+$ T cell help. Persisting infiltration led to podocyte dysfunction and glomerulosclerosis. $CD8^+$ T cell priming in the kidney lymph node as well as inflammation within the kidney required the presence of DCs, as demonstrated by ablation of $CD11c^+$ cells in NOH x CD11c-diphteria toxin receptor mice, which allow deletion of DCs upon injection of diphtheria toxin. Within infiltrates, proinflammatory cytokines and the chemokines CCL2, 3, 4 and 5 as well as their correlating receptors were detected on infiltrating leukocytes.

These findings demonstrate that T cells specific for glomerular antigens can induce kidney immunopathology and structural damage reminiscent of rapid and crescentic types of glomerulonephritis.

4. Introduction

4.1 T cell development and function

T cells take part in every major aspect of the adaptive immune response. In homeostasis, regulatory T cells actively tolerize other immune cells to prevent autoimmunity. During infection, $CD4^+$ T helper cells orchestrate immune effector functions by governing production of antibodies and activating $CD8^+$ T cells to become cytotoxic T lymphocytes that can specifically destroy infected cells by recognizing antigenic structures of the pathogen.

Initial activation of T cells is mediated by so called “professional antigen-presenting cells” such as dendritic cells (DC), B cells and to some extent macrophages or even specialized parenchymal body cells (e.g. liver sinusoidal endothelial cells).[1]

T cells in homeostasis and immunity

T cells, an abbreviation of the term "T lymphocytes", generally can be divided into three major subsets: $CD4^+$ T helper cells are key organizers of the adaptive immune response. Most immune reactions crucially depend on the presence of activated $CD4^+$ helper cells, which can guide the immune systems to tailor responses against pathogens as diverse as parasites, bacteria, fungi, viruses or even abnormal body cells such as cancer. $CD8^+$ cytotoxic T killer cells are specialized in detecting and removing infected or malignant body cells, which host pathogens inside the cells or have degenerated into tumour cells. Activated cytotoxic T lymphocytes can specifically detect those defective cells and remove them by cell lysis or apoptosis induction while leaving neighbouring cells, which have not been infected, intact. Immunosuppressive regulatory T cells, which are a specialized form of $CD4^+$ T cells, are responsible for silencing autoreactive immune cells in homeostasis and for shutting down immune reactions once they have cleared an infection to prevent collateral tissue damage by overshooting immune reactions.

Naïve, resting T cells circulate the blood and lymphoid organs in large numbers with each cell showing different and highly specific reactivities to distinct antigenic patterns. Each cell recognizes a specific peptide fragment based on their receptor, the T cell receptor (TCR). During T cell development in the thymus, each cell creates a highly individual receptor by a process called somatic recombination. Due to the unique structure of each TCR and the high numbers of T cells circulating the body, T cells can react to a broad spectrum of antigenic patterns. TCRs are used by the T cells as the main receptor for T cell activation. The ligand for the TCR is a complex of antigen presenting molecules called major histocompatibility complex (MHC) and small peptide structures (8-15 amino acids), which are being presented on the MHC molecules. In general, two different classes of cell bound MHC exist:

- MHC-I molecules are expressed on any cell throughout the body, presenting endogenous peptide fragments of proteins, which are synthesized within the cell and are responsible for the activation of $CD8^+$ T cells.
- MHC-II molecules are present almost exclusively on a specialized subset of cells called professional antigen presenting cells (APC) and are responsible for the activation of $CD4^+$ T cells. These cells sample antigens by phagocytosis, receptor mediated endocytosis or pinocytosis and present those antigens after degradation on MHC-II or by a process termed cross-presentation also on MHC-I, depending of the uptake mechanism and the intracellular compartment where the antigen is located to after uptake (e.g. vesicular compartments such as stable early endosomes or lysosomes).[2]

Since the genetic rearrangement of TCR-genes, which is known as somatic recombination, is a process that is virtually completely random, many developing T cells in the thymus show high levels of autoreactivity. Therefore, it is imperative for an organism to delete these cells before they leave the thymus and enter the periphery. In the thymus, developing cells are scanned by medullary thymic epithelial cells (mTEC) and selected in a two step process. To ensure that only T cells with functional TCR leave the thymus, T cell precursors are first selected for their ability to positively match their TCR to the MHC molecules on the mTEC (positive selection). In the second step, cells that show strong interactions with unloaded MHC are eliminated to prohibit unspecific T cell activation. Furthermore, mTEC are able to present a broad variety of proteins from any cell type throughout the body by using a specific transcription factor called AIRE[3], which activates transcription of a wide selection of organ-specific genes. T cells reacting to peptides generated from those proteins are identified as autoreactive and are also eliminated (negative selection).

Although this process, which is termed as *central tolerance*[4], prevents highly autoaggressive cells from reaching the circulation, not all self-reactive T cells are eliminated. T cells which are showing only low or intermediate reactivity to self antigens are allowed to leave the thymus, making it necessary for the body to apply additional mechanisms of tolerance induction to prevent activation of those cells and to suppress autoimmunity. This process, called *peripheral tolerance*[5, 6], is a multi-step mechanism that includes three different concepts, depending on the reactivity of the cells to the self-antigens.

Antigens which are either occluded from the immune system by physical barriers (so called immunological privileged sites, e.g. brain, eye cavity, testis or uterus)[7] or that are only detected with very low affinity are commonly being ignored by the immune system, not leading to an activation of immune cells. However, any access of the adaptive immune cells to immunological privileged sites generally leads to massive autoimmunity, showing that sequestered antigens cannot be used for the induction of peripheral tolerance.[8]

Antigens, which are readily accessible to the immune system but only lead to low-level activation of adaptive immune cells, induce a defective activation followed by a state of cellular paralysis termed anergy.[9] Those cells could only be reactivated by very strong signals, so that these cells would ignore self-antigens, but still could react to highly infectious pathogens. However, it is crucial for the organism to actively shut down those cells once the infection is cleared to prevent autoimmunity.[10]

Antigens that are recognized by adaptive immune cells with medium or high affinity lead to a state of transient activation of these cells.[11, 12] However, for full immunogenic activation it is necessary for the antigen presenting cells to provide further costimulatory signals, a concept that will be discussed in more detail below addressing the development and function of APC.

The improper activation of lymphocytes leads to a process called activation induced cell death (AICD).[5, 13] Lymphocytes that become activated in the absence of proper survival signals start dividing (clonal expansion), but shortly die after having undergone few rounds of proliferation (clonal exhaustion). These autoreactive clones would therefore be finally deleted from the system.[14]

Additionally to the tolerance mechanisms described above which could be seen as passive tolerance mechanisms, there is also induction of active tolerance either by specialized, tolerance inducing T cells (regulatory T cells)[15] or by soluble, tolerance inducing factors such as TGF-β or IL-10[16-18], which are secreted by various immune cells or tissue cells suppressing inflammatory reactions.

After having described the development of T cells and the concept of tolerance induction during homeostasis, the following passage will describe the events following immunogenic activation of T cells and their effector functions.

Generally, the first step of T cell activation is thought to happen in the secondary lymphoid organs (spleen, lymph nodes, specialized lymphoid tissues[19]).

Antigen presenting cells, mostly tissue dendritic cells, constitutively sample antigens and migrate from peripheral tissues to the lymphoid organs, where they present those antigens to bypassing T cells.[20, 21] Furthermore, antigen-presenting cells also reside in the lymphoid organs and sample antigens that enter the organs by the lymph flow. In the absence of inflammatory signals, those APC are functionally immature and lack costimulatory molecules that would lead to full immunogenic activation of the T lymphocytes. During an ongoing infection, APC receive maturation signals, enabling them to activate T cells and to trigger an adaptive immune response.[22, 23 24]

Activated $CD4^+$ T cells perform a variety of immune effector functions, depending on the costimulatory signals they receive. Generally, $CD4^+$ T helper cells can differentiate into three distinct subsets:

- Th1 cells are responsible for activating macrophages to clear bacterial infections residing in vesicular compartments such as *Mycobacterium tuberculosis*.[25] Secondly, they activate B cells to become antibody secreting plasma cells, producing IgG2a and IgG2b, which are highly efficient antibodies for opsonizing circulating antigens, marking them for phagocytic cells and activate complement.[26] Last but not least, Th1 cells activate APC to upregulate costimulatory molecules necessary for activation of $CD8^+$ cytotoxic T cells. Th1 cells require the presence of IL-12 and IFN-γ during their activation.[27]

- Th2 cells are responsible for the induction of immune responses against parasites, inducing a strong B cell activation and production of IgM and IgE, the latter being a crucial factor in the activation of mast cells, which together with basophilic and eosinophilic granulocytes form the main defence against parasite infections. Th2 cells require the presence of IL-4 during their activation.[28, 29]

- Th17 cells have only very recently been discovered as a third class of helper cells. Their immunological function is still a matter of debate, although it is becoming clear that they are induced early in adaptive immune response and seem to be involved in the defence against extracellular bacteria by recruiting and activating neutrophilic granulocytes to an infection. Th17 cells require the presence of IL-6 and TGF-β during their activation and probably also during their effector phase in the peripheral tissue.[30, 31]

Activation of $CD8^+$ T cells requires stringent control mechanisms due to their high potential for causing major tissue damage if activated inadvertently. Therefore, most antigens require a triage of activated DC, a matching $CD4^+$ Th1 cell and antigen specific $CD8^+$ T cells, before a CTL response is commenced.[32-34] In the first step, the DC has to be activated by inflammatory signals such as endogenous danger signals or toll like receptor ligands (licensing)[35, 36], inducing a maturation of the DC, leading to an enhanced migration of the cells from the periphery to the secondary lymphatic tissues[37] and upregulation of costimulatory molecules like e.g. B7.1 / B7.2 (CD80/86), which bind to the activating receptor CD28 on the T cell surface.[38, 39] Furthermore, mature dendritic cells also upregulate CD40 ligand, which is a crucial interaction partner in the cross-talk between $CD4^+$ T helper cells and APC.[40-43] Mature APC also stabilize antigen loaded MHC-II complexes on their cell

surface, therefore enabling the cell to efficiently present antigens they collected in the periphery over a period of several hours in the secondary lymphatic tissue.[21] This allows bypassing $CD4^+$ T cells, which randomly scan APC in the lymph nodes until they find an interaction partner, to detect DC carrying the matching antigen, which leads to a stable interaction between the DC and the T cell.[44] This interaction subsequently activates the $CD4^+$ T cell, which in turn also gives further activation signals back to the APC.[44] Fully activated DC upregulate further markers necessary for $CD8^+$ T cell activation such as 4-1BBL.[45] If such a matured DC encounters a matching $CD8^+$ T cell, these cells also form stable interactions lasting for several hours, leading to immunogenic activation of the $CD8^+$ T cell, which in turn starts proliferating and developing into CTL effector cells.[44]
Although most antigens require $CD4^+$ T cell help to induce efficient $CD8^+$ responses, some highly infectious antigens, e.g. viral antigens derived from Influenza or LCMV, are capable of activating DC in the absence of T cell help, inducing activated CTL. However, this activation is somewhat limited, since the $CD8^+$ T cells primed without T cell help fail to produce sufficient T cell memory responses.[46-48]

4.2 Dendritic cell function and development

Dendritic cells are the major fraction of antigen presenting cells responsible for the induction of T cell responses. DCs were described as early as 1973 by Steinman and Cohn[49], but it was not until the early 1980s that the cells were linked to their function in T cell activation.[50] Much has been learned since then about the development and different functional properties of those cells.

Today, two classes of DC are being described: *conventional dendritic cells (cDC)* are responsible for activating and guiding T cell responses as will be discussed in detail below; *plasmacytoid dendritic (pDC) cells* have been described as a sort of effector dendritic cell, responding to viral infections by secreting large amounts of anti-viral cytokines like type I interferons, but do not seem to play a major role in activation of naïve T cells.
The major hallmark for identifying dendritic cells besides their morphology is the expression of a distinct set of receptors. Conventional DC express high levels of CD11c, which is also known as the integrin $\alpha_x\beta_2$, a cell adhesion molecule binding to intracellular adhesion molecule-1 (ICAM-1) as well as a receptor for complement product iC3b (CR4).[51] DCs also express basic levels of MHC-II, marking them as professional antigen presenting cells and to some extent also low levels of CD80/CD86. Several markers have been identified in the past that characterize different functional stages of cDC, although it is still an open debate, to what extent these cells might also represent different independent lineages of DC.

Markers commonly used for differentiation of DC are CD8α, CD4, CD11b, DEC205 and also to some extent F4/80[52]. These markers are not exclusive for DC and can also be found on T cells (CD4, CD8) or monocytes/macrophages (CD11b, F4/80).[53] Although all cDC are thought to arise from a common bone marrow progenitor, these markers allow to differentiate between cells coming from a common lymphoid precursor (CLP) in the thymus and a common myeloid precursor (CMP) emigrating directly from the bone marrow.[54] Commonly, CLP derived cells express CD4 and/or CD8 and also show rearrangement of their Ig-HDJ gene cluster. Flt3 ligand (FMS-like tyrosine kinase 3 ligand) is a crucial factor to induce DC differentiation in homeostatic DC development.[55] If absent, CMP cells develop into monocytes/macrophages and CLP cells into T cells, B cells and natural killer cells.[54]

In the steady state, CLP as well as CMP give rise to a common pre-DC precursor, which is thought to be the main source of DC residing secondary lymphatic organs. Phenotypically, these precursor cells express CD117, CX_3CR1 and CD11b but not MHC-II, which they gain later during DC development. These pre-DC also lack markers commonly found on monocytes such as CD115, Gr-1 and F4/80, which can also give rise to DC in peripheral tissues and especially under inflammatory conditions.[54, 56-61]

Dendritic cells in peripheral tissues form a tight network of sentinel cells, continuously sampling and trafficking antigens to the lymphatic tissues to maintain peripheral tolerance or to activate adaptive immune cells in the case of an infection. Peripheral tissue DC derive from CMP cells as well as mononuclear cells. Therefore, they commonly express CD11b as well as CX_3CR1 together with CD11c and MHC-II.[56, 62-64]

During an inflammatory reaction, mononuclear cells are being recruited from the blood stream into the periphery, enhancing the innate immune response already at very early timepoints.[65] Blood monocytes consist of two different, distinct subsets, which can be discriminated by their expression levels of Gr-1 as well as the chemokine receptors CX_3CR1 and CCR2.[56] Gr-1^{lo}CCR$2^{lo}$$CX_3CR1^{hi}$ monocytes (*Gr-1^{lo} monocytes*) are described as steady state precursor cells for tissue resident macrophages and dendritic cells in the periphery. They constitutively traffic the blood vessels of peripheral tissues, whereas Gr-1^{hi}CCR$2^{hi}$$CX_3CR1^{lo}$ monocytes (*Gr-1^{hi} monocytes*) shuttle back to the bone marrow where they differentiate into Gr-1^{lo} monocytes.[60] During an ongoing inflammation, the Gr-1^{hi} monocytes also accumulate at the site of inflammation, a process that is highly dependent on chemokine receptors CCR2 and CCR6.[66] Chemokines are a large class of small proteins with high chemotactic potential, differentially regulating leukocyte traffic throughout the body, depending on the expression patterns of their target receptors. CCR2 has been described to mediate release of Gr-1^{hi} monocytes from the bone marrow as well as tissue infiltration, whereas CCR6 seems to be of some importance for guiding infiltrating cells to the site of inflammation once the monocytes have left the bloodstream.[66, 67] Recruitment of

inflammatory Gr-1^{hi} monocytes has been described in various model systems, e.g. artherosclerosis[68], allergic airway inflammation[69], peritoneal inflammation[56] or *Salmonella* induced gut inflammation.[70]

As professional antigen presenting cells, dendritic cells activate CD4$^+$ as well as CD8$^+$ T cells. As already pointed out above, various routes of antigen uptake and processing exist, determining the way antigen is presented on the cell surface.[71] Especially activation of CD8$^+$ T cells is based on specialized mechanistic features specific for APC, enabling these cells to present exogenous antigen on MHC-I molecules, which under normal circumstances have been thought to be reserved only for presentation of cell-endogenous antigens and loading in the endoplasmatic reticulum. This so called cross-presentation is achieved by shuttling antigens into special compartments termed stable early endosomes (SEE), which contain MHC-I and direct antigen processing and MHC-I dependent presentation of antigen on the cell surface.[2] Up to today, the best-defined subset of cross-presenting DC is characterized by the expression of CD8α^+ and DEC205$^+$. These cells are mainly found in secondary lymphatic tissues and are thought to be the major source for activation (either tolerogenic or immunogenic) of CD8$^+$ T cells.[72]

Dendritic cell function is highly dependent on the maturation state of the cell, which is determining the signals the DC can provide either by cell-cell contacts or by secreting soluble mediators such as cytokines. Immature dendritic cells express low levels of MHC, only little amounts of costimulatory molecules but various receptors for antigen uptake (e.g. scavenger receptors, mannose receptor or DEC205).[54] Immature DC can act as tolerance inducers by secreting IL-10, a cytokine inhibiting immunogenic activation of T cells and favouring activation of regulatory T cells[22, 73-75]. They also carry receptors for DC activation such as the Toll-like receptor family (TLR).

TLR were first described in 1996 by Lemaitre et al in *Drosophila melanogaster*, showing that TLR mutants were highly susceptible to fungi infection.[76] In the following, it became clear, that the TLR-receptor family was designed to detect a broad variety of microbial derived patterns, so called pathogen-associated molecular patterns (PAMP) specifically found only in microbial organisms. Therefore, these receptors can be described as pattern recognition receptors (PRR).[77] Such patterns include e.g. bacterial derived glycolipids such as lipopolysaccharide, double stranded RNA as found during replication of RNA viruses, CpG DNA motives which are specific for bacterial DNA or lipopeptides such as Pam3Cys found on bacterial cell surfaces. Since all these patterns are specific for microbial organisms, the receptors allow DC to distinguish between antigens derived from host cells and foreign antigens derived from potentially pathogenic organisms, a concept described by Charles Janeway in 1992 as infectious non-self versus non-infectious self discrimination.[78] However, this concept had to be revised over the past few years, since it has become clear, that not

any microbial encounter induces an immune response (e.g. in the gut which is harbouring a total of approximately 3kg of bacteria) and that even under sterile inflammatory conditions APCs can commence T cell responses. The refined model, termed the ‚danger- model' by Polly Matzinger in 1994[79], also includes the context in which antigen is taken up by the APC. Antigens taken up in the absence of tissue damage or tissue damaging infection would not be considered harmful and therefore would not induce activation and maturation of the APC, whereas necrotic cell death, early innate inflammatory mediators or complement factors could be sensed by the APC and would be detected as an alarm signal.[35, 80, 81]

Dendritic cells that have received maturation signals undergo major changes in cell morphology and function. While resting DC show a branched, dendrite morphology and take up large amounts of antigens from their surroundings, maturing DC retract those dendrites and upregulate chemokine receptor CCR7[82, 83], which targets them to migrate to high endothelial venules (HEV), which are the entry point to the lymphatic system. Also, they stop their antigen sampling activity and start upregulating MHC and costimulatory molecules, e.g. for Th1 induction the DCs upregulate CD40 and start secretion of IL-12, which is a Th1 promoting factor.

DCs in Glomerulonephritis

As T cells have been described to be involved in autoimmune kidney disease, the involvement of renal dendritic cells also has become a question of great importance due to the direct link between DC function and T cell activation. Kidney dendritic cells have first been described by Krüger et al in 2004.[52] Phenotypically, they resemble monocyte derived DC, expressing CD11c and MHC-II marking them as DC, intermediate levels of CD11b and F4/80 and also to some extent B7 molecules. Functionally, they show lower phagocytic activity compared to macrophages and no effector function in terms of NO production. However, they efficiently prime T cells in mixed allogenic lymphocyte reactions, proving their function as APC. Kidney DC show a wide and regular distribution in the tubulointerstitial space and upon NTN induced inflammation accumulate at the site of cellular infiltrates.[52] Kidney resident DC also express high levels of CX_3CR1, marking them as monocyte derived cells of the Gr-1^{lo} phenotype. The interstitial DC form a tight network, therefore making them perfect sentinel cells for sampling any antigen reabsorbed by proximal tubular epithelial cells.[62, 63] Kidney resident DC have been described to be tolerogenic in homeostasis but cells isolated from inflamed kidneys showed properties of maturing DC.[55] This view is further supported by studies showing that early depletion of dendritic cells in nephrotoxic nephritis, which is a model for rapid progressive crescentic glomerulonephritis, aggravates development of renal disease. Resident renal DC also induce IL-10 in $CD4^+$ T cells *ex vivo*, showing their potential to act as inducers for immunosuppression.[84]

In contrast, kidney resident DC have also been described to be the main producer of TNF-α, IL−6 and proinflammatory chemokines such as RANTES and MCP-1 in early renal ischemia-reperfusion injury, indicating functional duality in immunity and tolerance depending on their state of activation.[85, 86] The cross-talk between $CD4^+$ T cells and APC is one of the major tissue-damaging factors in renal ischemia reperfusion injury, since a blockage of B7 signalling ameliorates chronic organ damage.[87, 88] Allograft rejection is also induced by migration of kidney resident DC into the renal lymph node followed by priming of T cells against kidney tissue antigens. Since this migration is CCR7 dependent, blocking of CCL19 signalling, which is the chemokine ligand for CCR7, substantially prolongs allograft survival.[89]

In addition, the requirement of B7 signalling has been reported in other nephritis models. In NTN, blockage of CD80/86 leads to attenuated infiltration of leukocytes and attenuated development of glomerulonephritis.[90]

4.3 Chemokines and homing of immune cells

Chemoattractant proteins have first been described in 1987 with the discovery of IL-8. Since then, more than 50 of such chemokines have been discovered, most of them due to their association with inflammatory responses. The first chemokine receptor described was the IL-8 receptor in 1991.[91] Up to now, more than 20 different receptors have been identified, belonging to four different families, based on different Cystein-motives commonly found within the members of each family (C,CC, CXC,CX_3C). The chemokine network is an ancient system already found in *D. melanogaster* and is not only used for immune system organization and cellular homing of leukocytes, but also plays an important role in various developmental processes during ontogeny.[92] The chemokine CXCL12 (SDF-1), which binds to the receptor CXCR4, is involved in brain and cardiac formation as well as in B cell development and homing inside lymph nodes.[93] All known chemokine receptors belong to the receptor family of G-protein coupled 7-Helix transmembrane receptors.

Chemokines are major roadsigns in leukocyte traffic during homeostasis and inflammation. CCR7 is the most important receptor in recruiting cells from peripheral tissues into the secondary lymphatic system.[83, 94] It is commonly expressed on DC which are leaving the tissue and is markedly upregulated upon DC maturation.[83] CCR7 ligands CCL19 (ELC) and CCL21 (SLC) are expressed by endothelial cells of high endothelial venules, which are the entry points for leukocytes to the lymphatic system. Upon inflammation, CCR7 ligands are upregulated to enhance migration of the APC into the lymph nodes.[83] Naïve T cells also may enter the lymphatic system by using CCR7, however, CXCR3, CXCR4 and CXCR5 can substitute for CCR7 in T cell and NK cell traffic to LN.[95]

Chemokines also direct cellular locations inside tissues. CXCR4 signalling is essential in organization of lymph follicles, B cell homing and development. T cell homing into lymph follicles is mediated by CXCR5 / CXCL13 (BCA-1) signals similar to CCR7.[94] CXCL13 can be detected inside B cell follicles and mantle but not in T cell zones, therefore directing contact between B cells and T cells in the lymph node.[94, 96] Dendritic cells can also specifically attract naïve T cells by secretion of CCL19/CCL21 (ELC/SLC).[96] DCs also produce chemokines such as CXCL9, CXCL10, CXCL11 (MIG, IP-10, ITAC) upon activation leading to an attraction of Th1 cells via CXCR3, a process which might be highly relevant in CTL priming to provide efficient T cell help during CTL priming.[97] Antigen specific DC contact blocks T cells from exiting peripheral tissue and induces downregulation of CCR7 and upregulation of CCR5 and CXCR6.[98] Also, sphingosin-1-phosphate (S1-P) signaling, which is essential for exiting lymphoid tissues is downregulated to restrain cells in the lymph nodes during the initial priming phase. S-1P signalling is restored after approximately four cell divisions, allowing effector lymphocytes to exit the lymphatic tissues and migrate into the periphery.[95]
Certain chemokines have been associated with homing properties of leukocytes into peripheral tissues. In homeostasis, CCR4 and CCR8 are supposedly being used for skin homing of leukocytes.[99, 100] Cells migrating into gut tissue are commonly found to express CCR6. This tissue specific imprinting is mediated by DC coming from the target tissue. As an example, DC from Peyer's patches induce CCR9, Langerhans cells induce CCR4 and CCR8 in T cells.[95]
Another factor in tissue homing which is especially important for homeostatic migration of monocytes into peripheral tissues is the Fractalkin-receptor (CX_3CR1).[64] Fractalkin is produced by endothelial cells and is known to have strong adhesive effects on leukocytes and therefore allowing them to stick inside peripheral tissues.[101] Another feature described specifically for tissue resident CX_3CR1^+DC is their extensive ability to sample antigens by protruding long dendrites even through endothelial barriers sealed by tight junctions, e.g. the gut lumen, a process which has been described as antigen snorkelling.[64]

Different types of immune reactions are closely linked to chemokine expression patterns. Classical chemoattractant receptors such as C5a or C3a receptors recruit cells of the innate immune response such as neutrophils and eosinophils and also induce production of TNF-α and acute phase proteins.[95] Monocytes commonly express CCR1, CCR2, CCR6, with CCR2 playing a special role in emigration of cells from the bone marrow and invasion into inflamed tissues.[56, 58, 65, 67, 102]
T cells show differential expression of chemokine receptors depending on their functional state. Naïve T cells express CCR7 and CXCR4/CXCR5, guiding them to secondary lymphoid organs and bringing them into contact with APC. Activated $CD4^+$ T helper cells express

either CCR5/CCL5, CCL3, CCL4 (RANTES, MIP-1), CXCR3/CXCL10 (IP-10) if they are of Th1 type[101, 103] or CCR3/CCL11 (Eotaxin) and CCL13 (MCP-4) if they are of Th2 type.[104] CCR4 and CCR8 have also been reported to be linked to Th2 responses, but rather determines homing capacities to skin.[99] CCR3/CCL11 signalling is closely linked to allergic inflammation and induces recruitment of Th2 cells as well as eosinophils. Chemokines also polarize T helper cell responses. CCL26 actively agonizes CCR3 but antagonizes CCR2, CXCL9 agonizes CXCR3 but antagonizes CCR3.[95] CCR5 is also a chemokine receptor found on activated $CD8^+$ T cells.[101, 103] Once activated, T cells lose CCR7 and CXCR5 and gain CXCR3 and CCR5 or CCR3. Expression of these inflammatory chemokine receptors in T cells strongly depends on IL-12 signaling.[105] Memory T cells differentially express CCR7: central memory are $CCR7^+$ and home to secondary lymphoid organs, effector memory T cells are $CCR7^-$ and quickly develop into effector cells in the periphery if reactivated.[106]
Activated T cells also express inflammatory chemokines CCL3 (MIP-1α), CCL4 (MIP1-β) and CCR5 (RANTES) early upon activation.[95]

Tab. 1: *Chemokines and chemokine receptors expressed by leukocytes*

	HOMEOSTASIS		INFLAMMATION	
	Receptors	**Chemokines**	**Receptors**	**Chemokines**
DC	CCR1, CCR4, CCR5, (CCR7), CX_3CR1	CCL19, CCL21	CCR1, CCR2, CCR5, CCR6, CCR7	CCL19, CCL21, CCL17, CXCL9, CXCL10, CXCL11
Monocytes	CCR1, CCR2, CCR5, CCR6, CX_3CR1	-	CCR2	CCL2, CCL3, CCL4, CXCL9
B cells	CXCR4, CXCR5	CCL17, CCL19, CCL21		CCL17
T cells	CCR7, CXCR4, CXCR5, CCR4, CCR8 (skin)	-		
-Th1			CCR5, CXCR3	CCL3, CCL4, CCL5
-Th2			CCR3, CCR4	CCL13
-CD8			CCR5, CXCR3	CCL4, CCL5

Chemokines have also been linked to various immune mediated diseases. In experimental autoimmune encephalitis, a mouse model for multiple sclerosis, CCL2, CCL3, CCL4 as well as CXCL10 are readily detectable in neural lesions. CCL3 has been linked to the acute phase of the disease, whereas CCL2 has been reported to be more important for relapse.
In allograft rejection, the acute phase infiltrate consists of monocytes and neutrophils recruited by CXCL2 (MIP-2), KC and CCL. In the late phase, CXCL9, CXCL10 and CXCL11 as well as CCL4 and CCL5 lead to the infiltration of T cells. Knockout of these chemokine signals show significantly enhanced graft survival.[107] Development of allergic asthma highly depends on Th2/CCR3 activation. Rheumatoid arthritis is linked to CCR2/CCL2, but also the

block of CCR1/CCR5 signalling by the inhibitor met-RANTES is effective in suppressing the inflammation.[108] Pulmonary fibrosis depends on CXCR3 and IFNγ,[109] CXCR3 has also been described in the activation of human myofibroblasts.[110]

Chemokines in renal disease

Chemokines have also been identified to be involved in development of renal disease. After renal transplantation, antibody mediated CCL19 block prevents allograft rejection and enhances graft survival. However, the outcome is not enhanced in $CCR7^{-/-}$ recipients, indicating that migration of organ resident leukocytes facilitates graft rejection.[89]
$CXCR3^{+}$ cells are part of any renal infiltrate detectable in renal biopsies from patients with IgA nephropathy, lupus nephritis, and membranoproliferative glomerulonephritis.[111] *In vitro* studies show that tubular epithelial cells, which have been activated by TNF-α or IFN-γ, express T cell attracting chemokines CCL5 and CXCL10.[112] In nephrotoxic nephritis, CXCR3 ligands are also found in renal tissue. $CXCR3^{-/-}$ mice show significantly ameliorated disease development, however, T cells isolated from these mice proved to be fully functional in effector T cell development, indicating impaired trafficking rather than functional defect of T cells *in vivo*.[113]

CCR1 and CCR5 have also been reported to be expressed in glomerular and interstitial lesions from various patients with different forms of glomerulonephritis. Also, urinary excretion of CCL3, CCL4 and CCL5 could be detected, indicating increased expression due to the renal inflammation.[114] However, in mouse nephrotoxic nephritis, $CCR5^{-/-}$ mice show an aggravated disease phenotype, presumably due to an overshooting CCR1 reaction. Blockage of CCR1 signalling using the CCR1 inhibitor BX471 in $CCR5^{-/-}$ mice leads to an attenuation of renal disease, resulting in less infiltration of inflammatory cells into the organ and reduced crescent formation.[115]

Chemokine induced monocyte infiltration also seems to be of some importance in nephritis development. For example, in Apoferritin-Nephritis, a model for immune complex induced nephritis, $CCR2^{+}$ and $CCR5^{+}$ inflammatory monocytes are abundantly found in inflamed renal tissue.[116]

Podocytes have also been reported to express various receptors for both CC and CXC chemokines *in vitro* and *in vivo*; however, their involvement in renal disease development has yet to be determined.[117]

4.4 Glomerulonephritis disease phenotypes: an overview

To clarify the overall aim of this study, it is necessary to introduce the various forms of renal disease in detail and to elucidate the involvement of T cells in the different disease phenotypes. As summarized by Kurts, et al, there is evidence for the involvement of T cells in almost any form of renal disease. Furthermore, all different types of misguided T cell responses can induce glomerular disease, including cytokine mediated disease, autoantibody formation, immune complex deposition, activation of macrophages or $CD8^+$ T cells.[118] $CD4^+$ T helper cells can act as key players in all types of renal disease by activating either B cells or DC. T cells have been described in human diseases like IgA nephropathy, diffuse proliferative GN, crescentic GN, Lupus nephritis and anti-neutrophil cytoplasmic antibody (ANCA)-nephritis. Also, the loss of active immune regulation by regulatory T cells has been described as a possible cause for the induction of GN.[119] $CD8^+$ T cells seem to be involved in the pathology of some forms of nephritis, although the evidence is somewhat less clear compared to other mechanisms of renal autoimmune disease development. Their involvement has been described as pathogenic in Lupus nephritis[120], rat NTN[121] and experimental autoimmune nephritis.[122] However, the exact mechanisms of T cell mediated GN still remain an open debate.

The various forms of renal autoimmune diseases can be classified into four major groups, depending on the major cause of disease: 1. Allergies, termed type I hypersensitivity reactions, involving formation of autoreactive IgE and activation of mast cells. 2. Type II hypersensitivity reactions by formation of autoreactive, complement activating antibodies, e.g. IgM, IgG1 and IgG3; 3. Type III hypersensitivity reactions involve the formation of immune complexes. 4. Cellular responses such as delayed type hypersensitivity (DTH) are termed type IV hypersensitivity reactions including activation of $CD4^+$ and $CD8^+$ T cells.

Type I Hypersensitivity: Allergy

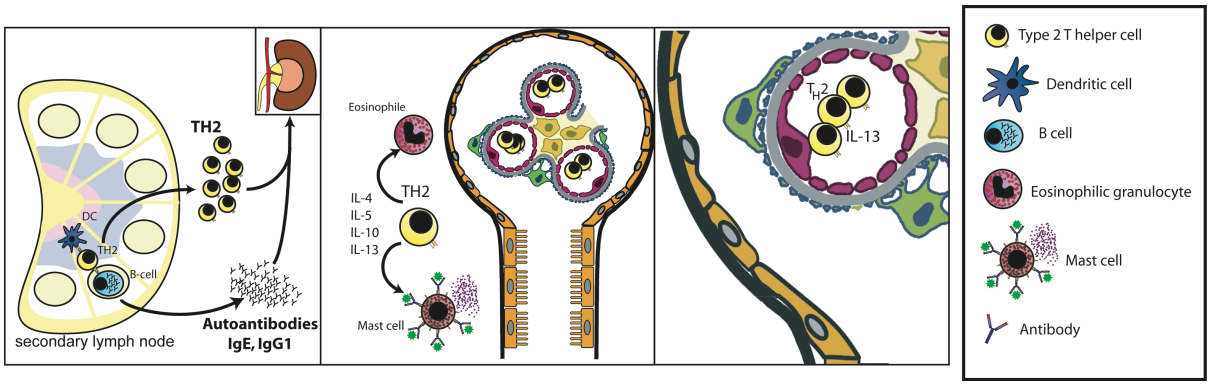

Fig. 1 *Th2-type response in renal disease.* $CD4^+$ T cells are activated and differentiate into Th2 helper cells, activating B cells to produce IgE (left). Th2 cells migrate to the kidney and together with activated Eosinophils and Mast cells can damage the podocytes by release of cytokines (left, right)

Little is known about the role of mast cell activation in renal inflammatory disease, although these cells are constitutively present in the kidney, albeit in low numbers. However, there is at least some evidence, that classical Th2 responses and IgE formation lead to cytokine mediated podocyte damage e.g. via IL-4, IL-10 or IL-13.[118, 123, 124] Podocytes express receptors IL4-Ra and IL13-Ra1/2 and show activation of STAT6 and NFκB signalling cascade *in vitro*, however, the functional consequences in vivo yet remain to be determined.[125, 126] Also, human podocytes express chemokine receptors CCR4, CCR8, CCR9, CCR10, CXCR1, CXCR3, CXCR4 and CXCR5 *in vitro* and are capable of IL-8 production, these factors may also influence podocytic stress responses induced by chemokine signals upon inflammation.[127]

Type II Hypersensitivity: Autoantibodies

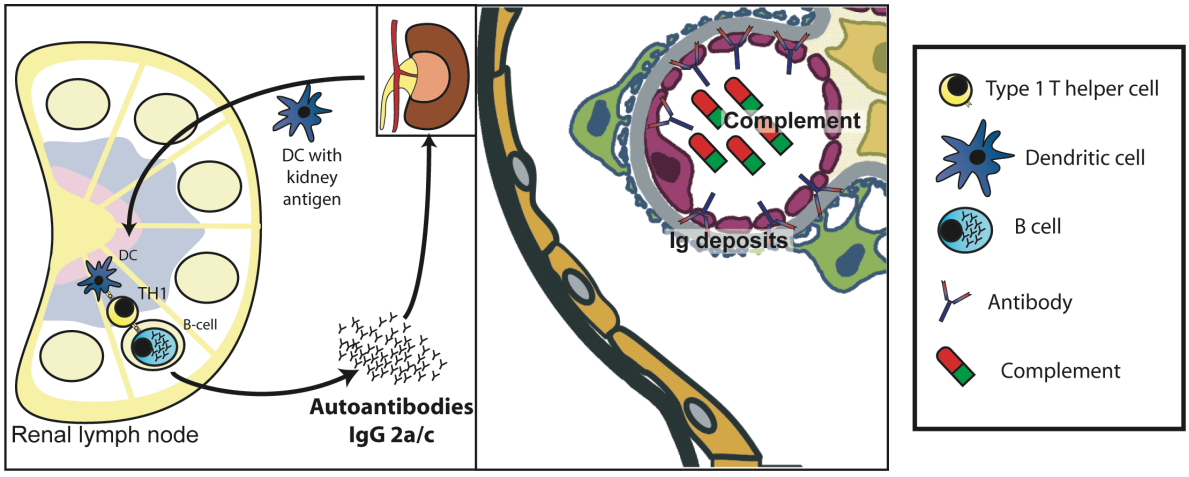

Fig.2 *Antibody mediated kidney inflammation.* $CD4^+$ T cells are activated and differentiate into Th1 helper cells, activating B cells to produce IgG2a/c (left). Autoreactive antibodies enter the kidney via the bloodstream and bind to their target structures, e.g. the basement membrane, and trigger complement activation (right).

The classical form of antibody induced renal disease is found in patients with the so called Goodpasture syndrome, mediated by antibodies directed against the α3 chain of type 4 collagen, which is an integral part of the glomerular basement membrane (GBM), causing severe kidney and lung autoimmunity. Since the epitope is so well defined, there are several rodent model systems to mimic anti-GBM nephritis by immunization against collagen α-chain. Although the necessity of the primary antibody response is undoubted, there is also evidence that local T cell responses are involved in disease progression as well, since adoptive transfer of antibodies alone is not sufficient to cause renal disease.[128] Similarly, in active Heymann nephritis[129], antibodies against renal cortical antigens are deposited between the GBM and podocytes, also triggering complement activation. Depletion of $CD8^+$ T cells seems to be beneficial in some cases, showing a possible role for cytotoxic T cell

responses in renal injury.[122] Another type of antibody mediated renal disease is the so-called IgA nephropathy. T helper cells are used in this case to class-switch antibody producing B cells to generate IgA. Depletion of $CD4^+$ T cells leads to reduced deposition of IgA and amelioration of disease, but no impairment of circulating IgA. Also, transfer of autoantibodies alone fails to induce nephropathy, suggesting an organ specific activation of T cells in the kidney. Similar to the anti-GBM model, depletion of $CD8^+$ T cells attenuates progression of disease.[130] However, IgA nephropathy is unlikely to be a classical type II hypersensitivity reaction, since only IgA1 induces weak activation of complement, therefore the immunopathological mechanisms most likely differ from anti-GBM GN and may well also involve T cell mediated immune responses.

Type III Hypersensitivity: Immune Complex formation

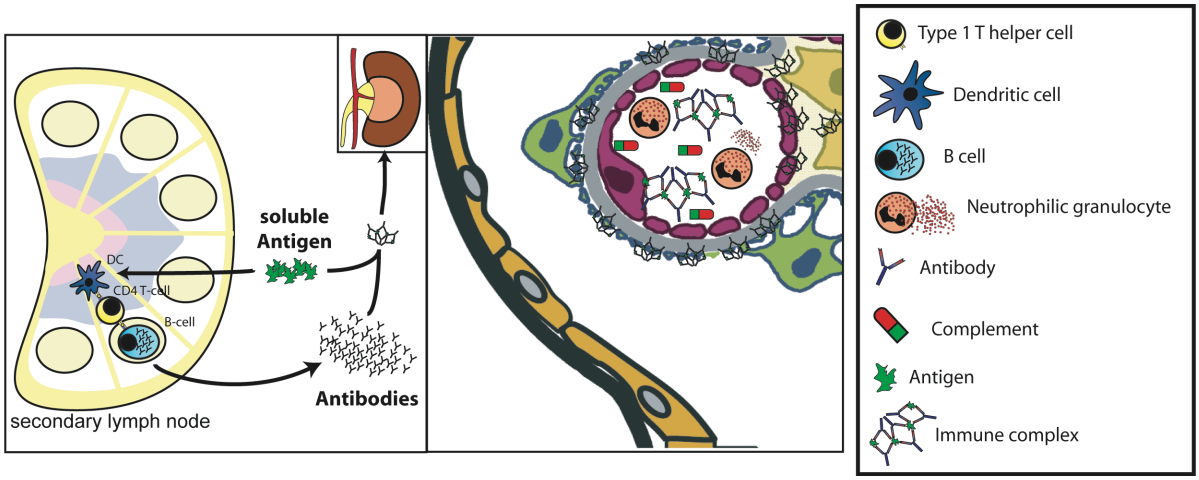

Fig.3 *Immune complex mediated kidney inflammation.* $CD4^+$ T cells are activated and differentiate into Th1 helper cells, activating B cells to produce antibodies against soluble antigens (left). Immune complexes enter the kidney via the bloodstream and precipitate in the basal membrane, activating complement factors and local macrophages and granulocytes (right).

Type III hypersensitivity reactions are immune reactions not targeting the kidney directly, rather the organ damage can be seen as a bystander reaction due to the local trapping of the immune complexes in the glomerular basement membrane. In membrane proliferative GN (passive Heymann Nephritis[131]), CD4 helper cells activate B cells to produce antibodies against soluble antigens, immune complex deposition in GBM leads to activation of complement and recruitment of phagocytes via MCP-1, RANTES, CCR1, CCR2, and CCR5.[132] Immune complexes act mainly via FCγRI and FCγRIII.[133] However, complement activation cannot be the only factor promoting disease progression, since it has been shown that C1q deficient mice are more prone to kidney disease in a model of accelerated GBM-nephritis.[134]

Also, a role for T cell mediated immunity has been suggested, since their depletion attenuates disease.[135-137] A special form of membranous nephritis is the post-streptococcal GN, where antibodies are formed against streptococcal antigens. These immune complexes then locate to the GBM and lead to recruitment of granulocytes. In the model of accelerated NTN, immune complexes are formed by immunizing animals against sheep immunoglobulins followed by a challenge with sheep IgG directed against kidney cortex antigens of the target animal[133, 134, 138]. In lupus nephritis, anti-DNA antibodies induce systemic immune complex formation. However, the progression of renal disease is highly dependent on $CD4^+$ T cells and cannot be explained solely by the activation of complement factors and macrophages. In fact, lupus nephritis seems to be based on a heterogeneous and complex immunopathology, involving mixed Th1/Th2 reaction[130, 139, 140], IFN-γ effector function[141-143] and possibly also loss of regulatory T cell function. It is most likely, that systemic lupus is a mixed reaction of type II, type III and possibly type IV hypersensitivity altogether, involving many different aspects of immune activation and most likely also $CD4^+$ and $CD8^+$ T cell responses. Similarly, in Wegener Granulomatosis, the formation of anti-neutrophil cytoplasmic antibodies (ANCA) lead to a systemic vasculitis also targeting the kidney, however, secondary responses in the kidney also depended on macrophage activation and a Th1 type T cell response.[144]

Type IV Hypersensitivity: Cellular immune responses / DTH

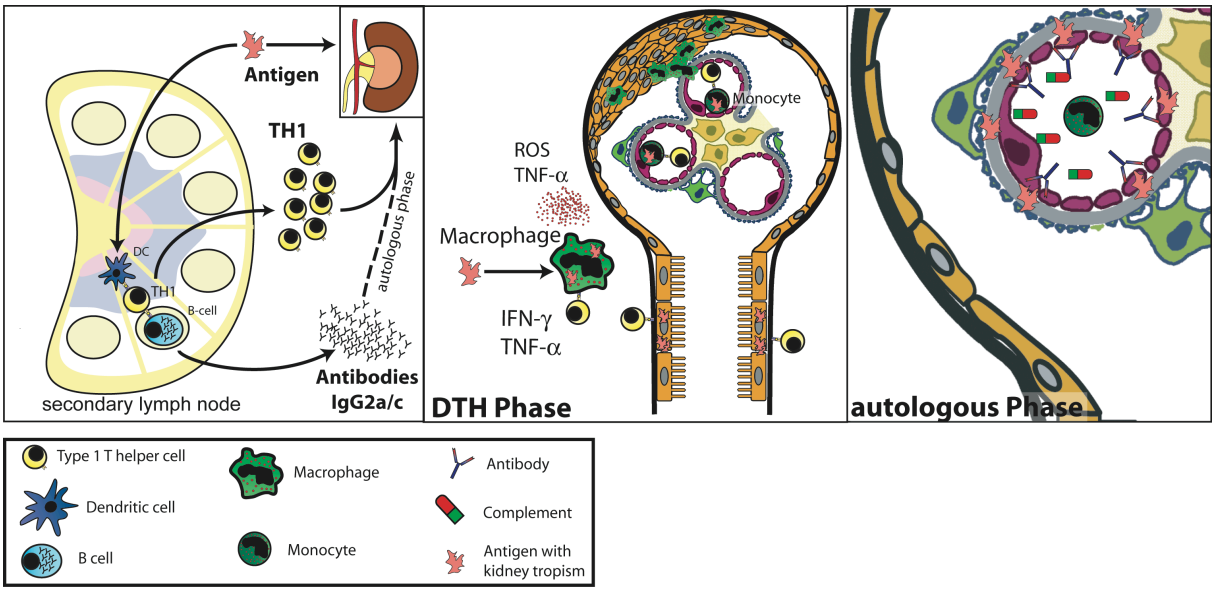

***Fig. 4** DTH response in renal disease.* $CD4^+$ T cells are being activated against antigens with kidney tropism and differentiate into Th1 type helper cells and travel to the kidney. Furthermore, these cells would then activate B cells similar to type II and III responses (autologous phase) (left). Activated $CD4^+$ T cells then activate local APC and phagocytic cells such as monocytes/macrophages, inducing a release of proinflammatory cytokines and reactive oxygen species (ROS) (middle). Autoantibodies travel to the kidney via the bloodstream and enhance cellular immune responses (right).

The involvement of T cells has been described in human rapid progressing GN already more than two decades ago.[145,146] The animal model of rapid progressive crescentic nephritis induced by injection of nephrotoxic sheep or rabbit sera (nephrotoxic nephritis or NTN) against renal cortex antigens is by far one of the most well studied renal disease phenotypes and has closely been linked to anti-renal T cell responses. In NTN, Th1 cells activate local macrophage response and trigger the release of ROS and TNF-α, leading to a type IVa hypersensitivity reaction of the DTH type. Ablation of macrophages in CD11b-DTR mice attenuates progression of crescentic GN, reducing crescent formation, proteinuria and serum creatinine and also renal fibrosis.[147] NTN highly depends on IL-12 signaling[148] and local antigen presentation on MHC-II not exclusively from hematopoietic cells but also from tubular cells or mesangial cells which upregulate MHC-II molecules upon inflammation.[149] Also, podocytes have been described to upregulate CD80/86 molecules upon LPS stimulation *in vivo*, suggesting a direct influence of the renal parenchymal cells on kidney infiltrating T cells.[150] However, NTN disease progression is highly dependent on CD80/CD86 signalling for leukocyte recruitment and glomerular injury. Blockage of CD80/86 by monoclonal antibodies leads to attenuated infiltration of leukocytes and less severe GN.[90] Also, CD80/86 blockage ameliorates chronic organ damage after cold ischemia/reperfusion injury.[87, 88]

Also, it is known that functional regulatory T cells can counteract anti-GBM nephritis.[119] The involvement of $CD8^+$ T cells is far less understood and seems to depend on the model organism used. Depletion of $CD8^+$ T cells was beneficial in rats but not in mice[121, 151] and MHC-I mediated antigen presentation in mice does not seem to contribute to renal disease progression.[152]

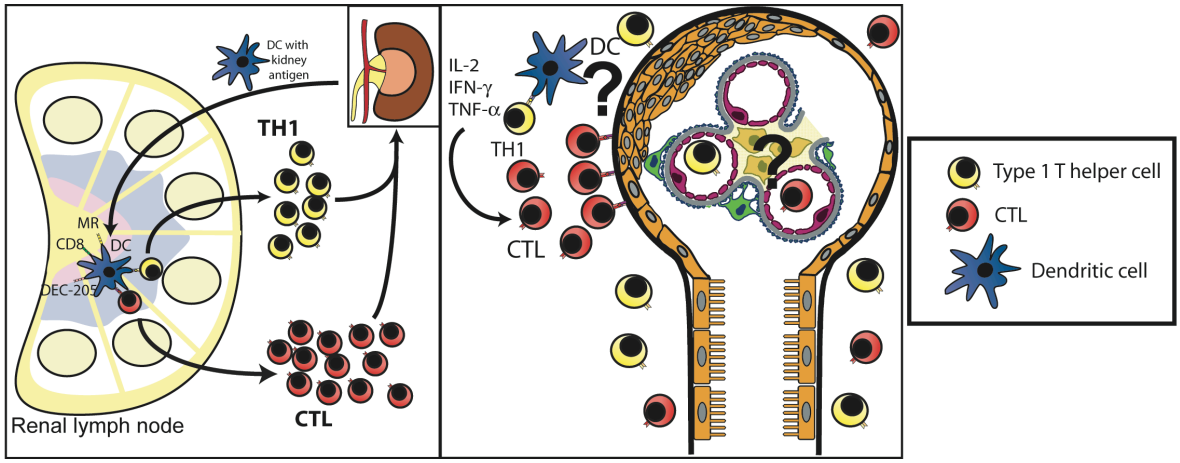

Fig. 5 *CTL response in renal disease.* $CD4^+$ and $CD8^+$ T cells are being activated against antigens with kidney tropism and differentiate into Th1 type helper cells and CTL and travel to the kidney. (left). Activated Local APC present target antigens to infiltrating $CD4^+$ T cells, leading to a secretion of proinflammatory mediators and chemokines, inducing further inflammation of cells and formation of cellular infiltrates. $CD8^+$ T cells infiltrate the renal tissue and perform CTL effector functions, targeting kidney cells.

Little is understood so far about the role of $CD8^+$ T cells in renal disease. As already pointed out above, $CD8^+$ T cells are likely to be involved in many renal autoimmune diseases to a variable degree, although the underlying mechanism yet remained unclear.

In lupus nephritis, a major fraction of kidney infiltrating lymphocytes persists of $CD8^+$ T cells and are linked to progression of renal disease.[120] Adriamycin (Doxorubicin), an anthracycline antibiotic used in cancer therapy, induces so-called Adriamycin nephropathy, primarily a non-immune proteinuric glomerulopathy, which mostly resembles focal segmental glomerulosclerosis. However, in late stages of the disease $CD4^+$ and $CD8^+$ T cells infiltrate the tubulointerstitium and may influence progression of disease.[153] Blockage of CD40 signalling by injection of monoclonal anti-CD40 antibodies (MR1) protects the kidney against lupus nephritis and Adriamycin nephropathy, leading to significantly reduced renal inflammation, expression of proinflammatory chemokines (MCP-1, RANTES), proteinuria and renal fibrosis.[154] In mesangioproliferative GN (anti-Thy1 Nephritis), the injection of the antibody against Thy1.1, a marker found on T cells as well as mesangial glomerular cells in rats induces mesangiolysis and compensatory cell growth. Interventional studies depleting $CD4^+$ and $CD8^+$ T cells showed an attenuation of disease, however the mechanistic basis is not understood.[155, 156]

The best evidence for a role of $CD8^+$ T cells in induction and progression of renal disease is the finding that CTL play a major role in primary interstitial nephritis[157-159], where $CD8^+$ T cells have directly been linked to progression of renal damage. However, the kidney seems to be somewhat better protected against cell mediated autoimmune reactions compared to e.g. pancreatic β-islet cells, which are a lot more susceptible to destruction by $CD8^+$ T cells.[160] It has been shown that renal tubular epithelial cells are able to induce tolerance in $CD4^+$ T cells.[161]

Interstitial nephritis development is highly dependent on the presence of reactive $CD4^+$ T cells, a hallmark also of the T cell induced GN in NOH mice as will be discussed below.

Taken together, these studies suggest that T cell effector functions seem to play an important role in the development of renal disease. The formation of periglomerular infiltration including T cells seems to be an early event in various forms of GN. Progression of these infiltrates may occur after rupture of Bowman's capsule followed by crescent formation and glomerulosclerosis.[162]

T cells produce effector cytokines like IFN-γ and TNF-α, which are directly linked to severity of GN development. IL-12 and TNF-α knockout-mice show attenuated renal disease. Also induction of Th2 responses by *in vivo* cytokine injection attenuates GN development.[163-167] Recruiting signals such as CXCR3/CXCL10, which is a T cell homing signal, have been described as essential factors in NTN.[113] Similarly, CXCR3 has been described in the

development of human GN as well[111] but can also express T cell attracting chemokines RANTES and IP-10 upon inflammation.[112] The role for IFN-γ signaling is less clear, a dual role has been described, either aggravating[166, 168] or ameliorating[138] renal disease, depending on timing and cosignals like IL-10.[169] Overall, the outcome of renal disease is directly linked to the strength of the cellular immune reaction and therefore the prognosis of renal disease.[170]

4.5 Aim of this study

The concept of autoimmunity has been described by Paul Ehrlich more than a decade ago as ‚horror autotoxicus', the misled activation of autoreactive effector cells as well as the formation of autoantibodies against self-antigens. Much has to be learned still about the underlying mechanisms, although there has been some recent major advances in understanding the cellular mechanisms that can lead to e.g. Diabetes type I[171, 172] and T cell mediated encephalomyelitis.[173, 174]

Renal autoimmune diseases are a complex and heterogeneous cluster of disease phenotypes, which can be divided into two major groups, namely glomerulonephritis (GN) and interstitial nephritis, regarding the microanatomical location of the inflammation.

Almost all parts of the immune system have been described to contribute to autoimmune kidney disease, but only for some such as autoreactive IgG, immune complex deposition and $CD4^+$ T cell mediated delayed type hypersensitivity (DTH) responses, the underlying mechanisms are beginning to be better understood.

Especially the role of T cells has been a matter of controversy for decades[145, 175-177] with evidence evolving that they may be involved in various forms of glomerulonephritis and interstitial nephritis to a variable degree. However, most of the evidence is either indirect or is coming from interventional studies that do not specifically target T cell responses, therefore the exact sequence of events of T cell mediated GN yet remains unsolved.

With the NOH mouse model it was possible for the first time to specifically investigate such T cell responses. In this thesis, the consequence of CTL mediated T cell damage as well as the functional role of T helper cells was addressed by adoptively transferring these cells into NOH mice. Also, the functional role of DCs in the development of T cell mediated nephritis was further investigated, depleting those cells at different disease stages and evaluating the outcome on renal disease development.

Development of GN was monitored by histopathological changes of the renal tissue architecture, immunohistochemistry and flow cytometry to quantitatively and qualitatively dissect the inflammatory reactions inside the organ and quantitative analysis of kidney filtration to assess renal loss of function induced by the inflammation.

5. Materials and Methods

Equipment

5417R (Eppendorf, Hamburg)

AbiPrism7000® Sequence detection system (Applied Biosystems, Darmstadt)

Analysenwaage Explorer (Ohaus, MA Bradford, USA)

Autoklav Infection control (Belimed GmbH, Mühldorf)

Axioskop (Zeiss, Jena, Germany)

Bestrahlungsgerät Biobeam2000 (MCP Medical International GmbH, Berlin)

CO2 begaster Inkubator Heraeus B 5061 EK-CO2 (Heraeus, Hanau)

Cryostat Leica CM1900 (Leica, Mainz)

Dispenser HandyStep (Brand GmbH, Wertheim)

ELISA reader MR5000 (Dynatech, VA Chantilly, USA)

Eppendorfpipetten 1-10µl, 5-40µl, 40-200µl, 200-1000µl, 1-5ml (Eppendorf, Hamburg)

12-Kanal-Pipette (Eppendorf, Hamburg)

FACS-Gerät FACS Canto II®; LSR II®, DiVa® (Becton-Dickinson, Heidelberg)

Flüssigstickstofftank XLC 810 HE (MVE Biological Systems, MN Burnsville, USA)

Hämozytometer Neubauer (Novoglas, Bern CH)

IVC Mauskäfige (Tecniplast, Hohenpeißenberg)

Julabo TW8 (Mitlacher, Mainz)

Labormikroskop DMIL (Leica, Hamburg)

Magnetrührer MR 3001 (Heidolph, Schwabach)

Mikroskopkamera BX-FLA F-View (Olympus, Hamburg)

Mikrotom RM2125 (Leica, Wetzlar)

Mikrowelle Micromat 241 (AEG, Nürnberg)

Mini-PROTEAN Tetra Electrophoresis System (Biorad, München)

Multifuge 3 SR (Heraeus, Hanau)

Nanodrop Spectrophotometer (Thermo Fisher scientific, Wilmington, DE, USA)

PALM® MicroBeam IP 230V Z laser microdissection microscope (P.A.L.M, Bernried)

ph-Meter PHM210 (Radiometer, Copenhagen)

Pipettierhilfe Pipetus standard / accu (Hirschmann, Herrenberg)

Rotlichtlampe Infrafil (Phillips, Hamburg)

Power supply EPS301 (Amersham, Straubing)

Sterile Werkbank Heraeus HeraSafe HS15 Air (Heraeus, Hanau)

Systemmikroskop IX71 mit U RFL-T UV-Lampe u. Fluoreszenzfiltern (Olympus, Hamburg)

Vortex Genie 2 (Bender & Hobein, Zürich, CH)

Wasserbad Typ 1083 (GFL, Burgwedel)

Wasserdeionisierungs-Anlage EASYPURE® II UV (Barnstead, MA Boston, USA)

Zentrifugen Megafuge 1.0R (Heraeus, Hanau)

Consumables

24x 50 mm (Menzel-Gläser)

18x 18 mm (Marienfeld)

Einfrierröhrchen 1,8ml Volumen (Nr.3-63401, Nunc, Wiesbaden)

Einmalkanülen 0,4 x 12mm Sterican (Braun, Melsungen)

0,7 x 30mm 22 G x 11/4 Luer-Lock Sterican (Braun, Melsungen)

0,8 x40mm 21 G x11/4 Luer-Lock Sterican (Braun, Melsungen)

Einmalspritzen 1/2/5/10/20ml (Braun, Sterican)

ELISA-Platten 96-Vertiefungen-Flachbodenplatte (Maxi-Sorp, Nunc, Wiesbaden)

Eppendorfreaktionsgefäße 2ml/1,5ml/0,5ml (Eppendorf, Hamburg)

FACS Röhrchen (BectonDickinson Labware, NJ., USA)

FALCON –Röhrchen 15 ml Bluecap Polypropylen (BectonDickinson Labware, NJ., USA)

50 ml Bluecap Polypropylen (BectonDickinson Labware, NJ., USA)

15ml PP, 50ml PP (Greiner, Frickenhausen)

Glaspipetten 2 ml, 5 ml, 10ml, 25ml Cellstar (Greiner,Nürtingen)

Objektträger SuperFrost/Plus 25x 75x 1mm (Menzel-Gläser)

Parafilm (American National Can, USA)

Pipettenspitzen 1-10µl (kristall) (Roth, Karlsruhe, Karlsruhe)

10 µl-100µl (gelb) (Roth, Karlsruhe, Karlsruhe)

100-1000µl (blau) (Roth, Karlsruhe, Karlsruhe)

Polyethylene membrane covered slides (P.A.L.M., Bernried)

Sterilfilter 0,2µm, steril, rotrand (Schleicher & Schuell,Dassel)

Stoffwechselkäfige (Tecniplast, Hohenpeißenberg)

Tissue-Tek Cryomold® Biopsy (Miles, USA)

Zellsieb Falcon 40µm Nylon, steril (Becton Dickinson, USA)

Zellkulturflaschen 25cm^2/50ml, 75 cm^2/250ml (IBS integra Biosciences)

Zellkulturplatten 6,12,24 Vertiefungsplatten, flach (CorningCostar, Bodenheim)

Reagents

Aceton (Merck, Darmstadt)

Acrylamid for SDS page (Roth, Karlsruhe)

Aluminiumhydroxid (Sigma, München)

Ammoniumchlorid (Roth, Karlsruhe)

Ammoniumpersulfat (Roth, Karlsruhe)

Bradford Reagenz (BioRad, München)

β-Mercaptoethanol (Sigma, München)

BSA Fraction V, low endotoxin cell culture grade, (Rinderserumalbumin) (PAA, Darmstadt)

CaliBRITE® APC-beads (BD, Heidelberg)

Carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Karlsruhe)

CD3/CD28 T cell stimulation beads (Invitrogen, Kalrsruhe)

Clodronate liposomes (Department of Molecular Cell Biology, Amsterdam, Netherlands)

Collagenase D (Roche, Mannheim)

Coomassie brilliant blue (Sigma, München)

Cresylviolett (Sigma, München)

Diaminobenzidin tablets (DAB) (Sigma, München)

Dabco (Sigma, München)

Dimethylsulfoxid (DMSO) (Roth, Karlsruhe)

Diphteria Toxin (Sigma, München)

DNAse I (Roche, Mannheim)

dNTPs (Invitrogen, Karlsruhe)

Ethylendiamin-tetraessigsäure Na2-Salz x 2xH2O (EDTA) (Sigma, München)

Ethylenglycol-bis(2-aminoethyl)-tetraessigsäure (EGTA) (Merck, Darmstadt)

Essigsäure (Roth, Karlsruhe)

Ethanol (EtOH) (AppliChem, Darmstadt)

fetales Kälberserum (PAN Systems GmbH, Nürnberg)

Formaldehydlsg. 39% (Sigma,München)

L-Glutamin 200mM (Gibco, Paisley, UK)

Glyzin (Roth, Karlsruhe)

Hämatoxilin solution (Sigma, München)

HEPES (Roth, Karlsruhe)

Histoclear (Roth, Karlsruhe)

Histokit (Roth, Karlsruhe)

Hoechst-33342 (Invitrogen, Karlsruhe)

Isopropanol (2-Propanol) (AppliChem, Darmstadt)

Mowiol (Hoechst, Bad Soden)

Natrium-Acetat (Roth, Karlsruhe)

Natrium-Azid (Roth, Karlsruhe)

Natriumcarbonat (Na2CO3) (Roth, Karlsruhe)

Natriumhydrogencarbonat (NaHCO3) (Roth, Karlsruhe)

Natriumchlorid (NaCl) (Roth, Karlsruhe)

Natrium-Citrat (Roth, Karlsruhe)

Natriumhydrogencarbonat (NaHCO3) (Roth, Karlsruhe)

Natriumdihydrogenphosphat (NaH2PO4 x 2xH2O) (Roth, Karlsruhe)

Natriumdodecylsulfat (SDS) (Roth, Karlsruhe)

NaOH, Plätzchen (Roth, Karlsruhe)

Neufuchsin (Merck, Darmstadt)

o-Phenylendiamin (OPD) (Sigma,München)

Ovalbumin, Grade V (Sigma,München)

Pikrinsäure (Sigma, München)

Penicilin (Merck, Darmstadt)

Perjodsäure (Sigma, München)

Proteinase Complete Inhibitor (Roche, Mannheim)

Proteinase K (Roche, Mannheim)

Random hexamer Primer (nvitrogen, Karlsruhe)

Reverse Transcriptase (Invitrogen, Karlsruhe)

Salzsäure (HCl), konzentriert (Merck, Darmstadt)

Saponin aus Tequilla bark (Sigma, München)

Schiff's reagenz (Merck, Darmstadt)

Schwefelsäure (H2SO4), 95% (Merck, Darmstadt)

SIINFEKL-peptide (Pineda, Berlin)

Sirius-Rot (Sigma, München)

Serum (Ziege) (Vector Labs, Peterborough, UK)

Streptavidin-Meerrettichperoxidase (Dianova, Hamburg)

Streptomycin (Sigma, München)

SYBRGreen® (Sigma, München)

TEMED (Roth, Karlsruhe)

Tissue Tek® (EMS, PA Hatfield, USA)

Tris-(hydroxymethyl)-aminomethan (Tris) (Roth, Karlsruhe)

Tris/HCl (Roth, Karlsruhe)

Trypanblau (Sigma,München)

Tween®-20 (Polyoxyehtylsorbitan-Monolaurat) (GERBU, Darmstadt)

Wasserstoffperoxid (H2O2), 30% (Merck, Darmstadt)

Xylol (Roth, Karlsruhe)

Antibodies

Polyclonal rabbit anti-OVA (home-made)

biotinylated goat anti-rabbit Ab (Vector Labs, Peterborough, UK)

biotinylated goat anti-mouse Ab (Dianova, Hamburg)

CD4 (clone GK1.5), CD8α (53-6.7), CD11c (HL3), I-Ab (AF6-120.1), CD11b (M1/70), Vα2 (B20.1), Vβ5 (MR9-4), CD69 (H1.2F3), CD86 (GL1), CD40 (3/23), IL-12p40 (C17.8), IFN-g (XMG1.2) (eBioscience, (San Diego, USA), Fc-block (24G2)

Ki-67 (SP6) (ThermoFisher, Dreieich)

IL-2 ELISA Rat-anti mouse monoclonal, Capture: JES6-1A12 Detection: JES6-5H4

Actin rabbit anti-mouse (20-33) (Sigma, München)

MACS antibodies: CD4 (L3T4), CD11c (N418)

"Kits"

Avidin / Biotin blocking kit (Vector Labs, Peterborough, UK)

ABC-Kit for Streptavidin-HRP Labeling (Vector Labs, Peterborough, UK)

ABC-Kit for Streptavidin-AP Labeling (Vector Labs, Peterborough, UK)

Fixation/Permeabilization Kit with BD GolgiPlug® Pharmingen (CA San Diego, USA)

RNeasy microRNA preparation kit (Qiagen, Hilden)

IFN-g ELISA kit, (R&D Systems, Wiesbaden)

Albumin ELISA kit (Bethyl Lab, Montgomery, TX, USA)

MLV-RT kit (Invitrogen, Karlsruhe)

Primers

CCR5 : fw-cctagccagaggaggtgagaca
rev-ccttgaaaatccatcctgcaa

CCR2 : fw-acctgtaaatgccatgcaagt
rev-tgtcttccatttcctttgatttg

CCL2 (MCP-1) : fw-ggctcagccagatgcagttaa
rev-cctactcattgggatcatcttgct

CCL3 (Mip-1a) : fw-tgaaaccagcagcctttgct
rev-gatctgccggtttctcttagtca

CCL4 (Mip-1b) : fw-tgctcgtggctgccttct
rev-gagggtcagagcccattgg

CCL5 (RANTES):fw-gcaagtgctccaatcttgca

rev-cttctctgggttggcacaca

Buffers and solutions

Bufferes were stored at room temperature if not indicated otherwise. Water was purified using a water deionizer and buffers were autoklaved if necessary.

ELISA coating buffer: 8,401g NaHCO3 in aqua dest. ad 1l, pH adjusted with NaOH to pH 8,2, storage 4°C

ELISA washing buffer: 0,1% (v/v) Tween20 was dissolved in 1x PBS, storage 4°C

ELISA blocking buffer: 1% (w/v) BSA was dissolved in 1x PBS, storage 4°C

Erythrocyte Lysis Puffer: 0,83% (w/v) Ammonium-chloride, 10mM Tris/HCl pH9,0 was added until final pH7,5 was reached, storage -20°C

FACS-buffer: 0,1% (w/v) BSA, 0,01% NaN3 in 1x PBS, storage 4°C.

4xNEM-buffer: 25mg NEM, 66%H2O, 33% Glycin, 0,1% Bromphenolblue

OPD-substrate buffer: 15,6g NaH2PO4 x 2xH2O and 14,7g Tri-Na-Citrat x 2xH2O were dissolved in aqua dest ad 500 ml and pH adjusted to pH5,0. 1mg/ml OPD and 1µl/ml H2O2 were added directly before usage.

PAS staining solution: 2% periodic acid in aqua dest

Paraformaldehyd (PFA): 4% 39% Formalin solution was diluted 1:10 using 1xPBS **Picro-Sirius red staining solution:** 0,1g Sirius red, 90mL picric adic (liquid),10ml aqua dest

Phosphate lysis buffer: 20mM NaPO4 pH8.0, 140mM NaCl, 3mM MgCl2, 0,5%NP-40. Proteinase complete inhibitor tablets and 1mM DTT were added freshly directly before usage.

10x Phosphate buffered saline (PBS): 402g NaCl, 78g NaH2PO4 x2H2O, aqua dest. ad 5 l. Working solution was diluted 1:10, storage 4°C

RT-buffer: 8µl 5xFS Buffer, 4µl 0,1M DTT, 2µl RNase out, 2µl MLV-RT

2xSDS-gel sample buffer: 250mM Tris/HCl pH6,8, 6,6% SDS, 40mM DTT in aqua dest

SDS-gel running buffer: 25mM Tris,190mM Glycin, 3,5mM SDS in aqua dest

1xTBS: 20 mM Tris and 150 mM NaCl were dissolved in aqua dest, pH was adjusted with HCl to pH7,6

TBST-buffer: 10mM Tris/HCl pH7,5, 140mM NaCl, 0,05% Tween20.

Trypan blue: 0,01% (w/v) Trypan blue and 0,001% (w/v) Na-Azid were dissolved in 1xPBS.

Western Blot buffer: 25mM Tris,190mM Glycin, in aqua dest

Cell culture media were purchased from Gibco (Invitrogen, Karlsruhe). RPMI1640 was used as standard cell culture medium.

Glutamin: L(+)-Glutamin was dissolved in 1xPBS to a final concentration of 200mM.

Fetal calf serum (FCS): ultralow endotoxin, to inactivate active complement factors, FCS was incubated for 30 min at 56°C, storage -20°C

Pen/Strep: Penicilin was dissolved in 1x PBS with a final concentration of 6mg/ml, Streptomycin was added with a concentration of 10mg/ml.

Standard cell culture media (used for all cell culture experiments): RPMI 1640, 10% FCS, 2mM L(+)-Glutamin, 6mg/ml Penicilin und 10mg/ml Streptomycin.

Methods

General mouse maintenance. C57/Bl6, NOH, OT-I Rag$^{-/-}$, OT-II and CD11c-DTR mice[178] were bred and maintained in the animal facilities of the University of Bonn (HET) under SPF conditions. Animals were housed in type2long IVCs and provided weekly with fresh water and food. Mice between 8 and 16 wk of age were used for experiments in accordance with local animal experimentation guidelines. All animals were backcrossed to C57/Bl6 for 10 generations before usage.

Generation of transgenic NOH mice. The 715 bp XhoI – HindIII fragment containing the rat insulin promoter was excised from the pOVA/HEL plasmid used to generate ROH transgenic mice[179], which contained cDNA for the human transferrin receptor membrane domain, for the ovalbumin (aa 161-407), the HEL gene and an SV40 poly A tail. It was replaced with the human nephrin promoter, which had been amplified from the plasmid nephrin/PCR2.1[180] using the primers GACAGAGGTGGGAGAATTACTT and ACAGCGCCCGCTGCCAGC, and was then ligated into the blunted 5.7 kb pOVA/HEL fragment. A 3.79 kb PvuI–BamHI fragment was cut out and injected into pronuclei of fertilized C57/Bl6 oocytes. The correct nucleotide sequence of the transgene was verified by sequencing.

Characterization of Ovalbumin expression in NOH mice. Tissue specific OVA expression was addressed by various methods. OVA antigen was either detected directly by immunohistochemical staining of various tissues, western blot analysis of tissue homogenates or indirectly by cross-presentation to OT-I cells in the lymphoid tissue.
For OVA-immunohistochemistry, kidneys were fixed in 4% formalin overnight and 1µm paraffin sections were cut. Antigen was retrieved with 5mg/ml proteinase type XXIV for 15min at 37°C. After 30min block in 5% goat serum, sections were incubated with homemade polyclonal rabbit anti-OVA Ab (1:500 in 5% goat serum) overnight at 4°C. Binding was visualized by biotinylated goat anti-rabbit Ab diluted 1:200 in 5% goat serum for 30min, and revealed by ABC-AP Kit followed by Neufuchsin and hematoxylin counterstaining, and analysis was performed using an Axioskop.
Western blot analysis was performed after preparing tissue lysates, mechanically dissolving the tissue in phosphate lysis buffer, incubating the samples for at least one hour on ice afterwards. Samples were centrifuged for 10min at 14.000rpm and supernatant taken (soluble protein fraction). Protein concentration was estimated by Bradford protein assay using a Nanodrop spectrophotometer. Equal amounts of total protein were incubated in 2x SDS-sample buffer and incubated at 99°C for 3min. 4xNEM was added and 20µl/sample was loaded on a 10% SDS-gel. Samples were run with 17mA for stacking and 34mA for separation. Western blot was performed by blotting the proteins on a PVDF membrane at

250mA and 80V for 90minutes. The membrane was blocked with 5% milk powder in TBST over night. The following morning, the blot was washed two times in TBST for 5-10 min and incubated with an OVA-specific polyclonal rabbit serum (1:5000 in TBST). Blots were washed two times for 10min in TBST and secondary anti-rabbit-HRP antibody was added, incubating the blot for 20-30minutes. Blots were washed four times for 10min in TBST. Detection was carried out by adding 400µl of ECL Western blotting substrate, incubating the blot for 2-3 minutes, removing surplus substrate and detecting luminescence by x-ray nefilm exposure. To ensure comparable protein amounts of the different tissues loaded on the gel, Actin was stained as in internal quantification control using Actin specific primary antibodies instead of rabbit anti-OVA serum.

Cross presentation was analyzed in various tissue draining lymph nodes and spleen by injecting CFSE-labelled OT-I cells *i.v.* into NOH mice, analyzing their proliferation 48hrs afterwards. In brief, OT-I cells were prepared from donor mice, isolating spleen and lymph nodes and preparing single cell suspensions. Cells were incubated in 10ml PBS+0,1% BSA, adding CFSE stock (5mM) in a ratio of 1:1000. Labelling was stopped by adding 40ml PBS+10% FCS, washing the cells two times before injection. Mice were injected with $2x10^6$ OT-I cells and proliferation was measured 48hrs later.

Isolation and transfer of cells. T cells were isolated for adoptive transfer from spleen and LN of OT-I and OT-II donor mice. Single cell suspensions were prepared by mashing the organs through a 50µm cell strainer. Isolates were centrifuged at 1400rpm for 5min and pellets resuspended in 2ml Erythrocyte lysis buffer for 7min. Cells were washed once with PBS and counted for living cells. OT-I and OT-II fractions were determined by flow cytometry analysis of a small aliquot, staining cells for Vα2, Vβ5, CD8α or CD4 respectively. Cells were injected using $5x10^6$ of each cell type if not indicated otherwise.

Isolation of primary DCs and T cells from kidney tissue. Kidneys were injected with RPMI1640 containing 1mg/ml CollagenaseD and 0.2mg/ml DNAseI and incubated for 1 hour, mechanically breaking up the tissue in smaller fragments in intervals of 20min using syringe pistils and vigorous pipetting. Cell suspensions were washed in PBS once to remove CollagenaseD and DNAseI and centrifuged at 1400rpm for 7min. Pellets were resuspended in Erythrocyte lysis buffer for 7min, washed once and resuspended in 5ml PBS. Undigested tissue fragments and kidney tubular cells were removed by sedimentation as described.[181] Samples were either taken directly for flow cytometry analysis or purified for *ex vivo* cocultures, using nanobead-labeled antibodies. Magnetic bead separation was done according to the manufacturer's instructions. Purity was 80 to 90%.

Cell culture. To generate activated OT-I and OT-II, cells were stimulated *in vitro* prior to adoptive transfer. Single cell T cell suspensions were isolated as described above. For activation of OT-I cells, SIINFEKL-peptide-loaded, irradiated splenocytes were added to the culture in a ratio of 10:1. In brief, splenocytes from 2-3 wt mice per OT-I mouse were prepared and resuspended in standard cell culture medium. For peptide loading, SIINFEKL-peptide was added with a final concentration of 100ng/ml. Cells were incubated at 37°C for 20min, centrifuged at 1200rpm for 5-7min and resuspended in RPMI1640 for irradiation. Cells were irradiated with an energy dose of 20-25Gy, centrifuged as described and resuspended in standard cell culture medium. OT-I T cells were cocultured with the splenocytes for 4-5 days and activation of T cells was measured by flow cytometry analysis of activation markers (e.g. CD69) prior to injection.

OT-II cells were isolated from donor mice as described above and single cell suspensions were prepared from spleen and LN in PBS. Cells were centrifuged as described, resuspended in standard cell culture medium with a concentration of approximately $10x10^6$ cell/ml and Ovalbumin was added with a final concentration of 1-2mg/ml, depending on the endotoxin level of the individual Ovalbumin stock. Cells were cultured for 4-5 days and activation of T cells was measured by flow cytometry analysis of activation markers (e.g. CD69) prior to injection.

Coculture experiments of DC isolated from NOH mice and OT-II cells were performed as follows. Kidneys from NOH mice were digested with CollagenaseD and DNAseI and DCs were prepared from the single cell suspensions as described above. OT-II single cell suspensions were prepared from spleen and LN of OT-II donor mice. Both cell populations were purified by magnetic associated cell sorting (MACS), using nanobead-labeled antibodies. Magnetic bead separation was done according to the manufacturer's instructions. Purity was usually 80 to 90% as determined by flow cytometry analysis. Cells were seeded in 96-well plates, coculturing $1x10^5$ DC with $2.5x10^5$ OT-II cells in a total volume of 250µl standard cell culture medium for 4 days. Cytokine production was measured by ELISA or intracellular flow cytometry analysis respectively.

Depletion of DC and Monocytes. To deplete $CD11c^+$ cells, mice were backcrossed to CD11c-DTR mice. For depletion, 4ng/g bodyweight of Diphteria toxin were injected *i.p.* into the mice. Depletion efficiency was assessed by flow cytometry analysis as described. Clodronate Liposome mediated depletion of phagocytic cells was accomplished by *i.v*. injection of 200µl of Clodronate Liposomes (kindly provided by N. van Rooijen, Amsterdam).

Immunization of mice. Immunization of mice was performed by weekly injections of mixtures containing 100µl Aluminium hydroxide + 100µg/mouse Ovalbumin in 100µl PBS. Mice were injected *i.p.*

Flow cytometry. Cells were isolated as described. For flow cytometry analysis, single cell solution of cells were resuspended in 100µl FACS-Buffer. Staining antibodies were added in a standard ratio of 1:400 for qualitative or 1:200 for quantitative expression analysis and incubated for 20-30min at 4°C. Cells were washed with FACS-Buffer and centrifuged with 1200rpm for 5min. Pellets were resuspended in 100-500µl FACS buffer ($5x10^6$ cells/ml estimated final concentration). 100ng/ml Hoechst-33342 was added for exclusion of dead cells. For quantitative cell count analysis, $2x10^4$ CaliBRITE® APC-beads were added as internal reference. Flow cytometry was performed on a FACSCanto® II or LSRII® and data analysis with Flow-Jo® software (Tristar, Ashland, OR). Cell sorting was performed on a FACSVantage®. For intracellular staining, cells were cultured for 5h with 1 µl/ml Golgi-Plug®. T cells were restimulated for 4hrs at 37°C with CD3/CD28 beads in 250µl standard cell culture medium before GolgiPlug® incubation. Cells were treated with 24G2 hybridoma supernant to block Fc receptors and stained for surface markers as described above. Stained cells were fixed with 2% PFA for 20min, washed with PBS and subsequently BD-Perm/wash® buffer (Intracellular staining kit). Cells were resuspended in 100 µl with BD-Perm/wash® buffer and incubated for 5min at 4°C to allow permeabilization. Antibodies for intracellular staining were added in a ratio of 1:200, stained for 20-30min and washed two times using BD-Perm/wash® buffer. Cells were resuspended in FACS buffer and analyzed as stated above.

Laser capture microdissection. 8µm cryosections of mouse kidney tissue were mounted on PET covered slides and prepared for alcohol based cresylviolet acetate staining by air-drying the sections for 1min and subsequent incubation for 2min in pre-cooled 75% EtOH. For staining, sections were dipped for 20s in 1% cresylviolet acetate dissolved in EtOH. Finally, slides were washed in 75% and 100% EtOH for 30s each to remove surplus cresyl violet. Sections were air-dried for 10min before microdissection. Tissue was excised using a PALM® MicroBeam IP 230V Z microscope for laser pressure catapulting as described previously.[182] Infiltrates were marked as indicated, cutting out mononuclear cells from the periglomerular space. Cells from interstitial areas from wt animals were isolated as controls.

ELISA measurements. Cytokine ELISAs were performed as two step-sandwich ELISAs using capture / detection antibodies for high sensitivity and specificity. ELISA plates were coated using 100µl/well of primary antibody solution (1:500 in ELISA coating buffer) over night at 4°C. Plates were washed three times using ELISA wash buffer and plates were blocked for 1h adding 200µl/well ELISA blocking buffer. 100µl of cell culture supernatants were added and incubated over night at 4°C. For development, plates were washed the following day for three times using ELISA wash buffer, biotinylated secondary antibody was added 1:500 in 100µl ELISA blocking buffer for at least two hours at room temperature or over night at 4°C. Plates were washed three times using ELISA wash buffer and Streptavidin-horse radish peroxidase was added 1:1000 in 100µl PBS, incubating plates for one hour. Plates were washed three times using ELISA wash buffer and 100µl OPD-Substrate buffer was added for ELISA development. To stop staining reaction, 100µl of 1M H_2SO_4 were added and samples were measured at 490nm. For quantification, standard dilutions were prepared in a range between 80-0.3125ng/ml for IL-2 and between 6-0.1875 ng/ml IFN-γ. All samples were measured in duplicates.

For OVA-specific IgG ELISA measurement from mouse sera, plates were coated over night at 4°C with Ovalbumin (1mg/ml stock, 1:40 in ELISA coating buffer). Plates were washed three times using ELISA wash buffer and plates were blocked for 1h adding 200µl/well ELISA blocking buffer. For titre determination, sera were added in 1:1 dilution series of at least 8 dilution steps, using 100µl/well. Serum samples were incubated over night at 4°C. For development, plates were washed the following day for three times using ELISA wash buffer, biotinylated goat-anti Mouse IgG was added 1:1000 in 100µl ELISA blocking buffer for at least two hours at room temperature or over night at 4°C. Plates were washed three times using ELISA wash buffer and Streptavidin-horse radish peroxidase was added 1:1000 in 100µl PBS, incubating plates for one hour. Plates were washed three times using ELISA wash buffer and 100µl OPD-Substrate buffer was added for ELISA development. To stop staining reaction, 100µl of 1M H_2SO_4 were added and samples were measured at 490nm. Titres were measured at an OD_{490} of 0.2.

Real time RT-PCR. RNA was isolated from sorted cells or tissues collected using laser microdissection by RNeasy microRNA preparation kit according to manufacturer's protocol. 20µl of RNA were mixed with 2µl random hexamer primers and 2µl dNTP mix and 16µl RT-buffer were added. Samples were incubated at 65°C for 5min for cDNA synthesis. Real time PCR was performed using the following settings: 40 cycles of 15sec denaturation at 95°C, 1min primer annealing and elongation at 60°C as described.[183] 1.5µl cDNA was used with 2.5µl (0.9µM) specific primers and 12.5µl of 2x Platinum SYBRGreen® qPCR Supermix. All samples were run in duplicates and normalized to 18S rRNA.

Histological analysis and scoring. For overview staining, tissues were fixed in 4% PFA overnight and embedded in paraffin by automated embedding. Sections were cut using a standard manual microtome. 5µm sections were stained with periodic acid-Schiff (PAS) or Sirius red. Sections were deparafinized by incubating them in Xylol, 100% Isopropanol and a descending EtOH row (100%, 96%, 80%, 75%, two times each). PAS staining was carried out incubating the slides in PAS staining solution for 30min. Samples were washed with water two times for 2-3min and stained with Schiff's reagent for up to 60min. Samples were washed in warm water for approximately 5min, counterstained with Haematoxylin for 5min and nuclei were stained blue by washing the samples three times for 2min in distilled water.
Sirius red staining was performed by Haematoxylin staining for 5min. Samples were dipped shortly in 1% HCl in EtOH and nuclei were stained blue by washing the samples three times for 2min in distilled water. Samples were incubated in Sirius staining solution for 20min. Surplus Sirius solution was removed by incubating the slides in 100% EtOH and Xylol for two times each. Samples were covered with Histokit for coverslipping.
Extent of mononuclear infiltration was semiquantitatively determined in PAS-stained sections using the following score: 0, no infiltration; 0.5 periglomerular infiltrates discontinuous or less than 3 layers; 1, continuous periglomerular infiltration with at least 2 cell layers; 2, severe infiltration with >4 layers; 3, severe infiltration plus crescents of Bowman's capsule or glomerular sclerotic lesions. A minimum of 50 glomeruli per section was counted.

For immunohistochemistry, 5-7µm cryosections were cut from Tissue Tek® embedded tissue samples snap-frozen in liquid nitrogen. Sections were fixed, incubating them for 7min in ice-cold Acetone. Sections were air dried for 15min and rehydrated in PBS. Samples were incubated using a Biotin/Streptavidin blocking kit according to manufacturer's protocols to block endogenous Biotin, which would result in unspecific staining. Unspecific protein binding was blocked by 30min incubation with 1% BSA in PBS. Biotinylated primary antibodies were incubated for 60min at room temperature or over night at 4°C and washed in PBS three times for 5min. ABC-Kit for Streptavidin-HRP labelling was added for 30min, slides were washed in PBS three times for 5min. DAB was dissolved in TBS according to manufacturer's protocol and 10µl H_2O_2 were added. Slides were incubated with DAB solution and staining intensity was checked every 30 seconds. The reaction was abrogated placing the slides in distilled water, washing them two times. Nuclei were stained using Haematoxilin staining, incubating the samples for 5min in Haematoxilin solution and washing them three times for 2min in distilled water. Sections were left to fully dry and were covered with Histokit for coverslipping.

Electron microscopy. Tissue samples were fixed for EM, using Karnovsky's fixative (4%PFA & 2%Glutaraldehyd) for at least two days. Samples were washed two times for 20min with 0,2M Cacodylat-buffer and further fixed with 2% (w/v) Osmiumtetroxid in Cacodylat-buffer for 2 hours. Samples were washed two times for 20min with 0,2M Cacodylat-buffer, dehydrated in 50% EtOH two times for 20min and contrasting reaction performed with Uranylacetat in70%- Ethanol for 45min. Samples were again dehydrated in an increasing EtOH gradient, incubating samples in 80%, 96% and Ethanol (abs) for 20min each. For tissue embedding, samples were placed in Propylenoxid two times for 30min and incubated in increasing Araldite concentrations, using Araldite : Propylenoxid 1 : 1 mix, 3:1 mix for 1h each and pure Araldite over night. Tissues were placed in gelatine capsules and polymerized for 48hrs at 60°C. Contrasting of ultra-thin sections was performed using Lead acetate according to Reynolds. Sections were 60-70nm and cut using an Ultracut UCT. Pictures were taken using a EM900 electron microscope.

Miscellaneous

Statistical analysis. T-test analysis was done using Prism Software (GraphPad, San Diego, USA). Results were given as mean ± standard deviation; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Urine collection. Urine was collected from mice over night using metabolic cages, providing the mice with fresh drinking water. To guarantee sufficient quantities of urine, all mice of a group were placed overnight in the same cage.

Creatinine measurement. Creatinine was measured using routine diagnostic methods according to Jaffé.[184] Creatinine clearance was calculated as follows:

$$\frac{c(Urine)\left[mg/dl\right]}{c(Blood)\left[mg/dl\right]} * V\left[ml\right] * \frac{1}{t\left[\min\right]}$$

6. Results

6.1 NOH mouse model

To investigate the role of specific T cells in autoimmune renal inflammation, Emma E. Hamilton-Williams generated a transgenic mouse expressing the model antigens Ovalbumin (OVA) and Hen egg lysozyme (HEL) together with the Transferrin receptor (Tfr) transmembrane domain under the podocyte specific Nephrin promoter. The construct (**Fig. 6A**) was injected into fertilized oocyte pronuclei of C57/Bl6 mice. To characterize the Nephrin-OVA/Hel mice (NOH), the expression of Ovalbumin on glomerular podocytes was assessed by immunohistochemistry (**Fig. 6B**). Staining revealed a specific expression of Ovalbumin in podocytes of NOH mice. Western blot analysis revealed expression of Ovalbumin in kidney tissue, but no other organ (**Fig. 6C**). Note that the NOH-Ovalbumin of NOH was slightly bigger compared to OVA due to the Tfr-HEL segment fused to the Ovalbumin.

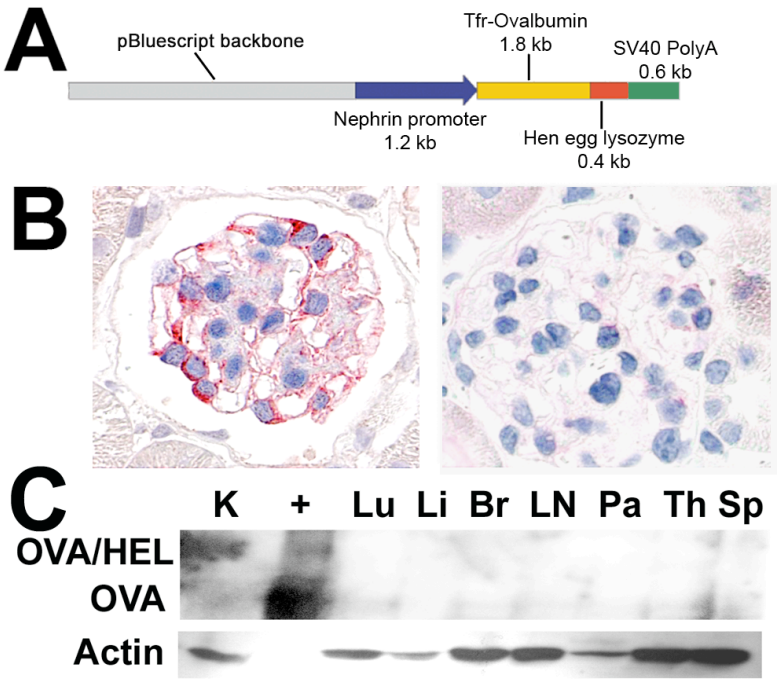

***Fig.6** Generation and characterization of NOH mice.* (**A**) Construct used for the generation of NOH mice. (**B**) Immunohistochemical staining of a glomerulus from a NOH mouse (left) and wt control (right). OVA expression was revealed using anti-OVA polyclonal rabbit serum and Alkaline phosphatase-Neufuchsin staining, nuclei were stained with Haematoxilin. Original magnification was x1000. (**C**) Western blot analysis of NOH mice. Tissue samples from indicated organs were taken and lysed. Proteins were loaded on an SDS-gel and blotted subsequently. 100ng of purified OVA was used as positive control (+). OVA was detected by an anti-OVA polyclonal rabbit serum followed by anti-rabbit HRP secondary Ab. (K=kidney, Lu=lung, Li=liver, Br=brain, LN=lymph node, Pa=pancreas, Th=thymus, Sp=spleen**)**

6.2 Activation of OVA-specific CD8$^+$ T cells in renal lymph node

To investigate the activation of T cells in the renal lymph node, CFSE-labelled OT-I T cells were injected into NOH mice and wt controls. Activation of OT-I cells could only be detected in renal lymph nodes of NOH mice but neither in other secondary lymphatic tissues nor in RLN of wt control animals. OT-I cells proliferated up to four cell divisions 2 days after intravenous injection of the cells and showed a significant upregulation of the early activation marker CD69, which was lost again subsequently from the cell surface during cell division (**Fig. 7**), consistent with previous studies.[185]

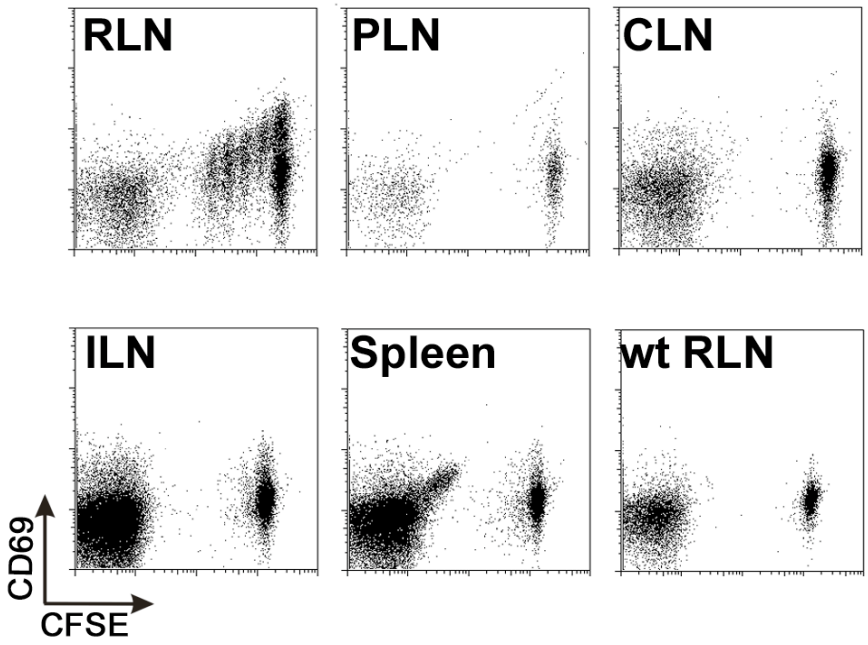

Fig.7 *Activation of OT-I cells was detected only in renal lymph node of NOH mice*. 2x10^6 CFSE-labelled OT-I cells were injected *i.v.* into NOH mice and wt controls. Mice were sacrificed on day 2 and lymph nodes and spleen were taken for analysis. T cell activation was determined by cell proliferation analysis and CD69 expression.

Furthermore, T cell activation was analyzed in a second line of transgenic NOH mice to rule out artificial effects induced by the transgene construction. Proliferation patterns in secondary lymphoid organs were essentially the same in both lines (**Fig. 8A,B**), albeit the activation was significantly lower in the second mouse line compared to NOH mice. These mice were therefore termed NOH^{lo} mice. Activation of OVA-specific CD4$^+$ T cells (OT-II) could not be detected *in vivo* (**Fig. 8C**) neither by proliferation nor by measuring upregulation of CD69 in either of the two mouse lines. Taken together, these findings suggest a kidney specific activation of T cells by the podocyte expressed Ovalbumin.

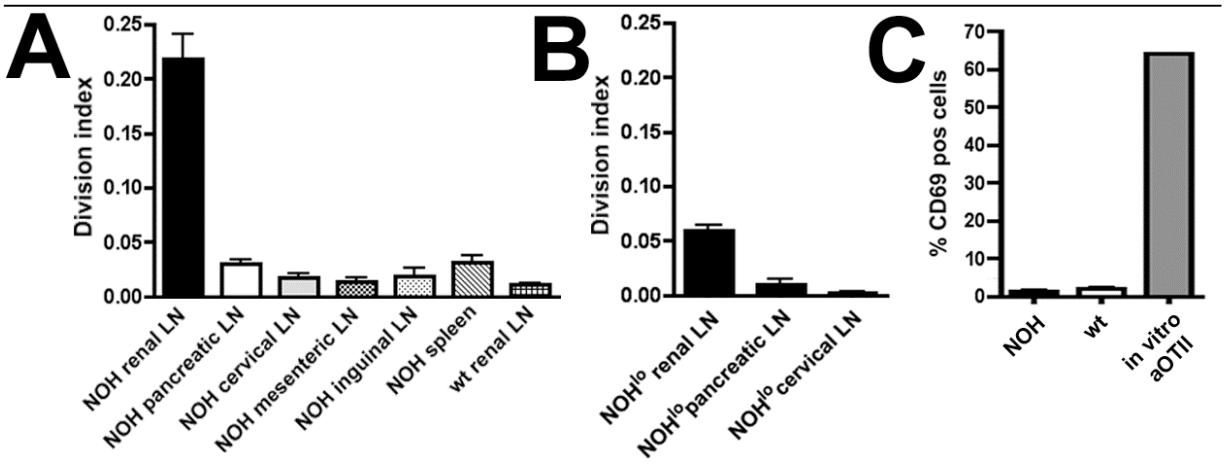

Fig.8 *Activation of OT-I cells but not OT-II cells in NOH and NOH^{lo} mice.* (**A**) Statistical analysis of data shown in Fig. 7. (**B**) Proliferation of OT-I cells in NOH^{lo} mice. CFSE-labelled OT-I cells were injected as described above and their proliferation in LN and spleen was determined 2 days later. (**C**) Activation of OT-II cells was analyzed the same way as before, CD69 upregulation was measured as a marker of cell activation. *In vitro* activated OT-II cells were used as positive control.

6.3 CTL release antigen from renal tissue and enhance priming of naive T cells in renal LN

In the next step, activated OVA-specific CTL were injected into NOH mice to test whether these cells are sufficient to induce kidney inflammation and glomerular damage. In contrast to other models, where activated OT-I cells were fully capable of mediating cytotoxic T cell damage[186], only little infiltration was detectable in NOH mice injected with *in vitro* activated OT-I cells. However, even small numbers of infiltrating CTL are sufficient to cause cytotoxic tissue damage as described previously for CTL mediated damage of pancreatic β-islet cells of RIP-mOVA mice. This damage would be accompanied by an enhanced release of antigen from dying podocytes that might then subsequently lead to an enhanced cross presentation of antigen and activation of naïve OT-I cells in the renal lymph node.[186] Indeed, also in NOH mice the activation of naïve OT-I cells was markedly increased after challenge with aOTI as seen by enhanced proliferation and increased upregulation of CD69 (**Fig. 9A-D**). The same experiment was performed in NOH^{lo} mice, which showed only very low levels of constitutive T cell activation. However, the cross-priming efficiency was markedly increased after challenge with activated OT-I cells also in these mice (**Fig. 9E,F**). These findings indicated that indeed the CTL seemed to be capable of damaging OVA-carrying podocytes and therefore releasing antigen for cross-presentation to naïve OT-I cells. However, at this stage it was not possible to rule out the alternative explanation, that the activated T cells provided proinflammatory signals influencing the maturation state of the dendritic cells, which would also lead to an enhanced cross-priming of naïve CD8$^+$ T cells.

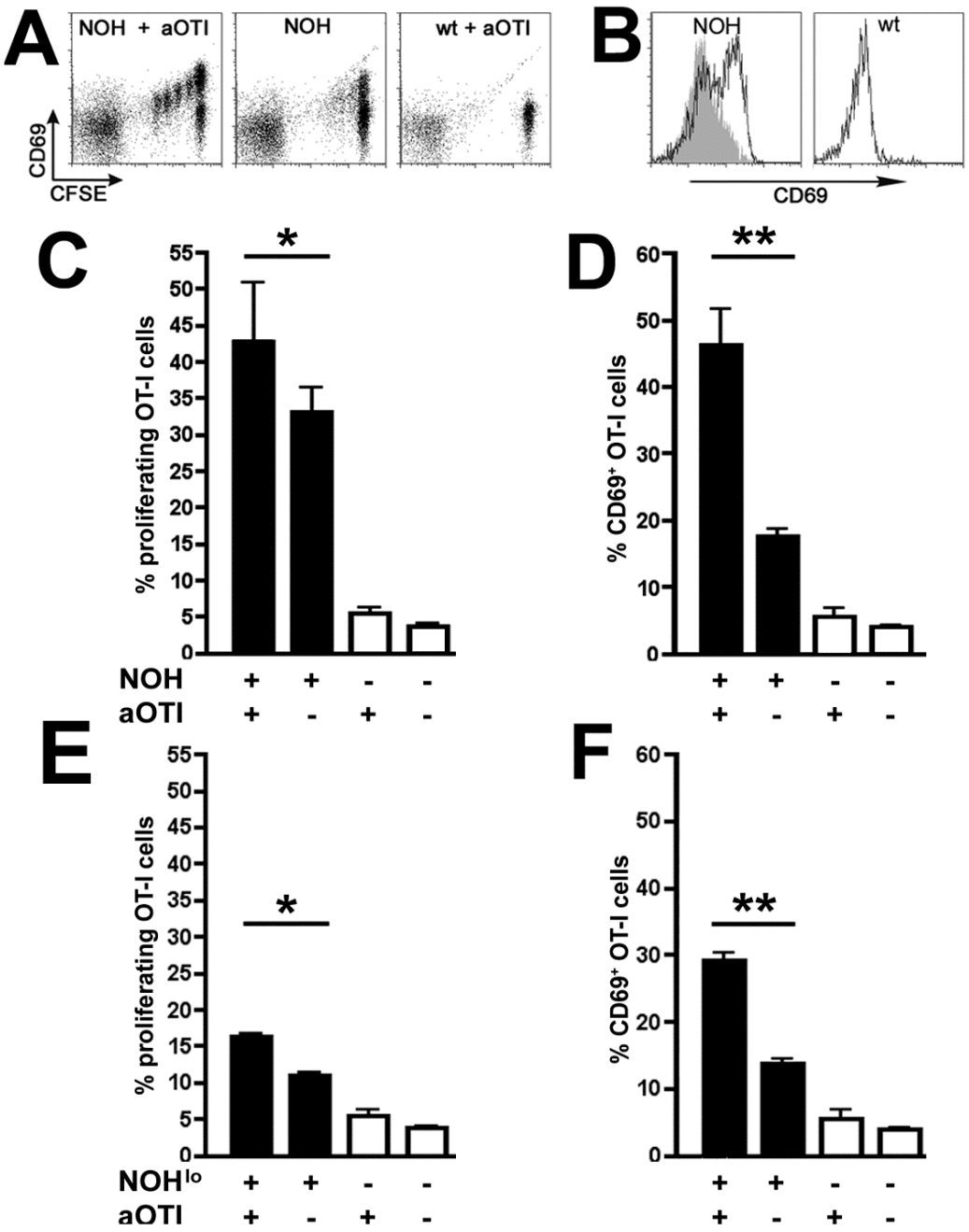

Fig. 9 *Activation of naive OT-I cells is enhanced by CTL-mediated damage in NOH mice.* 5x10^6 *in vitro* activated OT-I cells were injected into NOH mice and wt controls. 2 days later, naïve CFSE-labelled OT-I cells were injected and analyzed for activation as described above 2 days later. (**A**) Proliferation and CD69 expression of CFSE-labelled OT-I cells in the RLN of NOH and wt mice after challenge with *in vitro* activated OT-I cells. Data are representative for 2 individual experiments in groups of 3 mice. (**B**) Histograms of CD69 expression on adoptively transferred OT-I of unchallenged (filled histogram) or OT-I challenged (empty histogram) NOH mice compared to wt mice. (**C,D**) statistical analysis of 9A and 9B. Experiments were performed with 3 mice per group. (**E,F**) Same experimental setup as above performed in NOH^{lo} mice. Data are representative for 2 independent experiments in groups of 3 mice.

6.4 OT-I and OT-II T cells do not induce early DC maturation in RLN

To test whether the activated T cells also had an influence on the OT-I activation, DC isolated from the RLN were analyzed for the expression of costimulatory molecules. Neither activated OT-I (**Fig. 10A**), nor OT-II cells (**Fig. 10B**) induced significant functional maturation of DC as judged by expression patterns of CD40 and CD86. Since the activated OT-I cells failed to induce maturation of DC, we concluded that the CTL indeed released OVA antigen by cytotoxic damage.

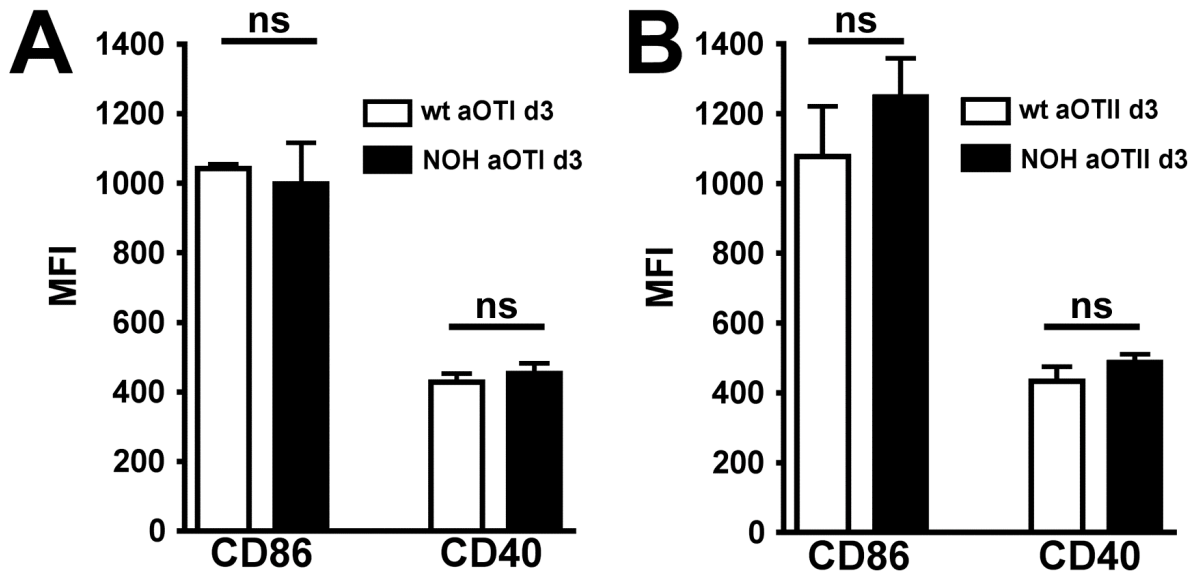

Fig. 10 *Maturation state of lymph node DC after adoptive T cell transfer.* DC were isolated from RLN of NOH and wt control mice 3 days after adoptive transfer of $5x10^6$ (**A**) activated OT-I cells or (**B**) activated OT-II cells. Cells were stained for CD11c and MHC-II ($I\text{-}A^b$) to identify DC and for CD40 and CD86 as activation markers. Results shown are representative for 2 independent experiments performed in groups of 3 mice.

6.5 Antigen released by CTL is presented to OT-II cells by renal DC

To investigate the effect of the CTL mediated antigen release directly in the kidney, the interaction between T cells and renal DC was assessed by an *ex vivo* coculture of renal DC and T cells after CTL challenge of NOH mice. Higher availability of antigen could lead to an enhanced T cell / DC interaction in kidney tissue, however, previous studies showed that the kidney does not harbour cross-presenting DC under steady state conditions.[187] Therefore it is likely, that mainly the interaction between T helper cells and DC is influenced by increased antigen levels. To test this hypothesis, DC were purified using magnetic associated cell sorting (MACS) of $CD11c^+$ cells from kidney single cell suspensions of NOH and wt mice with and without previous aOTI challenge. These cells were then cocultured with naïve OT-II cells and 4 days later T cell activation was measured by cytokine ELISA against IL-2 and IFN-γ.

IL-2 could not be detected in any of the cocultures (**Fig. 11A**). However, a significant increase of IFN-γ production could be detected in cocultures with DC from aOTI-challenged NOH mice (**Fig. 11B**), showing that indeed the ability of the renal DC was enhanced to prime T cell responses after release of antigen by the CTL. Taken together, these results demonstrated that CTL mediated damage leads to increased presentation of glomerular antigen.

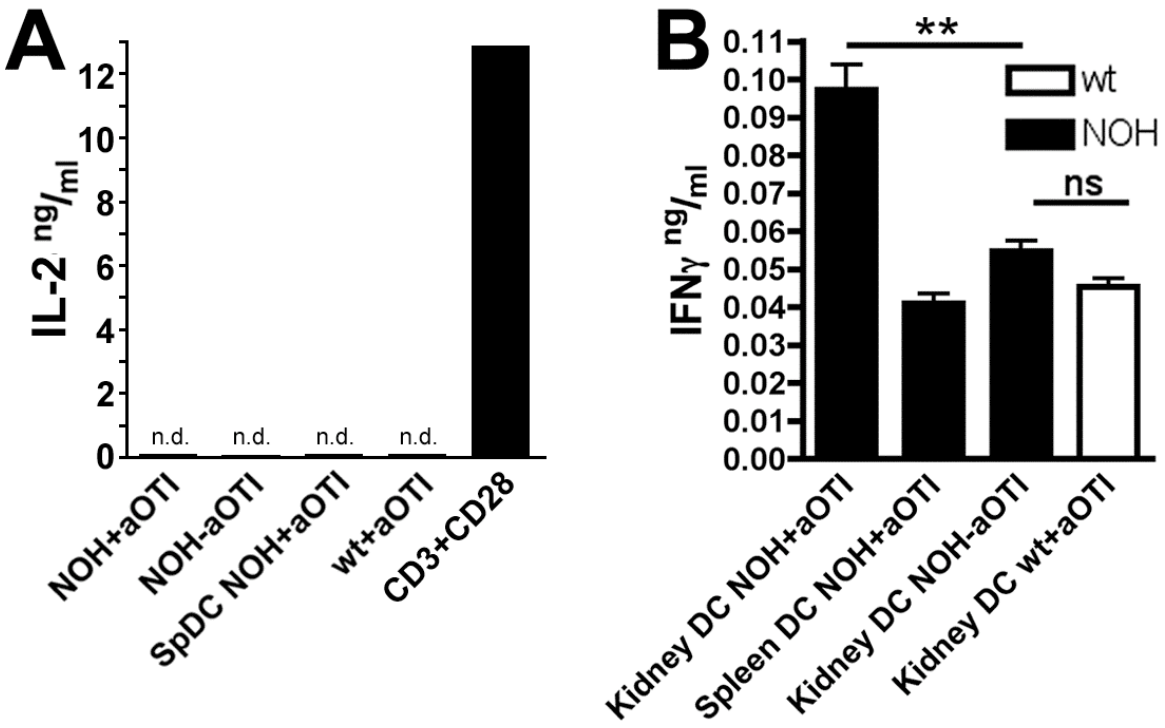

Fig. 11 *Coculture of DC and OT-II cells after challenge with activated OT-I*. DC were isolated from NOH and wt control mice 2 days after adoptive transfer of $5x10^6$ *in vitro* activated OT-I cells. Cells were purified by MACS and $2x10^5$ $CD11c^+$ cells were co-cultured with $4x10^5$ naïve OT-II cells. On d4, cell culture supernatants were taken and analyzed by ELISA for IL-2 and IFN-γ. (**A**) IL-2 ELISA. *In vitro* stimulated OT-II cells incubated with CD3/CD28 microbeads for 48 hrs were used as positive control. (**B**) IFN-γ ELISA . Means were calculated from three individual mice per group; ELISA measurements were done in duplicates. Results shown are representative for 2 independent experiments in groups of 3 mice.

6.6 Joint attack of CD8 and CD4 T causes renal inflammation

One possible explanation for the surprising finding, that even high numbers of activated OT-I cells only induced very mild renal immunopathology might be the lack of licensing by T cell helper cells.[188-190] As described earlier, T helper cells play a major role in the development of autoimmune kidney disease as it has been shown mostly in the model of serum induced nephrotoxic nephritis.[191-197] Since OT-II cells were not activated *in vivo* in the NOH mouse system, the cells were pre-activated *in vitro* in a culture with OVA-loaded splenocytes for

4 days. Virtually all activated OT-II cells showed an activated phenotype expressing CD69. Furthermore, the cells expressed IFN-γ but neither IL-4 nor IL-17, showing that the cells were of a Th1 phenotype as it has been shown in previous studies **(Fig.12)**.[198]

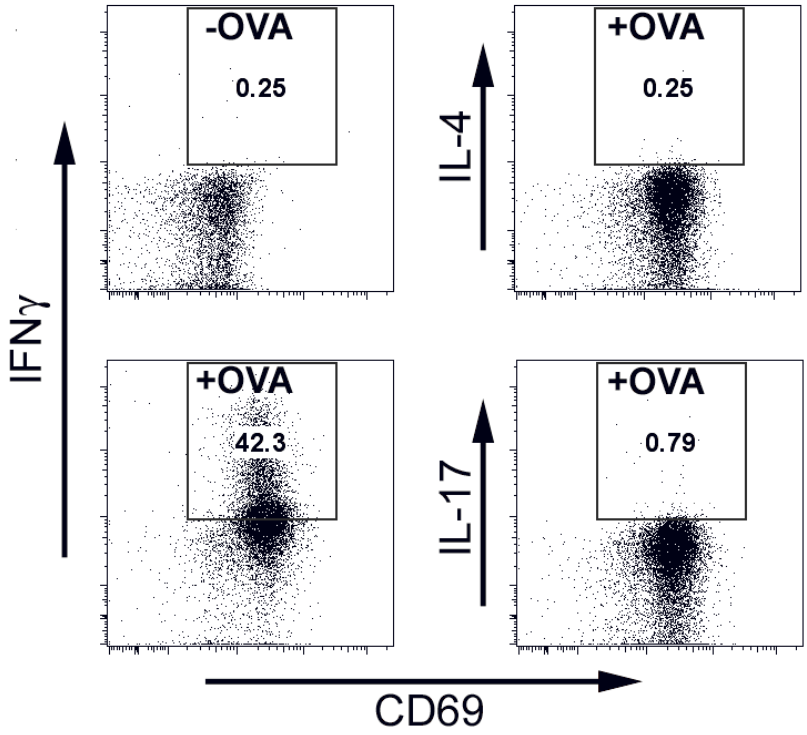

Fig. 12 *Cytokine expression pattern of in vitro activated OT-II cells.* Single cell suspensions were prepared from LN and spleen of OT-II donor mice. Cells were cultured in the presence of $2^{mg}/_{ml}$ Ovalbumin for 4 days. Cells were stained for CD4 as well as TCR-chains Vα2 and Vβ5 to identify OT-II cells and CD69 to analyze their activation state. Functional analysis was performed by intracellular cytokine staining.

The co-injection of pre-activated OT-II together with naïve OT-I cells resulted in a renal inflammation visible by formation of periglomerular infiltrates consisting of mononuclear cells on d7 (**Fig. 13A,B**). Between 15 and 20% of the glomeruli showed focal, periglomerular mononuclear infiltrates in all NOH recipient mice after 7 days, as opposed to wt controls, indicating that the infiltration was antigen-specific. No intraglomerular infiltration could be observed during that time-point, Glomeruli showed enlarged parietal cells and thickening of the Bowman's capsule membrane (**Fig. 13B**). Adoptive transfer of either naïve or activated OT-I or OT-II cells alone failed to induce any detectable immunopathology (**Fig. 13C,D**). These findings demonstrated that $CD8^+$ T cells together with $CD4^+$ T cells were able to induce an inflammatory reaction in the kidney, leading to an accumulation of cells around, but not within the glomeruli at early time points after adoptive transfer.

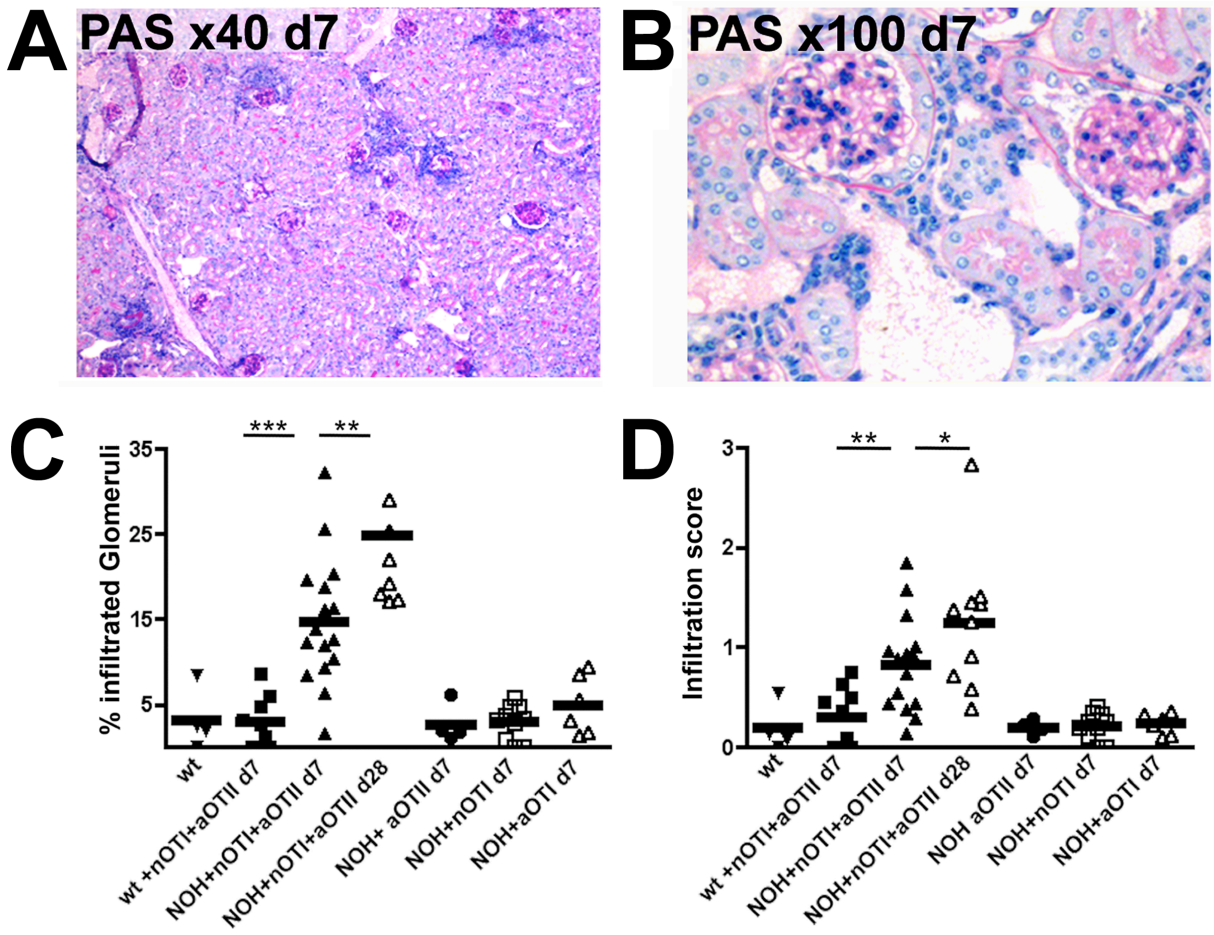

Fig. 13 *Immunopathology of NOH mice after adoptive transfer of OT-I and OT-II cells.* 5x10^6 naïve OT-I and 5x10^6 activated OT-II cells were injected alone or together in NOH and wt control mice. Kidney sections were stained with PAS. (**A**) Representative overview of infiltrated kidney from a NOH mouse. Original magnification x40. (**B**) High-magnification picture from the same mouse. Original magnification x200. (**C,D**) Semiquantitative analysis of kidney sections by histological scoring. The frequency (**C**) of glomeruli surrounded by mononuclear infiltrates was determined in PAS-stained kidney sections. Histological score (**D**) was defined as follows: 0, no infiltration; 0.5 periglomerular infiltrates discontinuous or less than 3 layers; 1, continuous periglomerular infiltration with at least 2 cell layers; 2, severe infiltration with >4 layers; 3, severe infiltration plus crescents of Bowman's capsule or glomerular sclerotic lesions. A minimum of 50 glomeruli per section was counted. Results are representative for 4 independent experiments in groups of 3-5 mice.

6.7 OT-II but not OT-I cells induce functional maturation of renal DC

To elucidate the outcome of the OT-II helper cell response, the functional properties of renal DC after challenge with activated OT-I and OT-II cells were further investigated. DC were isolated from renal tissue of NOH and wt control mice after injection of T cells and analyzed for the expression levels of costimulatory molecules. Furthermore, isolated cells were stained for the marker Gr-1, which has been described as a marker present on organ infiltrating

inflammatory monocyte subsets.[199-202] Upregulation of costimulatory molecules CD40 (**Fig. 14A,C**) and CD86 (**Fig. 14B,D**) was detectable only on DC from NOH mice, which had been challenged with activated OT-II alone or together with OT-I. Activated OT-I cells did not induce any maturation of DC (**Fig. 14A-D**). Notably, OT-II cells also lead to an antigen specific infiltration of $CD11c^{+}I\text{-}Ab^{+}Gr\text{-}1^{+}$ cells, but those cells were of a somewhat less mature phenotype, expressing only base levels of CD40 and CD86 (**Fig. 14A,B**). These findings demonstrated that DC activation was dependent on OT-II but not OT-I cells, and that the latter augmented DC activation, presumably by release of OVA antigen and therefore increased interaction between OT-II cells and DC.

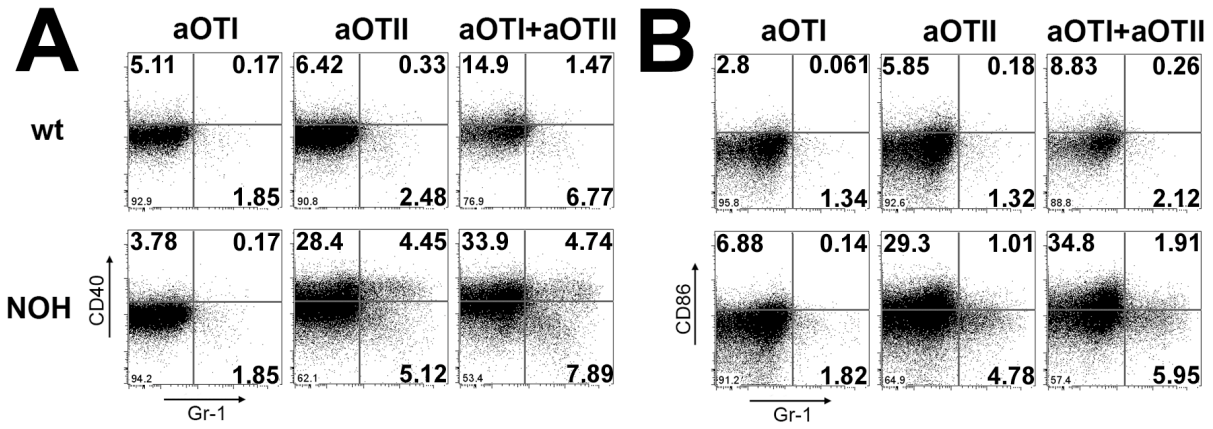

Fig. 14 *Renal DC activation by OT-II cells*. $5x10^{6}$ activated T cells were injected into NOH and wt control mice. DC were isolated from kidney tissue on d3 and stained for CD11c and MHC-II ($I\text{-}A^{b}$) to identify DC, for Gr-1 to distinguish between resident and inflammatory subsets and for CD40 (**A**) and CD86 (**B**) as activation markers.

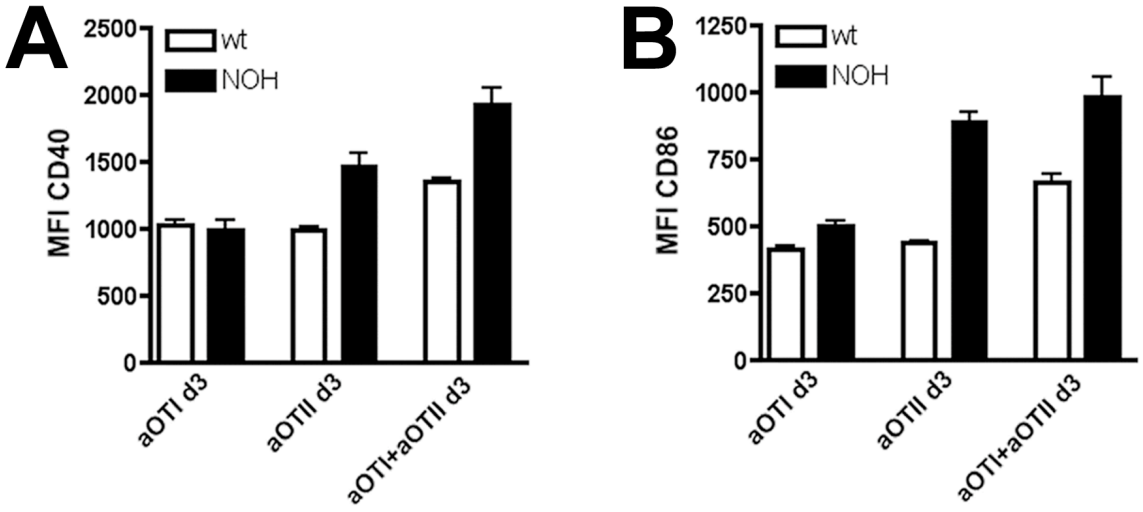

Fig. 15 *Statistical analysis of data shown in Fig. 14* (**A)** Statistical analysis of CD40 expression by MFI of total DC isolated from renal tissue. (**B**) Statistical analysis of CD86 expression of total DC isolated from renal tissue. Experiments were performed twice with individual groups of 3 mice.

In the next step, production of IL-12 by DC was analyzed, which has been described before as a key factor in renal inflammatory disease.[192, 193, 195] Mice were treated as described above and stained for IL-12 in DC. The IL-12 expression pattern was similar to the expression of costimulatory molecules and was highly dependent on the presence of activated OT-II cells **(Fig. 16)**. The activation of DC was even enhanced when activated OT-I cells were co-injected, presumably due to the higher amount of available Ovalbumin released by the CTL, enhancing the interaction between DC and OT-II cells.

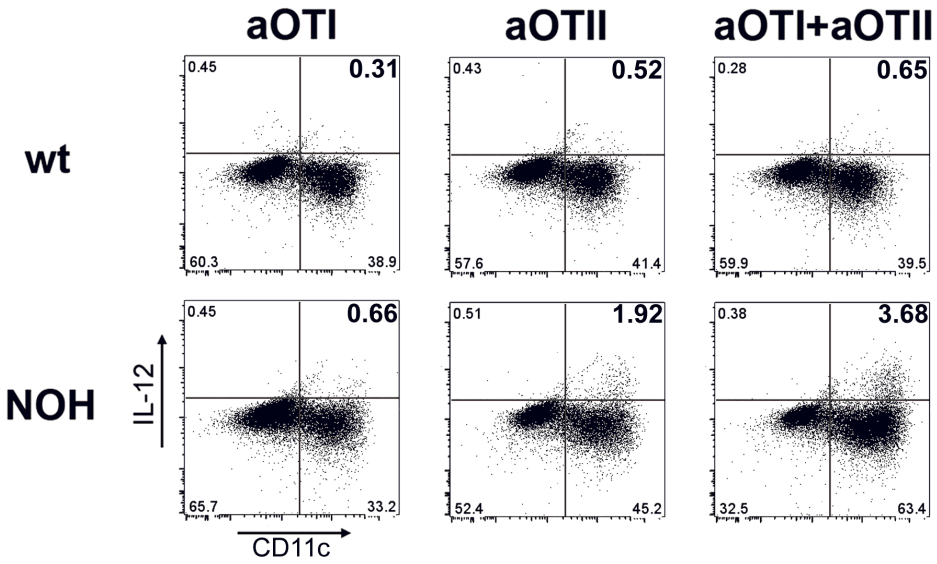

Fig. 16 *Renal DC produce IL-12 in an OT-II dependent fashion*. DC were isolated after adoptive transfer of T cells as described above. Cells were stained for CD11c and MHC-II (I-A^b) to identify DC, cytokine production was measured by intracellular IL-12 staining. Dot plots shown are representative of 2 independent experiments in groups of 3 mice.

6.8 Histological characterization of periglomerular infiltration

To elucidate the composition of the periglomerular infiltrates, immunohistochemical analysis was performed on kidney cryosections to identify the cellular components of the renal infiltration. The stainings were positive for CD8α (**Fig. 17A**), CD4 (**Fig. 17B**), CD11c (**Fig. 17C**), CD11b (**Fig. 17D**), MHC-II (I-A^b allotype) (**Fig. 17E**) and CD86 (**Fig. 17F**) within periglomerular infiltrates and therefore revealed a cellular composition of both T lymphocytes and monocyte/macrophage-like cells such as DCs and macrophages. CD86 staining indicated that infiltrating $CD11c^+$ cells were indeed DC-like cells. B220 was not detectable, indicating that neither B cells nor plasmacytoid DC were part of the infiltration. Similar to the PAS staining, no immune cells were detectable inside the glomeruli.

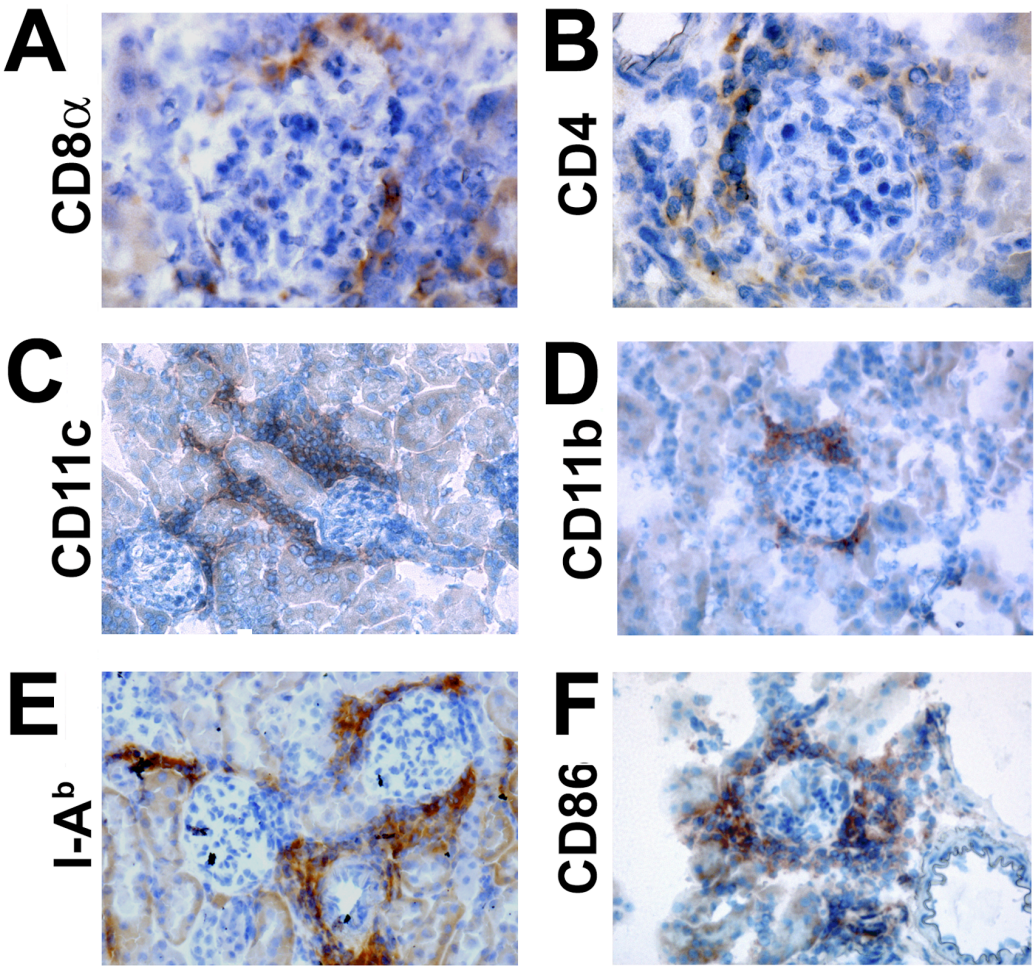

Fig. 17 *Immunohistochemical staining of periglomerular infiltration in NOH mice.* 5µm kidney cryosections NOH mice treated with $5x10^6$ naïve OT-I and activated OT-II cells. Mice were sacrificed 7 days after adoptive transfer of T cells. Histochemical stainings shown are representative for all mice carrying periglomerular infiltration. Staining was performed for (**A**) CD8α, (**B**) CD4, (**C**) CD11c, (**D**) CD86, (**E**) MHC-II ($I\text{-}A^b$) and (**F**) CD11b by using biotinylated mAbs followed by SA-HRP/DAB staining. Nuclei were stained with haematoxilin.

6.9 Infiltration of CTL into the kidney and activation of effector function depends on interaction between Th cells and DCs

The histological analysis of the infiltrates indicated a considerable influx of immune cells into the renal tissue. Quantitative flow cytometry analysis was performed to determine absolute numbers of infiltrating cells. OT-I cell numbers were about 10-fold increased in NOH mice injected with both OT-I and OT-II cells compared to wt controls (**Fig. 18A**). NOH mice, which had been injected with OT-I cells alone, showed even less OT-I cells in kidney as well as in spleen when compared to the wt controls in 3 out of 3 experiments (**Fig. 18A,C**). Functional activation of $CD8^+$ T cells was also highly dependent on OT-II cells, as shown by IFN-γ production of OT-I cells isolated from renal tissue (**Fig. 18B**). Infiltration was tissue specific, since neither the ratio (**Fig. 18C**) nor the absolute numbers of OT-I cells were increased in

the spleen of NOH mice compared to wt mice, indicating that the increase in cell numbers in the kidney was not due to a systemic expansion of the OT-I cells, but rather a site specific effect. Intrarenal OT-II cell numbers also increased in NOH mice, however this infiltration was independent of the presence of OT-I cells (**Fig. 18D**). These findings demonstrated that OT-I T cells were functionally dependent on the presence of $CD4^+$ T cell help and that OT-II T cells acted upstream of OT-I, since their infiltration did not depend on the presence of OT-I.

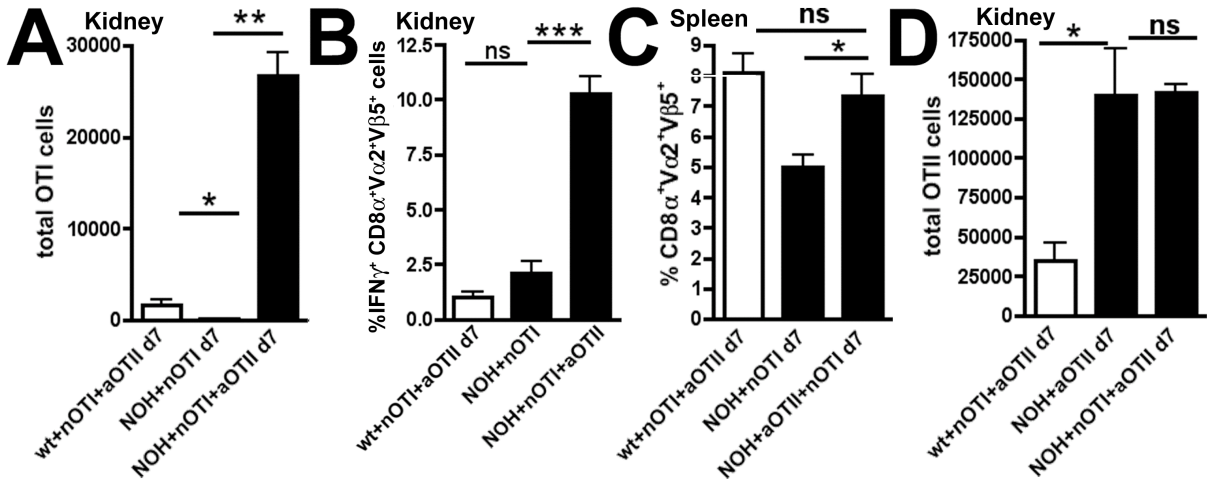

Fig. 18 *Infiltration of T lymphocytes into renal tissue of NOH mice*. $5x10^6$ naïve OT-I cells were injected alone or together with $5x10^6$ activated OT-II cells into NOH mice and wt control animals. Cells were isolated from renal tissue 7 days later and quantified by flow cytometry. (**A**) Total cell numbers of OT-I $CD8^+$ T cells isolated from kidneys of NOH mice and wt controls. (**B**) Intracellular staining of IFN-γ in OT-I cells isolated from kidney tissue of NOH and wt mice on d7. (**C**) OT-I T cell fraction isolated from spleen single cell suspensions. (**D**) Total cell numbers of OT-II $CD4^+$ T cells isolated from kidneys of NOH mice and wt controls. Results shown are representative for 2 independent experiments performed in groups of 3 mice.

6.10 aOT-II cells induce effector function of infiltrating OT-I cells

To investigate whether the kidney-infiltrating CTL were also functional effector cells, intracellular cytokine staining for IFN-γ was performed on OT-I cells isolated from renal tissue. Indeed, OT-I cells that had been co-injected together with *in vitro* activated OT-II cells showed significantly more production of IFN-γ after restimulation when isolated from renal tissue of NOH mice, but not from wt controls (**Fig. 19**). OT-I cells, which had been injected in the absence of OT-II help, showed no significant upregulation of IFN-γ. This result showed that the kidney infiltrating OT-I cells indeed were not only increased in number, but also were able to perform effector functions depending on the presence of cognate T cell help.

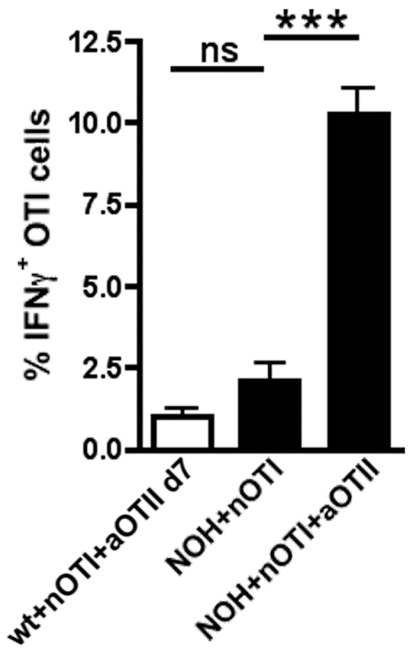

Fig. 19 *IFN-γ production of kidney infiltrating OT-I cells depends on OT-II*. 5x10^6 OT-I cells were injected alone or together with 5x10^6 activated OT-II cells into NOH mice and wt control animals. Cells were isolated from renal tissue 7 days later and quantified by flow cytometry. T cells were stained for CD8α, Vα2 and Vβ5 to identify OT-I cells. IFN-γ production was assessed by intracellular cytokine staining. Results shown are representative for 3 independent experiments performed in groups of 3 mice.

6.11 Increase of intrarenal OT-I cell numbers is independent of their local proliferation

To address the question whether OT-I cells underwent a secondary expansion in renal tissue[203] or were only recruited from the blood stream, proliferation of lymphocytes isolated from renal tissue was assessed by staining for the cell cycle protein Ki-67, which has been described as a marker for proliferating cells.[204, 205] Ki-67 was well detectable in *in vitro* activated OT-II cells on d3, which served as positive controls. In comparison, OT-I cells isolated from NOH mice never showed positive Ki-67 staining even if co-injected with activated OT-II cells (**Fig. 20 A,B**), indicating that there was no expansion of this cell fraction upon infiltration into the kidney tissue.

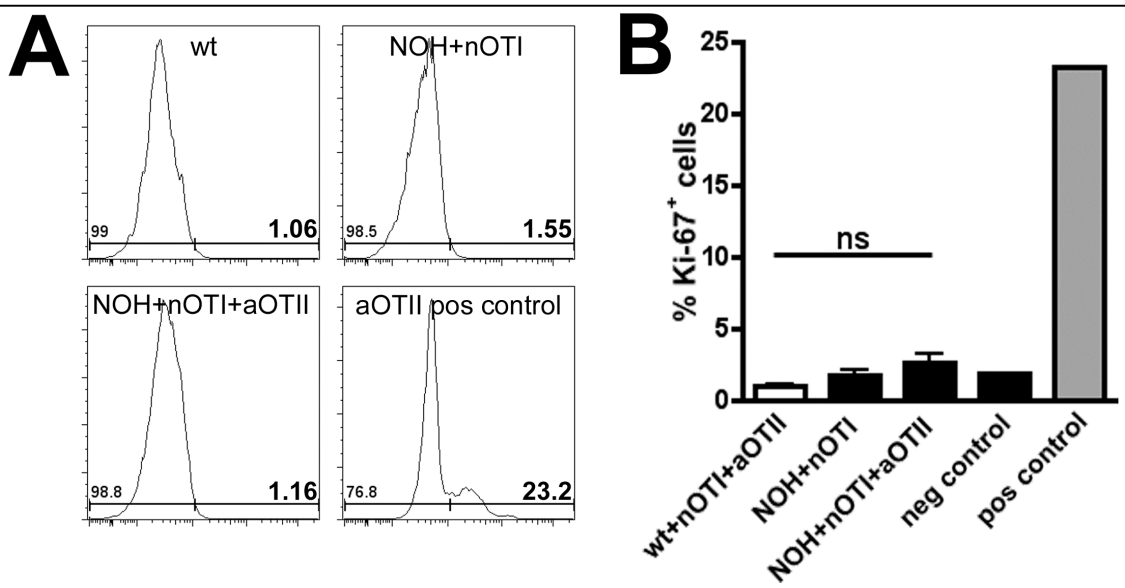

Fig. 20 *OT-I cells do not proliferate upon infiltration of NOH kidneys*. 5x10^6 naïve OT-I cells were adoptively transferred alone or together with 5x10^6 activated OT-II cells into NOH and wt control mice. 7 days after adoptive transfer, T cells were isolated from kidney tissue and stained for CD8α, Vα2 and Vβ5 to identify OT-I cells. Proliferation was measured by intracellular Ki-67 staining. *In vitro* activated OT-II cells were used as positive, naïve OT-II cells as negative control.

6.12 Conventional CD8α^- DCs are an abundant and essential component of the periglomerular infiltrate

DC represented another major component of the periglomerular infiltration. To further dissect the functional properties of these cells, renal DCs from NOH mice were isolated in the first step and compared with DC derived from wt animals regarding their phenotypical properties to rule out functional defects of these cells by the transgene. Indeed, renal DCs of NOH and wt mice showed comparable expression levels of phenotypical and functional DC markers such as CD11c, MHC-II (I-A^b), CD86 and CD40 (**Fig. 21**), indicating that the NOH transgene did not alter the renal DC. In the next step, DC-subtype analysis was performed. Cells were screened for CD8α expression, which has been described as a marker for crosspresenting DC.[206-208] CD8α^-CD11b$^+$ conventional DC were considered to be of myeloid origin and B220$^+$Gr-1$^+$ cells were considered to be plasmacytoid DC, representing cytokine producing effector DC[209] secreting e.g. type-I interferons. A 2-fold increase in the CD11c^{hi}CD11b^{hi} as well as the CD1c^{lo}CD11b^{hi} sub-population could be observed (**Fig. 22 A, Fig. 23**), indicating infiltration of conventional DC as well as macrophages. Also, these cells expressed the marker Gr-1 (**Fig. 22B, Fig.23**), which has been described as a marker for neutrophilic granulocytes but also short-lived inflammatory effector cells as stated above.[199, 200, 210] Notably, the amount of CD11c^{hi}CD1b^{int} cells was not altered significantly, indicating that these cells are likely to be the kidney resident DC.

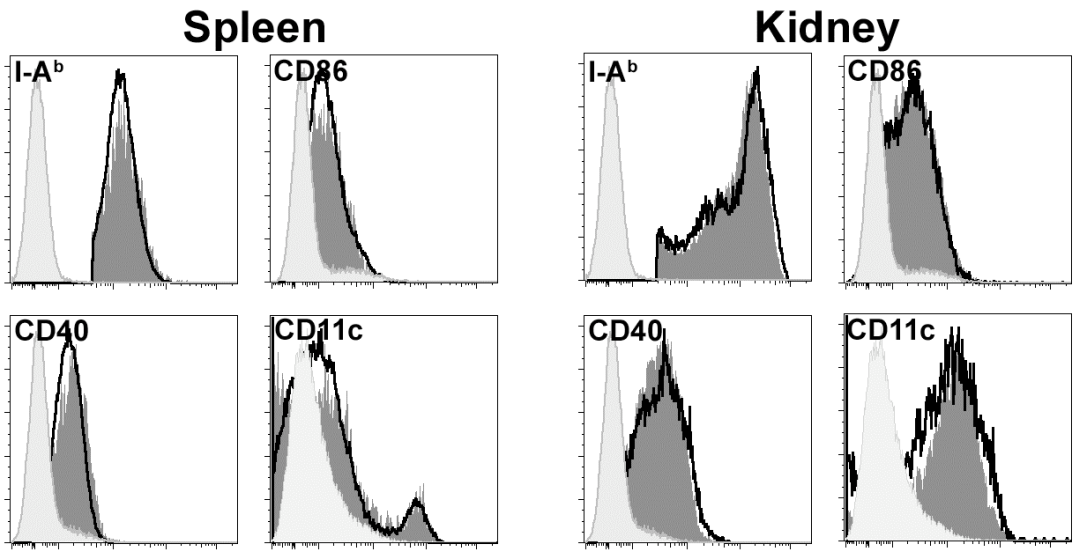

Fig. 21 *Phenotyping of DC isolated from NOH mice*. Renal and splenic DC were isolated from untreated NOH and wt animals and analyzed by flow cytometry. Histogram overlays were performed as follows: light grey histogram = unstained control, dark grey histogram = wt control, black line = NOH

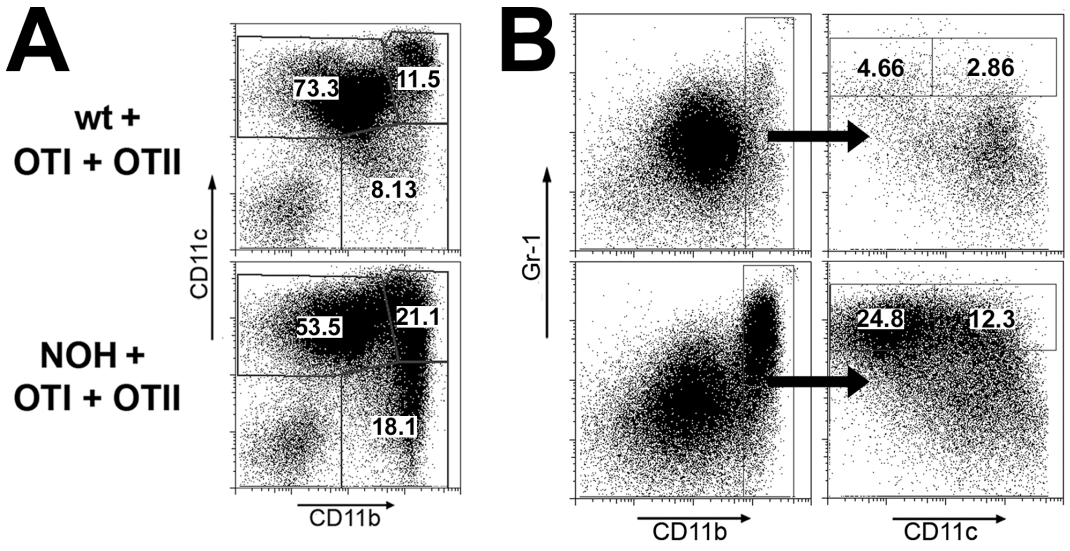

Fig. 22 *Conventional CD8α^- DC and Macrophages represent major subpopulations in kidney infiltrates.* NOH mice and wt controls were injected with 5x10^6 naïve OT-I and activated OT-II cells. 7 days later, DC and Macrophages were isolated from the kidney tissue and analyzed by flow cytometry. (**A**) Cells were stained for CD45 and MHC-II (I-A^b) to identify APC and for CD11c and CD11b to identify DC subtypes. (**B**) Expression of Gr1 on CD11b cells (left 2 dot-plots) and of CD11c vs. Gr1 on CD11b^{hi} cells (right 2 dot-plots) from **A.**

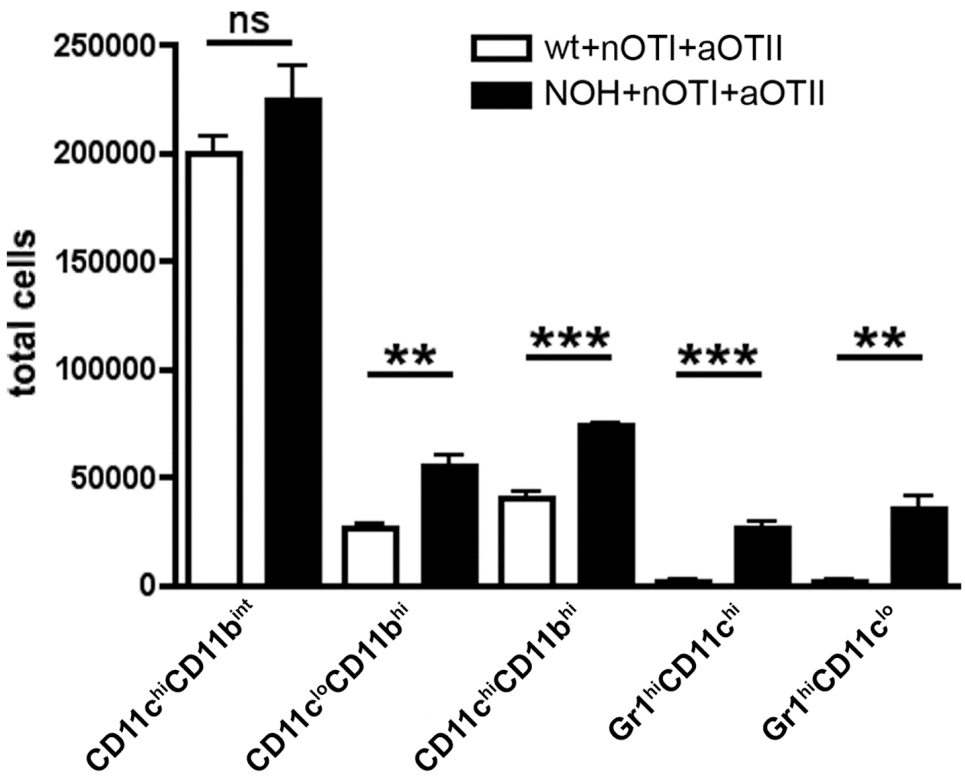

Fig. 23 *Statistical analysis of Fig. 22*. Results shown are representative for 3 independent sets of experiments performed in groups of 3 mice.

6.13 Inflammatory DC express lower levels of CX_3CR1 compared to kidney resident DC

Kidney resident DC have been described previously to form a tight network consisting of cells expressing high levels of CX_3CR1.[211] Inflammatory monocyte derived DC expressing high amounts of Gr-1 are also known to express lower levels of CX_3CR1. Therefore, DC isolated from NOH and wt mice were analyzed for CX_3CR1 expression 7 days after adoptive transfer of OT-I and OT-II T cells. Indeed, similar numbers of $CD11c^{+}I\text{-}Ab^{+}Gr\text{-}1^{lo}CX_3CR1^{hi}$ cells were found in kidneys of NOH mice challenged with OT-I and OT-II cells compared to wt animals (**Fig. 24A**), but in contrast a significant increase in $CD11c^{+}I\text{-}Ab^{+}Gr\text{-}1^{hi}CX_3CR1^{lo}$ cells could be detected upon T cell induced inflammation. (**Fig. 24B**). These findings further support the view, that T cells caused infiltration of inflammatory monocytes differentiating into inflammatory DC.

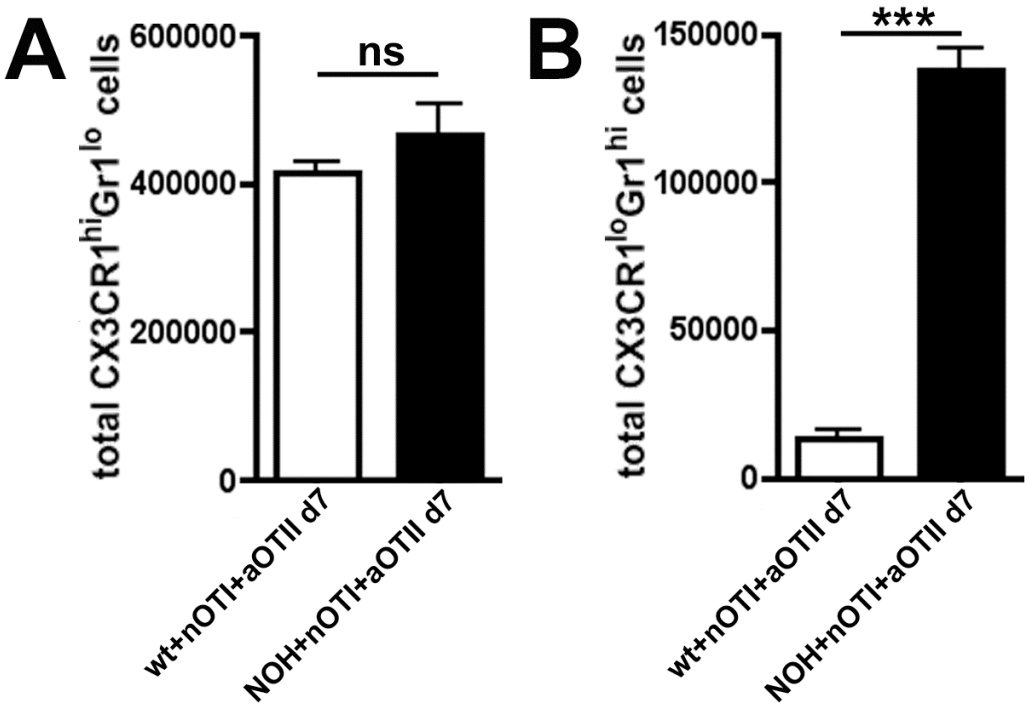

Fig. 24 *T cell mediated infiltration of $CX_3CR1^{lo}Gr\text{-}1^{hi}$ DC into kidneys of NOH mice*. NOH and wt mice were injected with $5x10^6$ OT-I and OT-II cells. 7 days after injection, DC were isolated from the kidney and analyzed by flow cytometry analysis. Cells were stained for CD11c and MHC-II ($I\text{-}A^b$) to identify dendritic cells and for Gr-1 and CX_3CR1 to distinguish between resident and infiltrating cells. **(A)** Total cell numbers of $Gr\text{-}1^{lo}CX_3CR1^{hi}$ cells **(B)** Total cell numbers of $Gr\text{-}1^{hi}CX_3CR1^{lo}$ cells.

6.14 Kidney infiltrating dendritic cells do not contain CD8α^+ DC or plasmacytoid DC

Next, renal infiltrates were analyzed for the presence of plasmacytoid DC or conventional $CD8\alpha^+$ DC. Therefore, mice were treated as described above, cells were isolated and stained for pDC-Markers Gr-1 and B220 and for CD8α on $I\text{-}Ab^+CD11c^+$ cells. Neither cell population was detectable in the DC fraction isolated from NOH, nor from wt animals on d7 (**Fig. 25**), indicating that these cells do not contribute to the early pathological steps of T cell mediated renal inflammation in our model. Notably, $CD11c^+CD8\alpha^+$ cells were readily detectable in the MHC-II negative cell fraction as will be described in more detail below.

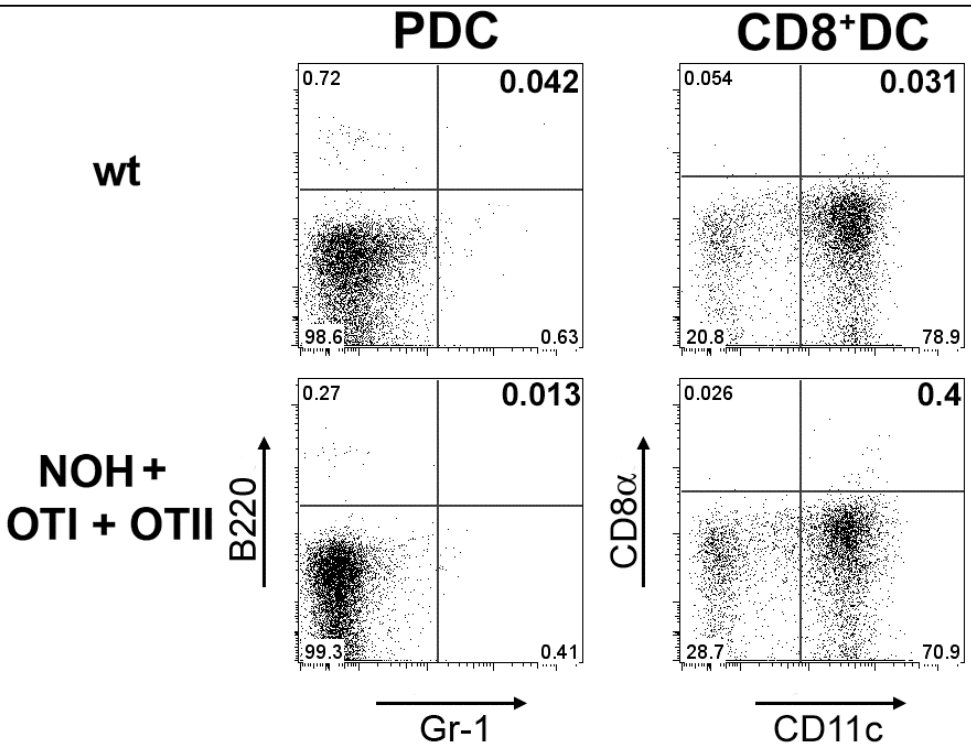

Fig. 25 *Neither pDC nor conventional CD8$^+$ DC are part of the periglomerular infiltration.* NOH mice and wt controls were injected with 5x10^6 OT-I and OT-II cells. 7 days later, DC and macrophages were isolated from the kidney tissue and analyzed by flow cytometry. Cells were stained for CD45 CD11c and MHC-II (I-A^b) to identify DC and co-stained for Gr-1 and B220 to identify pDC (left panel) or for CD8α to identify CD8α^+ DC (right panel).

6.15 CD8$^+$ T cells express CD11c upon kidney infiltration

To further investigate the finding of CD11c$^+$CD8α^+I-Ab$^-$ cells, these cells were analyzed to identify their origin and function. Notably, these cells expressed CD8α to similar levels than CD8$^+$ T cells. Therefore, cells were co-stained for CD3ε, which is a subunit of the TCR signalling complex and for MHC-II (I-A^b) as a marker for DC. Virtually all CD11c$^+$CD8α^+ cells expressed CD3ε but no MHC-II, marking them as T lymphocytes rather than APC (**Fig. 26A**). These cells have been described before as activated CTL[212-214], therefore also in our system these cells were T cells rather than CD8α^+ DC. Consistent with the previous results, the amounts of CTL as well as conventional DC isolated from kidney tissue were significantly increased in NOH mice compared to wt, whereas the amount of CD8α^+ DC was very low and remained unaltered upon infiltration (**Fig. 26B**).

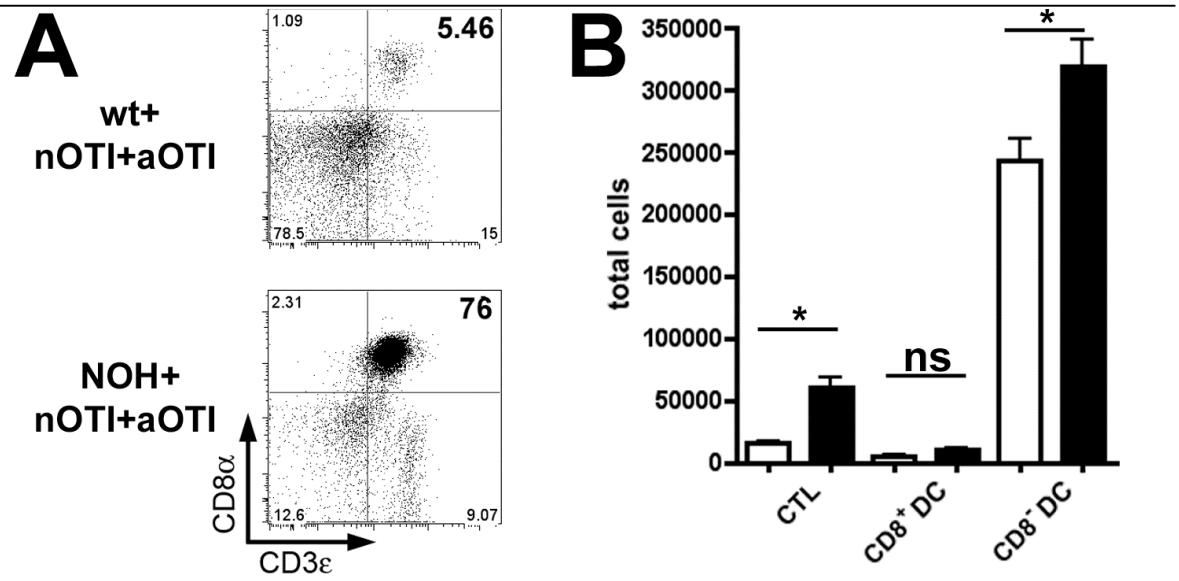

Fig. 26 *Kidney infiltrating CTL express CD11c.* NOH mice and wt controls were injected with $5x10^6$ OT-I and OT-II cells. 7 days later, cells were isolated from kidney tissue and stained for CD11c, MHC-II (I-A^b), CD8α and CD3ε. (**A**) Expression of CD8α vs. CD3ε on $CD11c^+$ cells. Dot plots shown are representative for 3 experiments performed in groups of 2-3 mice (**B**) Absolute cell numbers of kidney infiltrating cells. CTL were defined as $CD8\alpha^+CD3\varepsilon^+CD11c^+$ cells, $CD8^+$ DC were defined as $CD11c^+$I-$Ab^+CD8\alpha^+CD3\varepsilon^-$ cells, $CD8\alpha^-$ DC were defined as $CD11c^+$I-$Ab^+CD8\alpha^-CD3\varepsilon^-$ results shown are representative for 3 independent experiments performed in groups of 2-3 mice

6.16 Chronic persistence of periglomerular infiltrates causes structural and functional kidney damage

To investigate progression of renal disease, long term experiments were performed by injecting the mice with naïve OT-I and activated OT-II cells and analyzing them 4 weeks later. Surprisingly, the mice neither showed any histopathological damage, nor periglomerular infiltrates, nor could any infiltrating cells be detected by flow cytometry. Most likely, the short live-span of the activated OT-II cells without the proper reactivation *in vivo* may have been responsible for this phenomenon. To circumvent this problem, T cells were injected repetitively on a weekly basis, analyzing the mice 4 weeks after the first injection. In 2 out of 5 experiments, mice developed severe histopathological symptoms, showing pronounced tubulointerstitial infiltration by mononuclear cells, intratubular protein casts, focal tubular atrophy and focal segmental glomerular sclerosis with retraction of the glomerular tuft to the vascular pole. (**Fig. 27A,B**). Sirius red staining revealed fibrotic areas within the infiltrates (**Fig. 27C**). Also, staining for α-smooth muscle actin (α-SMA) revealed presence of myofibroblasts within periglomerular infiltrates (**Fig. 27D**). Also, staining for Collagen type IV revealed deposition of extracellular matrix proteins at the later stages of disease, but not at timepoints as early as d7 (**Fig. 27 E,F**). These findings indicated chronic tubulointerstitial damage and also to some extent development of renal fibrosis.

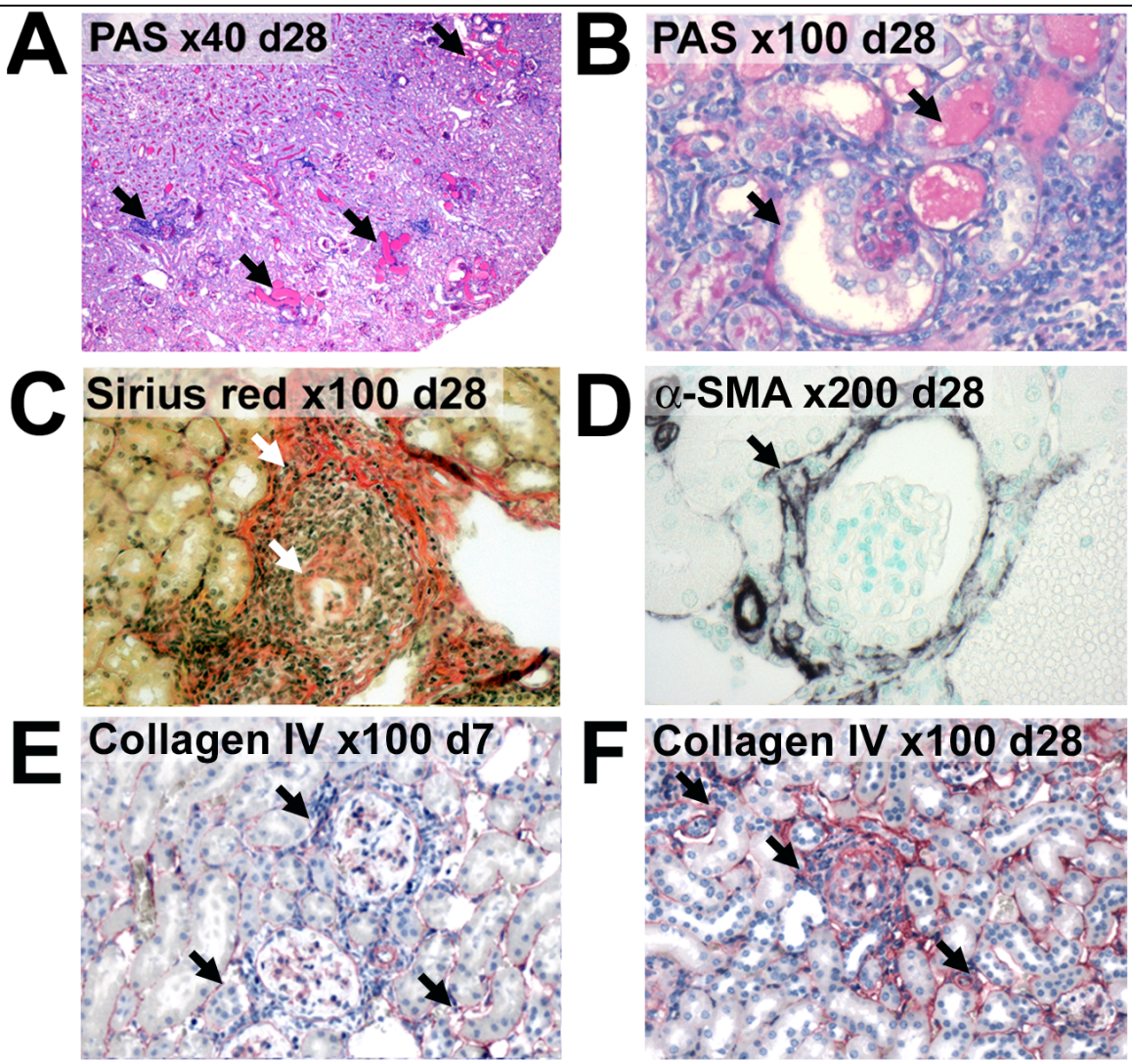

Fig. 27 *Repetitive OT cell injection causes functional and structural kidney damage in NOH mice.* $5x10^6$ naïve OT-I and $5x10^6$ activated OT-II cells were injected into NOH and wt control mice in weekly intervals. Mice were sacrificed after 4 wks and kidneys analyzed by histology. (**A**,**B**) PAS staining, originals as indicated. (**C**) Sirius red staining for fibrotic areas. (**D**) α-SMA staining by immunohistochemistry, α-SMA was stained with DAB, nuclei with methyl green staining. (**E**) Immunohistochemical staining of a Collagen type IV a NOH mouse on d7 after adoptive transfer (Collagen = red, nuclei = blue). (**F**) Collagen type IV staining of a NOH mouse on day 28. Collagen was stained using a polyclonal rabbit serum followed by Alkaline phosphatase - Neufuchsin staining, original magnification was x100. Typical results are shown for all histological pictures, mice were analyzed in groups of 3 mice.

6.17 Immune mediated kidney damage leads to albuminuria

Urinary protein excretion is commonly used as quantitative readout to display the amount of renal damage. In NOH mice treated with OT-I and OT-II cells, total protein levels only increased about 2-fold on day 7, which is relatively low compared to other disease models such as nephrotoxic nephritis, where proteinuria is increased up to 30-fold. Therefore,

albumin excretion was used as a more sensitive marker for renal disease. This method is commonly used in clinical diagnostics[215, 216] and has also been described in mouse models as marker for renal disease.[217] Albumin levels increased over time when OT-I and OT-II cells were co-injected, leading up to a 40-fold increase on d7 and a 400-fold increase on d28 (**Fig. 28A**). Injection of activated OT-I cells only led to a much lower increase in albuminuria up to d28, mice injected with activated OT-II cells showed no pathological protein excretion (**Fig. 28A**). SDS-gel electrophoresis of urine showed that increase of protein secretion was size selective. No significant amounts of higher MW proteins like e.g. Ig-class molecules could be found in the urine (**Fig. 28B**).

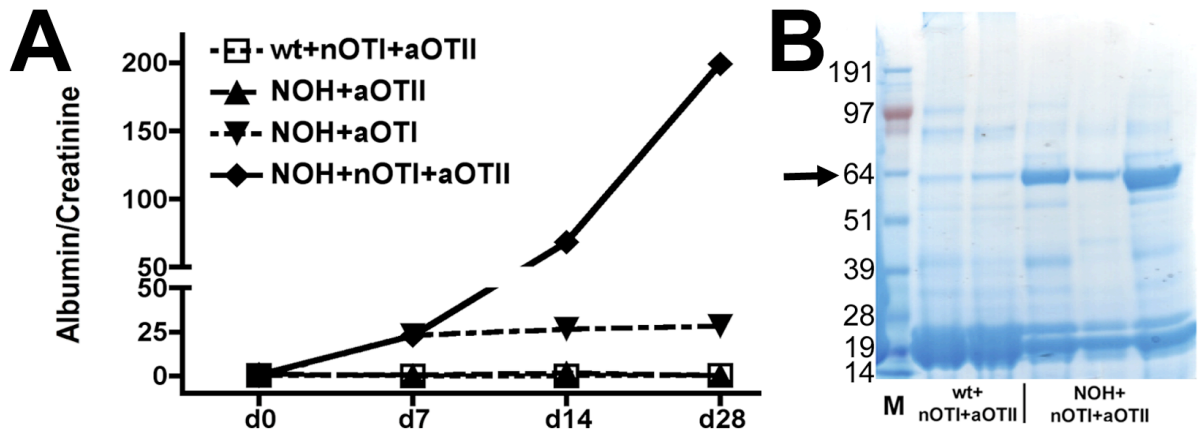

Fig. 28 *T cell mediated renal inflammation leads to albuminuria.* 5x10^6 OT-I and OT-II cells were injected into NOH and wt control mice as indicated. (**A**) Urinary Albumin levels were determined by ELISA at day 0, d7, d14 and d28, values were normalized against urinary creatinine. Mice from each group were pooled in metabolic cages over night to obtain sufficient amounts of urine for analysis. Results shown are representative for 2 individual sets of experiments. (**B**) native SDS gel electrophoresis performed on 50 µl urine samples collected from NOH and wt mice injected with 5x10^6 nOTI and aOTII cells on d7. Proteins were stained with Coomassie blue. Arrow indicates the size of Albumin. Results shown are representative for 2 independent experiments.

6.18 Renal inflammation does not alter creatinine clearance

Functional impairment of the kidney is accompanied by loss of filtration which can be measured e.g. by creatinine clearance, which is another standard clinical parameter to determine glomerular filtration. Standard laboratory values for mice range between 120-170 ml/min.[218]

As depicted below (**Fig.29**), creatinine clearance measurements showed now significant differences between wt, NOH and NOH^{lo} mice. We concluded that alterations in creatinine clearance were not strong enough to overcome the high variability of the assay.

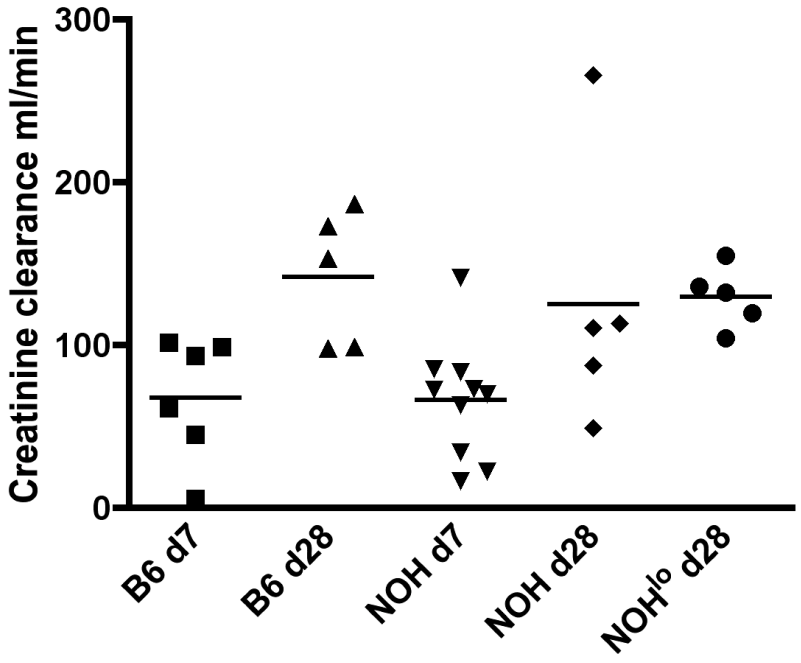

Fig. 29 *Creatinine clearance measurement.* 5x10^6 naïve OT-I and activated OT-II cells were injected into NOH, NOH^{lo} and wt control mice. On d7, mice were placed in metabolic cages o.n. for 12 hrs to collect urine samples. Sera were taken the following morning, urine sample volumes were measured and creatinine concentration was measured from sera and urine to calculate clearance values.

6.19 Ultrastructural analysis of periglomerular infiltration

Electron microscopy is commonly used to depict ultrastructural changes of the glomerulus. To address the role of periglomerular infiltration, EM-analysis was performed on NOH and wt control mice treated with OT-I and OT-II cells. Podocytes of the NOH mice appeared unaltered on day 7 (**Fig. 30A**), showing neither effacement nor fusion of foot processes. Mesangial cells as well as basement membranes also seemed unaltered (**Fig. 30B**). However, parietal cells of Bowman's capsule appeared activated with an increased number of organelles and oedematous cytoplasm (**Fig. 30B**). On day 28, some Glomeruli showed multiple cell layers of parietal cells, indicating crescent formation at later timepoints (**Fig. 30C**). Mononuclear cell infiltrates were clearly visible on day 7 (**Fig. 30B**) and day 28 (**Fig. 30C**), but were more pronounced and embedded in increased extracellular matrix at the latter time-point. Ultrastructural changes in podocytes were still not detectable even after 4 wks of renal inflammation. Glomerular capillaries showed regular endothelial cells, basement membranes and podocytes. Bowman's capsule membrane was thickened. However, intimate contacts between podocytes and parietal cells were detectable in multiple glomeruli (**Fig. 30B-D**). These findings demonstrated that primary damage of T cells was not due to a direct effect on the podocytes by infiltrating T cells, but nevertheless the lymphocytes still induced glomerular pathology.

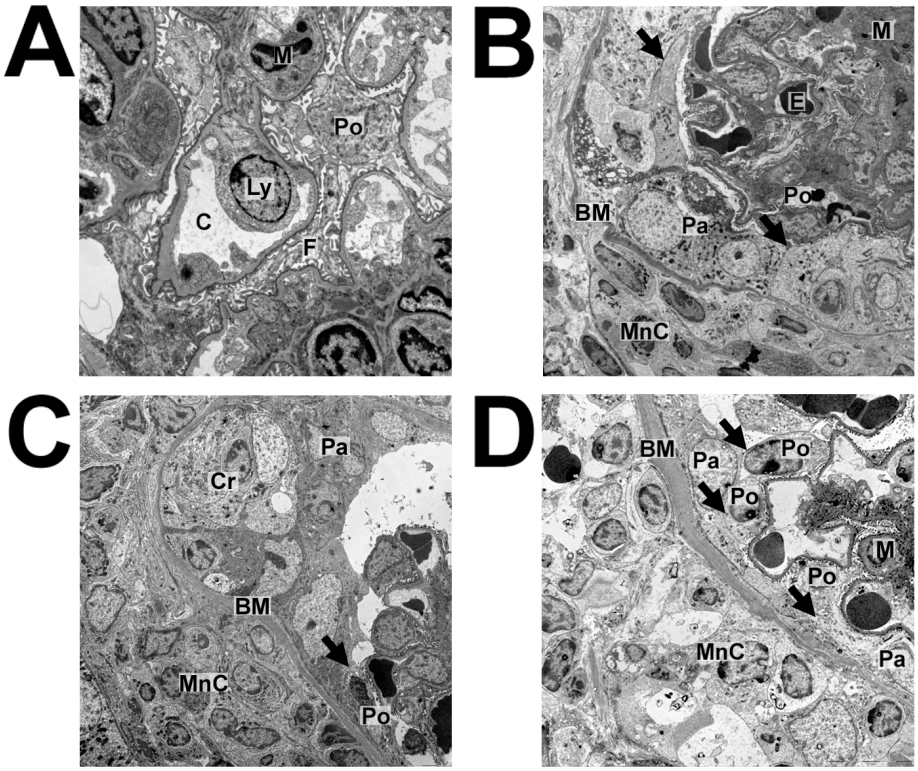

Fig. 30 *EM Analysis of periglomerular infiltration.* (**A,B**) Sections of NOH mice injected with $5x10^6$ naïve OT-I cells or $5x10^6$ activated OT-II cells 7d before analysis. (**A**) Capillary of the Glomerular tuft lined with podocytes. C = capillary, F = foot process, Ly = lymphocyte, M = mesangial cell, Po = podocyte (**B**) Parietal cells, basement membrane and mononuclear infiltrates. BM = basement membrane, E = erythrocyte, MnC = mononuclear cell, Pa = parietal cell. Arrows indicate podocyte-parietal bridges. (**C,D**) Sections of NOH mice injected weekly with $5x10^6$ OT-I cells or $5x10^6$ activated OT-II on day 28. (**C**) Parietal cells, basement membrane and mononuclear infiltrates, note formation of glomerular crescent and thickening of basement membrane, Cr = crescent. (**D**) Glomerulus on day 28 showing periglomerular infiltration, membrane thickening and various podocyte-parietal bridges (arrows).

6.20 Renal inflammation induces OVA expression in the parietal cell layer of Bowman's capsule

As described above, T cell induced inflammation leads to substantial changes in glomerular architecture. The intimate contact between parietal cells and podocytes has been described earlier[219] as well as a relocation of podocytes to the Bowman's capsule.[220] Both ways it would be possible that Ovalbumin would be present in the Bowman's capsule cell layer and therefore far more easily accessible for T cell mediated immune responses. To test whether OVA was present in the parietal cells, Immunohistochemical staining was performed on paraffin sections of NOH mice on d7 and d28 after adoptive T cell transfer. On d7 parietal cells expressed no or only little amounts of OVA (**Fig. 29A**). However, the antigen was readily detectable on parietal cells of infiltrated glomeruli at later timepoints (**Fig. 29B**).

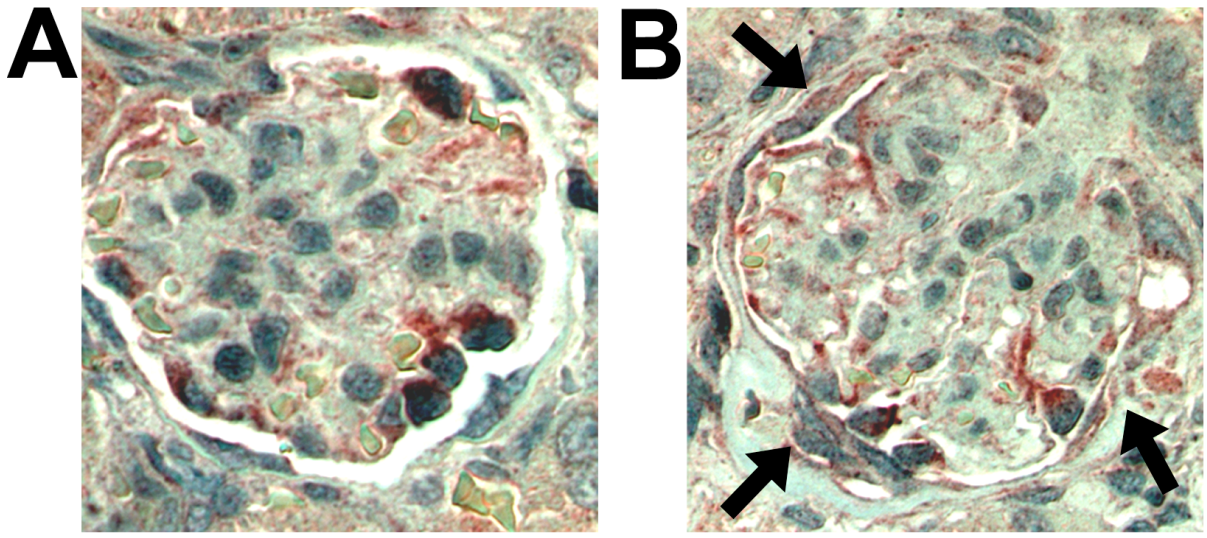

Fig. 29 *Ovalbumin expression in parietal cells of NOH mice.* (**A**) Immunohistochemical staining of a glomerulus from a NOH mouse on d7 after adoptive transfer of $5x10^6$ naïve OT-I and $5x10^6$ activated OT-II cells. (**B**) Immunohistochemical staining of a glomerulus from a NOH mouse on d28 after adoptive transfer of $5x10^6$ naïve OT-I and $5x10^6$ activated OT-II cells. Mice were repetitively injected on a weekly basis. OVA expression was revealed using an anti-OVA polyclonal rabbit serum followed by an Alkaline phosphatase-Neufuchsin staining, nuclei were stained with Haematoxilin. Original magnification was x1000.

6.21 Early depletion of DC and Macrophages leads to aggravation of renal inflammation

It is likely that homeostatic DC and infiltrating DC possess differential functional properties. As it has been reported earlier, homeostatic DC play an important role in cross-tolerance[207, 221, 222], whereas invading DC rather mediate inflammatory responses[201, 223] as it has been shown e.g. for resident kidney DC.[224] To investigate the role of the inflammatory DC, NOH were backcrossed to CD11c-DTR mice[225], which therefore could be used to selectively deplete $CD11c^+$ cells by injection of Diphteria toxin. Unfortunately, these mice developed severe side effects after injection of DT from d5 on. Only in 1 out of 4 experiments mice injected with OT-I and OT-II T cells developed a far more severe pathology after early depletion of DC on d0 compared to undepleted control animals; in the other experiments the mice died regardless if T cells were co-injected with the DT or not. Therefore, in the next step Clodronate-Liposomes (CloLip) were used, which depleted most of the circulating monocytes and also tissue-resident macrophages and DC.[226]

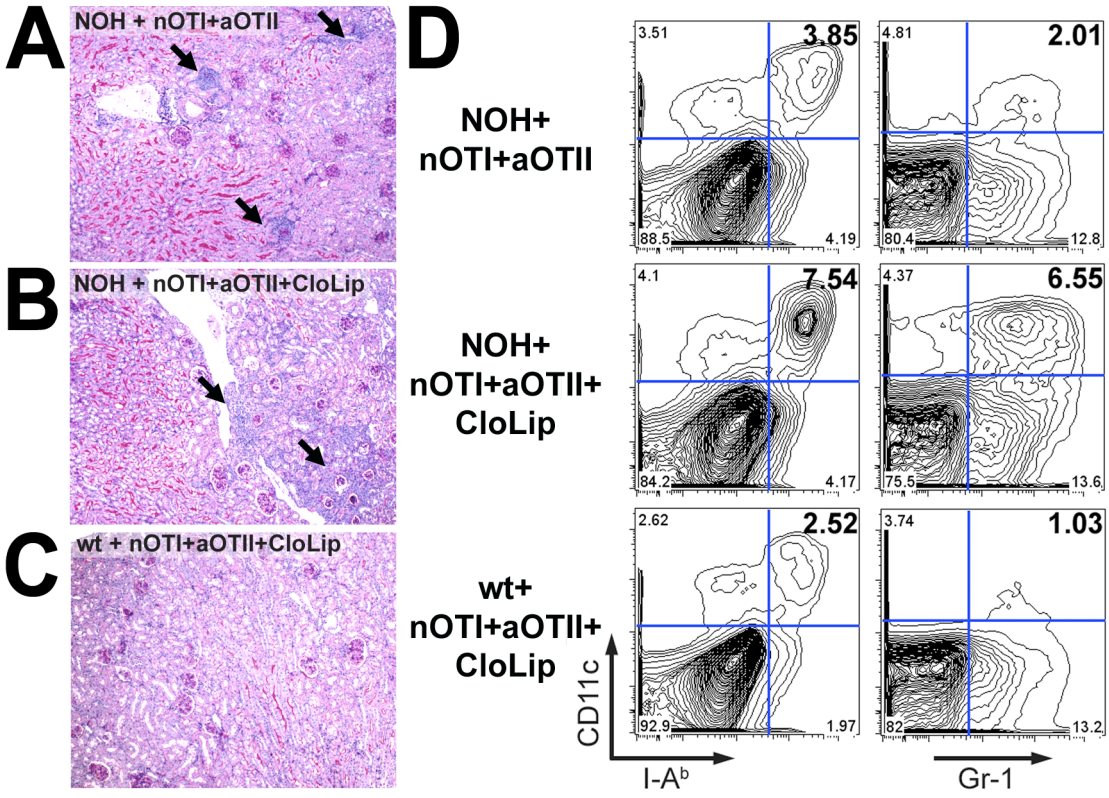

Fig. 32 *Histological and flow cytometry analysis of CloLip depleted mice.* NOH and wt mice were injected with CloLip followed by coinjection of 5x10^6 nOT-I and aOT-II cells. (**A-C**) PAS-histology, original magnification x40. Representative pictures of each group were taken. (**D**) Flow cytometric analysis of renal infiltrating DC/monocytes. Cells were stained for CD11c and MHC-II to identify DC and for Gr-1 to analyze infiltrating cells of monocyte origin. Representative dot plots are shown. Experiment was performed in groups of 3 mice.

Depletion of cells with Clodronate-Liposomes before adoptive T cell transfer resulted in an aggravated renal inflammation compared to undepleted NOH mice (**Fig. 32A,B**). This effect was not due to a general infiltration of cells, since wt control animals did not show any signs of renal inflammation (**Fig. 32C**). Moreover, not only even higher amounts of DC were present in the CloLip-treated NOH mice, but these cells also strongly expressed Gr-1, identifying them as inflammatory DC of monocytic origin (**Fig. 32D**). Furthermore, additionally to the inflammation of monocyte-derived cells, there was also a significantly increased infiltration of T cells detectable in CloLip depleted animals. Compared to undepleted NOH mice, which had received OT-I and OT-II cells, depleted mice showed a two-fold increase in OT-I (**Fig. 33A**) and a 1.5-fold increase in OT-II cell numbers (**Fig. 33B**). However, Clodronate-Liposomes also induced unspecific immigration of CD8$^+$ and CD4$^+$ T cells into the kidney to some extent (**Fig. 33A,B**). Taken together, these findings indicate that depletion of DC and other phagocytic cells in the kidney at early time points induced a severely aggravated renal immunopathology, leading to a massive infiltration of inflammatory monocytes as well as T cells into the renal tissue.

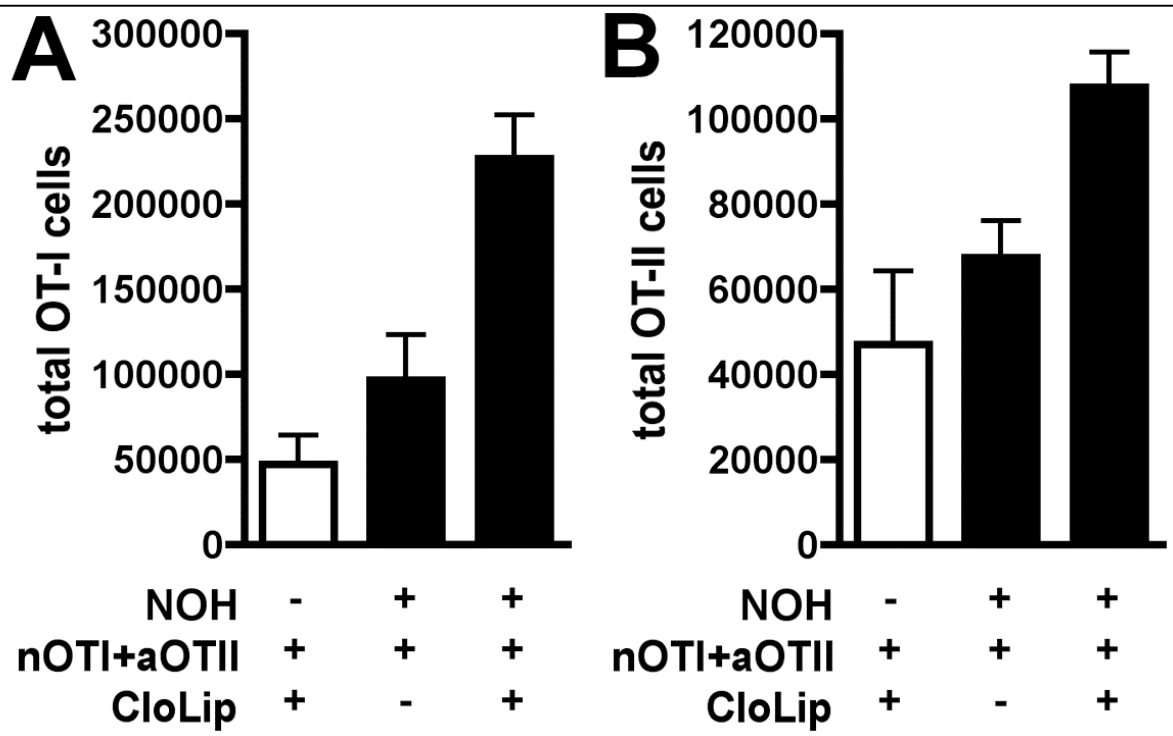

Fig. 33 *Infiltration of T cells after Clodronate-Liposome mediated depletion of phagocytic cells*. NOH and wt mice were treated as described above. T cell infiltration was assessed by flow cytometry analysis. Cells were stained for CD8α, Vα2 and Vβ5 to identify OT-I cells (**A**) and CD4, Vα2 and Vβ5 to identify OT-II cells. (**B**) $2x10^4$ CaliBRITE® beads were added as internal reference standard before flow cytometry analysis to determine total cell numbers. Experiment was performed in groups of 3 mice.

6.22 Depletion of DC abrogates priming of T cells in RLN

Early depletion of DC not only influences local immune responses in the target organ, but also priming of cells in the draining lymph node.[225, 227] The effects of the DC depletion on T cell priming were addressed by proliferation studies, depleting DC in the CD11c-DTR mice before transfer of CFSE-labelled OT-I cells, assessing their proliferation 2 days afterwards. Depletion efficiency was also determined by staining for $CD11c^+$ cells in the RLN. DC Depletion efficiency was about 85%. Proliferation of OT-I cells was almost completely abrogated in DC depleted NOHxCD11c-DTR mice but not in NOH or wt control animals (**Fig. 34**). To rule out unspecific effects of the DT or the CD11c-DTR transgene, DT treated NOH as well as undepleted NOHxCD11c-DTR mice were used as control groups. These controls showed no significant differences in proliferation compared to NOH mice (**Fig. 35**).

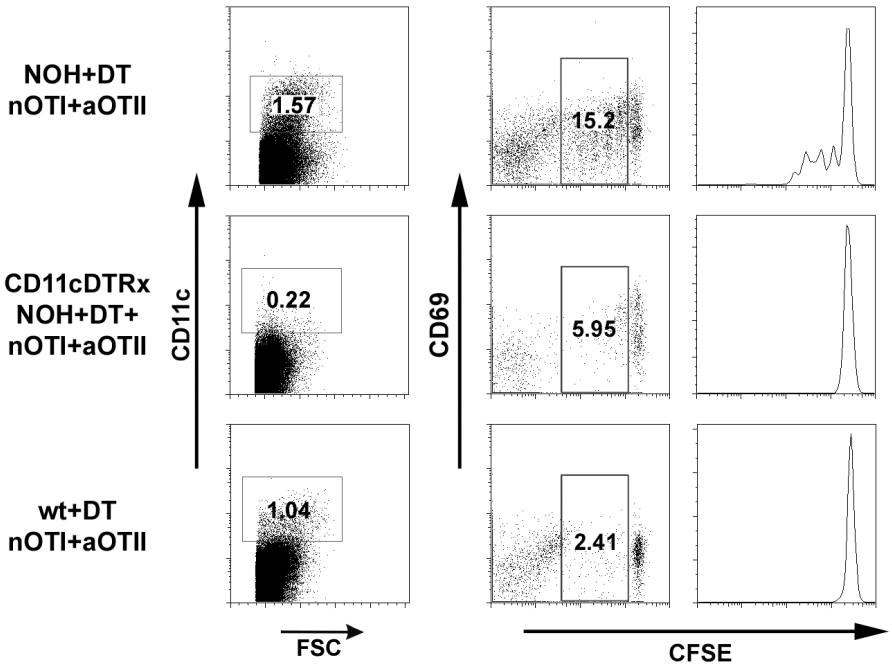

Fig. 34 *DC depletion and proliferation of OT-I T cells*. 2x10^6 CFSE labelled OT-I cells were injected into NOH, NOHxCD11c-DTR and wt control mice. DC were depleted by injection of 4$^{ng}/_{g}$ mouse weight Diphteria toxin *i.p.* on d0. DC depletion efficiency was determined 2 days later in the RLN. Proliferation was measured in CD8α^+Vα2$^+$Vβ5$^+$ cells. Histograms shown were gated only on CFSE positive cells.

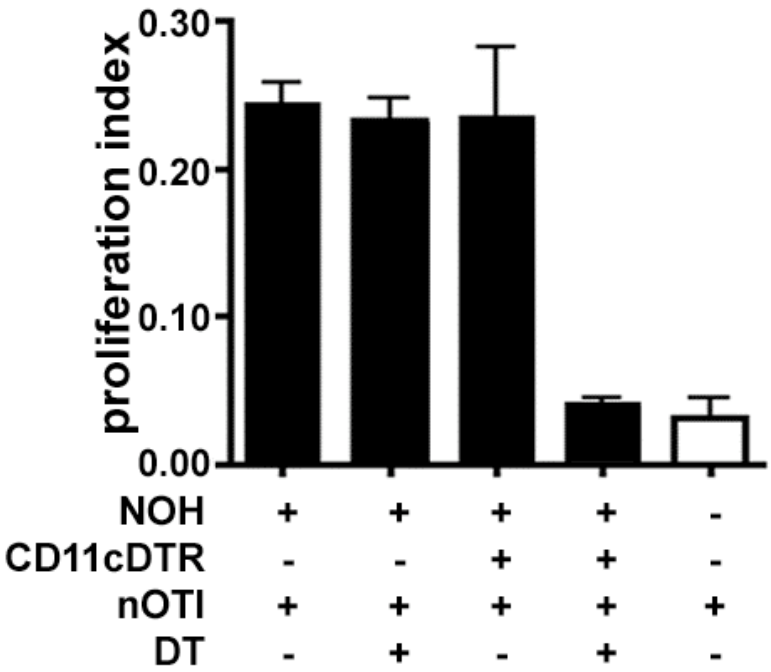

Fig. 35 *Statistical analysis of data shown in Fig. 34.* Depletion and proliferation assays were performed as described. Proliferation indices were calculated from total CD8α^+Vα2$^+$Vβ5$^+$ cells. Data shown are representative for 2 independent experiments in groups of 3 mice.

6.23 Depletion of DC in effector phase abrogates kidney infiltration

To test the effect of DC depletion at later time-points, DC were depleted in the same mice 5 days after adoptively transferring the T cells. It was likely that because of DC being a major fraction of the renal infiltrates, they would also be involved in the orchestration of the periglomerular infiltrates. NOHxCD11c-DTR mice showed cell infiltrates comparable to NOH mice when challenged with naïve OT-I and activated OT-II cells (**Fig. 36, 37C and 38A,B**). However, depletion of the $CD11c^+$ cells by DT injection 5 days after T cell injection resulted in a 90% loss of DC from the kidney (**Fig. 36**). This loss of dendritic cells from the kidney also resulted in disaggregation of the periglomerular infiltrates (**Fig. 37A and 38A,B**). Mice showed an almost complete clearance of the infiltrates (**Fig. 38A**) with a significant reduction also in severity of the singular infiltrates (**Fig. 38B**). Lack of infiltration was not due to an unspecific effect of the DT treatment or the CD11c-DTR transgene, since the correspondent control groups showed no reduced infiltration on d5 (**Fig. 37 B-D** and **Fig. 38 A,B**). These findings suggested that $CD11c^+$ cells were essential for the maintenance of periglomerular infiltrates and that the loss of these cells resulted in a rapid resolution of those infiltrates.

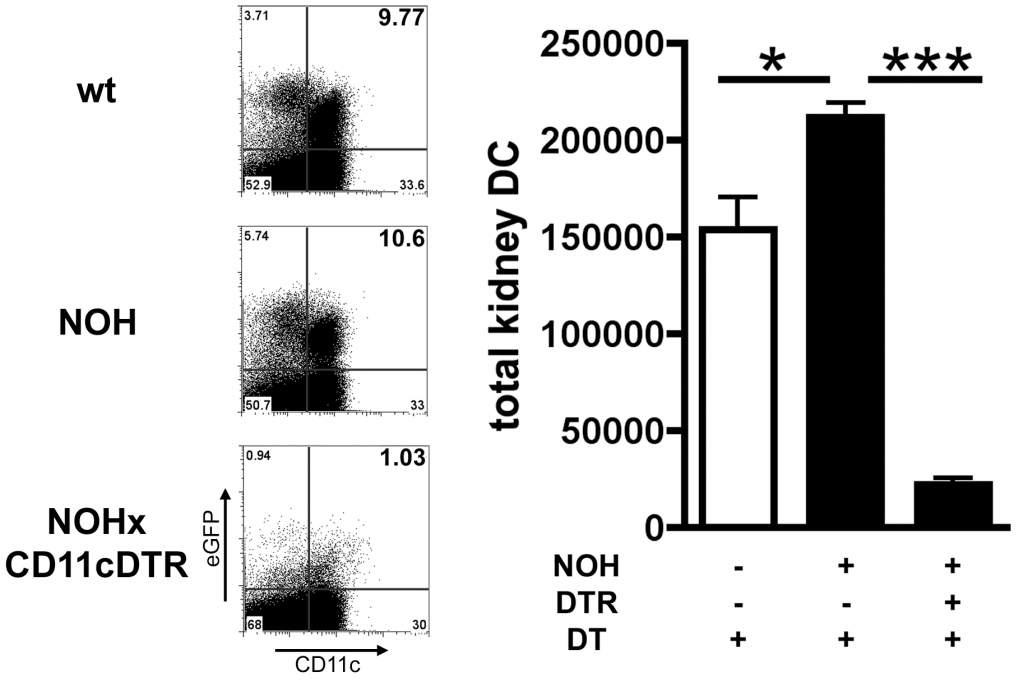

Fig. 36 *DT mediated depletion of $CD11c^+$ cells from kidney on d5 after adoptive transfer of T cells*. NOH, NOHxCD11c-DTR and wt mice were injected with $5x10^6$ OT-I and OT-II cells. On d5, mice were injected with DT. Kidneys were analyzed by flow cytometry for CD11c and eGFP to quantify DC present in the renal tissue. $2x10^4$ CaliBRITE® beads were added as internal reference standard.

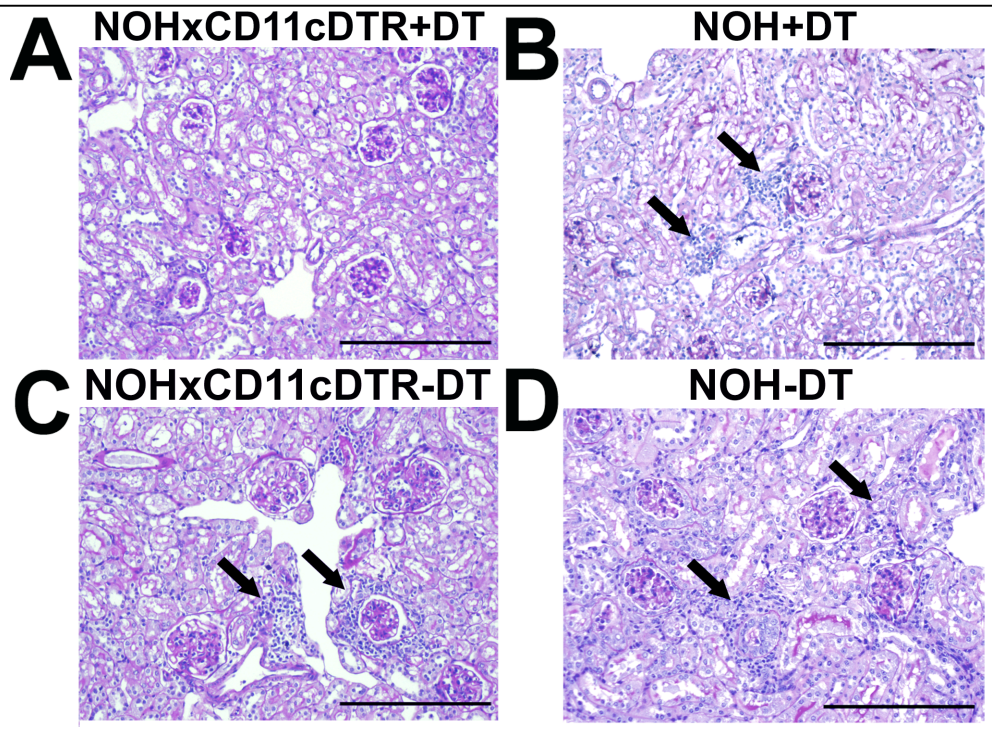

Fig.37 *Histology of mice after CD11c^{+} depletion on d5.* Mice were injected with 5x10^{6} naïve OT-I and activated OT-II cells. DT depletion was performed by injection of 4$^{ng}/_{g}$ mouse weight Diphteria toxin *i.p.* on day 5 as indicated. Mice were sacrificed on d7 and kidneys analyzed by PAS histology. Pictures shown are representative for two experiments performed in groups of 3 mice. (**A**) NOHxCD11c-DTR mouse injected with DT. Original magnification x40. (**B**) NOH mouse with DT injection. Original magnification x40. (**C**) CD11c-DTR mouse without DT injection. Original magnification x40. (**D**) NOH mouse without DT injection. Original magnification x40. All scale bars equal 400µm.

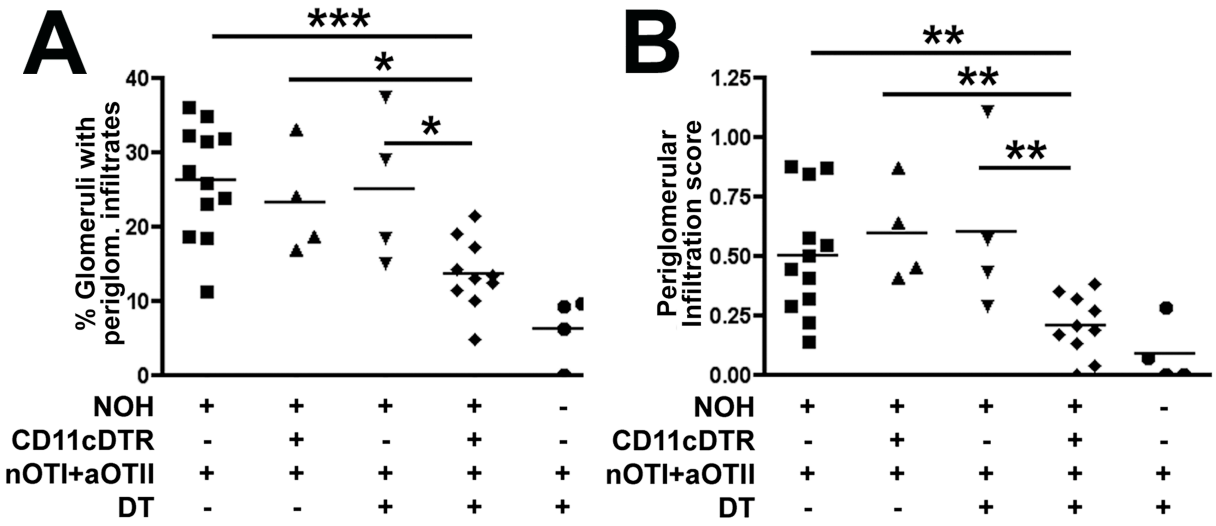

Fig. 38 *Statistical analysis of data shown in Fig. 37.* The frequency of glomeruli surrounded by mononuclear infiltrates was determined in PAS-stained kidney sections. Scoring of infiltration frequency and severity was performed as described in Fig. 13. Data shown represent 2 independent experiments performed in groups of 2-6 mice.

6.24 Immunization against OVA aggravates renal inflammation

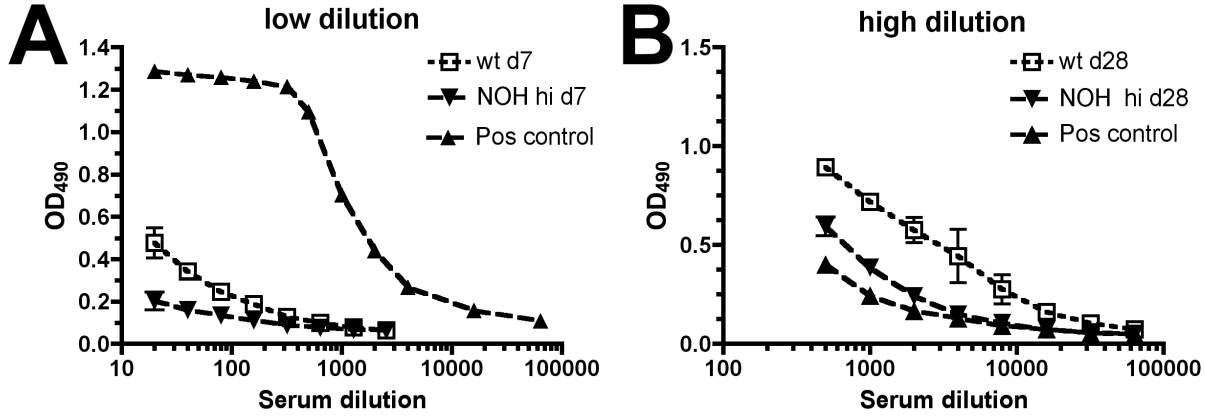

Fig. 39 *ELISA for OVA-specific IgG in mouse serum after adoptive transfer of nOT-I and aOT-II.* NOH and wt control mice were injected with $5x10^6$ naïve OT-I and activated OT-II T cells. Sera were taken on day 7 by bleeding from the tail vein. Injections were repeated weekly and mice were sacrificed on day 28. Sera were taken for analysis of OVA-specific IgG. IgG levels were determined by ELISA. Positive control sera were taken from wt mice immunized with OVA/Alum for 28 days as described above.

Long-term experiments performed with adoptive transfer of OT-I and OT-II cells not only led to cellular kidney infiltrates, but also induced OVA-specific IgG responses, probably by transferring minute amounts of either soluble or cell bound Ovalbumin from the activated OT-II cell cultures (**Fig. 39**). NOH mice showed almost no induction of anti-OVA IgG on d7 ($Titre_{0.2}$ 1:20), whereas wt animals already showed significantly increased levels of anti-OVA IgG at this early stage ($Titre_{0.2}$ 1:128) (**Fig. 39A**). On day 28, wt mice featured high titres of OVA-IgG (Titre 1:9000) and also NOH mice showed increased IgG-Titres (Titre 1:1500) if injected with OT-I and OT-II T cells on a weekly basis (**Fig. 39B**). Notably, the overall IgG response in NOH mice was approximately 5-fold lower compared to wt control animals at early as well as at late time-points, indicating that these mice are at least partially tolerant for Ovalbumin regarding their endogenous B cell repertoire. However, increased IgG levels indicated that at least at late stages of renal disease an involvement of humoral immune responses in renal damage could not be completely ruled out. These findings demonstrated that the adoptive transfer of T cells not only induced cellular infiltration into the kidney, but also lead to an OVA-specific antibody response, albeit NOH mice developed a somewhat weaker antibody response compared to wt control animals.

To test the hypothesis whether these OVA-specific antibodies contribute to the development of renal disease, NOH mice were immunized using *i.p.* injections of soluble Ovalbumin together with aluminum hydroxide which is known to be a mainly Th2 inducing adjuvant.[228]

Immunization of NOH and wt control mice was performed on d0 together with injection of OT-I and OT-II cells. OVA/Alum injections were then repeated in weekly intervals. Mice that had received T cells without being immunized additionally developed renal infiltrates from d7 on and showed an aggravated disease phenotype after 28 days. (**Fig. 40B and Fig. 41**). However, the severity of renal infiltration was markedly increased when mice were also immunized with OVA/Alum (**Fig. 40A and Fig. 41**). This effect could not have been due to the Alum-injection itself, since mice injected only with OVA/Alum did not develop any renal pathology (**Fig. 41**). Furthermore, the increased renal inflammation was not due to an enhanced activation of OT-I or OT-II cells alone, since OVA/Alum injected together with either of these cell types alone failed to induce periglomerular infiltrates (**Fig. 40C,D and Fig. 41**). Renal pathology was restricted to OVA-transgenic animals, indicating that the effect was antigen specific and not due to unspecific activation of the OVA-responsive T cells (**Fig. 39**). Taken together, these findings show that immunization of NOH mice with Ovalbumin contributes to the development of renal disease, but that antibodies alone are not sufficient to induce a renal immunopathology.

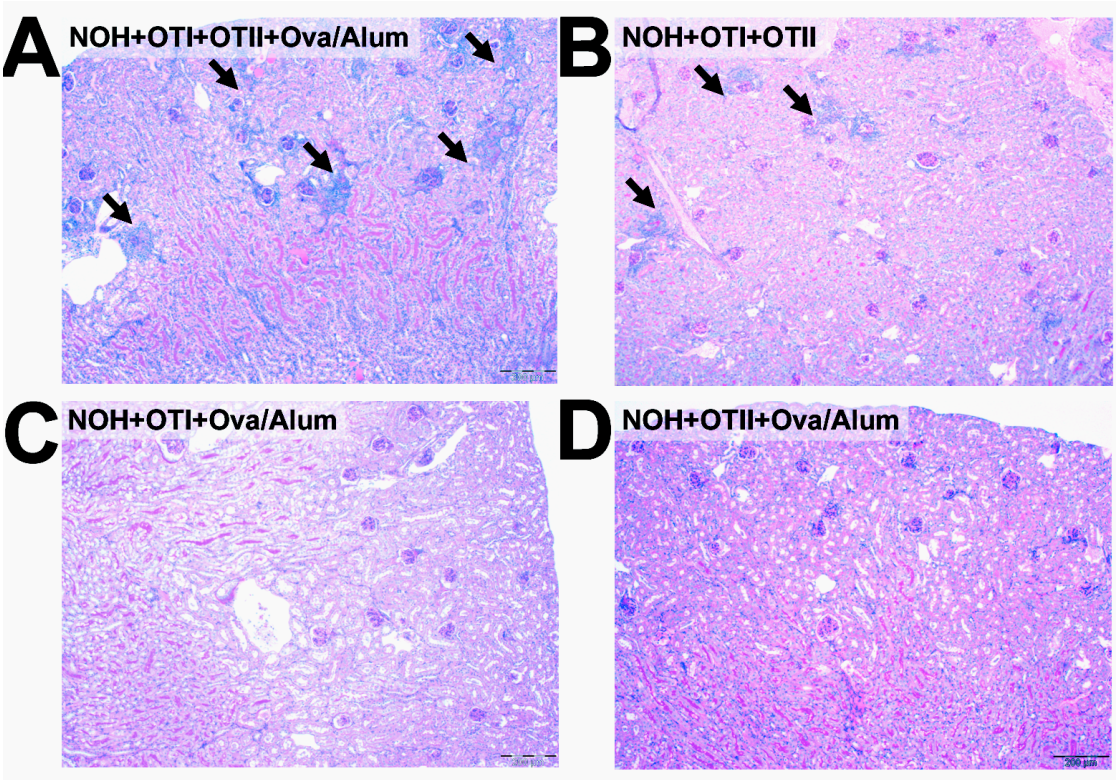

Fig. 40 *Histology of NOH mice after OVA/Alum immunization and T cell transfer.* NOH and wt mice were injected with $5x10^6$ OT-I and OT-II cells as indicated. Mice were immunized with OVA/Alum using 1:1 suspensions of $100^{\mu g}/_{ml}$ OVA and Alum. (**A**) NOH kidney on d28, injected with OT-I, OT-II and OVA/Alum. (**B**) NOH kidney on d7 after OT-I and OT-II injection. (**C**) NOH kidney on d28, injected with OT-I and OVA/Alum. (**D**) NOH kidney on d28, injected with OT-II and OVA/Alum. Experiment was performed in groups of 3-4 mice.

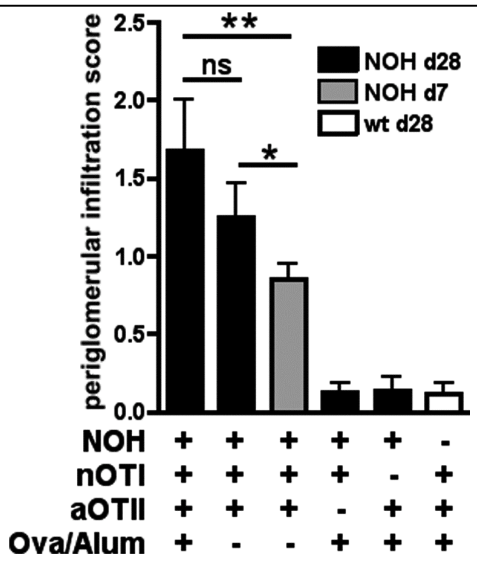

Fig. 41 *Histological score and statistical analysis of data shown in Fig. 40.* Mice were injected with 5x10^6 OT-I and OT-II cells as indicated in weekly intervals together with OVA/Alum immunization as indicated. Experiment was performed in groups of 3-4 mice.

6.25 CCR2 and CCR5 are expressed in periglomerular infiltrates

Chemokines are key players in cell recruitment and migration. Real time PCR analysis for various chemokines and chemokine receptors was performed to investigate which signals may be important for the development of periglomerular infiltrates. Mononuclear infiltrating cells and tubulointerstitial cells were isolated by laser catapult microdissection from NOH or wt control mice after adoptive T cell transfer. Cryosections of tissues from NOH and wt mice treated with naïve OT-I and activated OT-II cells were contrasted with cresylviolet and infiltrates were cut out as depicted below, interstitial cells were cut out as controls (**Fig. 42**).

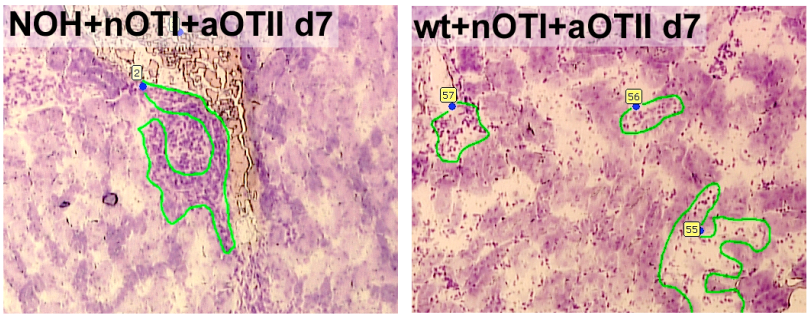

Fig. 42 *Cresylviolet staining of NOH and wt cryosections.* NOH and wt control mice were injected with 5x10^6 OT-I and OT-II cells. On day 7, mice were sacrificed and kidneys taken for cryoembedding in liquid nitrogen and cryohistology. 7µm sections were stained with cresylviolet and regions of interest were cut out by laser catapult microdissection as indicated. Only areas enclosed by the green lines were isolated for analysis. A minimum of 20 tissue fragments was collected per mouse. Representative pictures are shown; experiment was performed in groups of 3 mice.

In the next step, RNA was extracted from the excised tissue fragments and expression of chemokines and chemokine receptors was assessed. To quantify and normalize the varying amounts of total RNA isolated from the different tissues, 18S rRNA was used as internal reference. Differential chemokine expression was analyzed between groups of NOH and wt mice as shown in **Fig. 42**. Compared to cells isolated from wt animals, periglomerular infiltrates in NOH mice showed high expression levels of CCR5 (**Fig. 43A**) and also high levels of CCR2 (**Fig. 43B**) as well as their related chemokines CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES) (**Fig. 43A**) and MCP-1 (CCL2) (**Fig. 43B**).

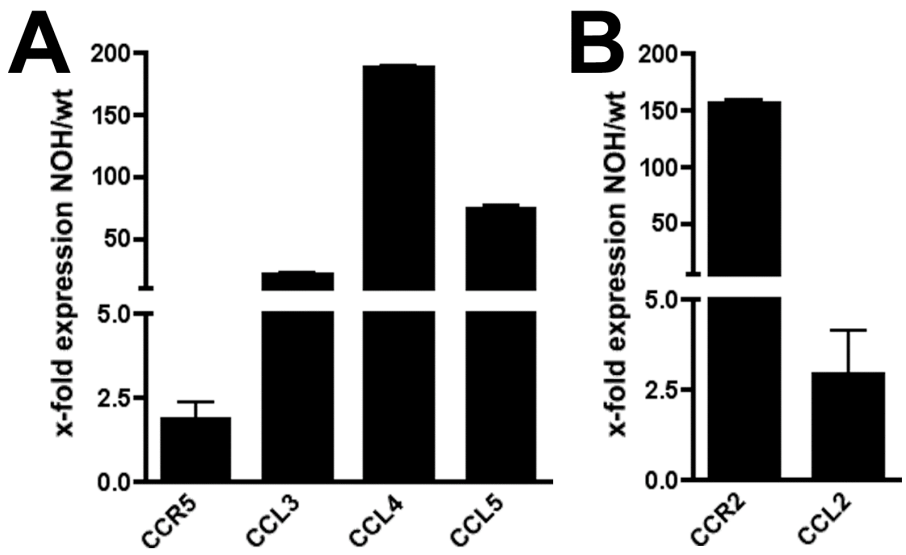

Fig. 43 *Increased mRNA expression of CCR5, CCR2 and their ligands in periglomerular infiltrates of NOH mice.* cDNA was synthesized from RNA isolated from dissected fragments shown in Fig. 42. Expression levels of chemokines and receptors were assessed by real time PCR. (**A**) Relative expression of CCR5 and CCR5-ligands compared between NOH and wt animals after adoptive T cell transfer. (**B**) Relative expression for CCR2 and CCR2 ligands compared between NOH and wt animals. Data shown are representative for 2 independent experiments performed in groups of 3 mice.

To further investigate the possible mechanisms of T cell infiltration into the tissue, OT-I and OT-II cells were sorted from renal tissue of NOH and wt mice and their expression profile for chemokines and chemokine receptors was analyzed. OT-I cells isolated from NOH mice showed about 7-fold increased CCR5 expression compared to cells isolated from wt animals (**Fig. 44A**). Notably, only few cells could be isolated from the control animals, which presumably were recirculating blood T cells rather than tissue infiltrating CTL. OT-II cells only showed little alterations of chemokine receptor expression with about a twofold increase in CCR5. However, OT-II cells expressed high levels of the CCR5 ligands CCL3 (MIP1-α) and CCL4 (MIP-1β) and also to some extent CCL5 (RANTES) (**Fig. 44B**). CCR2 and CCR7 expression remained unaltered in both OT-I and OT-II cells. (**Fig. 44A,B**)

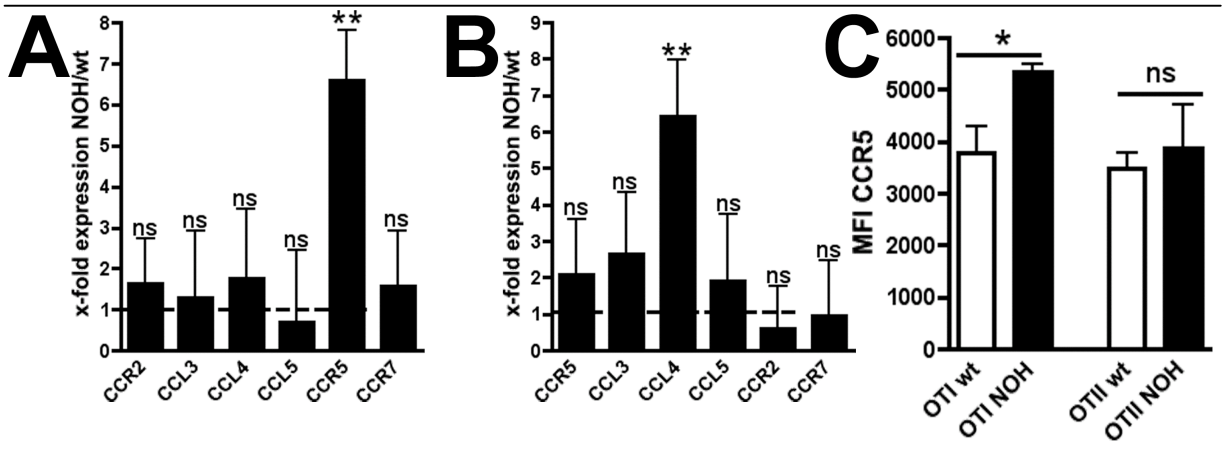

Fig. 44 *mRNA expression of CCR5, CCR5 ligands, CCR2 and CCR7 in OT-I and OT-II T cells*. NOH and wt control mice were injected with $5x10^6$ OT-I and OT-II cells. On day 7, mice were sacrificed and T cells isolated from kidney single cell suspensions by FACS sorting or flow cytometry analysis. (**A,B**) RNA was isolated from OT-I (**A**) or OT-II (**B**) cell fractions after sorting for CD8α, CD4, Vα2 and Vβ5 positive cells, transcribed into cDNA and analyzed by real time PCR for expression of chemokines and chemokine receptors. Data shown are representative for 2 individual experiments performed in groups of 3 mice. (**C**) T cells were stained for CD8α, Vα2 and Vβ5 to identify OT-I cells, for CD4, Vα2 and Vβ5 to identify OT-II cells and for CCR5 using a mouse monoclonal primary antibody followed by anti-mouse FITC secondary Ab. MFI were calculated to determine CCR5 expression levels. Experiment was performed in groups of 3 mice.

To test whether the elevated chemokine receptor expression also resulted in higher expression of the chemokine receptors on cell surface, T cells were isolated from renal tissue of NOH and wt control mice and stained for CCR5. OT-I cells showed a significant increase in CCR5 expression on the cell surface if isolated from NOH transgenic mice compared to cells isolated from kidneys of wt animals. OT-II cells did not show significant alterations in CCR5 expression similar to the mRNA expression levels (**Fig. 44B**). Taken together, these findings demonstrated that recruitment of $CD8^+$ T cells into the kidney tissue was likely to be a CCR5 mediated process and that $CD4^+$ T cells were capable of producing the analogous ligands. High levels of CCR2 and CCL2 were also present in the infiltrates as shown by laser microdissection, but were absent from both $CD8^+$ and $CD4^+$ T cells.

7. Discussion

Characterization of NOH mice and the induction of T cell responses

With the NOH model described in this thesis, it was possible to provide direct evidence for the ability of T cells to mediate glomerular injury similarly seen in other models e.g. for rapid progressive GN or MRL-lpr lupus nephritis. Until now, there has been only indirect evidence, leading to controversy about the involvement of T cells, in particular for $CD8^+$ T cells.

In contrast to previously existing GN model systems, the tissue specific expression of the OVA-HEL antigen in NOH mice allows to specifically investigate T and B cell responses by selective adoptive transfer. In this model $CD4^+$ and $CD8^+$ T cells jointly induced renal inflammation, which was characterized by primary periglomerular infiltrates containing T cells and APC at early timepoints. These infiltrates then could lead to progressive kidney damage when T cells were injected repetitively. The induction of renal disease was highly dependent on the intrarenal crosstalk between DCs, $CD8^+$ and $CD4^+$ T cells.
Consistent with previous studies, the kidney seemed to be very well protected against T cell mediated injury in the NOH model.[229] T cell activation under normal circumstances leads to cross-tolerance as previously demonstrated in other models expressing OVA, like the Rip-mOVA model, in which only fully activated CTL induced diabetes or renal inflammation.[229-233] However, OT-I cells could be effectively rescued by CD4 T cells, which were able to prevent cross-tolerance.[234-237]

Similar to the Rip-mOVA model, where OVA-antigen is expressed in tubular epithelial cells, glomerular antigen in NOH mice was cross presented in the kidney draining lymph node, where it lead to activation and proliferation of the highly reactive OT-I cells. However, MHC-II restricted antigen presentation was incapable of activating OT-II cells in NOH mice. This may be explained by low affinity of OT-II cells and their need of high antigen doses for activation, as previously suggested.[235]
The route of antigen uptake and transport to the renal lymph node was not addressed in this study. However, two possible mechanisms have been suggested in the literature: First, that small blood antigens are constitutively filtrated and are therefore reabsorbed from the primary urine by proximal tubular epithelial cells and subsequently transported by passive lymph flow and/or DC migration to the lymph node as it has been shown before for filtrated antigens.[238] In this case, podocyte-bound Ovalbumin shed from the cell surface would be reabsorbed and could serve for T cell priming by APCs. However, an attempt to detect Ovalbumin in the urine by western blot analysis was unsuccessful, presumably because the amount of antigen was below the threshold of detection.

The second mechanism suggested in the literature has been reported for CX_3CR1^+ DC, which represent also the major fraction of kidney resident DC. These cells have been shown to be capable of transepithelial antigen uptake, by extending protrusions through tight junctions into the intestinal lumen or the alveolar space, in order to sample antigen from otherwise inaccessible sites.[239, 240] However, although CX_3CR1 DCs are a major part of the interstitial DC residing in the kidney, dendritic protrusions into the intratubular space have not yet been reported. In addition to these two published mechanisms, it is conceivable that podocytes may relocate to Bowman's capsule and thereby permit adjacent interstitial DCs to directly acquire antigen. However, during homeostasis, OVA-positive cells were absent from the parietal epithelium. It remains to be examined whether this occurs in periglomerular inflammation.

Specific functions of $CD4^+$ and $CD8^+$ T cells in NOH mice

The inability of OT-II cells to become activated *in vivo* in this model had to be circumvented by their *in vitro* preactivation. Only OVA antigen doses above 100 µg/ml sufficed for such activation, giving rise only to Th1 type helper cells expressing IFN-γ if not additionally treated by cytokines. As suggested by previous studies, the type of T cell help may determine outcome of renal immunopathology. It has been shown, that Th1 cells are closely linked to development of rapid progressing forms of GN[241] leading to crescent formation and glomerulosclerosis[241-244], whereas Th2 cells rather induce nephrotic symptoms e.g. by inducing cytokine mediated podocyte damage.[245, 246] Similarly, also in our model the Th1 type response lead to a rapid progressing form of nephritis including infiltration of immune cells into the kidney, loss of glomerular filtration, crescent formation, tubular atrophy and glomeruloscerosis.

There is also evidence evolving demonstrating a possible role for the newly discovered Th17 $CD4^+$ T cells, however their involvement in nephritis induction has to be further elucidated. Th2 type responses have closely been linked to development of nephrotic syndrome, leading to severe proteinuria and podocyte damage by cytokines such as IL-13.[241, 244, 246, 247] However, a similar pathology could not be observed in NOH mice after adoptive transfer of T cells. *In vitro* differentiated Th2 cells also induced periglomerular infiltrates but no aggravated proteinuria or persisting renal inflammation.[248]

As shown by the injection of activated OT-I cells, $CD8^+$ T cells specific for a glomerular antigen were able to perform CTL effector functions, and to release antigens from damaged podocytes. NOH mice already showed constitutive activation of naïve OT-I cells, so that the increased antigen amount only induced moderate enhancement of T cell proliferation. Therefore, this experiment was performed also in the NOH^{lo} mouse line, which had been

generated using the same construct, but expressed lower levels of Ovalbumin, resulting in lower constitutive activation of OT-I T cells. When NOH^{lo} mice were challenged with activated OT-I cells, proliferation and CD69 expression of naïve T cells was increased in NOH^{lo} mice to a greater extent compared to NOH mice. No signs of DC maturation were evident in LN as well as in kidney after injection of activated OT-I shown by surface staining of CD86 and CD40, indicating that indeed the enhanced priming resulted from increased MHC-I presentation, rather than from higher costimulatory activation.

This indirect proof of the higher antigen presentation by renal dendritic cells induced by the CTL mediated damage was further supported by the *ex vivo* coculture experiments, showing increased activation of $CD4^+$ T cells by IFN-γ production. IL-2 could not be detected in the culture supernatant, presumably because of consumption by the activated OT-II cells, which require IL-2 as proliferation-promoting factor.

As already stated above, the infiltration and kidney damage induced by activated $CD8^+$ T cells alone was only minor in contrast to the pancreas-OVA model systems, where activated CTL completely destroyed pancreatic β-islet cells, and caused Diabetes type I.[249] Various possibilities exist why specific T cells were targeted less efficiently to podocytes than to pancreatic islets.

To mount a productive CTL response, it has been shown in various model systems, that tissue-bound antigens, which are being cross-presented, always induce cross-tolerance in the absence of $CD4^+$ T cell help and that only few strong exogenous antigens such as viral antigens are able to directly mount an immunogenic activation of $CD8^+$ T cells.[231 250-253] Furthermore, even activated CTL would distribute throughout the whole body, unspecifically infiltrating only peripheral tissues they could gain access to easily. Without inflammation, cells would not receive specific tissue homing signals such as adhesion molecules on activated endothelium or chemokine signals, making it difficult for the cells to identify their target tissue. Therefore, one possible role for the $CD4^+$ T cells may be the specific recruitment of the $CD8^+$ T cells into the kidney. An initial contact between the activated OT-II cells and tissue resident renal DCs which have acquired OVA-antigen would induce the release of proinflammatory signals, which would actively recruit other immune cells such as inflammatory monocytes, $CD4^+$ T cells or $CD8^+$ T cells into the kidney.

Another possible reason for the minor damage induced by the activated OT-I cells might result from sequestration of the OVA-antigen. Podocytes, which line the capillary loops of the glomerular capillary tuft, are well secluded and difficult to access for bypassing immune cells, since it has been shown that vascular endothelia and basement membranes constitute effective barriers restricting access of CTL to tissue bound antigens.[254] Similarly, tubular antigens also have shown to be sequestrated and therefore being protected against T cell

mediated immune responses.[255] Cells, which form the periglomerular infiltrates would have to pass both the external membrane of Bowman's capsule as well as the parietal epithelial cell layer. Only prolonged persistence of these infiltrates could lead to enhanced access of cells by release of e.g. matrix metalloproteinases (MMP), which would loosen up these barriers and therefore allowing the CTL access into the Glomeruli and to the podocytes, provided that glomerular leakage also leads to a defect of primary filtration and loss of urinary drain.[256] Another explanation of how renal disease progresses from periglomerular infiltration to glomerular damage may be a direct attack of the CTL on the OVA-expressing cells in the parietal layer of the Bowman's capsule. Although it is still yet unclear, how the OVA is sensed by the local DC in the onset of the disease, OVA-expressing cells could be observed at d28 the Bowman's capsule in infiltrated glomeruli. Either, parietal cells take up large amounts of Ovalbumin from the podocytes upon inflammation due to the intimate contact between podocytes and parietal cells[257] or the podocytes relocate to Bowman's capsule as it has been described recently in a model of experimental crescentic nephritis.[258] It is also possible, that inflamed parietal cells acquire podocyte-like properties. These cells would therefore be by far more accessible to the CTL, being only protected by the basal membrane.

Infiltration of the OT-II $CD4^+$ T cells was independent of $CD8^+$ T cells and just depended on the presence of OVA-antigen in the kidney, leading to an accumulation of OT-II cells. In contrast to the existing nephritis models relying on Th1 T cells like e.g. NTN-induced GN, the presence of the T helper cells alone was not sufficient to trigger a DTH-like immunopathology, showing the specific role for $CD8^+$ T cells in our model system.

$CD4^+$ T cells provide local activation signals, leading to an activation of the renal immune network as well as sending out recruiting signals by inducing a proinflammatory milieu. These signals led to a maturation of renal DC, inducing cell bound costimulatory molecules on the APC as well as secretion of proinflammatory cytokines such as TNF-α and IL-12. The presence of Gr-1^{hi} DC indicated recruitment of inflammatory monocyte precursors, although at least at the early stages of disease development these cells seem to be less mature compared to kidney resident Gr-1^{lo} DC. To follow the differentiation process of these monocyte-derived precursors into either DC-like or macrophage like cells, *in vivo* tracing experiments using congenic or fluorescent markers may facilitate following the development of these cells.

Since the infiltrating as well as the kidney-resident DC have been described as non-cross-presenting conventional $CD8^-CD11b^+$ dendritic cells[238], these cells could not provide signals to infiltrating CTL by MHC-mediated cell-cell contact. However, activation of those cells triggered release of IL-12, which has been described as an activating factor for CTL in peripheral tissues. For example, tumor infiltrating $CD8^+$ T cells are activated by local IL-12 to

perform anti-tumor effector functions.[259-261] Furthermore, IL-12 has been described as a protective factor for T cells against activation induced cell death (AICD).[262] In Toxoplasma gondii infection, IL-12 triggers IFN-γ production and cytotoxic activity in $CD8^+$ T cells.[263] In autoimmune thyroiditis, IL-12 is one of the major factors driving *in vivo* activation of effector T cells leading to inflammatory lesions of the thyroid.[264]

The periglomerular infiltrates were a major hallmark of T cell-induced nephritis in NOH mice. In human kidney biopsies from GN patients, similar infiltrates are frequently observed, but up to now the pathomechanism underlying their development remained unclear. The NOH model suggests that periglomerular infiltrates are closely linked to T cell mediated inflammatory reactions in the kidney, since the formation of the infiltrates was highly dependent on the presence of both OT-I and OT-II cells. Furthermore, $CD4^+$ as well as $CD8^+$ T cells were also abundantly present within the infiltrates together with mature dendritic cells and presumably also macrophages. This might be of diagnostic as well as therapeutic value, since selective inhibition of T cell activation or of cellular infiltration into the kidney may be more selective than unspecific anti-inflammatory treatments, such as corticosteroids.
The accumulation of cells in the kidney resulted from organ-specific recruitment rather than systemic expansion, further supporting the view that local inflammatory reactions drive the development of renal disease in NOH mice. $CD8^+$ T cells in the absence of $CD4^+$ T cells not only failed to infiltrate kidney tissue, but rather were also deleted from the system, indicating that $CD4^+$ T cells were vital for rescuing OT-I cells, and that the activation of OT-I cells in the absence of OT-II cells induced cross-tolerance. However, tolerance induction in kidney could not be observed directly due to the very low amount of OT-I cells that could be isolated from kidneys of OT-I treated NOH mice. Furthermore, functional activation of OT-I cells was highly dependent on the presence of OT-II cells as shown by intracellular measurement of IFN-γ production. However, since IL-2 could not be detected in OT-II cells neither by ex vivo ELISA nor by intracellular cytokine staining, the exact mechanism how the $CD4^+$ T cells activate the $CD8^+$ T cells remains to be determined, although IFN-γ as well as IL-12 have been shown to be able to stimulate $CD8^+$ T cells.[265, 266] However, IFN-γ has also been described as a regulatory factor for T cell responses, responsible for AICD[267] and that $CD11b^+$ cells responding to IFN-γ are also able to limit T cell responses *in vivo*.[266] Taken together, this might explain the necessity for the repetitive injection of OT-I and OT-II T cells to induce progressive renal inflammation to circumvent IFN-γ mediated tolerance induction, which may contribute to preventing overshooting T cell responses.

Local accumulation of OT-I and OT-II cells could be explained either by recruitment from the bloodstream or local secondary expansion inside kidney tissue. To address renal

proliferation of the T cells *in vivo*, cells were isolated from infiltrated organs and stained for Ki-67, which is a cell cycle protein of yet unknown function, which nevertheless can be used to detect dividing cells. No Ki-67$^+$ OT-I cells could be isolated from kidney tissue, indicating that inflammatory cells indeed invaded the renal tissue from the blood stream rather than undergoing local expansion.

The role of dendritic cells in T cell induced nephritis in NOH mice

Renal dendritic cells in homeostasis and inflammation only show properties of conventional CD8$^-$ DC. However, kidney resident dendritic cells can be distinguished from the inflammatory cells invading the renal tissue upon T cell induced inflammation. Infiltrating cells express higher levels of CD11c and CD11b and also show markers of inflammatory monocytes, expressing Gr-1 and low levels of CX_3CR1, which in contrast is highly expressed on kidney resident DC. Also, CD11b$^+$CD11c$^-$Gr-1^{hi} cells, presumably resembling macrophages, are clearly detectable in the infiltrates. Taken together, macrophages and dendritic cells were a major component of the renal infiltrates.

The absence of CD8$^+$ dendritic cells from kidney tissue in both homeostasis and inflammation suggested that the dendritic cells did not directly interact with the infiltrating CD8$^+$ T cells. Furthermore, absence of B220$^+$Gr-1$^+$ DC suggested, that also no plasmacytoid dendritic cells invaded the kidney upon inflammation and therefore DC effector functions like secretion of type I interferons was not involved in the induction of renal disease.

However, upon inflammation, CD11c$^+$CD8$^+$ DCs were clearly detectable in cells isolated from renal tissue. However, these cells were CD3ε^+ but not MHC-II$^+$, identifying them as T cells, rather than CD8$^+$ dendritic cells, a phenomenon already described by others.[268] One explanation might be the involvement of CD11c in the recruitment of the T cells into peripheral tissues. CD11c has been described to be a binding factor for leukocytes to activated endothelia and would therefore provide a homing signal to allow extravasation of cells at the site of inflammation.[269]

The outcome of CD11c$^+$ cell depletion or depletion of DC/macrophages by DTR or Clodronate-Liposomes varied depending on the timing of depletion versus adoptive T cell transfer. Early depletion of these cells abrogated effective priming of CD8$^+$ T cells in the renal lymph node between d0 and d2 after depletion. However, kidneys isolated on d7 after adoptive T cell transfer clearly showed an aggravated disease phenotype with high-number infiltrations of inflammatory monocytes as well as T cells into the renal tissue. Although early priming was impaired by the DT-mediated DC depletion, Clodronate-Liposome depletion

clearly showed that removal of phagocytic cells (resident DC / macrophages) before adoptive T cell transfer was proinflammatory. However, it is also clear that Clodronate-Liposome depletion resembles a rather unspecific approach for depletion of DC, only targeting subfractions that are highly phagocytic. Furthermore, systemic depletion of phagocytes leads to substantial body-wide cell death of phagocytes, presumably releasing proinflammatory danger signals, which may induce maturation as well as recruitment of leukocytes and also a prolonged imbalance between Gr-1^{hi} and Gr-1^{lo} monocytes. This view is also further supported by the unspecific infiltration of OT-I and OT-II T cells into the kidney of non-transgenic wildtype animals. Nevertheless, the data presented here is supported by a study showing that repetitive DT mediated depletion of $CD11c^+$ cells in a model of nephrotoxic nephritis also aggravated disease, showing the role of organ resident, anti-inflammatory DC.[270] It is likely, that the balance between regulatory DC and proinflammatory DC determines the outcome of tolerance and autoimmunity, although several other mechanisms such as local suppressive factors provided by the tissue, or infiltration by regulatory T cells might also play an important role.

In contrast, DT mediated depletion of $CD11c^+$ cells on d5 after adoptive T cell transfer led to an almost total resolution of renal infiltration and to 90% reduction of renal DC numbers. DT mediated depletion would target infiltrating $CD11c^+$ cells as well as organ resident DC; however, the almost complete loss of DC from the organ seemed to be sufficient to abrogate renal inflammation, showing the essential function of the DC in the T cell mediated kidney infiltration. This phenomenon cannot be explained by impaired priming of T cells, since on d5, a substantial amount of T cells had undergone priming and had developed into effector cells. It should be noted, however, that depletion of $CD11c^+$ cells also targets subsets of macrophages carrying CD11c, at least in the spleen.[271] Therefore also the effector cells responsible for DTH may be compromised. $CD8^+$ T cells, although carrying CD11c upon activation, would not be targeted by the Diphteria toxin, since they had been transferred from a DTR-negative OT-I donor.

Functional relevance of cell mediated renal inflammation in NOH mice

The model introduced in this study directly links T cell mediated renal inflammation to structural and functional damage of the kidney. T cells induced infiltration of inflammatory cells into the renal tissue, forming periglomerular accumulations of leukocytes. Glomeruli enclosed by these infiltrates showed stress reactions to varying degrees. Depending on the severity of the infiltration, parietal epithelial cells showed signs of activation, as evident by a marked increase in cell body size and accumulation of vesicular structures within the

cytoplasm, presumably being transcytotic vesicles. Furthermore, the parietal cells formed intimate contacts with glomerular podocytes, a pathological feature that has already been described in a model of accelerated NTN, leading to structural podocyte damage and crescent formation.[257] Direct podocyte damage such as foot process effacement or fusion could not be detected by ultstrastructural electron microscopy analysis, indicating protection of these cells from the T cell mediated attack at this early stage of disease. Also, there is only little evidence for deposition of immune complexes, arguing against a primary involvement of immunoglobulins in the development of renal inflammation.
In long term studies, where NOH mice were repetitively challenged with OT-I and OT-II cells, the primary immune infiltrates led to progression of disease, including destruction of the glomerular architecture and, equally important, structural damage to the tubular system.

The primary lesions described at the early stages of disease are commonly observed yet unexplained in various human kidney diseases, e.g. lupus nephritis[272], rapid-progressing GN[254] and in chronic allograft reaction following kidney transplantation.[273] From the data presented in this study, these infiltrates are likely to be part of the early events of cellular immune-mediated kidney inflammation, showing for the first time a possible role in the development of full-blown GN. It needs to be emphasized, however, that the immunopathology observed in the NOH model does not mimic any distinct human GN form in all respects. Nevertheless, it may help understanding the periglomerular inflammatory component that occurs in many GN entities.

The development of the renal pathology observed over time is similar in human disease as well as the according animal model systems, leading to formation of cellular crescents, protein casts, tubular atrophy and focal glomerular sclerosis. Also, the matrix deposition as well as the α-smooth muscle actin staining (α-SMA) suggested that there are myofibroblasts present in the inflamed regions, which led to deposition of extracellular matrix material and formation of microfibrotic areae. The origin and exact function of these cells still has to be determined and was beyond the scope of this study, although it has been suggested that epithelial cells might be able to redifferentiate into fibroblasts or that the cells might be of hematopoietic origin recruited by CCL2 signaling.[274, 275]
Functional damage of the kidney was assessed by measuring albumin excretion and creatinine clearance. Loss of renal function was only slowly developing over time, showing a gradual increase in urinary albumin without reduced clearance rates, showing that renal damage did not lead to terminal loss of function followed by renal failure during the time observed. Increased albumin secretion indicated selective loss of the podocyte barrier.[276] However, standard creatinine diagnostics are neither sensitive nor accurate enough, to

detect subtle changes in creatinine levels which would have been expected in the moderate renal inflammation in NOH mice and which could only be addressed by high-sensitive HPLC measurements.[277]

The role of antibodies in T cell induced nephritis in NOH mice

Since antibodies have been described to play a major role in various forms of kidney disease, it was important to address their functional involvement in the NOH nephritis model as well. The data presented in this study suggest that primary infiltration of immune cells is totally independent of immunoglobulin deposits in the glomeruli. In the long term experiments, however, at least a partial contribution of OVA-specific IgG could not be completely excluded, since serum samples taken from these animals showed gradual increase of OVA-specific IgG. This is not surprising, since activated OT-II cells would activate OVA-specific B cells, which have taken up Ovalbumin in the renal lymph node. Furthermore, in addition to the endogenous OVA expressed in the NOH mice, it could not be completely ruled out that minute amounts of Ovalbumin were cotransferred with the *in vitro* activated OT-II cells that would lead to the activation of B cell responses.

Although antibodies may contribute to the progression of renal disease in NOH mice after T cell induced kidney inflammation, they could not be the cause of renal tissue damage, since immunization experiments showed that antibodies alone or with either subset of T cells failed to induce nephritis in NOH mice. Furthermore, NOH mice show at least partial tolerance in OVA-antibody formation with titres approximately ten times lower compared to wild type animals, further supporting the view that antibodies are unlikely to be the leading pathomechanism in the NOH model.

These experiments further support the view, that neither antibodies directed against glomerular antigen nor activation of $CD8^+$ or $CD4^+$ T cells alone were sufficient for the induction of glomerular immunopathology in the NOH model.

Chemokine signalling in NOH mice

The specific recruitment and migration of inflammatory cells is a major hallmark of any cellular immune reaction. As shown in this study, $CD4^+$ T cells specific for a glomerular antigen specifically invade the renal tissue, sending out recruitment signals to attract other inflammatory immune cells such as monocytes or activated $CD8^+$ T cells, presumably by the induction of CCR2 ligands which have been described being responsible for migration of inflammatory blood monocytes[278-280] and CCR5 ligands, which have recently described to specifically guide $CD8^+$ T cells to sites of $CD4^+$ T cell-DC interactions in lymphoid organs.[281] CCR5 has also been described as a major factor in T cell mediated allograft rejection, e.g. in transplanted blood vessels[282] and has also been described to be involved in the development

of renal disease, yet the existing studies about the exact role are somewhat controversial.[279, 283] The present study suggests a direct involvement of CCR5-mediated $CD8^+$ T cell recruitment into kidney tissues, by showing enhanced expression of CCR5 on OT-I cells and CCL3, CCL4 and CCL5 on kidney infiltrating OT-II cells. T cell dependent recruitment has also been suggested by early studies of interstital nephritis models, yet the exact mechanism still had to be determined.[284] Direct proof of a role of CCR5 in recruitment of OT-I cells, however, would require blockade experiments, or the use of OT-I cells backcrossed to CCR5 knockout mice.

Summary and conclusion

With the NOH model, several new insights into cell mediated kidney diseases could be obtained, such as the first direct evidence for an involvement of $CD8^+$ T cells in the induction and development of glomerulonephritis, or the critical dependency on the functional presence of dendritic cells within the renal tissue. The defined onset of disease together with the slow progress made it possible to elucidate the sequence of events in high detail, by dissecting functional properties of T cells and DCs. The model also may be of some relevance to diagnostics of glomerulonephritis, because it offers a possible role for the involvement of periglomerular infiltrates in progression of renal disease, which are also commonly observed in human renal biopsies of nephritis patients.

In terms of a novel therapeutic approach, the NOH model has identified several new possible targets to break the chain of events in an ongoing T cell reaction in the kidney by targeting molecules like IL-12 or recruiting chemokines such as CCL3, 4 and 5. However, their degree of involvement in the development of GN has not been addressed in this study and therefore would need further investigation.

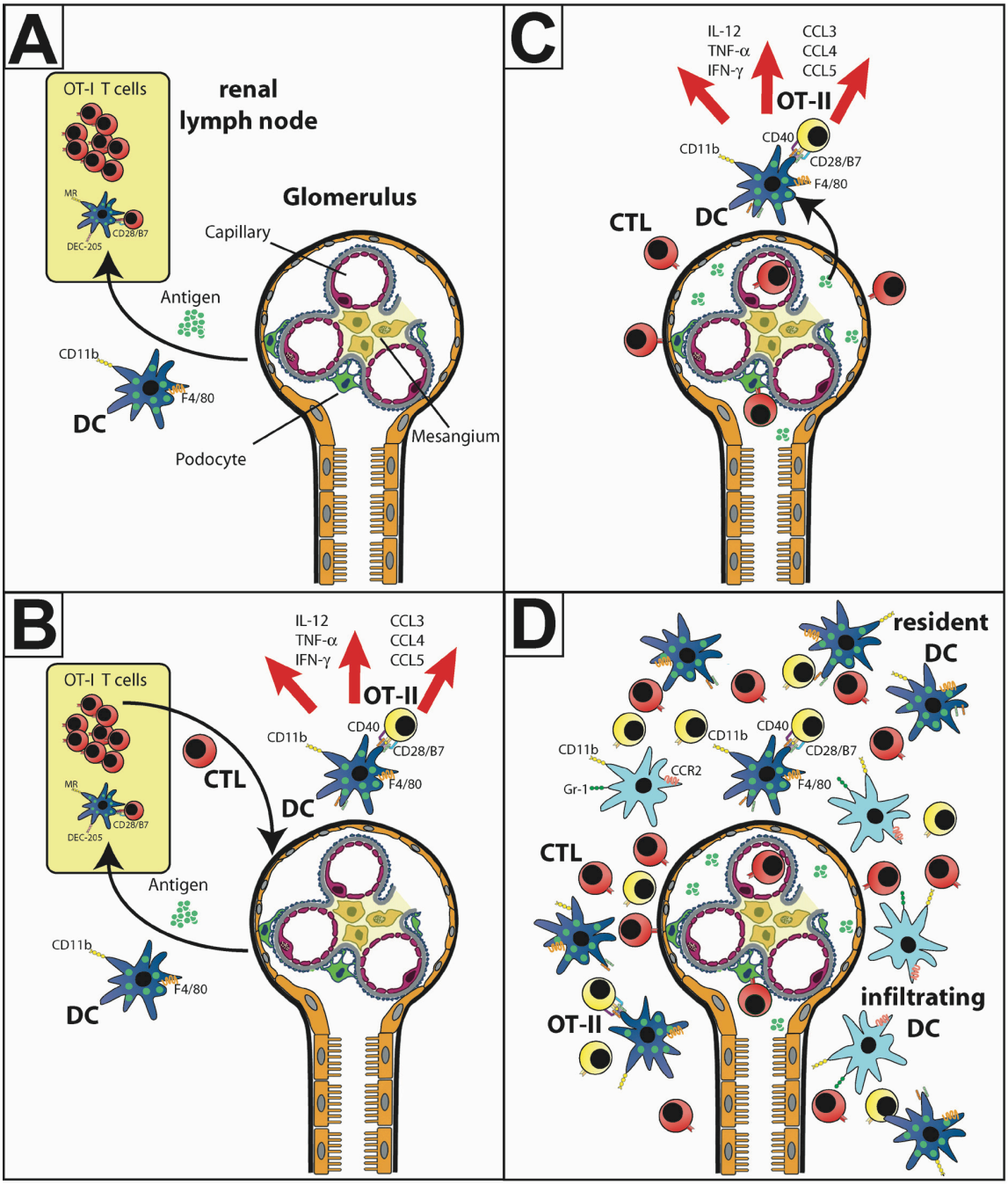

Fig. 45 *Development of T cell mediated glomerulonephritis in NOH mice.* (**A**) Renal antigen is being transported to the lymph node and leads to activation of T cells. (**B**) Activated T cells infiltrate the kidney. CD4 T cells contact local DC, this contact leads to production of proinflammatory signals and release of chemokines. (**C**) CTLs infiltrating the renal tissue induce cellular damage and lead to release of further antigen. (**D**) More T cells and inflammatory DC enter the renal tissue, forming periglomerular infiltrates that may subsequently progress to renal tissue damage and kidney fibrosis.

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Work experience

Apr 2002-Apr 2003 Research at the University of Auckland, New Zealand, School of biological sciences, Dept. for structural biology

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Apr 1998-Oct 1998 Research at Henkel KGaA, Düsseldorf, Germany, Dept. for bioorganic chemistry and organic synthesis

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Scholarships

2005-2009 PhD scholarship German National Academic Foundation (Studienstiftung des deutschen Volkes)

2002-2003 Travel grant for abroad studies from the German National Academic Foundation

2000-2003 Student scholarship German National Academic Foundation student scholarship

Publications

Heymann F, Meyer-Schwesinger C, Hamilton-Williams EE, Hammerich L, Panzer U, Kaden S, Quaggin SE, Floege J, Gröne HJ, Kurts C Kidney dendritic cell activation links glomerular injury with progression of renal disease (JCI, accepted for publication, May2009)

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Conference presentations

EFIS, 1st joint meeting, Paris Immunopathology mediated by T cells specific for a glomerular autoantigen

DGfI 37th annual meeting, Heidelberg Immunopathology of T cell-mediated glomerular kidney damage

DGfI 38th annual meeting, Wien CD8 T cells specific for a glomerular antigen induce renal inflammation in a CD4 help dependent fashion

Posters

EKRA-EDTA 2005 Basic Science-Young Investigator Forum

DGfI 36th annual meeting, Kiel Immune mediated nephritis induced by glomerulus specific T cells

DGfI 37th annual meeting, Heidelberg Immunopathology of T cell mediated glomerular kidney damage

ENII 3rd Summer school, Sardinia Immunopathology of T cell mediated glomerulonephritis

Hiermit bestätige ich, dass die vorliegende Arbeit selbständig von mir angefertigt wurde und ich alle verwendeten Materialien, Hilfestellungen, Veröffentlichungen und sonstige Hilfsmittel vollständig und korrekt angegeben habe. Zitate aus gedruckten und ungedruckten Inhalten habe ich als solche gekennzeichnet und mit den erforderlichen bibliographischen Angaben nachgewiesen.
Die eingereichte Dissertation wurde weder an einer anderen Fakultät oder Fachbereich zur Promotion vorgelegt noch bin oder war ich bereits im Besitz eines anderen Doktorgrades. Ich erkläre weiterhin, dass ich noch kein Promotionsverfahren erfolglos beendet habe. Gegen mich laufen keine Strafverfahren.

Bonn, Wednesday, 16. December 2009

Gez.

Felix Heymann