Dissertation

Immune Cells in Renal Disease: Regulatory T Cells in Nephrocalcinosis and TH9 Cells in Nephrotoxic Serum Nephritis

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Declaration

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of “Good Scientific Practice“.

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Abbreviations and Definitions

AAV	ANCA-associated vasculitis
ANCA	Anti-neutrophil cytoplasmatic antibodies
APC	Antigen-presenting cell
BALT	Bronchus-associated lymphoid tissue
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CD25	α chain of the IL-2 receptor
CD3	Cluster of differentiation 4 – T cell receptor
CD4	T cell co-receptor
CD45	Protein tyrosine phosphate receptor, type C
CD45.1	Isoform of CD45 – introduced transgenically for cell tracking
CD69	C-type lectin; upregulated during T cell activation
CKD	Chronic kidney disease
CKD-MBD	Chronic kidney disease – mineral and bone disorder
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
ELISA	Enzyme-linked immunosorbent assay
ESRD	End-stage renal disease
FCS	Fetal calf serum
FGF-23	Fibroblast growth factor 23
FSGS	Focal segmental glomerulosclerosis
GATA3	GATA binding protein 3
IFN	interferon
IL	Interleukin
IL-9-KO	Interleukin-9 knock out
KDIGO	Kidney disease – improving global outcomes

LTI	Lymphoid tissue inducer cell
Lyve1	Lymphatic vessel hyaluronan receptor 1
MALT	Mucosa-associated lymphoid tissue
MCN	Minimal change nephropathy
MPGN	Membranoproliferative glomerulonephritis
PBS	Phosphate-buffered saline
PIGN	Post-inflammatory glomerulonephritis
PRR	Pattern recognition receptor
PTH	Parathyroid hormone
Rorc2	RAR-related orphan receptor gamma 2
Sfpil1	Gene encoding the Pu.1 transcription factor; vital to TH9 differentiation
SHP	Schoenlein-Hennoch Purpura
Tbx21	T-box 21; master regulator of TH1 differentiation in mice
TH1	T helper type 1
TH17	T helper type 17
TH2	T helper type 2
TH9	T helper type 9
TLR	Toll-like receptor
TNF	Tumor-necrosis factor
Treg	Regulatory T cell
WT	Wildtype

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Zusammenfassung

Die vorliegende Dissertation beschäftigt sich mit der Rolle verschiedener T Zell Subpopulationen in zwei verschiedenen Modellen experimenteller Nieren-erkrankungen.

Als Nephrokalzinose werden ektop auftretende Verkalkungen im renalen Parenchym verstanden, wie sie unter anderem beim primärem Hyper-aldosteronismus, der akuten Phosphatnephropathie und der renal-tubulären Azidose auftreten. Um einen möglichen Beitrag verschiedener T Zell Populationen zur Pathogenese dieser Erkrankung zu untersuchen, verwendeten wir ein Mausmodell einer beschleunigten Nephrokalzinose und dystrophen kardialen Kalzinose in weiblichen DBA/2 Mäusen. $CD3^+$ T Zellen oder regulatorische T Zellen wurden in weiblichen DBA/2 Mäusen durch Injektion mit einem anti-CD3 oder anti-CD25 monoklonalem Antikörper depletiert und mit Isotyp-behandelten Tieren verglichen. Nach dieser immunomodulatorischen Behandlung, wurden die DBA/2 Mäuse für 9 Tage mit hoch-phosphathältiger Nahrung gefüttert und anschließend das Ausmaß der ektopen Kalzifikation mittels Mikro-Computertomografie quantifiziert. Die Effizienz der jeweiligen Depletion wurde mittels Flusszytometrie von Splenozyten überprüft. Die hoch-phosphathältige Nahrung ging mit dem deutlichen Phänotyp einer Nephrokalzinose und dystrophen kardialen Kalzinose einher. T Zell-Depletion führte zu einer signifikant stärker ausgeprägten renalen Kalzifikation. Weiters verschlechterte Treg Depletion den Phänotyp der akuten Phosphatnephropathie ebenfalls deutlich und führte auch zu signifikant höherer Mortalität. Die zwei immunmodulatorischen Behandlungen hatten keinen Einfluss auf das Ausmass der kardialen Verkalkung. Semiquantitiative histologische Auswertung von Alizarin-Rot gefärbten Nieren gestätigte die Mikro-CT Messungen.

Bei TH9 Zellen handelt es sich um eine rezent beschrieben T Zell Subpopulation, die sich durch die starke Sekretion von Interleukin 9 (IL-9) auszeichnet. Da es sich bei IL-9 um ein den TH2 Zellen zugeordnetes Zytokin handelt, wurden TH9 Zellen bis jetzt vor allem mit Erkrankungen des allergischen Formenkreises in Verbindung gebracht. IL-9 verstärkt die Aktivierung von TH17 Zellen und fördert Treg Funktion und Mastzellproliferation. Bis dato ist es unbekannt ob und welche TH9 Zellen in der Nephritis spielen. In der vorliegenden Arbeit, beschäftigten wir uns daher weiters mit der Rolle von TH9 Zellen in der nephrotoxischen Serumnephritis (NTS).

Es zeigte sich, dass sich in gesunden, nicht-nephritischen Mäusen TH9 Zellen vor allem in den Lymphknoten finden. Nach der Induktion des NTS Modells, zeigte sich eine signifikante Zunahme von TH9 Zellen und der TH9-assoziierten Chemokine CCL17 und CCL22 in der Niere. Die verhielt sich spiegelbildlich zu deren Abnahme in den sekundären lymphatischen Organen. Um die funktionelle Rolle von TH9 Zellen in der NTS genauer zu untersuchen, induzierten wir das Modell in IL-9 Knock-out (IL-9-KO) und Wildtyp (WT) Mäusen. Nach 14 Tagen, zeigten IL-9-KO Mäuse signifikant geringere Albuminurie, histologische Schäden und Leukozyteninfiltration in der Niere. Durch den Transfer von in vivo TH9 polarisierten naiven T Zellen, ließ sich der in WT Tieren beobachtete Phänotyp wieder komplett herstellen, v.a. in Bezug auf die renale Zellinfiltration.

Zusammenfassen zeigt die vorliegende Dissertation die bedeutende Rolle zweier T Zell Populationen in zwei verschiedenen Modellen experimenteller Nierenerkrankungen auf. Zunächst, untersuchten wir die entscheidende Rolle von T Zellen, vor allem die von Tregs in der Pathogenese der Nephrokalzinose und zeigen insbesondere die Tatsache auf, dass die begleitend auftretende Entzündung das Fortschreiten der ektopen Verkalkung in der Niere beschleunigt. Außerdem, zeigen wir, dass TH9 Zellen eine bedeutende Rolle in der Entstehung der NTS haben, was sich in unseren Versuchen durch die TH9 Migration zur Niere erklären lässt, was im weiteren Verlauf durch die Sekretion von CCL17 und CCL22 zur Rekrutierung von $CCR4^+$ Zellen führt.

Abstract

The present doctoral thesis examined the role of different T cells subsets in two different experimental models of renal disease:

Nephrocalcinosis is characterized by aberrant deposition of calcium in the kidneys and is seen in phosphate nephropathy, primary hyperparathyroidism, and distal renal tubular acidosis. To further evaluate the specific pathophysiologic role of T cells in ectopic calcification, we used DBA/2 mice that are prone to develop nephrocalcinosis and dystrophic cardiac calcinosis. Female DBA/2 mice were depleted of T cells or regulatory T cells (Tregs) using either an anti-CD3ε or an anti- CD25 monoclonal antibody and compared with isotype-treated controls, respectively. After this immunomodulation, the DBA/2 mice were given a high-phosphate diet for 9 days and the degree of calcification was assessed by microcomputed tomography. Successful depletion was confirmed by flow cytometry of splenocytes. In DBA/2 mice, the high-phosphate diet induced a phenotype of nephrocalcinosis and dystrophic cardiac calcinosis. T-cell depletion significantly increased renal calcification in microcomputed tomography. Concordantly, Treg depletion significantly deteriorated acute phosphate nephropathy and was associated with a significantly increased mortality rate. Immunomodulation had no impact on the amount of cardiac calcification. Semiquantitative histopathologic evaluations with Alizarin Red staining independently confirmed the respective radiologic measurements.

TH9 cells are a T helper cell subpopulation characterized by predominant secretion of interleukin 9 (IL-9). Functionally, TH9 cells have mainly been implicated in allergy. The cytokine IL-9 improves TH17 cell activation, regulatory T cell function and promotes mast cell proliferation. To date the role of TH9 cells in nephritis is unknown. In the present study, we therefore evaluated the role of TH9 cells in nephrotoxic serum nephritis (NTS).

In healthy mice, TH9 cells localize predominantly to the lymph nodes. Upon NTS induction, a gradual increase in TH9 cells and the TH9-secreted chemokines CCL17 and CCL22 in the kidney is paralleled by their decrease in lymph nodes and spleen. To evaluate the role of TH9 cells, we subjected IL-9 knock-out (IL-9-KO) and wild-type (WT) mice to NTS. After 14 days, IL-9-KO mice displayed significantly decreased albuminuria, histological changes and renal inflammatory cell infiltration. Upon transfer of in vitro polarized TH9 cells into IL-9-KO mice, the WT phenotype was restored. Importantly, TH9 transfer restored leukocyte infiltration.

In summary, the present doctoral thesis illustrates the pivotal contribution of T cells to renal pathogenesis across two experimental models of renal disease: Firstly, we suggest a pivotal role of T cells, particularly Tregs, in the progression of nephrocalcinosis and emphasize the fact that inflammation deteriorates the outcome in acute phosphate nephropathy. Secondly, we show that TH9 cells are essential for NTS, which may be explained by TH9 migration to the kidney and subsequent local secretion of CCL17 and CCL22 and $CCR4^{+}$ cell recruitment.

General Introduction

The immune system – an overview

The immune system consists of a large number of cellular and acellular compenents, which ultimately serve the purpose of maintaining the integrity of the organism. It depends on an intricately regulated balance of pro- and anti-inflammatory mechanisms, where any dysbalance may result in disease. While impaired immunity renders an organism more susceptible to infections and uncontrolled cell proliferation, a dysregulated, untargeted, over boarding immune response may ultimately cause autoimmune disease.(1)

In evolutionary higher organisms, the immune system is composed of two distinct systems: (i) the innate immunity, unable to mount pathogen-specific responses or adapt to changing threats, yet rapidly responsive to invading pathogens and (ii) the adaptive immune system, which relies on specific antigen recognition in order to develop a highly-specific immune response. These two intersect at various points and both complement one another.(1)

Innate immunity

Obstacles to pathogen invasion – surface barriers

The first task a potentially pathogenic microorganism has to fulfill in order to cause harm in an organism is to enter into said organism. As part of the innate immunity, several mechanisms have evolved which aim at impeding this invasion. These are especially deployed at potential entry sites, such as the skin, the gastrointestinal and genitourinary mucosa, and the respiratory tract. The skin is covered by the secretions of sebaceous glands, whose high content of fatty and lactic acids and therefore low pH poses several obstacles to invading pathogens.(2) The mucous membranes covering the body's internal sufaces are covered by mucus, in which many invaders are trapped and subsequently removed mechanically by sneezing, coughing or ciliary movements. The flushing functions of saliva, tears and urinary flow are complemented by a large variety of bactericidal molecules actively secreted into these fluids, lysozyme B in tears and saliva, hydrochloric acid in the gastric juice or lactoperoxidase in breast milk.

Habitats which are already occupied by a physiological flora and fauna are much harder to be colonized by invading microbes, thus the commensal microbiome of the human body is of great importance to immunity. Physiological microorganisms compete with invaders for nutrients and in some cases even actively secrete antimicrobial substances such as colicins.(1,2)

Soluble players in innate immunity

The complement system plays a vital role in the immediate defense against invading bacteria. It consists of relatively limited number of plasmatic proteinase-protein pairs, and mainly serves the functions of (i) immediate neutralization of invading pathogens, (ii) attracting leukocytes to the sites, where invasion occurs and (iii) clearing antigen-antibody complexes. The detrimental effects of over-boarding as well as inadequately low complement activation or inhibition can be considerable.(3) This is illustrated by the potentially life-threatening consequences of disorders such as paroxysmal nocturnal hemoglobulinuria, atypical hemolytic uremic syndrome or properidin deficiency. (3-8) It thus becomes apparent that the complement activation and dysactivation are finely controlled processes. The alternative pathway and lectin pathway of complement activation bear resemblance to the later discussed principle of *recognition of microbial non-self* in that they depend on the recognition of specific pathogen associated patterns. Spontaneous cleavage of certain complement proteins in the vicinity of bacterial cell walls declenches the alternative pathway, while mannose-residues which are bound by lectins is the first step in the activation of the lectin pathway of complement activation. The classical pathway of complement activation represents one of the important crossroads of the innate and adaptive immunity. Complexes of antibodies bound to their cognate antigen trigger this pathway. All three of these first steps in the complement cascade culminate in: (i) the formation of the membrane attack complex – a heterotetrameric protein pore complex which is incorporated into the bacterial cell membrane and allows the free diffusion of molecules across the cell membrane and (ii) the production of large quantities of chemotactic complement proteins guide leukocytes by means of concentration gradients to the site of complement activation.(3,5,6)

Innate Immune receptors

While both the innate and adaptive immunity are able to distinguish between self-antigens and non-self antigens and target the latter, they are distinctly different in how this is achieved.

A concept proposed by Charles Janeway and Ruslan Medzhitov (9) distinguishes three basic concepts of how the innate immune receptors accomplish accurate targeting of the innate immune response. In the case of *recognition of microbial non-self* the innate immunity uses patterns frequently encountered on the surface of microbes to target them. The down-regulation of major histocompatibility complex type (MHC) 1 proteins on the cell surface is frequently the consequence of viral infections or malignancy. These cases require the *recognition of missing self* in order to mark these cells for an innate immune response. In addition, cells react to viral infection or malignant transformation with a number of intracellular pathways ultimately resulting in apoptosis and the elimination of the potentially harmful cell. In cases where these intracellular pro-apoptotic pathways fail to result in apoptosis – frequently due to the event that triggered them in the first place – the *recognition of induced or altered self* is necessary to pick up extracellular signals which show that cell to be affected and target it for immune clearance.

Thus, the innate immunity depends on a limited number of germ-line encoded, widely expressed receptors to recognize certain molecular patterns. These receptors frequently interact with a relatively wide range of molecular patterns of a specific type and usually entail a fairly immediate response of the cell type expressing the receptor, which frequently is the release of an abundance of preformed, anti-pathogenic substances, or the ingestion of the invading pathogen in the case of phagocytes.(1,9-12) These pattern recognition receptors (PRR) are especially important for the *recognition of microbial non-self*. Toll-like receptors (TLR) recognize commonly found surface antigens on potentially harmful pathogens, such as lipopeptides on gram-negative bacteria, double-stranded RNA of RNA viruses.(12) Thus, the recognition of microbial non-self predominantly dependes on the recognition of foreign molecular patterns, while the recognition of loss of self and the recognition of induced or altered self frequently depends on the lack of a common pattern. Killer activating receptors on natural killer cells bind to a multitude of proteins

that are virtually ubiquitously expressed on any given cell type and trigger a pro-apoptotic signaling cascade. The presence of MHC type 1 molecules however, inhibits this cascade and thus conveys protection to cells which present an adequate level of self. (13,14)

Cellular components of the innate immunity

Phagocytes – cells capable of ingesting and digesting material – are a vital component of the innate immunity.(1) The three major types of phagocytes include macrophages, granulocytes and dendritic cells.(15) Macrophages are tissue-resident progeny of circulating monocytes and physiologically clear dying, dead cells or acellular material from their surrounding, e.g. in the spleen, where they remove senescent erythrocytes from the circulation.(15) They do, however, also fulfill important functions in the immunity against invading pathogens, in which case chemokine-guided migration of monocytic cells to sites of infections and rapid, cytokine-driven differentiation to inflammatory macrophages is a vital part of pathogen clearance. Helper T lymphocyte subsets are known to support macrophages in the clearance of intracellular pathogens, one of the many sites of interaction between the innate and adaptive immunities. Granulocytes, especially neutrophil granulocytes, the most potent member of the phagocyte family in terms of phagocytosis, are circulating cells, which are recruited to tissues in cases of infections and vitally contribute to pathogen clearance. Dendritic cells, on the other hand, are not immediately involved in the process of destroying potentially harmful agents, but are tissue-resident sentinels of their microenvironment. After ingesting a pathogen, immature tissue-resident myeloid dendritic cells mature and migrate to secondary lymphoid organs, where they present antigens they have previously encountered in their tissue of origin to the adaptive immunity, predominantly T lymphocytes. This represents an important intersection between the innate and adaptive immunities.(1,2)

Mast cells and basophil granulocytes share common bone-marrow progenitors. While mast cells are relatively long-lived, tissue-resident cells and are not to be encountered in peripheral blood, basophil granulocytes are short-lived and circulating cells.(16) Both of these cell types are capable of promptly releasing large quantities of preformed molecules, especially such molecules associated with the defense

against parasitic infections and allergic reactions. Apart from their established role in allergy-type reactions, mast cells have been implicated as one of the intersections of innate and adaptive immunity, since there is evidence of close collaboration of regulatory T cells with mast cells to establish peripheral tolereance.(17-19)

Natural killer cells form part of the lymphoid population and specialize in the clearance of cells which fail to display a proper level of self.(20) These cells are identified by natural killer cells using the principle of *recognition of missing self.*

The adaptive immunity

Primary, secondary and tertiary lymphoid organs

Primary lymphoid organs

Lymphocytes represent the main cellular component of the adaptive immunity and B lymphocytes are the producers of its soluble phase, the immunoglobulins. These B cells mature in the bone marrow, while mature, circulating T lymphocytes are the progeny of thymocytes. Thus, the bone marrow and thymus are referred to as primary or central lymphoid organs.

Secondary lymphoid organs

However, after emigrating from their respective tissue of origin, lymphocytes are dependent on the interaction with other cell types, most importantly antigen presenting cells (APC), in order to exert their function, which in very general terms, is the orchestration and very narrowly controlled regulation of a targeted acquired immune response.(21,22) After maturation and the deletion of autoreactive cells, B and T circulate only to regroup at a later point of time in the spleen and lymph nodes, the bronchus- (BALT) and mucosa associated lymphoid tissue (MALT). These peripheral, secondary lymphoid tissues provide lymphocytes with a constantly updated, representative image of the antigens currently encountered in their respective drainage sites via the influx of APCs with the lymph.(23) Secondary lymphoid organs are characterized by a very clear architecture with strong compartmentalization of B and T cells, APCs, stromal cells and cells of the blood and lymphatic vasculature.(24) This rigorously controlled architecture is dependent on the production of chemokine gradients – largely by the different kinds of stromal cells –

which guide cells to their respective niches within the lymph node. During ontogeny, secondary lymphatic organs are formed at strategic sites at precisely regulated points of time throughout the body. This is dependent on a relatively recently understood cell type: lymphoid-tissue inducer (LTI) cells. These $CD3^{-}CD4^{+}IL\text{-}7R^{+}$ cells, produce large quantities of cytokines of the lymphotoxin family and interact with mesenchymally-derived lymphoid-tissue organizer (LTO) cells to give rise to secondary lymphoid organs.(25,26)

Lymphatic vessels

The lymphatic vasculature plays an important role in the direction of lymph flow in a multitude of physiological and pathophysiological settings. During the course of their migratory path, lymphocytes enter secondary lymphatic tissue via specific types of blood vessels, high endothelium venules, and leave via lymphatic vessels. These lymphatic vessels are characterized by lymphatic vessel hyaluronan receptor (LYVE)-1 positive endothelium arise from initially very thinly walled lymphatic capillaries, which progressively merge forming larger collecting vessels and ultimately converge in much larger lymphatic vessels such as the collecting duct.(23) Afferent lymphatic vessels transport antigens and APCs to the lymph nodes, while efferent vessels allow activated cells to access higher lymph nodes and ultimately to reenter circulation via the thoracic duct into the subclavian vein.

Tertiary lymphoid organs

During the course of autoimmune driven inflammation in e.g. Hashimoto thyroiditis,(27) chronic microbial infection in e.g. borreliosis,(28,29) and chronic allograft rejection in heart and kidney transplants, (30,31) ectopic lymphoid structures have been shown to arise in a variety of tissues including synovial membranes, the thyroid, the thymus, the brain, the colon and small intestine, and the kidney.(23,32) These lymphoid structures are ectopic in that they occur in sites where lymphoid tissue is not encountered physiologically and are referred to as tertiary lymphoid organs. However, they bear close resemblance to secondary lymphoid organs, especially lymph nodes, in terms of their structural and cellular composition and the chemokines they are orchestrated by.(23) As to their function, tertiary lymphoid organs are thought of as a site of antigen presentation, T and B cell activation, as well as somatic hypermutation in B cells,(33) which implies their role as supporting

the tailored, locally confined immune response against invading pathogens, but also make them contributory factors to exacerbated autoimmune disease.(34,35)

Chemokines and chemokine receptors

The correct function of the immune system is above all dependent on the presence of the right cells at the right time and place. This is above all ensured via a multitude of chemotactical gradients, which allow for the targeted migration of specific cell types. Chemotactic cytokines (chemokines) can be secreted by secreted by virtually any resident or transient cell types, specifically podocytes, endothelial cells and mesangial cells in the kdiney.(36,37) Whether or not a specific cell type will migrate along a given chemokine gradient is determined by whether that cell expresses the cognate receptor for that specific chemokines. Four structural families of chemokines – CC-, CXC-, XC-, and CX-chemokines – encompass more than 35 family members.(36,38-40) While chemokines generally do only bind to chemokine receptors of their respective family, e.g. CCL19 and 21 will bind to CCR7, one chemokine may be promiscuous in that it will bind to several chemokine receptors, e.g. CCL5 which will ligate with CCR1, CCR3 and CCR5.(36)

Renal disease

Epidemiology and impact of chronic kidney disease

The 2012 Kidney Disease – Improving Global Outcome (KDIGO) clinical guidelines on the diagnosis and management of chronic kidney disease (CKD) define kidney disease as “as an abnormality of kidney structure or function with implications for the health of an individual, which can occur abruptly, and either resolve or become chronic”.(41) The diagnosis of CKD is then made based on the presence of these findings for a period longer than three months. The presence of kidney damage is ascertained on the basis albuminuria, urinary sediment abnormalities, imaging abnormalities, abnormal histology findings or a history of kidney transplantation.(42)

When applying current GFR-based definitions of CKD, the prevalence of CKD in the United States of America was 11.5 %, or 23 million patients.(43)

Chronically decreased renal function – CKD – goes along with an increased risk of end-stage renal disease (ESRD), cardiovascular disease and death:(44,45) the degree of increased morbidity and mortality in patients with renal insufficiency was impressively illustrated in a prominent study by Go and coworkers, who analyzed more than 1,1 million patients with non-dialysis dependent renal failure: In this study, after careful adjusting, there was a consistent inverse correlation between glomerular filtration rate (GFR) and overall and cardiovascular mortality as well as hospitalization rates, especially in stage CKD G3b and higher.(46) Early stages of CKD as well as ESRD thus go along with significant morbidity, health care system utilization and thus increased mortality and costs. Furthermore, CKD and ESRD patients frequently suffer from a number of comorbidities and report much lower quality of life than control populations with intact renal function.(42,44,45) Overall mortality in chronic kidney disease is thus dramatically higher compared to populations with normal renal function. This is largely due to excessive cardiovascular morbidity and mortality.(46) A number of factors are thought to contribute to this excessive mortality:(46-49) increased sympathetic activity along with activation of the renin-angiotensin-aldosteron system,(50,51) chronic inflammation, (52,53), oxidate stress,(54) proatherogenic uremic toxins,(55), endothelial dysfunction,(56) coagulation disorders,(57) renal anemia,(58) as well as the variety of disturbances in mineral and

bone metabolism (chronic kidney disease – mineral and bone disorder: CKD-MBD)(59-61) have been documented to play an extensive role in the setting of CKD.

Disturbances in phosphorus metabolism in chronic kidney disease: pathogenesis and impact

Since the first part of the present dissertation aims to elucidate the inflammatory aspect of the mechanisms leading to ectopic calcification in renal disease, phosphate metabolism and its disturbances in renal disease should briefly be evaluated: In the physiological aqueous milieu of the human body, phosphorus is exclusively encountered as anionic phosphate ($H_2PO_4^-$,HPO_4^{2-}). It is by far the most abundant anion in organism and accounts for around 1% of net body mass, with the intracellular outweighing extracellular concentrations by a hundred-fold.(62,63) Around 85% of overall phosphate is relatively statically bound into the hydroxyapatite of the bones and teeth, with around 14% being accounted for by phosphoesters, phospholipids and free phosphate anions of cell membranes, while merely 1% is found in the extracellular space. A large proportion of extracellular phosphate is found as free anionic phosphate and is therefore freely filtered across the glomerular size- and charge-specific barrier. Therefore, the kidney plays a paramount role in the maintenance of phosphorus homeostasis since prompt changes in tubular reabsorption allow for a fine regulation of phosphate balance in the setting of intact renal function. After glomerular filtration, phosphorus is almost exclusively reabsorbed in the proximal tubule. The membrane expression of the respective sodium phosphate antiporters is finely regulated by PTH, 1,25-OH vitamin D, as well as the phosphatonin fibroblast growth factor 23 (FGF-23).(64) In non-CKD individuals, the precise regulation of bone metabolism, intestinal phosphate and calcium absorption, as well as cellular phosphate homeostasis supplement renal phosphate handling.

Physiologically, a drop in extracellular calcium – due to decreased oral calcium uptake or increased calcium uptake in bone – decreases signaling of the calcium sensing receptor on the parathyroid gland, whereupon increasing amounts of PTH are released. PTH stimulates the release of calcium from bones and increases availability of active Vitamin D by increasing the activity of 1a-hydroxylase. This

enzyme converts 25-OH vitamin D to active 1,25-OH vitamin D, which increases intestinal and tubular calcium and phosphate reabsorption. Phosphate is thus released from the bones, reabsorbed from the primary glomerular filtrate and taken up from the intestinal lumen. Upon increases in the levels of free phosphate, osteocytes are stimulated to release FGF23, which counteracts the activity of vitamin D and PTH by a) increasing the fractional excretion of phosphate from the kidney, b) decreasing the amount of active vitamin D by downregulating 1a hydroxylase and upregulating 24-OH hydroxylase. Increased levels of circulating FGF-23 have been recognized as the earliest signs of disturbances in mineral and bone metabolism in CKD,(65,66) where the precise mechanism causing the initial rise in FGF-23 still remains elusive: in addition to phosphate retention,(67,68) early undetected alterations in vitamin D and PTH metabolism,(65,66) and renal deficiency in the FGF-23 coreceptor Klotho,(69,70) recent studies implicate direct injury to renal tissue as well as inflammatory mechanisms.(53,71,72)

Interestingly, early alterations in phosphate handling and metabolism during the course CKD do not result in alterations in serum phosphate levels but in changes in FGF-23 levels.(66,67,73) Due to an incomplete understanding of whether these early changes in mediators involved in mineral metabolism are causes of detrimental outcomes or potentially beneficial compensatory mechanism, these new findings have been slow to be integrated into clinical practice. Initially, rising levels of PTH during early CKD were thought to be the consequence of negative calcium balance, while rising FGF-23 levels were attributed to increased phosphate retention.(74) However, a cross-sectional analysis of more than 4000 CKD 2-4 patients showed that during early CKD, patients are well able to adequately translate higher levels of FGF23 into increase fractional phosphate excretion and thus to prevent phosphate retention with some patients even showing a negative phosphate balance.(66) Isakova and coworkers were further able to show a second phase of disturbed phosphate metabolism, which is found in patients with a GFR below 60 ml/min/1.73 m^2 body surface. This degree of renal impairment then entails even higher rises in circulating FGF-23, which are however unable to prevent a positive phosphate balance and hyperphosphatemia.

Glomerular disease

Primary glomerular disease is a heterogeneous group of diseases, including a variety of etiologies, initial clinical presentations, and impact on morbidity and mortality. The general incidence of primary glomerular disease is hard to ascertain due to varying standards for when to perform a renal biopsy and few studies with defined histological criteria for making a diagnosis. In a large meta-analysis including 40 studies on the incidence of primary glomerulonephritis performed between 1980 and 2010 the per year incidence varied between 0.5 and 2.5 cases / 100,000.(75) Despite this relatively small incidence, glomerulonephritis remains the primary renal diagnosis in roughly 10% of ESRD patients, thus an important source of morbidity and mortality. (75)

Apart from other clinical findings (e.g. edema, hypertension) and or parts of the history (e.g. family history of glomerular disease or concomitant hepatitis C infection), glomerular disease is most often suspected on the basis of urinary findings.(76) The presence or absence of hematuria with or without dysmorphic erythrocytes, red cell casts and the degree of proteinuria are all important factors which help in the differential diagnosis of a patient presenting with suspected glomerular disease. Despite the important contribution of history taking, physical examination, the evaluation of the renal histology by an experienced nephropathologist after kidney biopsy remains the diagnostic gold standard.(77,78) Table 1 illustrates different types of glomerular diseases according to the age at onset and the leading clinical syndrome, i.e. nephritic, nephrotic, or mixed nephritic-nephrotic.

age at presentation (years)	nephritic	nephrotic	mixed nephritic/nephrotic
<15	• PIGN • IGAN • thin-GBM • hereditary nephritis (e.g. Alport's) • SHP • Class I-IV lupus nephritis	• MCN • FSGS • Class V lupus nephritis	• Class V lupus nephritis • MPGN
15-40	• IGAN • thin-GBM • lupus nephritis • hereditary nephritis • RPGN • Class I-IV lupus nephritis	• FSGS • MCN • Diabetic nephropathy • Preeclampsia • IGAN	• MPGN • Class V Lupus nephritis • Fibrillary GN • Immunotactoid GN • IGAN
>40	• IGAN • RPGN • Vasculitis (e.g. AAV)	• FSGS • MN • Diabetic nephropathy • MCN • IGAN • Amyloidosis • Light chain deposition disease • Benign nephrosclerosis	• IGAN • Fibrillary GN • Immunotactoid GN

Table 1 – Types of glomerular diseases depending on age at presentation and leading clinical syndrome.

GN – glomerulonephritis; PIGN – postinflammatory GN; SHP – Schoenlein-Hennoch Purpura; MCN – minimal change nephropathy; FSGS – focal segmental glomerulosclerosis; AAV – ANCA-associated vasculitis; MPGN – membranoproliferative GN; RPGN – rapid progressive GN. Adapted from Rose BD. Pathophysiology of Renal Disease. New York: McGraw-Hill; 1987.

Clinical patterns of glomerular disease

For ease of clinical use, the evaluation of the urinary sediment and the extent of proteinuria in the patient suspected of having glomerular disease is broadly subdivided into the nephritic and nephrotic patterns.(77,79,80)

Nephrotic pattern – The hallmark of the nephrotic pattern of glomerular disease is protein excretion exceeding 3.5 g/day, frequently accompanied by lipiduria. Nephrotic patients tend not to have hematuria. If hematuria is present, this does commonly not include the presence of red blood cell casts. In the cases where these casts are present however, they may be the results of concomitantly present inflammatory glomerular diseases, i.e. glomerulonephritis.(76) Clinically, most underlying conditions causing a nephrotic urinary pattern may bring along an initial presentation ranging from asymptomatic, nephrotic-range proteinuria to full nephrotic syndrome with hypoalbuminemia, edema and dyslipidemia.(76) With the initial presenting symptoms frequently being limited to isolated edema, a large number of other abnormalities are associated with the nephrotic syndrome.

Nephritic pattern – The hallmark of the nephritic pattern of glomerular injury is hematuria, frequently involving the presence of red blood cell casts in the urinary sediment. The presence of acanthocytes, a specific type of dysmorphic erythrocyte in the urinary sediment is considered pathognomonic of glomerular bleeding in inflammatory renal diseases, especially if the degree of acanthocyturia reaches more than 5% of total urinary erythrocytes.(81) These cells are distinguished as ring like structures with plasma membrane protrusion and can most easily be observed using phase contrast microscopy. The degree of concomitant proteinuria in nephritic urinary findings varies according to the underlying condition, but can extend to the nephrotic range.

Nephritic Syndrome	Nephrotic Syndrome
• Hematuria • Salt and water retention • Edema • Arteria hypertension • Oliguria, anuria • Diffuse flank pain	• Proteinuria (>3.5g/24h) • Hypoproteinemia • Dysproteinemia • Hyerlipoproteinemia • Edema • Coagulation disorders • Infections • Acute kidney injury

Table 2 – Characteristics of acute nephritic as compared with nephrotic syndrome.

Adapted from ref. **(76)**

Anti-glomerular basement membrane disease

The first reported case of a 19-year-old patient, presenting with acute glomerulonephritis, who later died of acute glomerulonephritis was published in 1909 by Goodpasture.(82) Today, Goodpasture's disease describes the clinical entity of rapidly progressive glomerulonephritis, alveolar hemorrhaging and anti-glomerular basement membrane (GBM) antibodies. Strictly speaking, Goodpasture's syndrome is encountered whenever alveolar hemorrhaging and rapid progressive glomerulonephritis are concomitantly present, which would hence also apply in other vasculitides, e.g. anti-neutrophil cytoplasmatc antibody (ANCA)-associated vasculitis (AAV). Practically speaking Goodpasture's syndrome and disease are however used synonymously.(83)

Epidemiologically, anti-GBM disease is a relatively rare, however important cause and differential diagnosis of glomerular disease presenting as rapid progressive glomerulonephritis, since approximately 10 to 20% of all RPGN cases and about 5% of all primary glomerulonephritides are associated with anti-GBM disease.(76) Men are more frequently affected than women, and Caucasians more frequently than men. Occupational exposure to inhaled toxins in hairspray or spraypaint as well as

glue sniffing have been identified as a risk factor for the development of Goodpasture's disease.(84,85)

Initial clinical presentation if frequently late during the course of disease, after the onset of acute nephritis and/or lung hemorrhaging, since the initial symptoms are relatively unspecific and frequently involve weight loss, fever, and arthralgias.(83,86) More specific symptoms, such as hematuria, edema, and hemoptysis are often relatively late occurrences, where pulmonary complaints have been shown to frequenty precede renal involvement, which is especially well documented in smokers.(86) Renal disease in anti-GBM disease can however occur without signs of pulmonary hemorrhaging, where patients usually presents with glomerular hematuria and proteinuria, which rarely extends in the nephrotic range, and at later stages with signs of progressive acute or chronic renal failure such as worsening oliguria and hypervolemia due to fluid retention, as well as electrolyte imbalances.

Anti-GBM glomerulonephritis is the clinical entity most closely resembling the murine model of nephrotoxic serum nephritis and will thus be discussed here in more detail: In anti-GBM nephritis, circulating antibodies target components of the GBM and typically cause acute and rapidly progressive glomerular disease, typically associated with proliferative changes and crescent formation.(87) Pathomechanistically, a still elusive inciting event triggers the production of immunoglobulins most commonly directed against the non-collagenous domain 1 of the collagen IV alpha-3 chain, but can also target other collagen subtypes. (88-91) The two most common epitopes in Goodpasture's disease, EA and EB, are not accessible to immunoglobulins in the physiological setting, since they are within the quaternary structure of collagen IV. (90,92-94) It is currently presumed that external stimuli such as toxins in tobacco smoke induce a conformational change of the collagen hexamer, which renders the epitopes accessible to immunoglobulins, or even eventually gives rise to these epitopes as neoepitopes. (89,92,95) The specific pathogenicity of these circulating anti-GBM antibodies has been illustrated in passive transfer experiments, where immunoglobulins isolated from patients with Goodpasture's disease, caused a similar disease entity upon transfer into primates. (96) Additionally recurrence of disease linked to anti-GBM autoantibodies has been documented in ESRD patients suffering form Goodpasture's disease who received an allograft, although this is very rare and only a small proportion of transplanted

patients have recurrence of disease in their allograft. (97) While the pathogenicity of anti-GBM immunoglobulins has been well proven by the above mentioned evidence, there is recent data strongly implicating cellular autoimmune mechanisms into the pathogenesis of anti-GBM disease: as with any primarily antibody-mediated disease, antibody deposition is followed by a strong infiltration of various leukocyte subtypes and renal biopsy studies have confirmed significant monocytic and lymphocytic infiltrates and it has furthermore been shown that anti-GBM disease can be induced by adaptive transfer of T cells isolated from a nephritic rat.(98,99)

Part 1 - Regulatory T cells improve nephrocalcinosis but not dystrophic cardiac calcinosis in DBA/2 mice (71)

Introduction

Nephrocalcinosis is characterized by aberrant deposition of calcium in the kidney parenchyma and tubules, it is associated with tubular atrophy and interstitial inflammation, and it is seen in a multitude of clinical settings such as acute phosphate nephropathy, (100) primary hyperparathyroidism, (101) and distal renal tubular acidosis. (102) Unlike symptomatic urolithiasis, nephrocalcinosis is often asymptomatic and can lead to progressive renal failure and end-stage renal disease. (71,100,103)

In the largest biopsy registry study so far, Markowitz and coworkers analyzed more than 7300 native kidney biopsies and identified nephrocalcinosis as defined by diffuse tubular injury as well as ectopic calcium deposits in 31 cases. (100) While nephrocalcinosis is typically associated with periods of significant hypercalcemia, in this study the authors identified the administration of phosphorus-containing bowel cleansing agents prior to colonoscopy as a major risk factor for the development of an entity termed acute phosphate nephropathy, as 21 of the 31 reported cases were normocalcemic and had undergone such a procedure prior to presentation with acute kidney injury. The 21 patients identified as having acute phosphate nephropathy induced by extensive oral phosphate loads were largely female and white. Of note, only four of the patients had mild preexisting renal insufficiency with creatinine levels > 1.2 mg/dL prior to undergoing colonoscopy. They all presented with periods of acute kidney injury with a mean serum creatinine level of 3.9 mg/dL after a median of one month after colonoscopy. (100) At presentation, urinalysis of patients presenting with acute phosphate nephropathy was significant for mild proteinuria, and roughly half the patients presented with significantly elevated levels of serum phosphorus. Histologically, acute phosphate nephropathy was significant for mixed acute and chronic tubulointerstitial kidney disease as well as a few cases of acute tubular necrosis with unaffected glomeruli and mild to moderate concomitant tubulointerstitial inflammation. In all cases, there were diffuse tubular deposition of calcium and phosphate and in all but five specimens of nephrocalcinosis there was evidence of moderate to severe nephrocalcinosis. Eighty-five percent of patients with acute phosphate nephropathy were older than 51 years, and 60% older than 60 years. In

addition, roughly 75% of patients had a history of arterial hypertension and the overwhelming majority of these patients had previously been treated with agents that may potentially reduce renal perfusion, such as ACE-inhibitors or angiotensin receptor blockers. (100) On this basis, preexisiting renal disease and use of agents which interfere with renal microcirculation has been identified as a risk factor for developing phosphorus-bowel cleanser-associated nephrocalcinosis.

Nephrocalcinosis is not only the mere consequence of mechanical intra- or extrarenal urinary outflow obstruction, as it is also seen in a variety of both systemic and renal metabolic disorders. Furthermore, it is also actively modulated by diverse inflammatory repair mechanisms that lead to tissue remodelling, aberrant calcium deposition, and ultimately the loss of functioning renal parenchyma. The acute inflammatory response to calcium crystal precipitations in crystallopathies such as gout and pseudogout, but also in calcium oxalate nephropathy are generally initiated by the NLRP3 inflammasome via activation of caspase-1 and secretion of interleukin-1β. (104) Intrarenal dendritic cells and macrophages are essential regulators of these initial responses of innate immunity. They recruit further inflammatory cells for clearance of dead tissue, and initiate reparative responses by inducing interstitial fibrosis. (71,104)

Adaptive immunity, T lymphocytes and especially regulatory T cells (Tregs) play a central role in the modulation of renal inflammation. $CD4^+CD25^+Foxp3^+$ Tregs counteract proinflammatory cell populations such as TH1 and TH17 cells, orchestrate prolonged soft tissue remodeling und can thus control chronic inflammation. (105,106) Yet, the role of Tregs in the pathogenesis of ectopic calcification has not been systematically investigated. In the setting of nephrocalcinosis, which is accompanied by a strong and pathogenic innate inflammatory response, it is conceivable that pro-inflammatory effector T cells on the one hand as well as anti-inflammatory Tregs on the other hand significantly modulate the immune response. In order to shed light on this aspect of acute phosphate nephropathy, we used DBA/2 mice that are prone to develop nephrocalcinosis. (107) DBA/2 mice have an alternative splice variant of the *Abcc6* gene which encodes for an orphan transporter primarily located in the basolateral plasma membranes of hepatocytes and basal membranes of renal proximal tubules. (108-112) *Abcc6* mutations are associated with dysfunctional mitochondria and a decreased maximal respiratory capacity. (113)

These mice are thereby naturally susceptible to develop acute phosphate nephropathy and aberrant tissue calcification and ideally suited to study the effects of immunomodulation in these settings. (71)

Material and Methods

Study design

Female eight-week-old dilute-brown agouti 2 (DBA/2NCrl, hereafter referred to as DBA/2) mice were obtained from Charles River (Sulzfeld, Germany) and housed in a virus/antibody-free environment in the laboratory animal facility of the Medical University of Graz. DBA/2 mice develop nephrocalcinosis. (107,111) We chose to study female DBA/2 mice, because they are more prone to develop calcification when compared to their male littermates. (114,115) Mice were treated with either 10 µg anti-CD3ε monoclonal antibody (mAb; clone 145-2C11; Biolegends, San Diego, CA, USA), or isotype control antibodies administered intraperitoneally on five consecutive days. (116) After this pre-treatment, DBA/2 mice were set on a high-phosphate diet ad libitum (Altromin, Lage, Germany). In a parallel set of experiments, mice were treated with either 200 µg of an anti-CD25 mAb (clone PC61; BD Biosciences, San Diego, CA, USA) or an isotype control antibody administered intraperitoneally before setting them on the high-phosphate diet. (105,117,118) Injection of the anti-CD25 mAb leads to functional inactivation of Tregs. (119) The high-phosphate diet contained 20.2 g phosphorus, 9.4 g calcium, 0.7 g magnesium, and 500 IU vitamin D3 per kg. The standard chow contained 7.0 g phosphorus, 10.0 g calcium, 2.2 g magnesium, and 1000 IU vitamin D3 per kg. Mice were then followed for 9 days. Urine collection was performed during a fasting overnight stay in metabolic cages. All animal experiments were approved by Austrian veterinary authorities (BMWF-66.010/0047-II/3b/2012) and corresponded to the European Commission Directive 86/609/EEC. (71)

Flow cytometry

In order to prove the effect and duration of T cell and regulatory T cell depletion, we performed flow cytometry experiments of splenocytes 9 days after the initial immunomodulation. Single cell suspensions from spleen were stained for CD4 (clone RM4-5; BD Biosciences, San Diego, CA, USA), and CD25 (clone PC61.5.3; Immunotools, Friesoythe, Germany) according to the manufacturer's instructions. Data collection and analysis was done by FACS Calibur (BD Biosciences). (71)

Telemetry

ECG transmitters (Model ETA-F20, Data Science International, St. Paul, MN, USA) were implanted subcutaneously in the back space in avertin-anesthetised (0.025 mg/kg body weight) female eight-week-old DBA/2 mice. Body temperature was maintained at 37°C by a thermostatically controlled heating pad until the recovery from anesthesia. Five days were allowed for recovery before putting mice either on high-phosphate diet or standard chow diet and water ad libitum (n=5 mice for each treatment group). ECGs were recorded continuously (24-hours) after the surgical procedure in conscious, freely-moving mice kept in single cages with either normal chow or high-phosphate diet (starting on day 6 after surgery). ECG signals were acquired at a sampling rate of 1 kHz. (71)

μ-CT quantification of aberrant calcification

Ectopic calcifications were quantified at the end of each experiment ex vivo by scanning explanted organs using Inveon micro-computed tomography (Siemens, Munich, Germany), as recently described by Le Corre et al. (120) Briefly, the source was set at 60 keV, and 500 μA. The spatial resolution was 26.3 μm on-a-side cubic voxel. After acquisition on an Inveon Acquisition Workplace 1.5.0.28, each raw data set was reconstructed using Inveon Research Workplace 3.0 software package for two-and three-dimensional models. The measurements of aberrant calcification were representative of the entire organs and covered the whole heart and kidney, respectively. Thus, we can definitely exclude any sampling error. For calcification quantification, total organ volume was defined as the volume of tissue with voxel intensity between -444 and +3000. Amount of radiopaque tissue was determined as the volume of tissue with voxel intensity value between -244 and +3000. The absolute amount of radiopaque tissue was defined as volume of calcified renal or cardiac tissue, whereas the relative amount of radiopaque tissue was defined as the proportion of calcified volume from the whole kidney or heart and was given in %, respectively. (71)

Inductively coupled plasma mass spectrometry

The freeze-dried kidney samples (10-30 mg) were digested with nitric acid in a microwave-heated autoclave (UltraCLAVE III, EMLS, Leutkirch, Germany). The temperature was ramped in 45 minutes to 250°C and kept at this temperature for 45 minutes. After cooling the samples were transferred to 50 mL polypropylene tubes. The calcium and phosphorus concentrations were determined with an inductively coupled plasma mass spectrometry (Agilent 7500ce, Agilent Technologies, Waldbronn, Germany) at a mass-to-charge ratio of 43 for calcium, and 31 for phosphorus. The accuracy of the results was validated with the reference material bovine muscle (RM 8414, NIST, Gaithersburg, ML, USA). (71)

Histopathological evaluations

Frozen sections of kidneys and hearts were evaluated for calcification by performing von Kossa and Alizarin Red stains using standard protocols. For von Kossa staining, paraffin sections were incubated with 0.1% silver nitrate solution under UV light and then rinsed in 5% sodium-thiosulfate solution (both Merck, Darmstadt, Germany). Alizarin Red staining was performed by incubating rehydrated paraffin sections in 2% Alizarin Red S solution (Sigma, St. Louis, MO, USA) followed by rinsing in acetone, and acetone xylene. Furthermore, organs were evaluated for T cell and macrophage infiltration. Formalin-fixed hearts were embedded in paraffin, cut in 4-µm sections and stained with periodic acid Schiff’s rection (PAS) employing standard staining techniques. Macrophages were stained using rat anti-mouse CD68 antibodies. For the detection of CD4+ T cells, rat anti-mouse CD4 mAb (Serotec, Oxford, UK) were used. Biotin-conjugated goat anti-rat IgG antibody (Jackson Immuno Research Laboratories, West Grove, PA, USA) was used as a secondary antibody, followed by incubation with an streptavidin-biotin complex and subsequent development with 0.4% 3-amino-9-ethylcarbazole for 6 min and counterstaining with Gill's Hematoxylin No. 3 (Vector, Burlingame, CA, USA). (71)

Biochemical and molecular biological evaluations

Biochemical analysis included serum electrolytes and urea (Roche Diagnostics, Mannheim, Germany), serum Fgf23 (Immutopics, San Clemente, CA, USA), and parathyroid hormone (ALPCO, Salem, NH, USA). Urinary neutrophil-gelatinase associated lipocalin (Lcn2) was quantified using a commercially-available enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA) and normalized for urinary creatinine concentration using a spectrophotometric assay (Sigma) based on the Jaffe-reaction. Both were measured on a FLUOstar Omega photometer (BMG Labtech, Ortenberg, Germany). Real-time PCR for Foxp3, Il10, Tgfb1, Il6, Tnf, Tbx21, Rorc2 and Gata3 was performed to evaluate cytokine changes and master regulator expression of various TH-populations. Total RNA was isolated from the kidney using Trizol (Invitrogen, Carlsbad, CA, USA) according to a standard protocol. Thereafter, 2 µg of total RNA was reverse-transcribed using Superscript III Transcription Kit (Inivtrogen) and random primers (Roche, Basel, Switzerland). Real-time PCR was performed in duplicates on a CF96 real time detection system (BioRad, Vienna, Austria). For quantification of Foxp3, Tnf, Il10, Tgfb1, Il6, Tbx21, Rorc2, and Gata3 the gene expression assays Mm00475162_m1, Mm00443258_m1, Mm00439616_m1, Mm03024053_m1, Mm00446190_m1, Mm00450960_m1, Mm01261022_m1, and Mm00484683_m1 were used, respectively. Hprt gene served as house-keeping control and was assessed using SYBR green (Invitrogen) and the following primers: forward 5’ GCT TCC TCC TCA GAC CGG TTT TTG C 3’; reverse 5’ ATC GCT AAT CAC GAC GCT GGG ACT G 3’. (71)

Statistical analysis

Results from animal experiments are presented as mean ± SEM. Normal distribution of the data was assessed by the Kolmogorov-Smirnov test with Lilliefors correction. Differences between groups were compared by either nonparametric Mann-Whitney U-test or unpaired Student’s t-test as appropriate depending on the distribution of the tested variable. Gata3 expression was correlated with the relative amount of renal calcification by computing the Pearson correlation coefficient. Differences in mortality rates were assessed by the log rank test. A value of $p< 0.05$

was considered significant. Statistical analysis was done with GraphPad Prism 6.0 for Macintosh (GraphPad Software, La Jolla, CA, USA). (71)

Results

Acute phosphate nephropathy in DBA/2 mice

Macroscopically DBA/2 mice on high phosphate diet rapidly developed a cachectic phenotype with scruffy fur, ducked body posture and markedly slower movements compared to mice fed standard chow diet (Fig. 1)

Figure 1 – Effect of high phosphate diet on DBA/2 mice.

After nine days on high phosphate diet (right side), female DBA/2 mice displayed a markedly different habitus compared to littermates on standard chow diet (left side). They were markedly cachectic with scruffy fur (upper panels), and upon necroscopy showed extensive cardial and renal calcifications, which were absent in standard chow-fed mice.

DBA/2 mice on standard chow showed no aberrant calcification (Fig. 2). On the other hand, feeding a high-phosphate diet to eight-week-old DBA/2 mice induced a remarkable phenotype of cortical nephrocalcinosis within nine days (Fig. 2). The histopathological evaluations with *von Kossa* (Fig. 2A) and Alizarin Red (Fig. 2B) stainings and Periodic acid Schiff's reaction (Fig. 2C) of the kidneys revealed a radial anatomical distribution of the renal precipitations that followed the renal tubular architecture and were most pronounced beneath the renal fascia and within the renal cortex. Immunohistochemical stainings for $CD68^+$ macrophages and $CD4^+$ T cells

revealed considerable infiltration by both cell types, which colocalized with precipitations (Fig 2D, E).(71)

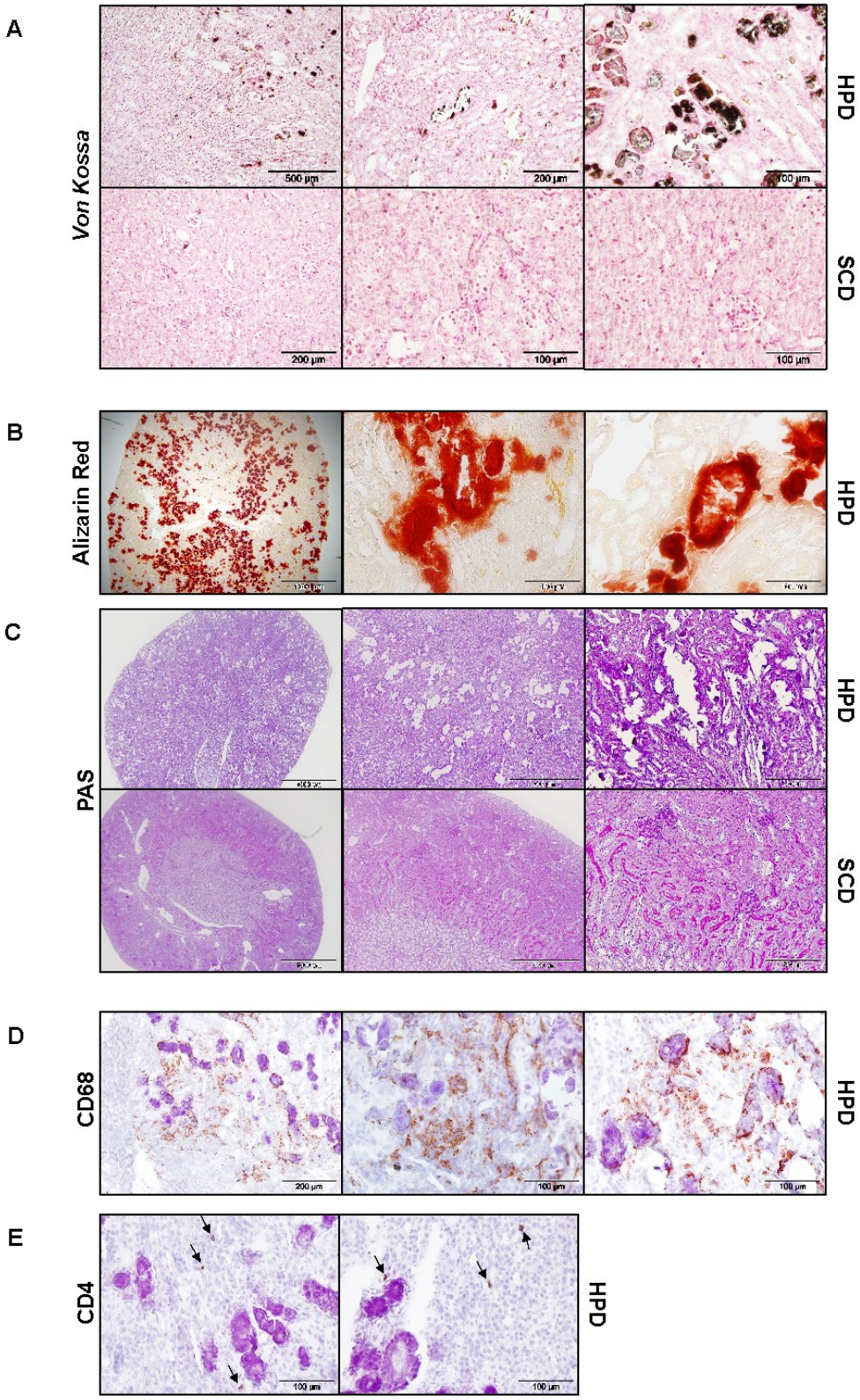

Figure 2 - ***Nephrocalcinosis in DBA/2 mice fed with high-phosphate diet.***

DBA/2 mice fed a high-phosphate diet (HPD) developed a clear phenotype of nephrocalcinosis within 9 days, whereas controls on standard chow diet (SCD)

showed no histopathological signs of renal calcifications. The histopathological evaluation with (A) *von Kossa*, (B) Alizarin Red stainings, and (C) PAS reaction revealed a radial distribution of the renal calcifications that were most pronounced beneath the renal fascia and within the renal cortex. Immunohistochemistry for (D) CD68 positive macrophages and (E) CD4 positive T cells showed colocalization of inflammatory infiltrates with renal precipitations. Magnifications: (A) 40x, 200x, 400x; (B) left column 40x, all other 400x; (C) left column 40x, middle column 100x, right column 200x; (D) left column 200x; middle and right column 400x; (E) 400x.

Micro-computed tomography confirmed the localization and distribution of renal precipitations (Fig. 3A-D). Thus, compared to previous data by our group on acute phosphate nephropathy in *Lepr*^{db} mice, DBA/2 mice on high-phosphate diet developed a similar phenotype in a considerably shorter period of time. (71,117)

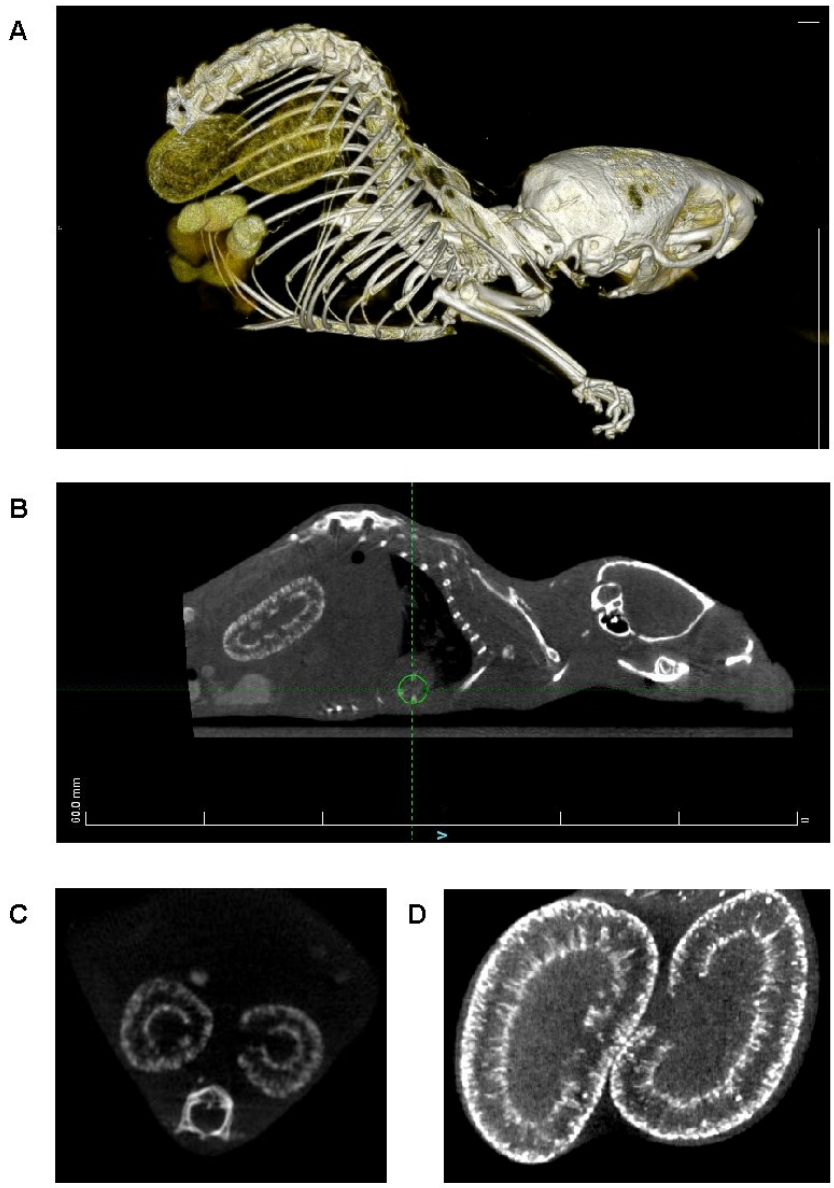

Figure 3 - ***Computer-tomographic assessment of nephrocalcinosis in DBA/2 mice*****.**

Microcomputed tomography confirmed the histopathologic assessment of nephrocalcinosis in DBA/2 mice on high-phosphate diet. (A) Whole-body imaging and three-dimensional reconstruction with false color display of aberrant calcifications (yellow) shows the prominent renal calcification. Representative (B) parasagittal and (C) coronal whole-body imaging confirms the radial distribution of renal calcifications in situ. To perform high-resolution microcomputed tomography of calcified organs, (D) both kidneys of a DBA/2 mouse on high-phosphate diet were explanted and imaged ex vivo, which confirmed the radial distribution of intrarenal calcifications within the cortex following the tubular architecture.

CD3-depletion deteriorates nephrocalcinosis

In order to investigate the role of T cells in inflammation and progression of nephrocalcinosis, we treated female DBA/2 mice with either 10 µg anti-CD3ε mAB or isotype control for 5 consecutive days. After this pre-treatment, both CD3-depleted DBA/2 mice and their respective isotype-treated controls were set on the identical high-phosphate diet for 9 days, thus excluding a possible impact of the dietary composition on the amount of nephrocalcinosis. (108,121) The mice were sacrificed after this short period and the kidneys and hearts were evaluated for precipitation of calcium hydoxyapatite crystals. Flow cytometry experiments from splenocyte single cell suspensions demonstrated the effect and duration of T cell depletion (Fig. 4A). The 2D and 3D images obtained with micro-computed tomography of the explanted kidneys allowed us to quantify the severity of the soft tissue calcification by measuring the absolute and relative amount of radiopaque tissue in single organs. Interestingly, the systemic depletion of T cells by anti-CD3ε antibody pre-treatment resulted in a significantly higher amount of renal radio-opaque calcium hydroxyapatite precipitations when compared to isotype-treated controls (44.3±7.5% vs. 61.3±5.9%; p=0.022; Fig. 4B). Semi-quantitative histopathological evaluations with Alizarin Red stainings independently confirmed these radiological measurements (Fig.4C). (71)

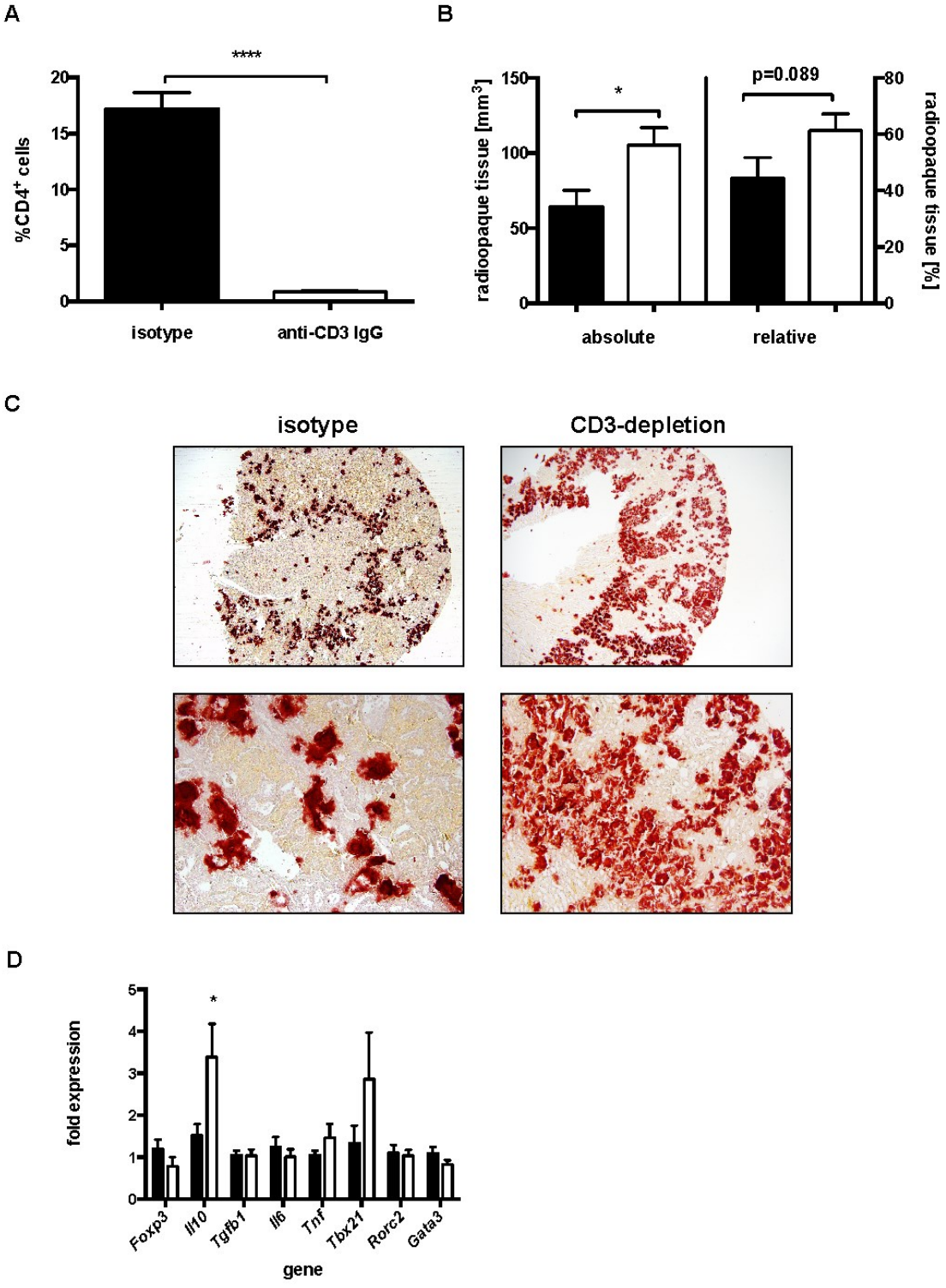

Figure 4 - ***Nephrocalcinosis in T cell-depleted DBA/2 mice.***

(A) Flow cytometry of splenic single cell suspensions were stained for CD4 in order to prove the effect and duration of T-cell depletion (n=4; white bars) when compared to controls (n=3; black bars). The percentages of CD4 positive T cells are shown as means ± SEM. (**B**) Voxel intensities of the native µ-computed tomography

images of the explanted kidneys were used to calculate the absolute and relative amount of renal calcification. Female DBA/2 mice that were depleted of T cells by anti-CD3ε pre-treatment had a significantly higher amount of calcified renal tissue when compared to isotype-treated controls (means ± SEM; n=9 (isotype group)/10 (CD3-depletion group); p=0.022). (**C**) Semiquantitative Alizarin Red stainings of kidney sections independently confirmed deterioration of nephrocalcinosis in CD3 depleted animals. Representative sections are shown. Magnifications: upper row 40x, bottom row 200x. Two independent experiments were performed. **(D)** Renal expression of mRNA in T cell depleted animals (n=10; white bars) and isotype-treated controls (n=9; black bars). * p<0.05, **** p<0.0001

Chemical analyses of renal calcifications by mass spectrometry showed significantly higher renal phosphorus contents in CD3-depleted DBA/2 mice when compared to isotype-treated controls (66.7±5.9 vs. 49.7±5.4 µg/mg dry weight; n=10 per group; p=0.048), whereas the renal calcium contents were not statistically different between the two treatment groups (105.1±11.3 vs. 73.0±10.6 µg/mg dry weight; p=0.055). Collectively, the systemic absence of T cells resulted in an increased severity of renal calcification. Therefore, we hypothesized, that the anti-inflammatory subpopulation of regulatory T cells might be involved in the progression of acute phosphate nephropathy. (71)

Regulatory T cells blunt calcium deposition in nephrocalcinosis

After having demonstrated that the mere presence of T cells was a prerequisite for controlling the acute phase of ectopic calcification, we next studied the role of anti-inflammatory regulatory T cells (Tregs) in our animal model of nephrocalcinosis. We treated the female DBA/2 mice with one intraperitoneal injection of a Treg depleting anti-CD25 antibody or an isotype control. After pre-treatment, DBA/2 mice were again set on the same high-phosphate diet for 9 days. The mice were sacrificed after this short period and the renal and cardiovascular tissues were evaluated for precipitation of calcium hydoxyapatite crystals. In order to prove the effect and duration of Treg depletion, we performed flow cytometry experiments at the end of the experiment (Fig. 5A). The depletion of Tregs resulted in a significantly higher amount of renal radio-opaque calcium hydroxyapatite precipitations as compared to isotype-treated controls (47.3±4.6% vs. 59.9±3.2%; n=15 mice per group; p=0.039; Fig. 5B). Semi-quantitative histopathological evaluations with Alizarin Red stainings

independently confirmed theses radiological measurements (Fig. 5C). Chemical analyses of renal calcifications by mass spectrometry displayed significantly higher renal phosphorus contents in CD25-depleted DBA/2 mice when compared to isotype-treated controls (72.1±4.6 vs. 57.1±3.7 µg/mg dry weight; n=5 per group; p=0.045), whereas the renal calcium contents were not statistically different between the two treatment groups (128.4±11.4 vs. 94.5±8.8 µg/mg dry weight; p=0.060). The systemic depletion of Tregs was paralleled by a significantly lower expression rate of *Foxp3* and *Gata3* in renal tissue (Fig. 5D), and by significantly higher serum Fgf23 levels (Fig. 4E). (71)

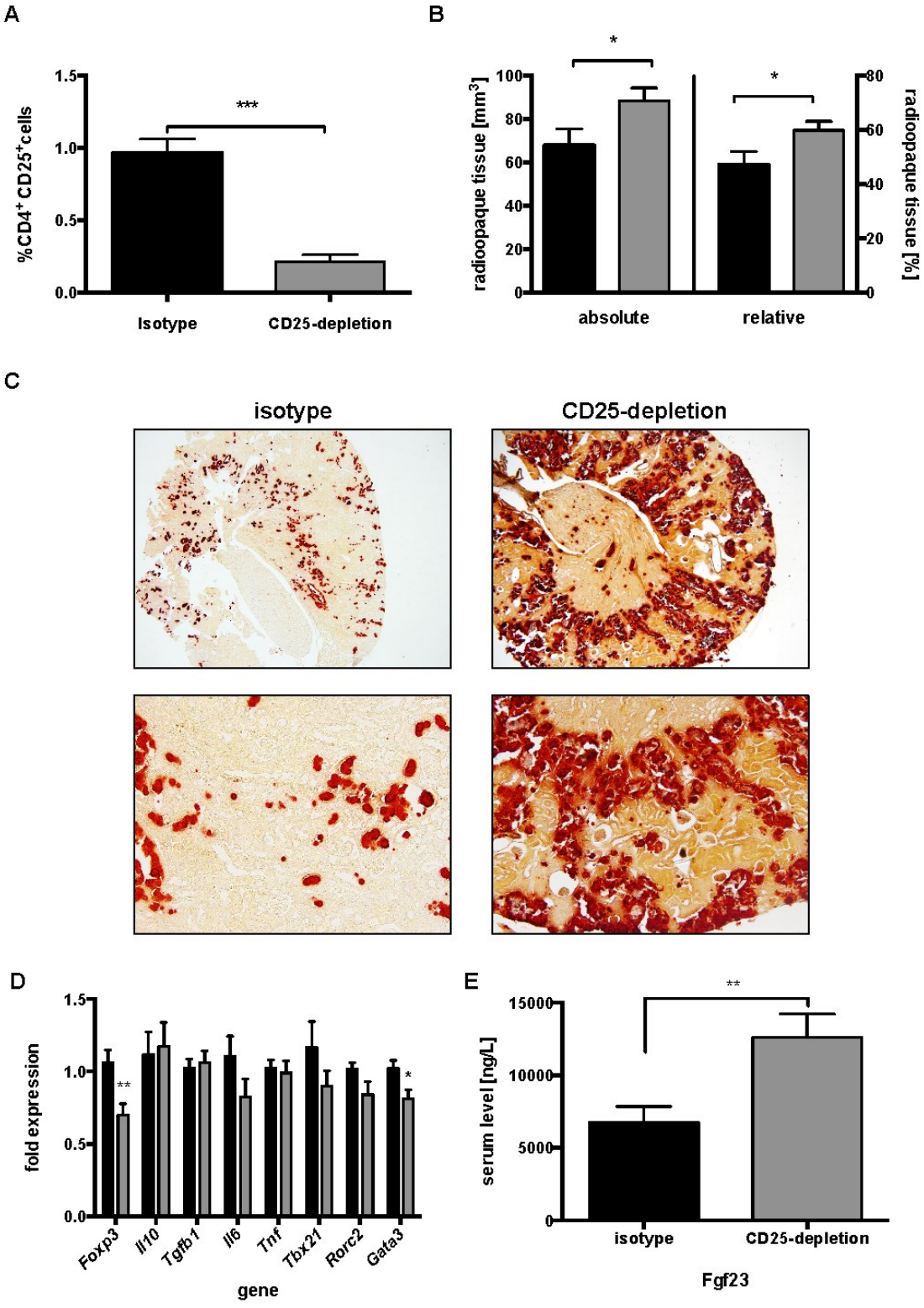

Figure 5 - ***Nephrocalcinosis in Treg depleted DBA/2 mice.***

(A) Flow cytometry of splenic single cell suspensions were stained for CD25 in order to prove the effect and duration of Treg depletion (n=9; grey bars) when compared to controls (n=9; black bars). The percentages of $CD4^+CD25^+$ T cells are shown as means ± SEM. **(B)** Voxel intensities of the native µ-computed tomography

images of the explanted kidneys were used to calculate the amount of renal calcification. Female DBA/2 mice that were depleted of Tregs had a significantly higher relative amount of calcified renal tissue when compared to isotype-treated controls (n=15 per group; p=0.039). (**C**) Semiquantitative Alizarin Red stainings of kidney sections independently confirmed deterioration of nephrocalcinosis in CD25 depleted animals. Representative sections are shown. Magnifications: upper row 40x, bottom row 200x. (**D**) These more severe renal precipitations in Treg depleted animals were associated with a significantly lower expression of *Foxp3* and *Gata3* (n=15 per group; p=0.020) and (**E**) a significant increase in serum Fgf23 levels (n=15 per group; p=0.005), respectively. Three independent experiments were performed. * p<0.05, ** p<0.01, *** p<0.001

Foxp3 is the master regulator in the development and function of Tregs, whereas Gata3 is the master regulator of TH2 differentiation. Thus, the lower renal expression of *Foxp3* in Treg-depleted animals confirms the effect and duration of Treg depletion the kidney, whereas the lower expression of *Gata3* in Treg-depleted animals indicates a shift from TH2 toward TH1 response. Furthermore, we found a significant inverse correlation of *Gata3* levels with the amount of renal mineral deposits throughout the entire sample of mice studied in our experiments (r=-0.3; p=0.037; n=49). There were no significant differences in serum parameters, such as urea, parathyroid hormone, calcium and phosphate levels between Treg depleted animals and isotype-treated controls, respectively (Table 1). (71)

	Control N=9	CD3 depletion N=10		Control n=15	CD25 depletion n=15	
Urea (mg/dL)	190±24	199±14	n.s.	265±31	292±35	n.s.
Calcium (mmol/L)	1.6±0.1	1.5±0.1	n.s.	2.9±0.2	3.3±0.3	n.s.
Phosphorus (mmol/L)	5.4±0.5	4.4±0.4	n.s.	6.3±0.6	6.7±0.8	n.s.
Ca x P ($mmol^2/L^2$)	8.1±1.4	5.8±0.9	n.s.	19.5±3.0	25.3±5.0	n.s.
Fgf23 (ng/L)	5141±806	5426±1017	n.s.	6736±1068	12617±1545	0.005
Pth (ng/L)	563±72	1015±275	n.s.	984±225	1059±162	n.s.

Table 3 - ***Serum biochemical parameters of DBA/2 mice on high phosphate diet.***

Since renal retention parameters were highly elevated after 9 days on high phosphate diet, we speculated that more sensitive parameters of renal damage might more accurately reflect the increase in renal calcification in Treg depleted mice. To this end, we determined urinary neutrophil-gelatinase associated lipocalin (Lcn2). Lcn2 is a marker for acute kidney injury, which has repeatedly been shown to sensitively reflect renal damage through a wide spectrum of clinical and experimental settings. (122,123) DBA/2 mice were partly anuric after 9 days of high phosphate diet. Therefore, we collected urine samples 3 days after putting Treg-depleted DBA/2 mice and respective isotype-treated controls on high phosphate diet (n=4 per group) and measured Lcn2/creatinine ratio. Interestingly, we found a significant higher urinary Lcn2/creatinine ratio in Treg-depleted animals when compared to isotype treated controls (9.03 ± 3.05 µg/mg vs. 1.99 ± 0.47 µg/mg; p=0.029).

Nephrocalcinosis is associated with increased mortality and dystrophic cardiac calcinosis

Next, we investigated whether the accelerated course of nephrocalcinosis after immunomodulation had any impact on the survival rate in our animal model.

Therefore, we again depleted DBA/2 mice of either T cells or Tregs and set them on high-phosphate diet ad libitum. Both, T cell and Treg depletion were not only associated with a deteriorated acute phosphate nephropathy, but also with a significantly increased mortality rate when compared to isotype treated controls ($p=0.004$) (Fig. 6A). DBA/2 mice are also prone to develop dystrophic cardiac calcinosis. Therefore, we analysed cardiovascular tissue for aberrant calcifications and performed telemetric electrocardiographic recordings. In our experimental setting, DBA/2 mice did indeed develop dystrophic cardiac calcinosis (Fig. 6B-C) that was associated with both negative chronotropic and negative dromotropic effects as assessed by telemetry (Fig. 6D). Continuous ECG telemetric readings displayed normal heart rhythm with progressive reduction of heart rate followed by a gradual prolongation of atrioventricular nodal conduction, ultimately developing into atrioventricular conduction block preceding death in the DBA/2 mice fed with high-phosphate diet. Micro-computed tomography 3D reconstructions of the explanted hearts were used to quantify the severity of the cardiac calcinosis. However – in contrast to the clear effect on nephrocalcinosis – neither the systemic depletion of T cells by anti-CD3ε pre-treatment nor the depletion of Tregs by anti-CD25 antibody significantly altered the amount of radio-opaque precipitations in hearts (Fig. 6E and 6F). (71)

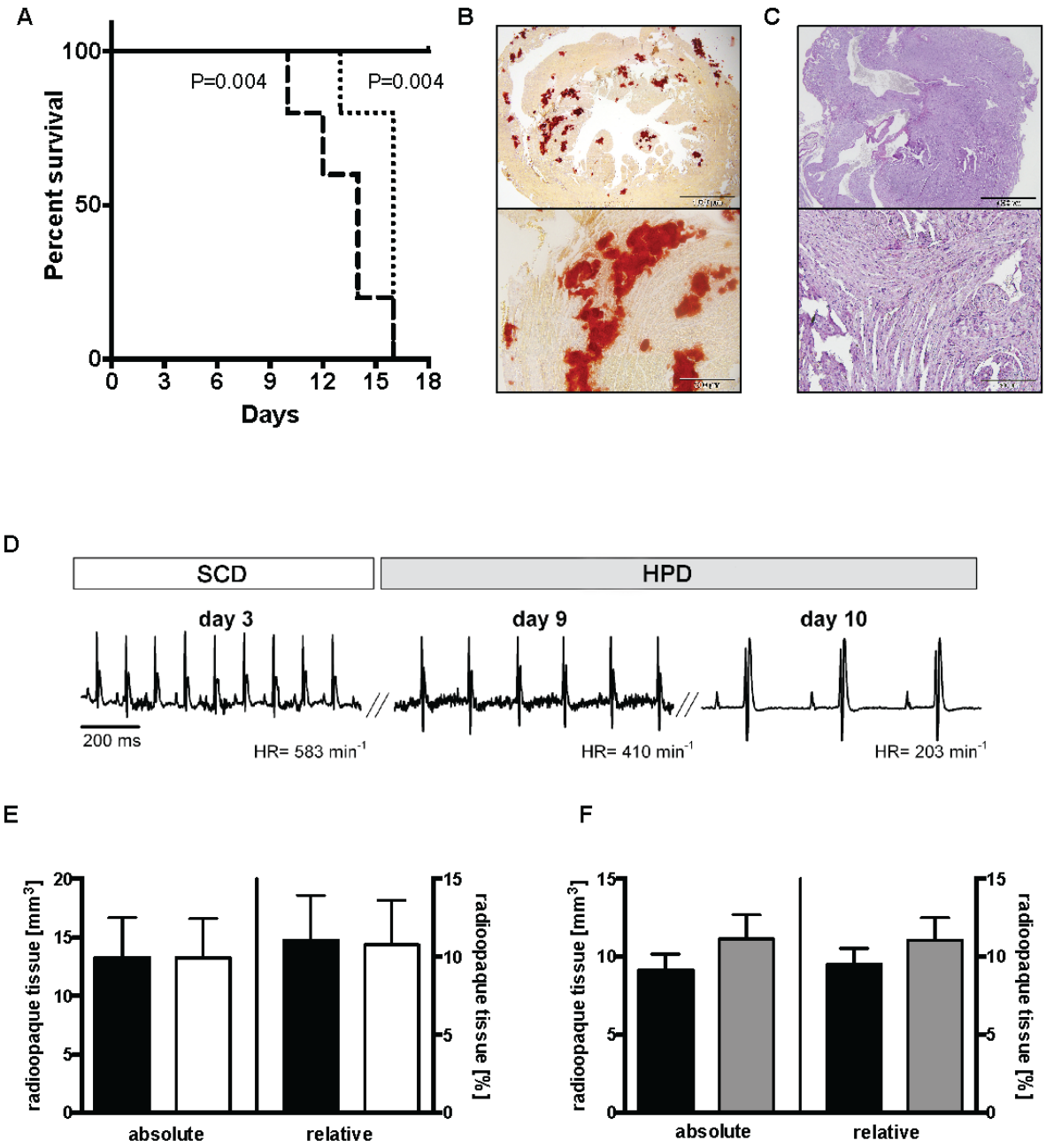

Figure 6 - ***Mortality rate and dystrophic cardiac calcinosis in DBA/2 mice.***

(**A**) Kaplan Meier survival curve of DBA/2 mice on high-phosphate diet that were either depleted of Tregs (dashed line), T cells (dotted line) or treated with isotype control (solid line) (n=5 per group; P-values versus isotype control). High-phosphate diet was also associated with dystrophic cardiac calcinosis. Cardiac calcifications were visualized by (**B**) von Kossa staining, and (**C**) PAS reaction. (**D**) ETA-F20 transmitters were implanted in anesthetised female eight-week-old dba2 mice 5 days before putting them on high-phosphate diet. Telemetric electrocardiographic

recordings were continuously recorded in freely-moving mice and revealed both negative chronotropic and negative dromotropic effects in mice suffering from dystrophic cardiac calcinosis (n=5). Representative electrocardiograms from one animal before and after putting it on high-phosphate diet are shown. (**E**) Voxel intensities of the native µ-computed tomography images of the explanted hearts were used to calculate the absolute and relative amount of cardiac calcification. Female DBA/2 mice that were depleted of T cells by anti-CD3ε pre-treatment (white bars) had a similar amount of calcified cardiac tissue when compared to isotype-treated controls (black bars) (n=10 per group). (**F**) Moreover, there was no significant difference in the amount of calcified cardiac tissue between DBA/2 mice that were treated with a Treg-depleting anti-CD25 antibody (grey bars) or an isotype-control (black bars) (n=15 per group; *p*=0.28). Magnifications: (B)&(C) upper panel 40x, bottom panel 200x.

Dicussion

Similar to other crystallopathies, acute phosphate nephropathy might be seen as passive deposition of an oversaturated ion product where inflammation is just a circumstantial phenomenon. Our data suggest that the course of acute phosphate nephropathy is actively modulated by these inflammatory repair mechanisms that lead to tissue remodelling, aberrant calcium deposition, and ultimately the loss of functioning renal parenchyma. The present findings are also in line with Mulay et al., who have recently shown acute calcium oxalate nephropathy to be attenuated by therapeutic Il1 antagonism. (104) Thus, nephrocalcinosis is not exclusively a passive precipitation of a supersaturated ion product, but an actively modulated process of tissue remodelling, where inflammation is not just an innocent bystander. Up to date, treatment of nephrocalcinosis was directed at correcting the underlying cause. Immunomodulation may nicely complement this therapeutic approach and withhold deleterious renal inflammation, tissue remodelling, loss of renal function and chronic kidney disease. Naïve CD4 positive T cells have been shown to differentiate into one of several distinct phenotypes of T helper cells (TH), such as TH1, TH2 or Treg. Each of these lineages has been shown to be regulated by distinct transcription markers, to express distinct cytokines, and to play detrimental and protective roles in various pathological processes. (124) In previous work by our group, we showed that leptin-

receptor deficient $Lepr^{Db/db}$ mice develop acute phosphate nephropathy when kept on a high phosphate containing diet for eight weeks. In the present work, we first aimed to establish a more rapid model of acute phosphate nephropathy. (71) In previous studies, using uremic DBA/2J or DBA/2NCrl mice, other investigators performed surgery to reduce functioning nephron mass before they put mice on high phosphate diet to induce ectopic calcficiation. (107,114,117,125,126) It is interesting to note, that in the present study without performing renal mass reduction surgery, calcification and onset and progression of acute phosphate nephropathy and ectopic calcification was much more rapid than in other published studies which used the same strain of mice, yet different substrains. It is unclear, what the etiology for more rapid onset of calcification and uremia in the present study was. Most previously conducted studies do not provide data on the precise substrain of DBA/2 mice used, and mostly used high phosphate diet containing 1.5% phosphorus, while the present study used 2% phosphorus diet. It is thus possible, that the Charles River substrain of the DBA/2 inbred mouse when using 2% phosphorus containing diet shows more rapid calcification than the other models used to study ectopic calcification in the DBA/2 model. (127,128)

Inflammation has been shown to play a vital role in a number of diseases, which are not classically considered to be autoimmune-mediated such as atherosclerosis. (129-132) However, to date there is very little data on the role of inflammation in ectopic calcification. (133) To test whether the concomitant inflammatory response plays any causative role in the development of nephrocalcinosis and dystrophic cardiac calcinosis, we designed the present study. After first documenting a strong influx of inflammatory cells into calcified cardiac and renal tissue we conducted T cell and Treg depletion experiments to test whether T cells control inflammation in this setting.

In our hands, T cells and particularly regulatory T cells play a central role in withholding renal inflammation in acute phosphate nephropathy and, thus, attenuate nephrocalcinosis. In the Treg-depleted animals, we observed a shift from TH2 toward TH1 response reflected by a significantly decreased renal mRNA expression of the master regulator of TH2 differentiation *Gata3*. (134) Furthermore, we found a significant inverse correlation of *Gata3* transcription levels with the degree of renal

calcification, which further corroborates our hypothesis that the TH2 lineage may play a role in protecting against aberrant calcification.(71)

Treg depletion led not only to increased renal calcification as shown by the higher calcium score of the kidneys in micro-computed tomography but also to a significant increase in serum Fgf23 levels. In this context these higher Fgf23 levels are most likely a reflection of the increased renal damage in Treg-depleted animals. Most importantly, this accelerated course of acute phosphate nephropathy after Treg depletion was also associated with a significantly increased mortality rate. Thus, the present data reconfirm previous clinical studies showing that elevated circulating Fgf23 levels were associated with increased risk for end-stage renal disease and death in patients with chronic kidney disease. (135,136) In patients with chronic kidney disease, there was also a cross sectional association of Fgf23 with left ventricular dysfunction, (137,138) vascular dysfunction (139) and higher levels of inflammatory markers. (53) In our hands, the continuous telemetric ECG recordings in DBA/2 mice suffering from nephrocalcinosis and dystrophic cardiac calcinosis revealed progressive negative chronotropic and negative dromotropic changes that ultimately led to cardiac arrest and death. However, the present experimental design did not allow to discriminate whether Fgf23 serum levels mediated these electrophysiological effects. (71)

Treg depletion had a clear impact on the amount of nephrocalcinosis, but did not increase cardiac calcificion in our experimental setting. Treg have been shown to ameliorate angiotensin II-induced cardiac damage, (140,141) but cannot withhold cardiac remodelling in dystrophic cardiac calcinosis. The differential regulation of aberrant calcification in different organs may reflect the influence of strain-specific modifier genes on the complex pathologic process of mineralization. (111,120,142) Thus, the present data derived from a single mouse strain cannot be easily extrapolated to other mouse strains and warrant further investigations. (71)

Taken together, the present study not only corroborates evidence that tissue remodelling in nephrocalcinosis is accompanied by inflammation, (104,117) but also provides first evidence that the degree of intrarenal aberrant calcium deposition is dependent on this inflammatory response. Moreover, our data suggest a pivotal role of T cells and particularly Tregs in the progression of nephrocalcinosis, and

emphasize the fact that intrarenal inflammation deteriorates the outcome in acute phosphate nephropathy. (71)

Part 2 – T cell-derived IL-9 is proinflammatory in nephrotoxic serum nephritis

Introduction

T helper (TH) cells were long thought to be a family consisting of TH1 and TH2 cells, characterized by specific transcription factors T-bet and GATA3 and secretion of IFNγ or interleukin 4 (IL-4) and IL-10, respectively. This model has been complemented with other TH populations, especially the diametrically opposing TH17 and regulatory T cells (Tregs). TH17 cells exert proinflammatory effects in diseases that were long considered to be TH1-dependent, such as inflammatory bowel disease. In contrast, Tregs exert anti-inflammatory and tolerogenic activity in cancer, infection and autoimmunity.

The understanding of the contribution of various T cell populations to the pathogenesis of glomerulonephritis have been refined significantly by a large number of studies of murine nephrotoxic nephritis. According to current data, the kinetics of T cell recruitment to the kidney and secondary lymphatic organs are finely orchestrated and decisively influence the degree of renal disease. The initial inflammatory trigger – in the case of nephrotoxic nephritis, the deposition of heterologous antibodies on the glomerular basement membrane – recruit a number of innate immune cells to the kidney. Neutrophil granulocytes are the earliest mediators of renal damage after antibody deposition, but their maintained influx crucially depends on the production of IL-17A by renal γδ T cells. (143) $CD3^+CD4^-CD8^-NK1.1^-$ T cells, γδ T cells, as well as TH17 cells have all been documented to secrete IL-17A, yet in the early phases of disease, γδ T cells are the most prominent producers of this neutrophil recruiting cytokine. (143) Mice deficient in γδ T cells or in whom γδ T cells are selectively IL-17A deficient show significantly decreased levels of renal neutrophils and nephritis severity. (143)

Mast cells are also prominent among recruited cells in the early phases of immune response to a glomerular antigen. (144-146) Different mast-cell deficient mouse strains have been used to study the role of these cells in renal inflammation, however these studies have yielded partially conflicting results by providing both evidence of detrimental(144) as well as protective effects (146,147) of mast cells. Judging from a number of studies on the roles of mast cells in other autoimmune-mediated disease models, such as experimental autoimmune encephalitis (148) or

experimental arthritis, (149) the most likely explanation of mast cell function in nephritis implies mast cell support of Treg function. (18,148,150) However, the currently available studies on mast cells in renal inflammation are similar in that the investigators used mouse models of mast cell deficiency which are based on the disruption of the receptor tyrosine kinase kit. Numerous studies have shown that the Kit-dependent mast cell deficiency models show a number of other aberrancies, such as aberrant myelopoiesis, (151) and one study prominently documented that some of the roles attributed to mast cell could not be reproduced in Kit-independent models of mast cell deficiency. (150)

The first wave of T cell infiltration is dependent on the activation of immature renal dendritic cells, which secrete large amounts CXCL16.(152) Its cognate receptor, CXCR6, is highly expressed on renal invariant NKT cells, which have been shown to act immunoregulatory in the first week of murine crescentic GN, as CXCR6-deficient mice, show significantly decreased recruitment of iNKT cells and severely more pronounced renal disease. (152) At this early point, if the immunoregulatory balance tips towards a proinflammatory milieu, large amounts of CCL20 recruit pathogenic, CCR6-expressing TH17 cells to the kidney. (153) These cells are dependent on the activity of RORγt (154), and – by means of CXCL1 dependent recruitment of $CXCR2^+$ neutrophils – are early mediators of renal injury in murine, crescentic glomerulonephritis. After this initial phase of TH17-dependent cell recruitment, increased levels of CXCL9 attract $CXCR3^+$ IFNγ-producing TH1 cells. (155,156) Similar to any other adaptive immune response, targeted responses by T cells against specific renal epitopes are dependent upon priming by T cells. Activated dendritic cells upregulate CCR7, which enables them to translocate to the secondary lymphoid organs where they present previously encountered renal antigens to T cells. Some groups also suggest that the main location of dendritic cell-mediated T cell activation in murine nephrotoxic serum nephritis is the periglomerular space, since renal dendritic cells mainly localize to this are during nephritis. (157) In the setting of upregulated co-stimulatory molecules and secretion of cytokines such as IL-12 and IFNγ, T cells are then activated and subsequently attract increased numbers of monocytes to the kidney. Different subsets of these infiltrating $CD11b^+$ monocytes thereafter differentiate into other proinflammatory cell types such as TNFa-, IL-6-, and nitric oxide-secreting macrophages or profibrotic fibrocytes. (158-160)

In parallel to the overwhelming, pathogenic and detrimental T cell activation in crescentic glomerulonephritis, a compensatory activation of regulatory T cells takes place. (106) In the first work to document a protective role of CD4+CD25+Foxp3+ regulatory T cells in glomerulonephritis, our group showed that the adoptive transfer of magnetically sorted regulatory Tregs significantly alleviated the course of murine nephrotoxic serum nephritis. (106) Here, Tregs were shown to significantly decrease disease indices by impacting upon the infiltration of leukocytes in the kidney and by decreasing TH1-associated cytokines such as IFNγ. Interestingly, there was no difference to in the B cell response in mice that had received Tregs as compared to that were injected with control cells. (106) Others have confirmed this initial description of the role of regulatory T cells in glomerulonephritis by showing that the ablation of endogenous regulatory T cells significantly deteriorates the course of nephritis. (161,162) Also, the systemic depletion of Tregs at different time points significantly increased the systemic TH1, as shown by higher levels of IFNg in splenocyte restimulation assays. (161) To date, the precise time course and location of the Treg response in inflammatory renal disease remain a matter of debate. Adoptively transferred Foxp3^{EGFP+} Tregs were initially shown to preferably migrate to the spleen and draining renal lymph nodes, i.e. the secondary lymphoid organs and not to the kidney. (106) Subsequently, it was shown that the targeted migration of regulatory T cells to the draining lymph nodes is of paramount importance for their immunosuppressive effect. (163) CCR7-deficient mice are significantly more susceptible to nephrotoxic serum nephritis and even display increased mortality compared to CCR7-competent animals. The chemokine receptor CCR7 and its ligands, the two homeostatic chemokines CCL19 and CCL21 are crucial for the correct homing of a large variety of leukocyte types – including Tregs and dendritic cells – to lymph nodes. Worse renal disease in the setting of CCR7-deficiency has been traced back to erroneous migration of Foxp3+ regulatory T cells to the kidney, which can be mitigated by adaptive transfer of CCR7+ regulatory T cells. (163) In this study, CCR7-deficient regulatory T cells showed equal in vitro suppressive capacity to wild type Tregs, but were unable to migrate to lymphoid tissue and therefore infiltrated the kidney at an earlier point of time and to a higher extent as compared with CCR7-competent cells. (163) An earlier study, however, has shown that the suppressive effect of Tregs in experimental nephritis, also depends to a large extent on the expression of CCR6. During the course of nephritis, the sole known ligand of

CCR6 – CCL20 – is strongly upregulated and guides Tregs as well as TH17 cells to the kidney. Considering data on the strong proinflammatory role of TH17 cells in the model, the fact that mice with CCR6-deficiency display significantly worse nephritis during the heterologous phase of nephrotoxic serum nephritis was surprising. (153) The lack of CCR6-signaling on regulatory T cells significantly reduced the numbers of renal Tregs, which was reversible by the administration of CCR6-competent Tregs.

Thus, many facets of the contribution of TH1, TH2, as well as Treg cells have been elucidated. TH9 cells represent a fairly recent addition to the T helper cell family. (164-166) IL-9 – initially described as a T and mast cell growth factor – has long been known to be secreted by T lymphocytes. (167-170) Since TH2, TH17 and Treg have been shown to secrete IL-9, (17,171-173) the *in vivo* existence of a distinct IL-9-secreting TH subset was a matter of debate. However, recent studies have corroborated the existence and *in vivo* relevance of TH9 cells. (167-170,174-178) Hence, TH9 cells are understood to develop from naïve CD4+ T cells and TH2 cells under the influence of TGFβ and IL-4, with their gene expression profile known to be dependent on STAT6, GATA3, IRF4, Batf and Pu.1. (17,157,171-173,179,180) Pu.1 is induced during the incubation of naïve T cells with TGFβ and directly controls IL-9 transcription. In addition, the epigenetic profile of TH9 polarized cells has been shown to be mediated by Pu.1. (180) Recent data demonstrated that TH9 cells contribute to anti-tumor immunity (175,177,180), allergy and anaphylaxis (179-181), and autoimmunity. (182-184) However, the pleiotropic effects of IL-9 are not limited to T cells and mast cells, since the IL-9 receptor is similarly expressed on other cells, including epithelial, hematopoietic progenitor, and smooth muscle cells. (185) Studies on the role of IL-9-secreting cells in autoimmune disease have largely shown a pro-inflammatory role. (186) Nonetheless, partially conflicting data exist: In experimental autoimmune encephalomyelitis, IL-9 has been shown to act both pro-inflammatory by activating mast cells and TH17 cells, (167,168,170) and regulatory by promoting Treg function. (171) Our group has previously studied the involvement of IL-9 in the interaction between mast cells and regulatory T cells in our setting. As discussed above, mast cell deficient animals were previously shown to be more susceptible to experimental nephritis, (147) while the adoptive transfer of regulatory T cells was able to abrogate disease progression. (106) Initial evidence of an involvement of mast cells into the effect of Tregs in this setting came from the observation that Treg transfer also increased the numbers of mast cells in the draining lymph nodes in the

model of nephrotoxic serum nephritis. (187) Further studies showed that mast cell deficiency rendered the effect adoptive Treg transfer inert, since the transfer of CD4+CD25+ cells into Kit^{W}/Kit^{W-v} mast cell-deficient mice did not show the suppressive effect on autoimmune mediated kidney damage observed in $Kit^{+/++}$ mast-cell competent mice. Thus, the presence of mast cells is a prerequisite for the immunosuppressive effect of Tregs, since the in vitro suppressive potential of mast cells isolated from mast cell deficient and competent mice did not differ. (187) At this point, the interaction of mast cells and Tregs to achieve regulatory effects had been described in a model of chronic skin graft rejection and had been linked to the secretion of IL-9 by Tregs.(17) On the basis of this observation, further experiments proved that the secretion of IL-9 by regulatory T cells is instrumental in attracting mast cells to the draining lymph nodes, where they can then suppress disease: Both, the concomitant administration of an IL-9 blocking antibody and Tregs as well as IL-9-deficient Tregs rendered the abolished the effect of the adoptive Treg transfer seen in the wild type setting. (163,187)

While the contribution of TH1, TH17 and Treg cells to the pathogenesis of the T cell-dependent NTS is well-established, (157) the relevance of TH9 cells in inflammatory kidney diseases is unknown so far. In the present study, we examined the contribution of IL-9 producing T cells to immune-mediated glomerular disease.

Methods

Induction of accelerated nephrotoxic serum nephritis (NTS)

C57BL/6 were obtained from Charles River (Sulzfeld, Germany), while IL-9-KO mice were kindly provided by Andrew NJ McKenzie (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom) and CD45.1-transgenic mice on C57BL/6 background (B6.SJL-Ptprca Pepcb/BoyJ) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Eight to 10 week-old male animals were used in all studies. Accelerated NTS was induced as described previously. (188) In brief, mice were pre-immunized subcutaneously with 100µL of 2 mg/mL rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) dissolved in incomplete Freund's adjuvant (Sigma, St. Louis, MI, USA). After 3 days, heat-inactivated rabbit anti-mouse glomerular basement membrane antiserum was injected in the tail vein. All animal experiments were authorized and performed in accordance with Austrian law (BMWF-66.011/0105-II/3b/2011).

Detection of urinary albumin and creatinine

Urinary albumin was determined using a sandwich ELISA (Bethyl Laboratories, Montgomery, TX, USA) using a well established protocol.(189) Briefly, a high-affinity 96 well plate (Thermo Scientific, Waltham, MA, USA) was coated with 1µg/mL goat anti-mouse albumin antibody in sodium bicarbonate coating buffer [1.59g Na_2CO_3, 2.93g $NaHCO_3$ in 1L double distilled water] and left to adhere overnight at 4°C. After three-fold washing using PBS-Tween [16g NaCl, 0.4g KH_2PO_4, 2.88g Na_2HPO_4*2 H_2O, 0.4g KCl, 1 mL Tween 20 (Sigma) in 2L distilled water with pH adjusted to 7.4] In a next step unspecific binding sites were eliminated using 0.5% bovine serum albumin (BSA, SERVA Electrophoresis GmBH, Heidelberg, Germany) in PBS-Tween solution. Thereafter, the sample (mouse urine, serial dilutions ranging from 1:100 to 1:100000 or 1:1000000 on day 7 or 1:10000000 on day 14 respectively) and standard (mouse albumin reference serum, 1 µg/mL, 0.1 µg/mL, 0.03 µg/mL, 0.01 µg/mL, 0.001µg/mL} dilutions are applied to the coated wells and allowed to adhere for 2h at room temperature. After repeated washing, a secondary horseradish-conjugated anti-mouse albumin antibody (Bethyl) was applied to the plate at a concentration of 0.02 µg/mL and again left to incubate for 2h at room temperature. Thereafter substrate solution (2 mg 3.3'5.5'-Tetramethylbenzidine [Sigma] in 100 µL

dimethylsulfoxide [Sigma] in 9.9 mL sodium-acetate buffer [0.82 g $C_2H_3NaO_2$ in 100 mL double distilled water with 1.05 g citric acid in 50 mL double distilled water adjusted to pH 4.9]) is applied to the washed plate. The reaction is then stopped 2 M H_2SO_4. Urinary creatinine was quantitated spectrophotometrically using a picric acid-based assay (Sigma) based on the Jaffé reaction and calculated according to the equation.

$$\text{Creatinine (mg/dL)}=(OD_{Sample\_initial}-OD_{Sample\_final})/(OD_{Standard\_initial}-OD_{Standard\_final})*0.3$$

IL-9 levels in supernatants of TH9-polarized cells were determined using ELISA (eBioscience, San Diego, CA, USA)

Histological and immunohistochemical assessment of renal pathology

Formalin-fixed paraffin-embedded tussue was cut in 4µm sections. Renal sections were then stained with periodic acid and Schiff's reagent (PAS). In all cases a minimum of 50 equatorial glomerular cross sections was evaluated as described previously. (106)

A three-layered immunoperoxidase staining technique was applied to detect macrophages and T cells in the kidney. T cells and macrophages were stained with rat anti-mouse mAB (clones YTS191.1 [CD4], KT15 [CD8], A3-1 [F4/80], FA11 [CD68]; both from Serotec, Oxford, UK). A semi-quantitative scoring system was used for infiltrating F4/80+ and CD68+ cells: 0 = 0 to 4 positive cells, 1+ = 5 to 10 positive cells, 2+ = 11 to 50 positive cells, 3+ = 51 to 200 positive cells, and 4+ = more than 200 positive cells per low-power field. For the detection of CD4+ and CD8+ T cells, we used rat anti-mouse mAbs (clone YTS191.1 and clone KT15, both from Serotec). T cells were quantified by counting the number of positive cells in 6 adjacent high-power fields of renal cortex and medulla. All samples were blinded before evaluation.

Detection of circulating mouse anti-rabbit IgG

Circulating anti-rabbit IgG was assessed using a semi-quantitiative ELISA: 96 well plates (Greiner, Kremsmuenster, Austria) were coated with 100 µg/mL rabbit IgG (Jackson ImmunoResearch Laboratories Inc.) in carbonate/bicarbonate buffer (pH 9.5). After blocking with BSA, plates were incubated with serial doubling dilutions of

mouse serum. Bound mouse IgG was detected using HRP conjugated goat anti-mouse IgG (Dako, Glostrup, Denmark).

Reverse transcription (RT) quantitative polymerase chain reaction (PCR)

Total RNA was isolated using TRIzol (Sigma) according to a standard protocol. Thereafter, 2µg of total RNA were reverse transcribed using Superscript III Transcription kit (Invitrogen, Carlsbad, CA, USA) and random primers (Roche, Basel, Switzerland). Real-time PCR was performed on a CFX96 real-time system (BioRad, Hercules, CA, USA). For linear amplification of β-actin and HPRT (reference genes) iSYBR Master mix (BioRad) was used. For quantification of T-bet, IFNγ, TNFα, GATA3, IL-10, IL-17, RORγt, Foxp3, Pu.1, CCL17, CCL22, and CCR4 Taq Man mastermix (Life Technologies, Grand Island, NY, USA) and respective Taq Man probes were used (See Table for all primers and Taq Man probes used).

In vitro differentiation of TH9 cells

In vitro differentiation of murine TH9 cells was performed as described before. (179) Shortly, we isolated naïve T cells from spleens of healthy mice using a CD4+CD62+ MACS isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany). Cells were stimulated with 3 µg/mL plate-bound anti-CD3 (clone 145-2C11, BD Biosciences, San Diego, CA, USA) and 5 µg/mL anti-CD28 (clone 37.51, Biolegend, San Diego, CA, USA) in RPMI1640 with 10% FCS and 50 IU/mL penicillin/streptomycin and 50 µM 2-mercaptoethanol (all Life Technologies). Cells were TH9-polarized in the presence of 20 ng/mL mouse IL-4, 100 U/mL mouse IL-6, 1 ng/mL TGFβ (all Immunotools, Friesoythe, Germany) and 20 µg/mL anti-IFNγ (clone XMG1.2, eBioscience). After polarization, dead cells were removed using a dead cell removal kit (Miltenyi Biotech) and $3x10^5$ intensively washed TH9 cells were injected intravenously into mice the day before antiserum administration.

Flow Cytometry

TH9 cells were evaluated in lymph nodes and kidneys of C57BL/6J mice by using an in vivo Brefeldin assay as described before. (190) Shortly, mice received 0.25 mg Brefeldin A (Sigma) intraperitoneally. After 6 hours, mice were sacrificed and organs were harvested. Single cell suspensions from lymph nodes and kidneys were obtained. After staining for CD4+ cells, cells were incubated with Cytofix/Cytoperm (BD Biosciences) for 20 min at 4°C. Intracellular IL-4 and IL-9 were stained using respective antibodies (clones 11B1 and MH9D1, both eBioscience).

In vitro differentiated TH9 cells were analyzed for their intracellular IL-4 and IL-9 content after a 12-hour stimulation with CD3/CD28 (BD Bioscience) using a similar staining protocol.

Kidney, liver, lymph node and spleen single cells suspensions were stained for CD45.1 using a fluorescently labeled antibody (clone A20, eBioscience). Data collection and analysis was done on a FACS Calibur using CellQuest and FlowJo software (all BD Biosciences).

Statistics

Normal distribution of data was assessed using the Kolmogorov-Smirnov test. In the case of two groups, normally distributed data was compared using Student's t test, while non-normally distributed data was analyzed using the non-parametric Mann-Whitney-U Tests. Three groups were compared using one-way ANOVA. When significant differences were detected, post hoc testing was done using Dunett's multiple comparison test where the IL-9-KO group was tested against the two other groups. In all cases, a two tailed $p<0.05$ was considered significant after adjustment for multiple testing. All analyses were done using GraphPad Prism 6.0b (GraphPad Software, LaJolla, CA, USA).

Results

TH9 cells are scarcely present in the healthy kidney

As a first step, we sought to localize TH9 cells in steady state conditions in healthy mice without NTS. To that end, we quantified TH9 cells in the lymph nodes and kidneys of healthy mice using co-staining of CD4 and intracellular IL-9 by flow cytometry, as well as gene expression analysis for Pu.1 – a crucial regulator for the TH9 phenotype, which is expressed at much higher levels in TH9 cells compared to TH1, TH2, and TH17 cells and upon activation potently induces IL-9, CCL17 and CCL22 expression. (180) We found that prior to NTS induction, TH9 cells are only scarcely detectable in renal tissue: in contrast to the lymph nodes only very few CD4+IL-9+ T cells could be seen in the kidney (Figure 7A). In parallel, there was a significantly higher expression level of Pu.1 mRNA in the lymph nodes compared to the kidney (Figure 7B).

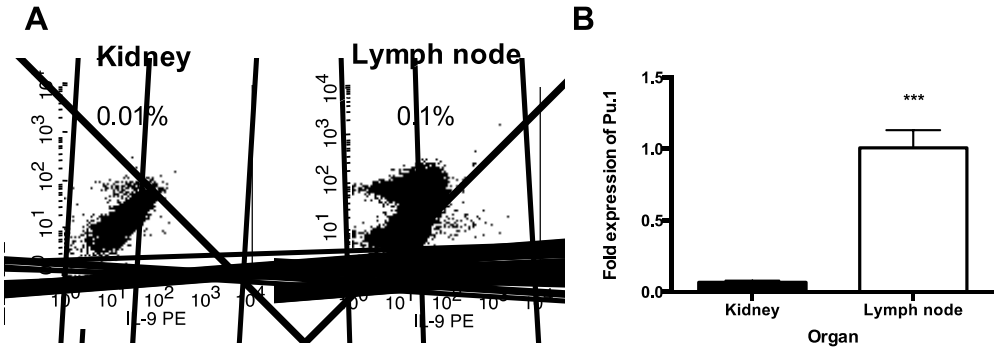

Figure 7 - ***In healthy mice, TH9 cell localize predominantly to the secondary lymphoid organs.***

(A) Intracellular staining for Il9 was performed in leukocytes isolated from kidneys and lymph nodes. (B) Relative mRNA expression analysis for Pu.1 from kidney and lymph node homogenates. n=3 per group; ***p<0.001

Renal TH9 cells increase during NTS

After determining that TH9 cells are hardly present in healthy renal tissue, we studied the TH9 cell distribution after NTS induction. Pu.1 gene expression was

found to be up-regulated in the kidney, starting on day 7 with an even higher increase on day 14 after disease induction (Figure 8A). Interestingly, we did not find a significant increase in Pu.1 mRNA expression 7 and 14 days after induction in lymph nodes or spleens (Figure 2A). Accordingly, gene expression levels of the Pu.1-dependent chemokines CCL17 and CCL22 (180) were also increased in the kidney (Figures 8B and C), whereas they decreased in secondary lymphoid organs. In addition, the main chemokine receptor for CCL17 and CCL22 – CCR4 (182,184) – was found to show a strikingly similar regulation pattern to its binding partners (Figure 8D).

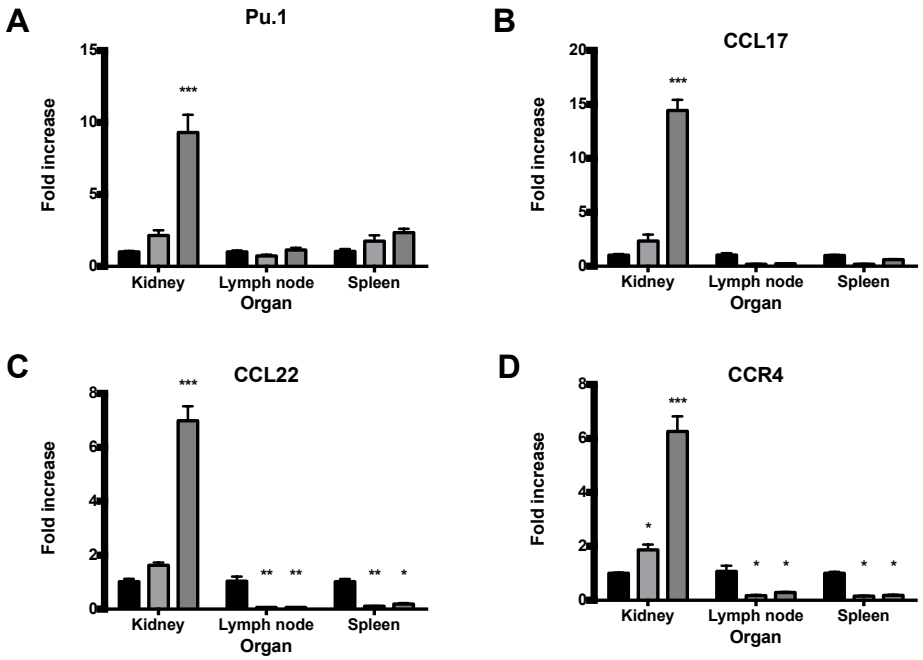

Figure 8 - ***TH9 cells localize to the lymph nodes during the course of nephrotoxic serum nephritis.***

mRNA expression levels of (**A**) Pu.1, (**B**) CCL17, (**C**) CCL22, and (D) CCR4 were evaluated in kidney, lymph nodes and spleen of mice on days 0 (black bar), 7 (light grey bar), and 14 (dark grey bar). n=3 per group; adjusted p-value *<0.05, **<0.01, ***<0.001

IL-9-deficient animals are protected from NTS

In order to further elucidate the involvement of IL-9 in glomerulonephritis, we induced NTS in IL-9 knock-out (IL-9-KO) and wildtype (WT) mice. Seven and 14 days after induction, IL-9-KO mice displayed significantly less albuminuria when compared to WT controls (Figure 9A). Accordingly, glomerulosclerosis quantified by PAS scoring was significantly less pronounced in the IL-9-KO animals (Figures 9B). While WT mice showed mild to moderate hypercellularity, occasional crescent formation as well as significant depositions of PAS-positive material in the glomeruli (Figure 9C), these changes were significantly less pronounced in IL-9-KO animals (Figure 9D). The autologous phase of NTS is characterized by a significant influx of CD4+ and CD8+ T cells as well as macrophages into the kidney. In line with a less pronounced nephritic phenotype in IL-9-KO mice, we found markedly decreased numbers of CD4+ and CD8+ T cells as well as F4/80+ and CD68+ macrophages in the kidneys of these animals compared to WT controls (Figures 9E and 9F). To study the effect of IL-9-deficiency on the infiltration by T cell subsets, we evaluated the mRNA expression of various cytokines and transcription factors. Interestingly, IL-9-KO displayed decreased gene expression levels of the TH1-associated genes T-bet, IFNγ, and TNFα (Figure 3G) the TH17-associated cytokine IL-17a (Figure 9H), as well as the TH9-associated transcription factor Pu.1 (Figure 9I). Furthermore, the two TH9-associated chemokines CCL17 and CCL22 showed significantly lower gene expression in the kidneys of IL-9-KO animals when compared to those of WT animals, as did their cognate receptor CCR4 (Figure 9I).

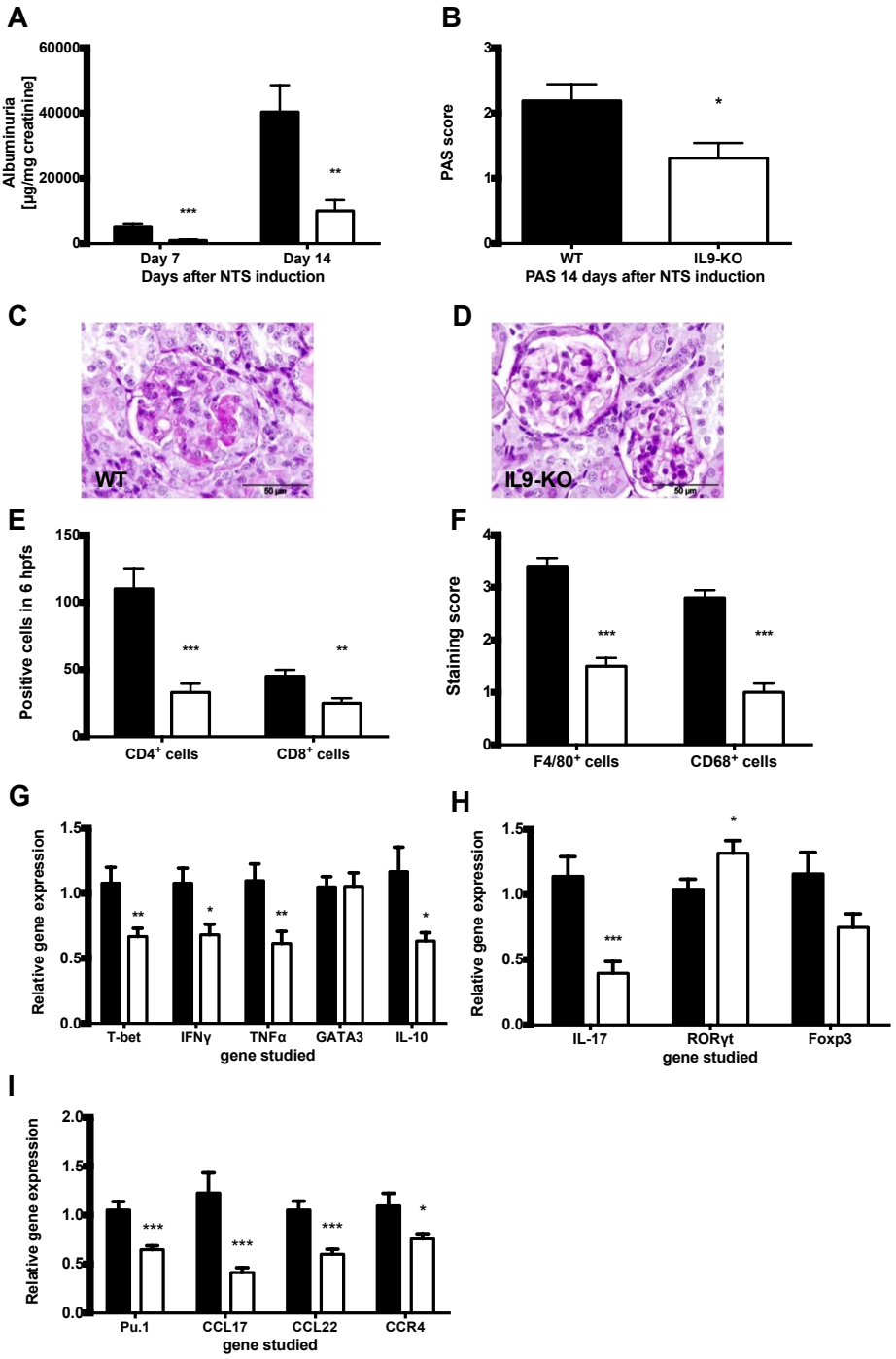

Figure 9 - *IL-9-KO mice are protected from nephrotoxic serum nephritis.*

(**A**) Urinary albumin excretion, (**B,C,D**) glomerular PAS score, inflammatory cell infiltration by (**E**) $CD4^+$ and $CD8^+$ T cells and (**F**) macrophages was significantly attenuated in IL-9-KO (white bars; n=14) mice compared to WT (black bars; n=17) mice. Renal mRNA expression analyses of (**G**) TH1-, (**H**) TH17 and Treg, and (**I**) TH9-associated genes showed a significantly less-pronounced up-regulation of several proinflammatory genes. *p<0.05, **p<0.01, ***p<0.001;

Of note, IL-9 deficiency did not alter the B cell response of the NTS model, which was reflected by comparable levels of anti-rabbit IgG in IL-9-KO and WT animals (Figure 10).

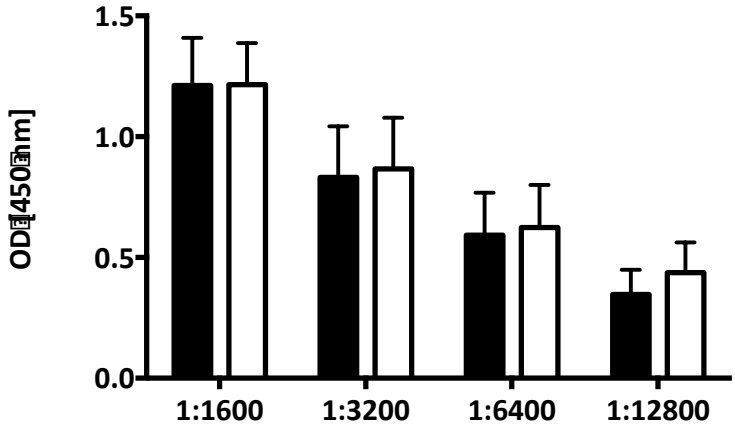

Figure 10 ***IL-9-KO mice and WT mice show comparable levels of circulating serum anti-rabbit IgG.***

Representative results of anti-Rabbit IgG ELISA from IL-9-KO mice (white bars; n=5) and WT mice (black bars; n=5), after 14 days of nephrotoxic serum nephritis. The x-axis denotes serial serum dilutions.

T-cell derived IL-9 is crucial for NTS pathogenesis

Since it is known that IL-9 is not only expressed by TH9 cells, but also by Tregs and TH17 cells, which have both repeatedly been shown to be involved in the pathogenesis of NTS (106,191), we next investigated whether TH9-derived IL-9 indeed promotes NTS. Therefore, we in vitro differentiated TH9 cells from naïve T cells to reconstitute IL-9-KO animals with these cells and subsequently subjected the animals to NTS. In vitro polarized cells produced and secreted high amounts of IL-9, as detected by ELISA and flow cytometry (Figure 11).

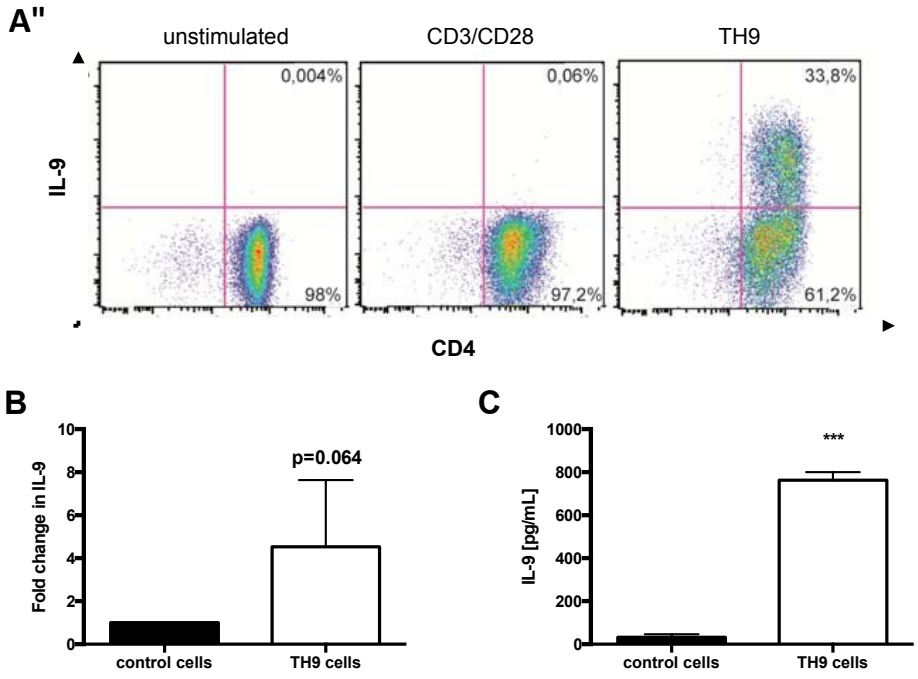

Figure 11 - ***In vitro polarized TH9 cells show distinct features of the TH9 phenotype.***

(A) TH9 cell were polarized in vitro and analyzed for their intracellular Il9 content by means of flow cytometry. The isotype control as well as stainings for IL-9 and IL-4 are shown. (B) mRNA expression analysis for IL-9 was performed in in vitro polarized Th9 cell as well as stimulated, unpolarized naive T cells. (C) IL-9 concentrations in supernatants were determined for TH9 polarized as well as unpolarized cells. ***p<0.001

Transfer of TH9 polarized cells into IL-9-deficient recipients largely restored the WT phenotype. TH9-recipients showed significantly higher albuminuria and glomerular PAS score when compared to IL-9-KO mice (Figure 12A and B). Importantly, TH9 cell transfer reestablished infiltration by CD4+ and CD8+ T cells as well as CD68+ macrophages to the levels seen in WT mice subjected to NTS (Figure 12C-G). The profoundly proinflammatory effect of TH9 cells in NTS was also supported by gene expression studies performed with the kidneys of IL-9-KO animals treated with in vitro polarized TH9 cells (Figure 12H): The gene expression levels of TH1-, TH17-, and TH9-associated cytokines, chemokines and regulators were

restored to levels seen in WT animals. Importantly, the expression levels of the chemokines CCL17 and CCL22 and their cognate receptor CCR4 were also elevated in TH9 recipients when compared to IL-9-KO animals without cell transfer.

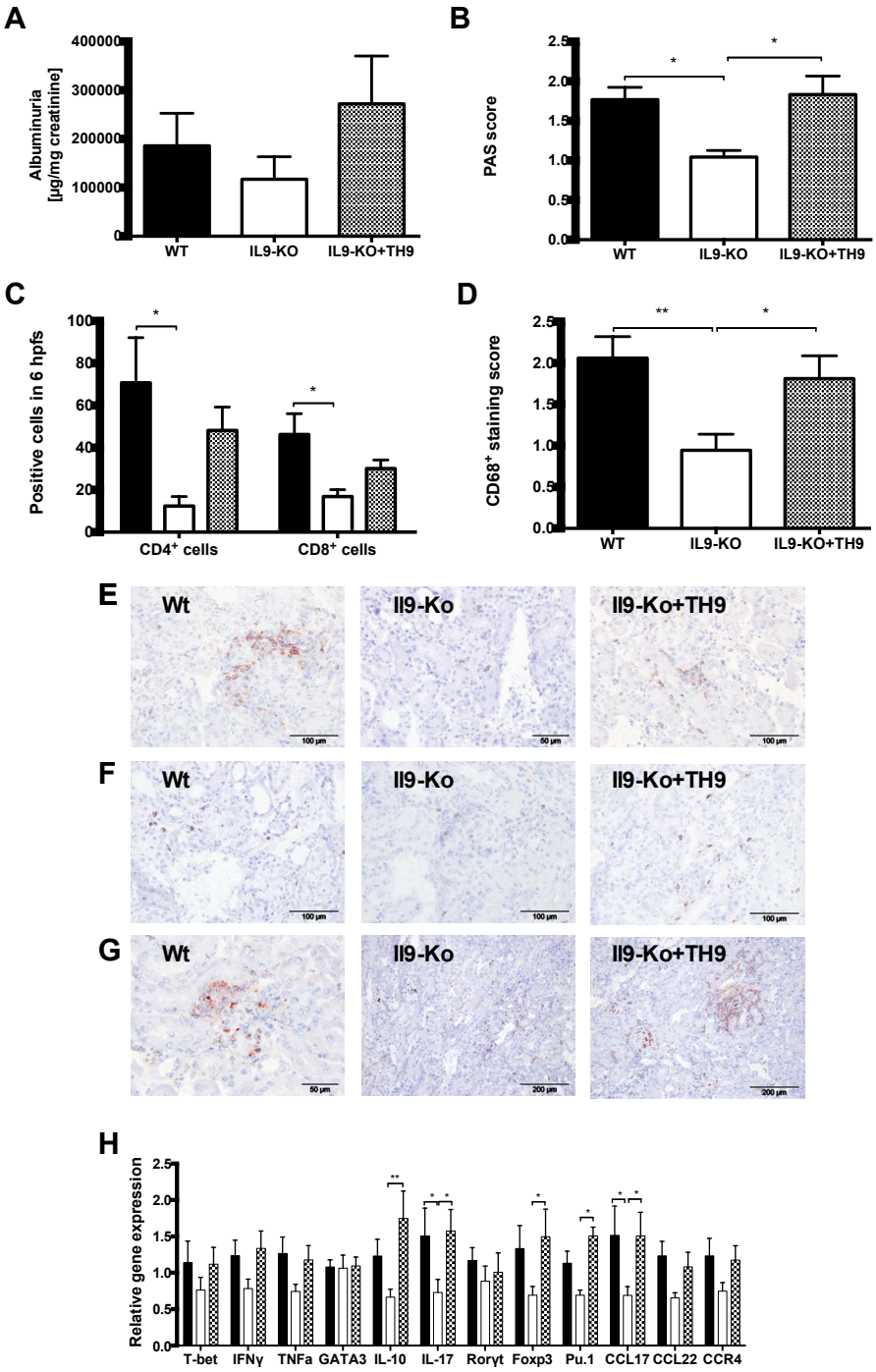

Figure 12 - ***Transfer of in vitro polarized TH9 cells restores the wildtype phenotype in IL-9-KO mice.***

IL-9-KO mice received $3x10^5$ in vitro polarized TH9 cells on the day before administration of the nephrotoxic serum (checked bar; n=9) and were compared to

Wt (black bar; n=10) or IL9-KO mice (white bar; n=8). (**A**) Albuminuria, (**B**) glomerulosclerosis, (**C**) T cell and (**D**) macrophage infiltration were restored to Wt levels in Il9-Ko mice receiving TH9 cells. Representative figures for (**E**) CD4, (**F**) CD8, as well as (**G**) CD68 stainings are shown. (**H**) Renal mRNA expression was assessed in the three groups and compared to WT mice. *p<0.05, **p<0.01

In a next step, using TH9-polarized naïve T cells from CD45.1 transgenic C57BL/6 mice, we aimed to trace adoptively transferred TH9 cells upon transfer. Unfortunately, we were unable to trace the transferred cells in kidney, liver, spleen, or lymph nodes 14 days after NTS induction by performing flow-cytometry.

Discussion

Immune-mediated glomerular disease is dependent on the time-dependent orchestration of leukocyte recruitment from the circulation. As has repeatedly been shown, T cells play an important role in this process, in so far as specific T cell subsets exert distinct pro- as well as anti-inflammatory roles by facilitating the recruitment of other leukocytes or altering their function. (157) To our knowledge, the present study provides the first direct evidence of the role of IL-9 producing TH9 cells in glomerulonephritis.

According to the current understanding, TH9 cells are implicated in a variety of pathological processes. In allergic inflammation TH9 cells are involved in airway hyperresponsiveness, as well as in the recruitment of mast cells and eosinophils and they have also repeatedly been shown to play a crucial role in the defense against worm infestations. (166) Tumor-associated TH9 cells on the other hand have been documented to exert potent anti-tumor effects in melanoma and lung cancer models, where the proposed mechanism was again the local production of chemokines induced by IL-9.

The understanding of TH9 cell contributions to the pathogenesis of autoimmune-mediated diseases is less clear: While these cells were initially thought to be regulatory, further studies in murine colitis and peripheral neuritis models showed that IL-9-producing CD4+ cells were clearly proinflammatory in this setting. (164) Reports on TH9 cells and IL-9 in experimental autoimmune encephalomyelitis indicate a more complicated pattern: while adoptively transferred antigen-specific TH9 cells induced significant disease and IL-9 blockade was able to ameliorate central nervous system pathology (168), IL-9 itself was shown to augment Treg function and the interruption of IL-9 signaling lead to a decrease in their capacity to suppress disease. (171)

Little is known about the contribution of IL-9 secreting T cells, specifically TH9 cells, to glomerulonephritis. We have previously shown that Tregs are crucially dependent on IL-9 to exert their immunosuppressive effect in NTS. (187) Having shown as a first step that during NTS TH9-associated markers are increasingly expressed in the kidney and decreasingly expressed in the secondary lymphoid organs, we speculated that TH9 cells might exert their effect in the end organ, the

kidney. This is in line with several reports from models of autoimmune disease as well as cancer, which all provide evidence of TH9 cell infiltration during the course of the disease. (168,175,177) To our surprise, we then found IL-9 deficiency to lead to a significantly attenuated NTS phenotype. This went along with a pronounced decrease of the chemokine receptor CCR4 as well as its cognate chemokines CCL17 and CCL22 in kidney from IL-9-KO animals. Both CCL17 and CCL22 are TH9-associated chemokines, whose transcription has been shown to be strongly dependent on one of the most important regulators of the TH9 phenotype, Pu.1. (180,192-194) CCR4 in turn is expressed on a large proportion of leukocytes including but not limited to monocytes and macrophages (195), neutrophils (196), and several T cell subsets. (197) This allows us to speculate that TH9 cells may facilitate the migration of CCR4+ cells during NTS. We can however not rule out that the CCR4 expression may reflect TH9 cells themselves, as these cells have recently been shown to express high levels of CCR4. (198)

We then showed that the protection from NTS in IL-9 deficient mice could be abrogated by in vitro polarized TH9 cells, which in turn restored the expression of CCL17, CCL22 and CCR4 to levels comparable to WT mice.

In addition to previous reports on the role of TH9 cells during early induction of anti-tumor immune response in cancer (175,177), our observations suggest that TH9 cells may be early mediators of leukocyte recruitment into the kidney. This may in part be the reason why we were unable to trace them two weeks after induction. Furthermore, there are other cells reported to secrete IL-9, among which TH2 and Tregs, mast cells and innate lymphoid cells are the most relevant IL-9 producers. (186) However, our observation that the WT phenotype can be rescued with TH9 polarized T cells highlights the proinflammatory importance of T cell-derived IL-9 in nephritis and indicates that the protective effect of IL-9 deficiency in our model is predominantly due to lack of T-cell derived IL-9.

We have previously provided evidence that Treg-derived IL-9 is of crucial importance for the immunosuppressive function of Tregs, by showing that IL-9-deficient Treg were unable to suppress NTS while their in vitro function was not impaired. (187) In consideration of these previous findings, our current data indicating a proinflammatory effect of IL-9 in NTS are all the more surprising and

nicely illustrate the diversity and pleiotropy of this cytokine. The simple observation that IL-9-KO animals are protected from NTS allows us to speculate that – in the early phase of GN – the proinflammatory role of IL-9-secreting TH9 cells outweighs the immunosuppressive importance of IL-9-secreting Tregs.

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Publications:

1. Eller K, Schroll A, Banas M, Kirsch AH, Huber JM, Nairz M, et al. Lipocalin-2 expressed in innate immune cells is an endogenous inhibitor of inflammation in murine nephrotoxic serum nephritis. PLoS ONE. 2013;8(7):e67693.
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