Dissertation

IN SEARCH FOR NEW THERAPEUTIC APPROACHES IN KIDNEY DISEASE: INVESTIGATIONS INTO THE ROLE OF THE SPLEEN IN NEPHROTOXIC SERUM NEPHRITIS

submitted by

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Statutory Declaration

I hereby declare that this thesis 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 thesis. Due acknowledgement has been made in the text to all other material used throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice at the Medical University of Graz".

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Eidesstaatliche Erklärung

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Abbreviations and Definitions

AKI - Acute kidney injury

Anti-GBM - Anti glomerular basement membrane

APCs - Antigen presenting cells

BMI - Body mass index

CAR cells - CXCL12-abundant reticular cells

CKD - Chronic kidney disease

CLP - Common lymphoid progenitor

CMP - Common myeloid progenitor

CMP - Granulocyte/macrophage progenitor

CRS - Murine cryptidin-related sequence

dH_2O - Distilled H_2O

DM - Diabetes Mellitus

EMH - Extramedullary haematopoiesis

ESRD - End stage renal disease

FSGS - Focal segmental glomerulosclerosis

GC - Germinal center

GFR - Glomerular filtration rate

GN - Glomerulonephritis

HEVs - High endothelial venules

HSCs - Haematopoietic stem cells

ICAM-1 - Intracellular adhesion molecule

LRP - Lineage-restricted progenitors

LSKs - Lineage-negative, Sca-1-positiv and c-kit-positive cells

LT-HSC - Long-term haematopoietic stem cell

Lyve-1 - Lymphatic vessel endothelial hyaluronan receptor 1

MAC - Membrane attack complex

MALT - Mucosal-associated lymphoid tissue

MEP - Megakaryocyte/erythroid progenitor

MHC - Major histocompatibility complex

NETs - Neutrophil extracellular traps

NFκB - Nuclear factor kappa-light-chain-enhancer of activated B cells

NLRs - Nucleotide-binding oligomerization domain (NOD)-like receptors

PAMPs - Pathogen-associated molecular patterns

PAS - Periodic-Acid-Schiff

PD-1 - Programmed death-1

Prox1 - Prospero-related homeobox 1

PRRs - Pattern recognition receptors

RBC - Red blood cells

RLRs - Retinoid acid-inducible gene I (RIG-I)-like receptors

RPGN - Rapid progressive glomerulonephritis

SLOs - Secondary lymphoid organs

SLPI - Secretory leukocyte protease inhibitor

ST-HSC - Short-term haematopoietic stem cell

TLOs - Tertiary lymphoid organs

TLRs - Toll-like receptors

TMB - 3,3´,5,5´-Tetramethylbenzidine

Treg - Regulatory T cell

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Abstract in German

Einleitung: Viele PatientInnen mit Glomerulonephritis müssen auch heute trotz vorhandener Therapiemöglichkeiten mit dem Eintreten eines terminalen Nierenversagens rechnen. Vorhandene therapeutische Möglichkeiten und vor allem Nierenersatzverfahren sind mit multiplen Komplikationen sowie einer deutlichen Einschränkung der Lebensqualität verbunden. Aus diesem Grund werden experimentelle Tiermodelle wie das der nephrotoxischen Serum Nephritis (NTS) verwendet, um neue therapeutische Optionen, aber auch pathophysiologische Grundlagen der Erkrankung zu erforschen. Sekundär lymphatische Organe wie der Lymphknoten werden in der NTS als Orte von Immunregulation und -initiation, aber auch der Dysregulation von peripherer und zentraler Immuntoleranz gesehen. Die Rolle der Milz war bisher im Modell der NTS allerdings unvollständig geklärt. Im Rahmen dieser Arbeit wurden deshalb funktionelle und strukturelle Veränderungen der Milz im Verlauf der NTS, sowie der Effekt einer operativen Splenektomie in diesem Modell evaluiert.

Methoden: Gesunde C57BL/6 Mäuse sowie Mäuse mit NTS an Tag 14 und Tag 28 wurden hinsichtlich Histopathologie und in die Milz infiltrierender Zellen evaluiert. Zusätzlich wurden durchflusszytometrische Daten für hämatopoetische Vorläuferzellen von gesunden, immunisierten und nephritischen Mäusen an Tag 14 erhoben. Weiters erfolgte eine Splenektomie oder Schein-Operation in C57BL/6 Mäusen und die NTS wurde nach einer zweiwöchigen Rekonvaleszenzphase induziert.

Ergebnisse: Im Verlauf der NTS zeigte sich bis Tag 14 eine stetige Zunahme von Milzgewicht und - größe. Allerdings zeigten splenektomierte Mäuse keine Unterschiede hinsichtlich Albuminurie an Tag 7 und 14 sowie PAS-positiven Ablagerungen im Vergleich zu scheinoperierten Mäusen. In die Niere infiltrierende $CD4^+$ und $CD8^+$ T Zellen, $CD68^+$ Makrophagen und $Ly6G^+$ neutrophile Granulozyten präsentierten sich unverändert zwischen den beiden Gruppen. Im Serum zirkulierende Maus anti-rabbit IgG, IgG Subklassen sowie rabbit IgG Titer waren gleich ausgeprägt. Allerdings zeigte sich eine über den Zeitverlauf der NTS bis Tag 28 zunehmende Anämie, welche sich nach Splenektomie bis Tag 28 zunehmend ausgeprägter präsentierte. Während $CD4^+CD69^+$ und $CD8^+CD69^+$ Zellen in der Milz nicht zur ihrer Gewichtszuname beitrugen, fand sich ein

deutlicher Anstieg an F4/80$^+$ Zellen als Indikatoren der roten Pulpa. Ebenso fanden sich vermehrt Megakaryozyten (CD41$^+$) und erythroide (Ter119$^+$) Vorläuferzellen im Sinne einer extramedullären Hämatopoese in der Milz immunisierter und nephritischer Mäuse. Während Zellen der erythroiden Linie im Knochenmark abnahmen stand die Ausbildung der extramedulläre Hämatopoese in der Milz in Zusammenhang mit der CXCR4/CXCL12 Achse. Dies konnte durchflusszytometrisch für nephritische und immunisierte Mäuse bestätigt werden. Die inflammatorischen Zytokine IFN-γ, Il-6 und TNF-α zeigten sich im Serum immunisierter und nephritischer Mäuse an Tag 14 erhöht.

Schlussfolgerung**:** In dieser Arbeit konnte gezeigt werden, dass die Milz keinen Beitrag zur Pathogenese der NTS leistet jedoch als Ort extramedullärer Hämatopoese fungiert. Diese extramedulläre Hämatopoese steht in Zusammenhang mit inflammatorischen Zytokinen, welche im Rahmen chronisch inflammatorischer Prozesse einen suppressiven Effekt auf das Knochenmark ausüben.

Abstract in English

Introduction: Despite the availability of different treatment options, many people suffering from glomerulonephritis today still face the burden of end stage renal disease. Experimental models like the model of nephrotoxic serum nephritis (NTS) serve as tools for the evaluation of possible new treatment targets. In NTS, secondary lymphoid organs are recognized as places of immune regulation. Although the lymph node has been well characterized as such, the role of the spleen in this model was incompletely understood so far. Therefore, this work aimed to elucidate the functional and structural changes in the spleen in the course of NTS, as well as the effect of splenectomy in this model.

Methods: Healthy C57BL/6 mice and mice with NTS on day 14 and day 28 were evaluated for histopathology and spleen infiltrating cells. Flow cytometric data for haematopoietic precursors of healthy, immunized and nephritic mice on day 14 were evaluated. Further, mice were splenectomised or sham-operated and subsequently NTS was induced.

Results: Anaemia and a gradual enlargement and weight gain of the spleen were seen over the course of NTS until day 14. However, splenectomised and sham-operated mice did not show differences in albuminuria on day 7 and day 14 and PAS-positivity in glomeruli. In line, numbers of kidney infiltrating $CD4^+$ and $CD8^+$ T cells, $CD68^+$ macrophages as well as $Ly6G^+$ neutrophils were unchanged between the two groups. Also serum anti-rabbit IgG did not differ between splenectomised and sham-operated nephritic mice. The enlargement and increase in spleen weight was not attributable to $CD4^+CD69^+$ and $CD8^+CD69^+$ leukocytes as measured by quantitative flow cytometry. Immunohistochemical stainings of spleens for $F4/80^+$ cells showed a marked increase of the red pulp. Finally, extramedullary haematopoiesis was detected by means of immunohistochemical stainings for CD41 and Ter119. Flow cytometric analysis provided evidence of erythroid cell increase in the spleen depending on the CXCR4/CXCL12 axis. Further, immunized and nephritic mice displayed increased levels of serum IFN-γ, Il-6 and TNF-α.

Conclusion: The spleen is not essential for the development of NTS, but is a place of extramedullary haematopoiesis in this model. Proinflammatory cytokines like IFN-γ contribute to this process by means of suppressive activity on the bone marrow.

Introduction

The Immune System

In order to protect individuals against infection, the overall excepted percept of a functional immune response comprises at least four criteria: immunological recognition, immune effector functions, immune regulation and immunological memory (2). Specific cells belonging to the innate and the adaptive immunity carry out these tasks. While innate immunity functions as a first barrier for pathogens and has the ability to defend the host against intruders, it cannot fulfil specific antigen dependent actions. Additionally to fighting pathogens, the adaptive immune system patterns a memory setup in case of reinfection (2). Specific functions with an emphasis on the major paradigms of immunity, clonal selection and pattern recognition (3), as well as cellular components of innate and adaptive immunity will be discussed hereafter.

Innate immunity

First barriers

Mechanisms of innate immunity can defend the body from intruders quite efficiently, thereby protecting the adaptive immunity from excessive service. At the very beginning of a microorganism's invasion into a host, several immediate actions can be fulfilled by the immune system before more specific steps like the recognition of non-self antigen are initiated.

The first barriers intruders have to overcome are mucus producing mucosal barriers and epithelial cells. Epithelial cells form a lining between outside and inside, which makes them perfect contestants for first immune responses. Barrier cells may also protect the environment by the secretion of antibacterial enzymes like lysozymes on the one hand (2), and antimicrobial peptides on the other hand (4). These peptides include defensins, murine cryptidin-related sequence peptides (CRS), cathelicidins and secretory leukocyte protease inhibitor (SLPI) (4). Defensins are cationic, short peptides rich in cysteine and can be classified in α-, β-, and θ-defensins (5). Defensins act via disruption of the cell wall of microorganisms, but can also promote RNA, DNA and protein synthesis inhibition. Besides their function in defence against bacterial, viral and fungal infections, defensins also seem to be involved in adaptive immune regulations, as well as inflammation, cytokine and chemokine production (5). CRS-Peptides form a family of homologous defence peptides which are produced by paneth cells in the gut and are divided into two main groups – CRS1C and CRS4C, based on their cysteine pattern. They have the ability to form homo- and heterodimers, which help to sustain their antimicrobial activity and to increase their potency against microorganisms (6). Cathelicidins are a group of peptides consisting of a family of over 30 members (7). They are bioactive against Gram+ and Gram- bacteria, but have additional activities as proteinase 3 processes mature LL-37 and cathelin. While cathelin also acts as a proteinase inhibitor, LL-37 acts via pore formation in cell walls (7). SLPI is a member of the whey acidic protein family and is known to be an inhibitor of enzymes like neutrophil elastase. It also inhibits nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), which is important for a variety of immune regulatory and proliferation responses, highlighting SLPI as a player in homeostasis

regulation at barrier sites (8). Besides the protease activity, SLPI has antimicrobial and antifungal as well as antiviral properties at epithelial barrier sites (8).

The complement system

Although the complement system cannot be assigned to the innate immune system only, it clearly proves its importance for the innate immune system based on its contribution to the initial defence mechanisms of microorganisms. Nevertheless, important features of the complement system in adaptive immunity exist. Over 100 years ago, the complement system was found to be a complementary feature in the serum, operating in defence situation (9). It is well known today that this system consists of over 30 proteins found in the serum and interacting receptors (10), whereby soluble components are mainly produced by hepatocytes (9).

There are three possible pathways of complement activation, namely the classical pathway, the alternative pathway and the lectin pathway. The common feature all of these pathways share is the activation of the central complement protein C3. Following this activation, C5 is cleaved and the membrane attack complex C5b-9 is formed, which initiates cytolysis (9,11).

The classical pathway was the first pathway described and is initiated by C1q binding mainly to antibodies bound to foreign surface (12), but also to cell walls of microorganisms, C-reactive protein and more (11). After a conformational change in C1q and the cleavage of serine proteases, these proteases cleave C4 and C2, and finally the C3 convertase C4bC2a is formed. The C3 convertase then cleaves C3 into a large (C3b) and a small (C3a) component. Binding of C3b to the C4bC2a complex builds the classical pathway C5 convertase (C4bC2aC3b) (13). The classical pathway C5 convertase has the ability to initiate the terminal complement pathway by cleavage of C5 into C5b and C5a (9,11,12).

In case of the lectin pathway, mannose-binding lectin and ficolins are binding to cell surfaces of microorganisms without the need of antibody presence (14). Although it utilises a different mode of recognition, it finally results in the formation of the same C3

convertase as in the classical pathway, although serine proteases generating C4bC2a differ (15).

The alternative pathway is activated by fragments of microorganisms, tumor cells, plastic surfaces and more (11). All foreign activating surfaces lack the possibility of regulation of this pathway, which is possible for mammalian host cells (9). The alternative pathway seems to be responsible for the largest proportion of complement activation products and is active at low levels also in the healthy individual (9,16), thus guaranteeing a rapid response. It relies on spontaneous low-grade hydrolysis of a C3 thioester bond, which can then bind factor B and becomes cleaved by factor D. This cleavage results in the formation of the alternative pathway C3 convertase C3bBb, which has the ability to cleave C3 into C3a and C3b like the classic C3 convertase (9,16). Rapid generated C3b constitutes an amplification loop (17) for the amplification of C3b, which is produced in the classical and the lectin pathway (9). Finally, after an additional C3 molecule is bound to C3bBb, the alternative pathway C5 convertase C3bBbC3b is built, encompassing the same functionality as the classical pathway C5 convertase (9).

All three pathways lead to the formation of C3 convertase and C5 convertase. Ultimately, C3a and C5a are produced by cleavage. They constitute potent anaphylatoxins per se and promote – via interaction with their G-protein coupled receptors C5aR and C3aR (18) – vasodilation and vessel permeability. Further, C5a is a chemoattractant (18) for many cells like neutrophils and macrophages, but also for B and T cells and both C5a and C3a can act as a trigger of oxidative burst in neutrophils, eosinophils and macrophages (16).

Finally the terminal pathway of complement is initiated by cleavage of C5, which results in the formation of a C5b-6 complex (19). This complex binds C7 and further produces the C5b-7 complex (9). After the binding of C8 to the C5b-7 complex, C5b-8 is formed and binds to C9. Unfolding of C9 in the membrane enables C5b-8 to bind several C9 molecules, leading to polymerisation and the formation of the trans membrane pore complex membrane attack complex (MAC) C5b-9 (9). This final insertion of the C5b-9 MAC complex causes cell death and promotes inflammation (16).

It is described that the complement system can be activated by other pathways than the classical, the lectin and the alternative pathway, mainly including coagulation and fibrinolysis pathways. The classical pathway can be activated by FXIIa (20) and C3 can be cleaved by thrombin, kallikrein and plasmin (9). Further, C4b and C3b can – apart from their role in the classical, lectin and alternative pathway - directly bind immune complexes and target cells covalently. This opsonisation leads to interaction with complement receptor 1, which can be found on red blood cells (21) and further these opsonized cells are rapidly delivered to liver and spleen, where they will face phagocytosis by macrophages and Kupffer cells (16).

Importantly as the complement system comprises potent effector functions, there are several regulatory strategies in order to keep the host from self injury (16). While C1 inhibitor inactivates steps in the lectin pathway and complement factor H and I regulate the alternative pathway in the plasma, there are also membrane-bound regulators like membrane cofactor protein, CR1 and decay-accelerating factor, but also the endothelial expressed thrombomodulin (16). Concerning the terminal pathway, the C5b-9 MAC is regulated in the fluid phase and on the cell membrane via the CFH-related protein 1, which prevents MAC formation and CD59, which inhibits C9 (16). Deficiencies in these regulatory mechanisms lead to deregulation of the complement system and are associated with several different diseases.

When considering the three pathways of complement activation and describing the complement system's role as an arm of humoral immunity, opsonisation and subsequent phagocytosis lead to an enhanced pathogen clearance on the one hand. On the other hand inflammatory cytokine and chemokine production can be promoted in cells of the innate immune system by the complement system and immune complexes and cell debris are cleared by the complement system, thereby preventing autoimmunity (22). But as mentioned initially, there is also a role for the complement system in adaptive immunity and the complement system therefore has a function as a bridge between innate and adaptive immunity. Humoral immunity can be regulated by complement dependent B cell enhancement via its function through complement receptors on B cells (23). This enhances signalling over the B cell antigen receptor and consequently provides survival signals (10). Further, antigen can be localized to follicular dendritic cells in lymphoid follicles, thereby initiating chemokine mediated organization of B cell follicles (10). Also T cell immunity is

influenced by the complement system (24), as C3 was shown to be compulsory for the priming of $CD4^+$ and $CD8^+$ T cells by antigen presenting cells (APCs), which likely prefer the uptake of C3-coated particles (10). As even regulatory T cells (Tregs) were found to be influenced by the complement system via interaction with the complement regulator CD46 (10,25), it is likely that many functions of the complement system on adaptive immune mechanisms are not revealed yet.

Cellular innate immunity

Several different cell types are considered cellular components of the innate immunity and utilise for example pattern recognition. Beneath classical resident cells like epithelial cells, which we defined as a first barrier for microorganisms when entering the host, the group of myeloid innate immune cells is of utmost interest and importance in innate immunity. Monocytes, macrophages, neutrophils, eosinophils, basophils, dendritic cells and platelets belong to this group and fulfil special functions in innate immunity like phagocytosis (22). Additional important cells worth mentioning at this place as they play central roles in innate immunity are mast cells, natural killer cells and γ/δ T cells.

Mononuclear phagocytic cells are monocytes, but compared to macrophages they seem to have less phagocytic activity. Indeed, they were long seen as simple precursors of macrophages, because monocytes can differentiate into macrophages after leaving the bone marrow where they originate. However, this idea has changed over the last years as it became obvious that the situation was more complicated than previously thought. On the one side it is now known that monocytes can differentiate into other cells like monocyte derived dendritic cells (22). On the other side and apart from the differentiation potential of monocytes into other cell types than macrophages, human and mouse monocytes are categorized into several groups based on their expression of surface markers. $CD14^{++}$ $CD16^-$ monocytes represent the most abundant monocyte population and have proinflammatory and microbial roles (26,27). They are also known as classical monocytes with their murine equivalent represented by the marker expression $CD11b^+CD115^+Ly6C^{hi}$ (26). Human monocytes further encompass intermediate $CD14^{+\ +}CD16^+$ monocytes with proinflammatory roles and non-classical $CD14^+$ $CD16^{++}$ monocytes with roles in patrolling. In mice, there is only one other monocyte population known besides classical

monocytes - $CD11b^{+}CD115^{+}Ly6C^{low}$ with patrolling function and functions in tissue repair (26). Macrophages themselves are thought to mainly but not only originate from monocytes. As today it is known that there are anti-inflammatory, immune suppressive macrophages existing besides inflammatory macrophages, their functions include immune suppression besides killing and phagocytosis (22).

Neutrophils, also part of the group of myeloid innate immune cells, infiltrate tissue and constantly look out for potential targets. Besides phagocytosis, chemokine production and the thereby evolving immune-regulatory function, the formation of neutrophil extracellular traps (NETs) was described recently and has been discussed extensively as a mode of bacterial killing other than phagocytosis. This concept involves degranulation of neutrophils and release of toxic components after activation into the extracellular area (28). Nuclear chromatin is released from the cell, which thereby faces necrosis through rupture of cell membranes (28). NETs have further been associated with autoimmune diseases, cancer and many other pathologic circumstances (28).

Also known for their phagocytic functions, eosinophils can be found in peripheral tissue. They are associated with Th2 immunity and allergic diseases (22).

Other cells associated with allergic diseases and parasitic infections are basophils, which have also been shown to contribute to bacterial clearance via the formation of extracellular traps (29).

Dendritic cells form a bridge between the innate and the adaptive immune system via their presentation function of antigen (30). Platelets are megakaryocyte-derived and have been shown to induce cytokine cascades, leukocyte recruitment and additionally express molecules like CD40L and TLRs, which both may modulate innate immune responses (31).

Mast cells possess effector functions in innate immune responses as they express several classes of pattern recognition receptors (PRRs), thereby promoting microorganism clearance. Mast cells have a key function in host defence as a source of bioactive amines and constitute a sole tissue source of histamine and heparin. While histamine released by degranulation leads to increased vascular permeability and blood flow, heparin regulates

mast cell granule storage and release (32). They can also attract other cells of the innate immunity like neutrophils, eosinophils and macrophages (33). Besides this function in innate immunity, modulating effects of mast cells on T and B cells were described as well (33).

Natural killer cells have originally been described as cells of the innate immunity due to their cytolytic action. However, it is noticed today that natural killer cells encompass features reaching as far as immunologic memory, which qualify them attributable to adaptive immunity as well (34).

Compared to the vast majority of T cells, which have a TCR composed of the two glycoprotein chains alpha and beta and are called α/β T cells, γ/δ T cells express an alternative T cell receptor and are less common in the peripheral blood. They can produce several cytokines, show cytotoxic activity and seem to use their TCR as a PRR. γ/δ T cells are recognized as another cell population bridging innate and adaptive immunity (35).

Pattern Recognition

These very first responses described above are recognized as immediate very fast actions of the innate immune system. The second step of pattern recognition - right before adaptive immune responses are initiated - can also be described as a part of innate immune response. Almost three decades ago, C.A. Janeway described a fundamental principle of innate immune recognition, whereby he was able to complement the clonal selection theory, which already existed by that time (3,36). He defined PRRs, which recognize foreign pathogen-associated molecular patterns (PAMPs), but are not clonally distributed on T lymphocytes (36). Cells of innate immunity like dendritic cells and phagocytes, but also epithelial cells use this system to identify self from non-self antigen (37).

There are two major groups of PRRs, namely toll-like receptors (TLRs) and cytosolic PRRs. TLRs are glycoproteins with an extracellular or luminal ligand-binding domain. If one of the 10 currently in humans identified TLR binds a PAMP or binds it through an intermediated PAMP-binding molecule, intracellular signal transduction is initiated. PAMPs recognized by TLRs derive from bacteria, viruses, fungi and protozoa (38). While

TLRs can be found on a variety of cells, the most important cells for pattern recognition bearing TLRs on their surface are macrophages, dendritic cells and B lymphocytes (38). As intracellular pathogens or internalized parts of them can not be sensed by TLRs, receptors called retinoid acid-inducible gene I-like receptors (RLR) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) independent of TLRs fulfil this action (39). When it comes to PAMPs, similar PRR mechanisms can identify and recognize PAMPs, which are evolutionarily conserved structures on pathogens, and might originate from completely different origin and composition. Importantly, a single pathogen is recognized by different PRRs using various PAMPS, which ensures a rapid and potent response (38). PRR-PAMP ligation induced signalling promotes proinflammatory cytokines and IFNs, as well as transcriptional gene expression regulation and posttranscriptional mRNA regulation (38,39). This concept changed the perception of innate immunity and lead to an understanding of the system, which comprises more complex and specified features than previously thought.

Adaptive immunity

While the existence of innate immunity is established as being more elemental, as it can be found in all species, vertebrates have developed adaptive immunity throughout evolution (30). The reason for this development was the need to defend pathogens more effectively in case they overcome innate immunity functions in the first place, especially if the individual is infected by the pathogen repeatedly (30). So adaptive immunity can be considered as being important for immunologic memory and the defending of antigen in the late phase of infection (38).

Thymus derived T lymphocytes and bursa or bone-marrow derived B lymphocytes have the ability to detect antigen and respond specifically (40). In a first place their progenitors rearrange their T cell receptors and B cell receptors by the rearrangement of immunoglobulin variable (V), diversity (D) and joining (J) segments. As this random rearrangement might lead to the production of autoreactive cells, these cells are either inactivated or deleted right away (40). As for T cells, if they survive positive and negative selection (41), they differentiate into $CD4^+$ or $CD8^+$ cells after a double-positive stage, depending on whether their TCR recognizes MHC class I (CD8) or class II (CD4). Cells surviving this selection process may enter into the blood and circulate through lymphoids in order to meet antigen, whereby anatomical lymphatic pathways build their guidance in order to circulate between lymphoid tissue and the blood stream. APCs take up antigen, process it and finally transport it to lymphoid tissue. There, utilising the major histocompatibility complex MHC class I and II, TCRs are able to recognize antigen presented by APCs like dendritic cells, B cells or other cells bearing phagocytic properties (40). Notably at this point, other than T lymphocytes, which primarily recognize pre-digested antigen, antibodies derived from B cells can later on recognize intact epitopes.

As first described over 50 years ago, the clonal selection theory further states that a clonal production of those cells possessing the accurate receptor for the antigen occurs. Also, these cells will not only be effector cells, but there will also be a group of cells bearing memory functions (42,43).

Importantly, T cells need second stimuli in order to get activated. These stimuli come from coreceptors, of which the CD28 pathway was one of the very first pathways

described. The ligands CD80 and CD86 can be found on APCs and their ligation to CD28 on $CD4^+$ T cells is required for proper activation (44). While this function seems important already during priming processes, the binding of B7 homologous receptor to inducible costimulator (ICOS) on T cells is likely important for reactivation processes (45). There are many more T cell costimulatory molecules described today (46), which are important for cell differentiation, proliferation and survival. At this point the inducible programmed death-1 (PD-1) shall be mentioned as an inhibitor of proliferation. Upon ligation with PD-L1 on monocytes, dendritic cells and some tissue cells, T cell proliferation is inhibited (45). Also B cells typically require support in order to get activated. CD40 expressed on B cell surfaces is required to ligate with CD154, which can be found on T cells for adequate B cell proliferation (45). Although there are many more coreceptors on B cells, two important coreceptors of B cells shall be mentioned here namely - CD19 and CD22. While CD22 on B cells acts inhibitory through its ligation with sialoglycoconjugates on T cells, CD19 has some functional overlap with CD40 when association with phosphoinositol 3-kinase and Vav occurs (45).

The fate of T cells after differentiation

Naive $CD8^+$ T cells are activated by antigen presented via MHC class I found on every nucleated cell and form cytotoxic T lymphocytes, which can effectively lyse infected cells (47). Further, they can differentiate into central memory T cells and effector memory cells, which, unlike effector cells, survive for a long time and ensure a more rapid effector immune response in case of reinfection (47). This principle can be found in $CD4^+$ T cells as well, however they have the ability to differentiate into several subtypes of $CD4^+$ T cells.

It is accepted today that CD4 differentiation can not be only explained by the T helper type 1 and T helper type 2 (Th1/Th2) dichotomy, but instead several CD4 subpopulation evolve, which were primed under different cytokine milieus, and are bearing distinct transcription factors resulting in subtype specific cytokine production profiles (47,48). While Th1, Th2 and Th17 cells are primarily targeting antigen, the role of regulatory T cells is best described by an immunomodulatory function in which self-tolerance is induced (48). Follicular helper T cells provide support for B cells in antibody production and the relative recently described subtype of Th9 cells has been shown to lead to both,

beneficial and detrimental functions in the body (48,49). Table 1 summarizes the most important features of human $CD4^+$ T cell subsets described.

T cell subset	Characteristic cytokines	Characteristic transcription factor	Function
Tfh (follicular helper)	IL-21	BCL6	B cell help
Th1	IFN-γ	T-bet	Protection against intracellular pathogens
Th2	IL-4, IL-5, IL-13, IL-9	GATA-3	Protection against extracellular parasites
Th9	IL-9	PU.1	Protection against extracellular parasites
Th17	IL-17, IL-22, IL-26	RORC2	Protection against extracellular bacteria and fungi
Treg (regulatory T cell)	TGF-ß	FOXP3	Maintenance of self-tolerance
Tr1 (type 1 regulatory)	IL-10	?	Inhibition of immunopathology

Table 1 – Characteristics of relevant human $CD4^+$ T cell subsets. Adapted from Geginat J. Frontiers in Immunology, 2014; 5:630.

B cells and the germinal centers

If B cells are activated by antigen in secondary lymphoid organs, germinal centers (GC) arise (50). There, class switch recombination or isotype-switching, somatic hypermutation, which is considered as mutation of antibody encoding DNA and increased affinity selection of BCRs through affinity maturation, which describes the production and selection of antibodies with higher affinity, are characteristic events (51). Most mature B cells are found in lymphoid follicles in secondary lymphoid organs, responding to T cell – dependent antigen, which is bound to follicular dendritic cells and either become plasma cells or enter GCs (50,51). In addition to GC B cells, B-1 and marginal zone B cells have been identified as being subsets of B cells with certainty at least in the murine system. However, all of these B cell subtypes contribute to antibody responses and can differentiate into plasma cells, which are known as cells with effector function of humoral immunity (51). GCs are also the place where memory B cells are generated. These cells have the ability to survive after antigen challenge and can turn into plasma cells rapidly if needed in case of repeated antigen challenge (51).

Chemokines and chemokine receptors

The concept of chemokines and chemokine receptors describes the interplay of certain protein molecules and cognate receptors of these proteins. Chemokines can be secreted by a variety of cells and their receptors are typically found on cells that can move towards increased concentration of the chemokine. This movement of cells along a chemoattractant gradient is also known as chemotaxis (52). There are many physiological and pathological circumstances especially concerning the immune system, to which chemokines may contribute. For example, they also play roles during embryogenesis, inflammation and cancer metastasis (53). Over 40 human chemokines have been identified with about half the number of chemokine receptors (53).

In a classic way, chemokines signal through G protein-coupled receptors on the surface of different cells. Subsequently, different signal transduction cascades are activated. Chemokines are typically either known for their inflammatory or constitutive behaviour, or they express both features. Depending on the first two cysteine residues, chemokines are grouped into four families: CXC, CC, C and CX_3C chemokines (53). Additionally, chemokine receptors without functional G protein-coupled receptor signalling called atypical chemokine receptors have been described. These receptors are also called decoy receptors, because although they are structurally similar to signalling chemokine receptors, their function can be seen in scavenging and buffering of chemokines (54). Duffy antigen receptor for chemokines (DARC), D6, CCX-CKR and CXCR7 are members of this family. Although these receptors do not hold signal transduction features, they are now also seen as important players in chemokine function and regulation (54,55).

Lymphoid Organs

Primary lymphoid organs

The thymus, fetal liver and bone marrow are defined as primary lymphoid organs. The fetal liver is important during development, where it constitutes an early colonization site for haematopoietic progenitors and haematopoietic stem cells (HSCs). The bone marrow is the place of common lymphoid progenitor cell production. Two functional compartments can be distinguished in the bone marrow. One supports haematopoiesis and contains haematopoietic stem cells (56). The second compartment is a stromal compartment housing mesenchymal stem cells (56,57). After committing to the T cell lineage, lymphoid progenitor cells leave the bone marrow and home in the thymus where they differentiate into functional, self-tolerant naive T cells. From here, T cells can migrate into the periphery (57). The thymus involutes around the time of young adulthood (57).

Secondary lymphoid organs

Spleen and lymph nodes as well as tonsils, adenoids, Peyer's patches and nasal/bronchial-associated lymphoid tissue, which belong to the group of mucosal-associated lymphoid tissue (MALT), are all recognized as secondary lymphoid organs (SLOs) (58). Concerning the development of secondary lymphoid organs, it is accepted that on the one hand cytokine and chemokine actions are important and on the other hand interactions between several cell types are required. For the formation of SLOs, the entity of IL-7R$^+$CD3$^-$CD4$^+$CD45$^+$ inducer cells interacts with stromal VCAM1$^+$LTßR$^+$ lymphoid tissue organizer cells (59). Another crucial point for the development of SLOs is lymphatic vessel development. There, lymphatic vessel endothelial hyaluronan receptor 1 (Lyve-1) expression is found already at an early point of lymphatic vessel building. Further, after Lyve-1 expression can already be found and respective endothelial cells are considered as competent, the transcription factor prospero-related homeobox 1 (Prox1) can guide these lymphatic progenitors for the lymphatic programme (60). Generally, SLOs share a basic, common organisation (61).

Lymph nodes, on which example this organisation can best be explained as follows, can be found strategically placed over the body (58). Surrounded by a capsule, the outer cortex houses B cells and follicular dendritic cells, while T cells and dendritic cells can be found paracortical and macrophages are to be found in the inner medulla and the subcapsular sinus (61). Leukocytes may enter lymph nodes via afferent lymphatic vessels, which characteristically are the route used by antigen-laden dendritic cells. Another way for cells and antigen to enter lymph nodes is via arterioles into the capillary bed. High endothelial venules (HEVs) are postcapillary venules, which have the ability to slow down the lymphocyte flow with the help of adhesion molecules (61).

Chemokines and chemokine receptors are of utmost importance concerning organisation in the lymph node. HEVs express the chemokines CCL19 and CCL21, which allow T cell transmigration into the paracortical area via interaction with intracellular adhesion molecule 1 (ICAM-1). Also, T cells express the receptor for CCL19 and CCL21, namely CCR7. B cells on the other hand are attracted by CXCL13 into B cell follicles, as they express the cognate receptor CXCR5. It also happens that GCs develop there. Further

highlighting the importance of chemokine interactions within SLOs, changes in the expression of chemokine receptors also enable T and B cells to change their position within the lymph node (61).

Looking at spleen architecture, one will find high similarity to lymph node architecture when observing the area called the white pulp, as the spleen can be subdivided in areas of white and red pulp. While the white pulp holds lymphoid characteristics and functions, the red pulp is an area of dead cell removal and iron storage as well as turnover, and therefore it is known as a tissue with haematological function (61).

Tertiary lymphoid organs

Tertiary lymphoid organs (TLOs) develop in mature individuals in response to chronic inflammatory stimuli. Importantly, places they arise in are typically defined as non-lymphoid tissue, indicating lymphoid tissue would not be expected there under steady state conditions (58). Still, TLOs show high structural and functional similarity to secondary lymphoid organs like the lymph node, with the exception of missing encapsulation of TLOs, while a capsule can always be found in lymph nodes (62). Apart from the missing capsule, HEVs, lymphatic vessels, germinal centers and leukocyte subtype organisation can be found in TLOs (63). HEV development thereby is, besides the production of inflammatory cytokines and chemokines, a hallmark of TLO formation (58).

Functionally, TLOs seem to be not only involved in B cell proliferation with germinal center formation and the development of memory and plasma cells (64), but also in antigen presentation and lymphocyte activation (63). These functions support the idea of TLOs in fulfilling different functions by forming a local microenvironment in close proximity to infected tissue or tissue involved in autoimmune processes. While during infection, local antigen presentation in TLOs definitely prevents the spreading of pathogens, it is likely that TLOs promote disease activity in autoimmunity though some studies suggested the involvement of TLOs in immuneregulation (58). Besides autoimmunity and infection, TLOs appear during graft rejection (61).

Haematopoiesis

The formation of blood cells is a lifelong process, which depends on HSCs. HSCs are stem cells on top of a progenitor hierarchy, which further differentiate into mature red blood cells, megakaryocytes, monocytes and macrophages as well as neutrophils and lymphocytes (65). HSC numbers can be affected by multiple factors like death or proliferation, survival, differentiation or self-renewal. These actions ultimately cause HSC expansion or HSC decline (66). During development, another important site of haematopoiesis besides the fetal liver and the bone marrow is the yolk sac. The first wave of 'primitive' blood production of mainly red blood cells in the embryo takes place there. Soon however, this primitive system is replaced and the 'definitive' haematopoiesis in adults takes over (65), which includes both, erythropoiesis and myelopoiesis. Stem cells require several transcription factors in order to fulfil their function already at an early, primitive stage. Table 2 summarizes the hierarchy of haematopoiesis.

<table>
<tr><td colspan="6">LT-HSC
($LIN^-KIT^+SCA1^+CD34^{lo}FLK2^{lo}$)
Stem cells with self-renewal capacity</td></tr>
<tr><td colspan="6">ST-HSC
($LIN^-KIT^+SCA1^+CD34^{hi}FLK2^{lo}$)
Multipotent progenitors</td></tr>
<tr><td colspan="6">MPP
($LIN^-KIT^+SCA1^+CD34^{hi}FLK2^{hi}$)
Multipotent progenitors</td></tr>
<tr><td colspan="4">CMP
Oligopotent progenitors</td><td colspan="2" rowspan="2">CLP
Oligopotent progenitors</td></tr>
<tr><td colspan="2">MEP
Oligopotent progenitors</td><td colspan="2">GMP
Oligopotent progenitors</td></tr>
<tr><td>Lineage-restricted progenitors “LRP”</td><td>Lineage-restricted progenitors “LRP”</td><td>Lineage-restricted progenitors “LRP”</td><td>Lineage-restricted progenitors “LRP”</td><td>Lineage-restricted progenitors “LRP”</td><td>Lineage-restricted progenitors “LRP”</td></tr>
<tr><td>Erythrocytes
Effector cells</td><td>Platelets
Effector cells</td><td>Granulocytes
Effector cells</td><td>Macrophages
Effector cells</td><td>T cells
Effector cells</td><td>B cells
Effector cells</td></tr>
</table>

Table 2 – Hierarchical differentiation of HSCs LT-HSC (long-term haematopoiectic stem cell); ST-HSC (short-term haematopoietic stem cell); MPP (multipotent progenitors); CMP (common myeloid progenitor); CLP (common lymphoid progenitor); MEP (megakaryocyte/erythroid progenitor); GMP (granulocyte/macrophage progenitor); LRP (lineage-restricted progenitor). Adapted from Sharpless NE. Nature Reviews Molecular Cell Biology, 2007, 8(9):703-13.

HSCs are found on the very top of the haematopoietic cell hierarchy. Typically most of the multilineage HSCs can be determined by their appearance within the group of lineage-negative, Sca-1-positiv and c-kit-positive cells (LSKs). In order to subdifferentiate HSCs and their more committed progenitors, other markers like CD34, CD150 and flk2 are regularly used. From the unrestricted, multipotent HSCs, MPPs arise, which themselves can give rise to the oligopotent CMPs and CLPs. T and B lymphocytes, as well as natural killer cells develop from CLPs, while the lineage-restricted GMPs and MEPs either differentiate to granulocytes or megakaryocytes or platelets and erythrocytes. Notably, dendritic cells have been reported to arise from both, CMPs and CLPs (67).

Factors determining the fate of HSCs and lineage commitment have been found in the expression of several transcription factors (65,67). Erythroid/megakaryocyte and myeloid differentiation are for example connected with the expression of GATA-1 and PU.1 (65). It is, however, important to note that a wide variety of transcription factors, which were originally believed to be restricted to more committed cells only, can be found in HSCs (67) and that lineage reprogramming in terms of redirection towards another lineage through forced expression of transcription factors might occur (65).

An extremely important part of stem cell biology yet poorly understood is the stem cell niche, which refers to the surrounding microenvironment of stem cells in their close proximity. These cells are said to have influence on HSCs as well, and the best-described niches are of course within the bone marrow. Erythroblast islands, which consist of a central resident macrophage, have been found to be essential microniches for the production of red blood cells (68). Also, endosteal surface osteoblasts and sinusoidal endothelium were found in close association to HSCs (67). While these permeable sinusoids are considered to serve in leukocyte trafficking, the vascular niche constituted by arterial blood vessels is less permeable and thereby maintains HSCs in a low reactive oxygen species state (69). This implies different regulatory functions of distinct vascular types in the bone marrow (69).

A critical chemokine/chemokine receptor pair for HSC homeostasis and maintenance within the bone marrow niche is CXCL12/CXCR4. CXCL12 has a role in retaining HSCs within the bone marrow (70). The perivascular niche within the bone marrow is also the

place where CXCL12-abundant reticular (CAR) cells expressing high CXCL12 levels can be found in close proximity to HSCs (71).

Extramedullary haematopoiesis

Extramedullary haematopoiesis (EMH) is defined as blood cell production outside the bone marrow (68). A crucial idea in the understanding of EMH is the reactivation or development of stem cell niches in places like spleen and liver providing the required surrounding, but theoretically also in many other tissues like myocardium, lung, renal tissue, skeletal muscle, small intestine or lymphoid and adipose tissue (68). Spleen and liver are two frequently reported sites of EMH. Typically for EMH, nucleated red blood cells and erythroid precursors as well as megakaryocytes and signs of mitosis can be found in different spleen areas and it is known that EMH might look different in terms of diffusion, nodularity and focal distribution depending on the underlying cause (68).

Four major causes might lead to EMH (68), of which the first is bone marrow failure. The very classical form of bone marrow failure is primary myelofibrosis, which - like myelofibrosis secondary to polycythaemia vera or essential thrombocythaemia - is a clonal myeloproliferative disorder and might lead to pancytopenia, bone marrow fibrosis, splenomegaly and many more symptoms (72). In the end, the balance and location of haematopoiesis is disturbed by a shift to new or reactivated stem cell niches by aberrant proliferating HSCs (68). The second cause of EMH is seen in myelostimulation, whereby a need for increased blood cells triggers a natural response. One trigger can be hypoxia due to anaemia with subsequent upregulation of erythropoietin in the kidney (68). Tissue inflammation, tissue injury and tissue repair constitute the third entity of the four EMH causes. It is considered as de novo haematopoiesis, because the environment developing within sites of inflammation, injury and repair constitutes a surrounding favourable of serving as a niche for EMH (68). Mediators like cytokines contribute as well and the whole process is based on the capability of HSCs in blood migration and engraftment of targets (73). The fourth at this point only theoretical subentity of EMH is the abnormal chemokine production found locally or systemically where this unusual generation promotes EMH (68). In clinical practice, differential diagnosis in the patient should always consider haematopoietic neoplasms like myeloid sarcomas, but also myeloproliferative disorders and myelodysplastic syndromes when EMH is diagnosed (74).

Kidney Disease

Prevalence of kidney disease

Abnormal kidney function but also abnormal structural kidney features play into the definition of kidney disease. Health is affected in a definite way, which is also determined by whether kidney disease occurs abruptly and resolves or does not resolve and thereby becomes chronic (75). While acute kidney injury (AKI) is considered as sudden loss of kidney function, which might resolve but might also be fatal, chronic kidney disease (CKD) is today recognized as a global health burden, considering the risk of end stage renal disease (ESRD) development with the need for renal replacement therapy. According to cross-sectional analyses of The National Health and Nutrition Examination Surveys, the prevalence of CKD 1-4 increased over the last observation periods in the United States. While the prevalence for these stages was 10% for the observation period between 1988 and 1994, it increased to 13.1% in the observation period between 1999 and 2004 (76). This increase was mainly due to high prevalence of stage CKD 3. Further increase could not be observed in the following periods (77). After the incidence of ESRD was rising until 2010 in the United States, there seems to be a plateau with 114,813 incident ESRD cases in 2012 (77). However, these findings should always be adjusted for geographic region, age, race and ethnicity and primary causes of ESRD. Regarding the European population, this becomes obvious as prevalences of CKD 1-5 vary between 3.31% in Norway and 17.3% in northeast Germany (78).

Incidence rates for AKI are widely discussed as the variation in reported numbers is high, very likely due to missing analysis and documentation of serum creatinine values and thereby diagnosis of AKI (79,80). However, incidences of AKI as high as 1811 per million population have been reported (81).

CKD

The conceptual understanding of the CKD model includes initial kidney damage, which is followed by a decrease in glomerular filtration rate (GFR) and finally results in kidney failure (82). This process takes a long time including a latency period without symptoms, after which CKD complications lead to late onset of symptoms (82). This model also plays into definition criteria of CKD. These criteria include kidney damage over a period of more than 3 months, whereby kidney damage is defined as structural or functional abnormality of the kidney and can present with or without GFR decrease, or the presence of GFR<60 ml/min/1.73 m^2 over a period of more than 3 months, with or without the presence of kidney damage (83). The period of 3 months was introduced in order to discriminate acute from chronic kidney disease.

Structural and functional abnormalities include urine abnormalities such as albuminuria, abnormalities in blood and imaging as well as a history of kidney transplantation. Out of these, albuminuria thereby reflects one of the most important pathological findings, as it reflects increased glomerular permeability and has been shown to be an early marker of kidney damage due to diabetes, hypertension and glomerulopathies. Importantly, it is known that high albuminuria is associated with progression of renal and cardiovascular disease thereby leading to adverse outcomes. Vice versa slow progression can be achieved by albuminuria-decreasing therapies (82). GFR on the other side is the well-accepted indicator of kidney function. While young people rarely show low levels, these low levels of GFR are paired with an increased complication rate as for example increased cardiovascular morbidity and mortality (82). Clinical practise utilizes creatinine based GFR equations, however the use of serum creatinine values alone is invalid.

Today, CKD is classified by cause, GFR category and category of albuminuria as follows: GFR categories are G1 (≥90 ml/min/1.73 m^2, normal or high), G2 (60-89 ml/min/1.73 m^2, mildly decreased), G3a (45-59 ml/min/1.73 m^2, mildly to moderately decreased), G3b (30-44 ml/min/1.73 m^2, moderately to severely decreased), G4 (15-29ml/min/1.73 m^2, severely decreased) and G5 (<15 ml/min/1.73 m^2, kidney failure) (84). Emphasis should be put to the separation of CKD stage 3 into CKD G3a and G3b. The introduction of these two groups owes its rationale to an increase in mortality rate within

stage 3 (85). Specifically, the risk of death, cardiovascular events and hospitalization increases tremendously between CKD G3a and G3b (86). The subdivision into these two groups therefore aims to detect patients at high risk immediately.

Albuminuria ranges are divided into A1 (<30 mg/g creatinine, normal to mildly increased), A2 (20-300 mg/g creatinine, moderately increased) and A3 (>300 mg/g creatinine, severely increased) (84). Symptoms usually occur only when CKD is already progressed.

While laboratory findings reveal increased serum creatinine values, abnormal urine protein findings might be obvious. When further advanced, edema, anaemia, abnormal blood electrolytes, mineral bone disease, hypertension and signs of uraemia such as nausea, vomiting and a confusional state. The burden of these complications, the high prevalence of CKD in the overall population as well as being a major global health issue, highlight the need for knowledge on disease origin and risk factors which might lead to CKD and ESRD.

Today, diabetes mellitus (DM) is recognized as the leading cause of CKD and ESRD (87). Hyperfiltration and molecular changes cause pathological changes and ultimately, diabetic nephropathy. In this context, albuminuria has high significance, since it is a main predictor of mortality in type 2 DM, even if cardiovascular risk factors and GFR decrease are evaluated separately (88). Thus, considering that many patients with type 2 DM already show albuminuria at the time of diagnosis (89) and as the high percentage of 50% of patients with type 2 DM will develop diabetic nephropathy, a high percentage of these patients will progressively loose kidney function (87). A not less important risk factor for the development of CKD is hypertension, however an important consideration is the fact that hypertension might also be a consequence of CKD. The relative risk of developing ESRD increases dramatically with increasing severity of hypertension (90,91). Therefore, hypertension not only leads to GFR lowering, but also is a strong independent risk factor for ESRD. Mechanisms contributing to renal damage due to hypertension can be seen in the systemic load of blood pressure, its degree of transmission to kidney vasculature and finally the local susceptibility to barotrauma (92). Mechanical stretch of glomerular capillaries and mesangial cells develops from the increased pressure load. As a result, repair mechanisms are initiated, which might lead to glomerulosclerosis (92). Low birth

weight causing low numbers of nephrons can lead to intraglomerular hypertension in the remaining nephrons and can therefore be seen as an independent risk factor (93). Itself also standing in close relationship to hypertension, however being a modifiable risk factor, obesity has been shown to be related to the development of CKD as well. Obesity (BMI $\geq$ 30 kg/m^2) and even overweight (BMI $\geq$ 25 kg/m^2) at age 20 lead to a three-fold and three to four-fold increase in the risk of developing CKD, respectively (94). Gender, ethnicity and age have to be considered when evaluating risk factors. It is known that men develop ESRD more frequently than women and so do African Americans as compared to Caucasians (87). As renal function declines with age (87), elderly are more likely to suffer from CKD, especially as they might not have the same starting position as younger individuals when it comes to renal events on top of anyway decreased renal function. Besides several genetic factors such as those causing polycystic kidney disease, also family history excluding hereditary disorders seems to be a relative risk factor for the development of CKD (87). However, whether this stands in association with socioeconomic factors or not yet described genetic factors cannot be said. Socioeconomic status as well as a history of smoking will have some impact on the development of CKD. Last but not least, the third most common kind of kidney disease encompasses the group of glomerulonephritides, which will be discussed in the next chapter.

Glomerular disease and glomerulonephritis

Definition and classification

Glomerular disease encompasses a wide variety of diseases affecting the glomerulus, such as membranous nephropathy, podocytopathies and thrombotic microangiopathy. This part will focus on one subclass of glomerular disease, namely glomerulonephritis (GN). The diverse group of GN describes several pathologic states, which lead to an inflammatory state of the glomerulus, although through different mechanisms. Based on kidney biopsy findings, GN can be classified into several entities: immune-complex GN, pauci-immune GN, antiglomerular basement membrane (anti-GBM) GN, monoclonal Ig GN and C3 glomerulopathy (95). A focus on pathogenesis rather than biopsy allows the broader classification of glomerular disease including GN into three groups. The group of primary glomerulopathies includes minimal change disease, idiopathic membranous nephropathy, IgA nephritis, primary focal segmental glomerulosclerosis (FSGS), acute poststreptococcal glomerulonephritis and anti-GBM GN. Secondary glomerulopathies are defined by the presence of a multisystemic disease with impact on the glomerulus and include diabetic nephropathy, lupus nephritis, antineutrophil cytoplasmatic antibody (ANCA)-associated vasculitis, amyloidosis, infection-related glomerulonephritis and type 1, 2 or 3 cryoglobulinemia. Finally the group of secondary FSGS is defined by sclerosis due to loss of nephrons and resulting overperfusion of remaining glomeruli and includes glomerular diseases with severe nephron loss, but also nonglomerular kidney diseases (96).

Pathogenic type	Specific Disease Entity	Pattern of Injury
Immune-complex GN	IgA nephropathy, IgA vasculitis, lupus nephritis, infection-related GN, fibrillary GN with polyclonal Ig deposits	Mesangial, endocapillary, exudative, membranoproliferative, necrotizing, crescentic, sclerosing or multiple
Pauci-immune GN	MPO-ANCA GN, proteinase 3-ANCA GN, ANCA-negative GN	Necrotizing, crescentic, sclerosing or multiple
Anti-GBM GN	Anti-GBM GN	Necrotizing, crescentic, sclerosing or mixed
Monoclonal Ig GN	Monoclonal Ig deposition disease, proliferative GN with monoclonal Ig deposits, immunotactoid glomerulopathy, fibrillary GN with monoclonal Ig deposits	Mesangial, endocapillary, exudative, membranoproliferative, necrotizing, crescentic, sclerosing or multiple
C3 glomerulopathy	C3 GN, dense deposit disease	Mesangial, endocapillary, exudative, membranoproliferative, necrotizing, crescentic, sclerosing or multiple

Table 3 – Classification of GN Adapted from Sethi S. JASN, 2015, 27(5): 1278-87.

Table 3 describes specific disease entities and injury patterns of GN classes. Polyclonal IgG deposits, which can be accompanied by complement deposition, are characteristic for immune-complex GN. IgA nephropathy, lupus nephritis, fibrillary GN but also post infectious GN count into this class. Typical pattern of glomerular injury in immune-complex GN may be mesangial proliferative, endocapillary proliferative, exudative, membranoproliferative, necrotizing, crescentic, sclerosing, or a combination of these pattern (95). The name pauci-immune GN stems from the absent or low IgG deposition

pattern found histologically. However, serologic evidence of ANCAs can be found in 80 to 90% of patients. For patients with existing ANCAs, antibody targets can be found to be myeloperoxidase or proteinase 3. Within the 2012 Revised International Chapel Hill Consensus Conference Nomenclature of Vasculitis, ANCA GN was defined into the categories of microscopic polyangiitis, granulomatosis with polyangiitis and eosinophilic granolumatosis with polyangiitis (97). Necrotizing, crescentic, sclerosing or multiple patterns of glomerular injury can be found (95). In anti-GBM GN, typical linear deposits of immunoglobulins but also C3 are found on the GBM (98). A necrotizing/crescentic pattern is typical for patients suffering from anti-GBM GN (95). A monoclonal gammopathy or paraproteinaemia is the underlying cause of monoclonal Ig GN, in which monotypic Ig deposits are found in the glomeruli (99) in most patients. Necrotizing, sclerosing, crescentic, mesangial and diffuse proliferative, as well as the most common membranoproliferative pattern can be found (95). In C3 glomerulopathy, glomerular C3 deposits are dominant (100) and may be categorized by electron microscopy. The variable pattern includes mesangial, endocapillary, exudative, membranoproliferative, necrotizing, crescentic, sclerosing or multiple of these appearances (95).

Clinical presentation and differential diagnosis of glomerular disease

Besides the presence of edema and elevated blood pressure, the most common diagnostic tool used for examination, diagnosis and classification of glomerular diseases in clinical practise is the urinary sediment. The presence of glomerular haematuria and/or proteinuria thereby directs the assumption of glomerular disease. Most importantly, the entities of nephritic and nephrotic sediments are defined and used in clinical routine, although they represent a spectrum of features rather than a strict classification into two groups. Nephritic sediment is defined by the presence of >5 red blood cells per high power field and the presence of at least one acanthocyte, red cell cast or mixed red cell/white cell cast. Acanthocytes and cast formation are hallmarks of glomerular haematuria, as they indicate their passage through the glomerular filtration barrier. Acanthocytes are characteristic due to distortion after this passage and can be identified by their distinct form and shape by standard and phase contrast microscopy. Cellular casts are the result of red and white blood cells being forced through the filtration barrier and might also be found in the urine of nephritic patients (96). Nephrotic proteinuria in the adult individual is

defined as urinary protein excretion >3.5 g/24 hours. The diagnosis of severe nephrotic syndrome is vastly described by proteinuria >10 g/24 hours, serum albumin <2.5 g/dl and the existence of severe edema (96). Hyperlipidaemia, lipiduria (101) and abnormal coagulation accompany these findings.

While the underlying disease of nephrotic syndrome is typically a noninflammatory glomerulopathy and severe nephrotic syndrome is a characteristic of diseases like idiopathic membranous nephropathy and minimal change disease, nephritic sediments can for example typically be found in rapid progressive forms of GN (RPGN). RPGN particularly is associated with signs of glomerulonephritis such as haematuria, proteinuria and red-cell casts, but the hallmark of this disease is crescent formation in glomeruli. It is defined by a rapid loss of renal function, which might lead to renal failure within a very short time period (102).

Differential diagnosis should in any case consider the degree of existing proteinuria in a patient and as a next step focus on the presence or absence of nephritic sediment (96). Based on the critical importance in time efficiently finding the correct diagnosis for a patient's condition if considering glomerular disease in order to provide correct treatment, broad testing including kidney biopsy, routine laboratory values and testing for specific markers may be employed. Table 4 and Table 5 summarize the most important directions in order to determine correct diagnosis in patients initially presenting with overt or minor proteinuria.

Overt proteinuria (>1 g/24h)			
Nephritic sediment		**No nephritic sediment**	
Evidence of multisystem disease	**No evidence of multisystem disease**	**Evidence of multisystem disease**	**No evidence of multisystem disease**
• Diabetic nephropathy • SLE GN Class II-V • ANCA-related vasculitis • Infection-related GN • Cryoglobulinaemia Types I, II, III • Thrombotic microangiopathy, Antiphospholipid syndrome, Scleroderma • Henoch-Schönlein purpura • Goodpasture's disease • Renal atheroembolism • Fabry's disease	• IgA nephritis • Alport's syndrome • Acute poststreptococcal GN • Idiopathic MPGN Type I or C3 glomerulopathy • Fibrillary/immuno-tactoid GN • Monoclonal immune deposition disease • Proliferative GN with monoclonal IgG deposits • Idiopathic thin/thick GBM disease	• Diabetes mellitus • SLE-GN Class V • Amyloidosis • Scleroderma • Mitochondrial disease • Fabry's disease • IgG4 disease	• Idiopathic membranous nephropathy • Minimal change disease • Primary FSGS • Renal tubular light chain crystal deposition • Nail-Patella syndrome • Collagenofibrotic (Type III) nephropathy • Dent's disease • C1q glomerulopathy

Table 4 – Differential diagnosis of glomerular disease in patients with overt proteinuria Adapted from Hebert LA. Am J Nephrol, 2013; 38(3):253-66.

<table>
<tr><th colspan="3">Minor proteinuria (<500 mg/24h)</th></tr>
<tr><th colspan="2">Nephritic sediment</th><th>No nephritic sediment</th></tr>
<tr><th>Evidence of multisystem disease</th><th>No evidence of multisystem disease</th><td rowspan="2">Orthostatic (postural) proteinuria
Exercise-induced proteinuria
Hypertensive nephrosclerosis in (African ancestry)
Mild forms of diseases listed for patients with overt proteinuria, no nephritic sediment and evidence or missing evidence of multisystem disease</td></tr>
<tr><td>Diabetic nephropathy
Mild forms of diseases listed for patients with overt proteinuria, nephritic sediment and evidence of multisystem disease</td><td>Idiopathic thin or thick GBM disease
IgA nephritis
Alport's syndrome
Mild forms of diseases listed for patients with overt proteinuria, nephritic sediment and no evidence of multisystem disease</td></tr>
</table>

Table 5 – Differential diagnosis of glomerular disease in patients with minor proteinuria Adapted from Hebert LA. Am J Nephrol, 2013; 38(3):253-66.

Resident cellular drivers of glomerular disease pathology

The proliferation of resident cells and infiltration by leukocytes, both resulting in increased glomerular cellularity, are the main characteristics of glomerulonephritis (95). It is, however, not only leukocytes, which will be discussed in the context of GN in the next chapter, but also resident kidney cells that play a major part in this pathology. While many resident cells have distinct roles under physiologic conditions, these roles may change in disease.

Under steady state conditions, mesangial cells contribute to the maintenance of glomerular structure, but also to mesangial matrix homeostasis. In IgA nephropathy, where mesangial IgA deposition is present, mesangial proliferation and injury can be found, leading to remodelling. Mesangial hypertrophy on the other hand is observed in diabetic nephropathy. Podocytes represent differentiated cells and lack the ability of regeneration. If podocytes are lost, the glomerular filtration barrier will be affected and this loss also contributes to the establishment of glomerulosclerosis. While podocytes can be affected by many causes, two primary manifestations are minimal change disease and FSGS, where apoptosis and foot process effacement can be found, respectively. Loss of podocytes and detachment from the GBM is also found in diabetic nephropathy (103). Glomerular endothelial cells are responsible for selective permeability and filtration under physiologic conditions, based on the existence of fenestrations in the glomerular endothelium. Classically, endothelial damage is found in rapid progressive forms of GN. Fenestration loss is seen in lupus nephritis class 3 and 4 for example. Inflammatory stimuli induce even transcellular holes and widening of cell-cell junctions as seen in haemolytic uraemic syndrome, as these cells are known to be extremely sensitive to complement induced injury. Parietal epithelial cells line the Bowman's capsule. Other than podocytes, parietal epithelial cells have the ability of proliferation. Crescent formation and apoptosis are promoted by their proliferation in RPGN (103).

Anti-GBM GN and the murine model of NTS

Anti-GBM disease is a well defined cause of RPGN and the most aggressive form of glomerulonephritis (104), although it occurs rather rarely. Anti-GBM disease is caused by autoantibodies against the GBM, distinctively the α3 chain of type IV collagen. The two sequences E_a and E_B have been described as conformational autoantitopes (105). If RPGN occurs in conjunction with lung haemorrhage, the disease is referred to as Goodpasture's syndrome (106). Autoantibodies are considered to be pathogenic, as also the levels of circulating antibodies are correlating with renal disease pathology (107).

Pathologically, in about 95% of patients, crescents can be found at the time of diagnosis (104) and linear IgG staining via immunofluorescence is used for diagnosis of anti-GBM disease (108), highlighting the importance of renal biopsy for adequate diagnosis of the disease. To date, corticosteroids, cyclophosphamide and plasmapheresis represent applied therapeutic options. However, prognosis is poor especially for patients in need for dialysis over the course of the disease (108). This emphasizes the need for precise knowledge of pathologic processes in order to find more efficient therapeutic targets. The need to study these pathologic processes required for a system combining the main features of human crescentic glomerulonephritis in an easy accessible format.

Representing the closest to the human equivalent of anti-GBM disease, nephrotoxic serum nephritis (NTS) is a widely studied murine model of glomerulonephritis. It is induced by the injection of anti-GBM serum either produced in sheep or rabbit. Immunisation against IgG from the antiserum host may be performed in order to accelerate the disease (109). However, different protocols of disease induction can complicate the direct comparison of results. B cells contribute to many kidney pathologies via production of antibodies, which is of special interest in IgA nephropathy, but also in multiple myeloma (103). Importantly, the pathogenesis of NTS was described to be based on a delayed-type hypersensitivity reaction (110,111). Many important findings in the field of glomerulonephritis have been described in this model.

Leukocytes play an immense role in the pathogenesis of glomerular disease, especially when it comes to RPGN. In this context, leukocytes can be beneficial for disease resolution, but may also exacerbate pathology. $CD4^+$ T cells - especially Th1 and Th17

subsets - are, generally speaking, associated with macrophage and neutrophil attraction to the kidney. Th1 dependence in this model is well established (112) and Th1 cells are recruited to the kidney in a CXCR3 dependent manner (113) which further impacts macrophage infiltration (113). Interestingly, macrophages may act proinflammatory as M1 macrophages or anti-inflammatory as M2 macrophages in kidney disease. The Th17 $CD4^+$ cells are also involved in the pathogenesis of the disease. Th17 cells express the CC chemokine receptor (CCR) 6 and infiltrate kidneys of mice with NTS. Il-17A mediates the expression of C-X-C motif chemokine ligand 5 (CXCL5) by renal tubular cells which further leads to CXCL5 dependent neutrophil infiltration (114). Neutrophils are the most abundant cells in most glomerular diseases and can directly damage the glomerular filtration barrier through cytokines, proteinases and reactive oxygen species (103). However, neutrophils responding to CXCL5 are not the first neutrophils entering the stage during this process. Neutrophils migrating to CXCL1 were shown to be recruited in an early phase of NTS, which is not T cell-dependent. Interestingly, these early neutrophils mainly show a glomerular pattern (114). Conflicting data exist about the role of cytotoxic $CD8^+$ T cells and whether their action is proinflammatory (115,116), at least in the experimental setting of GN. In NTS, DCs fulfil their function in recruitment and activation and intriguingly, display proinflammatory properties on the one hand, while anti-inflammatory properties were found on the other hand (117). Early depletion of DCs aggravated NTS thus highlighting a renoprotective effect, which is possibly due to the induction of IL-10 production either by T cells or DCs themselves (118). Interestingly, at later stages of NTS and due to proteinuria, DCs capture more antigen leading to increased presentation to T cells and thereby expressing increased co-stimulatory molecules and proinflammatory cytokines, which ultimately favours inflammation (119). DCs are not the only cell population connected with antiinflammatory properties in NTS. Immunoregulatory mechanisms ascribed to the $CD4^+CD25^+foxp3^+$ Tregs take place in secondary lymphoid organs (120). Tregs attenuate NTS (120), and CCR7 expression on Tregs is paramount for their correct localization in the draining lymph node (121). Tregs produce IL-10 and in one study were even found in the kidney (122). Mast cells exert their function differentially in glomerular kidney disease, and act pro- and antiinflammatory, depending on whether they are found in the kidney or in secondary lymphoid organs (103), respectively. In NTS, they were found to act in an anti-inflammatory way (123,124). Their predominance in draining lymph nodes led to the finding that mast cells are recruited there by IL-9 produced from Tregs, and finally act there in conjunction with Tregs in a

immunoregultaory way (125). Additionally Stat3-programmed Th17-specific Tregs were found to limit Th17 cell action which is done via CCR6-mediated migration of Stat3-programmed Th17-specific Tregs (126). Finally, regulatory B cells have been described in GN, but this IL-10 producing cell type did not prove to play a substantial immunoregulatory role in the NTS model (127) and in a model of spontaneous systemic chronic lupus nephritis (128). There are many more cells included in the pathogenesis of the disease. γ/δ T cells for example, which represent only a small number of all circulating T cells, have also been shown to be pathogenic in NTS (129). Even innate lymphoid cells were found to be crucially involved in the pathogenesis of NTS. CXCR6 expressing invariant natural killer T cells are recruited via CXCL16 secretion by DCs early in the course of the disease. The attracted cells attenuate the disease most likely due to IL-4 and TGFβ expression (130,131).

Hypothesis and aims

The spleen encompasses various functions like antibody production and resolution, but also storage of red blood cells and lymphocytes, platelets and monocytes. Based on these diverse capabilities, the spleen could have either beneficial or detrimental effects in the pathogenesis of NTS. The null hypothesis therefore was that the spleen does not contribute to pathogenesis of the disease, and that splenectomy does not influence disease development as compared to sham-operated controls. The alternative hypothesis was that the spleen contributes to the pathogenesis of the disease, and that splenectomy influences disease development as compared to sham-operated controls.

Hence there were three primary aims defined: (A) to characterise the immunologic processes in the spleen in NTS (B) to evaluate the effects of splenectomy on NTS development and thereby (C) to define the extent of contribution of the spleen to NTS development.

Material and Methods

Induction of NTS and mice

For all experiments, C57BL/6J mice were used. These mice were either obtained from Charles River Laboratories (Sulzfeld, Germany) or Himberg (Vienna, Austria). Used mice were 8 to 12 weeks old and of male gender. They were kept in a virus-free environment at the Medical University of Graz. NTS experiments were induced as described previously (132): Mice were preimmunized with 2 mg/ml rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) dissolved in incomplete Freund´s adjuvant (Sigma-Aldrich, St. Louis, MO, USA) with addition of desiccated, nonviable Mycobacterium tuberculosis H37a (Difco Laboratories, Detroit, MI, USA) subcutaneously in the foot. Three days thereafter, 100 µl/mouse of heat-inactivated rabbit anti-mouse glomerular basement membrane serum, which was prepared as described previously (133), was injected intravenously via the tail vein (1).

Splenectomy and study design

C57BL/6J mice (8 to 12 weeks old) were used for splenectomy experiments. 6 - 8 mg/kg bodyweight xylazine and 90 - 120 mg/kg bodyweight ketamine were used to anesthetize mice. After a sickle-shaped incision was made on the central abdomen the spleen was exposed while carefully avoiding bowels and intestines. Next, preparation of the vascular bundles was performed and subsequently two ligations were placed on each bundle. Thereafter, the bundles were cut in order to remove the spleen. Sham-operated mice underwent the opening and preparation procedure as well, but without removal of the spleen (1). After a two-week period of recovery under close postoperative monitoring of the mice, NTS was induced and mice were either killed 14 or 28 days after disease induction.

Urinary albumin detection

For determination of urinary albumin excretion, a double-sandwich ELISA was used as reported previously (1,120). 96-well plates were coated with goat anti-mouse albumin antibody A90-134A-5 (Bethyl Laboratories, Montgomery, TX, USA) at a concentration of 1 µg/ml and incubated at 4°C overnight. The following day, plates were blocked with 0,5% BSA (SERVA Electrophoresis GmbH, Heidelberg, Germany) in PBS including 0,05% Tween20 after three washes in PBS/0,05% Tween. Urine samples and mouse albumin standard (Sigma-Aldrich) were pipetted in triplicates at 100 µl/well. Mouse urine samples were diluted in serial dilutions ranging from 1:100 to 1:100000 (day 0), 1:1000 to 1:1000000 (day 7) and 1:10000 to 1:10000000 (day 14). Standard was diluted to achieve concentrations of 1 µg/ml, 0,1 µg/ml, 0,03 µg/ml, 0,01 µg/ml and 0,001 µg/ml. After two hours of incubation at room temperature, plates were washed again three times and subsequently HRP-conjugated goat anti-mouse albumin antibody A90-134P-7 (Bethyl) was applied as a secondary antibody and incubated for another two hours. After three washes in PBS/0,05% Tween, 1 mg 3,3´,5,5´-Tetramethylbenzidine (TMB) completed with TMB-Buffer and 1% H_2O_2 was used as a substrate and the reaction was stopped using 50µl 2M H_2SO_4. Optical density (OD) was measured at 450 nm. A blank value was subtracted from raw data values and final concentration was determined using a four parameter logistic curve.

Urinary creatinine detection

Urinary creatinine was evaluated photometrically using a commercially available picric acid-based kit (Sigma-Aldrich) (1). Alkaline picrate solution was prepared using picric acid and sodium hydroxide in a 1:6 ratio. Urine samples diluted 1:10 in distilled H_2O (dH_2O) and creatinine standard were pipetted in triplicates on 96-well plates and incubated with alkaline picrate solution for twelve minutes at room temperature before OD was measured at 490 nm. Subsequently, acid reagent was added and optical density was read a second time after five minutes incubation at room temperature at OD 490 nm. Raw data were blank corrected, and calculation of creatinine values (mg/dl) was done using the following equation:

Creatinine (mg/dl)= (INITIAL Sample OD – FINAL Sample OD) / (INITIAL Standard OD – FINAL Standard OD)*0,3 (1).

Evaluation of kidney histopathology

For fixation, kidneys and spleens were placed in 4% formalin for two days. Thereafter, tissue was processed in ethanol and finally embedded in paraffin. Kidneys and spleens were cut in 4µm sections on a rotation microtome. Periodic-Acid-Schiff (PAS) and Haematoxylin-Eosin (HE) stainings were both performed after deparaffinization and hydration in Xylene and Ethanol. Briefly, for PAS (Sigma-Aldrich) stainings, kidney slides were immersed in Periodic Acid Solution for 5 minutes with subsequent rinses in dH_2O. Thereafter, slides were immersed in Schiff's Reagent for 15 minutes followed by washing in running tap water. Finally, slides were counterstained in Gill's Hematoxylin III (Sigma-Aldrich). For HE stainings, slides were stained with Gill´s Hematoxylin and Eosin, each step followed by subsequent washing steps. Finally, slides were dehydrated, cleared and mounted in anhydrous mounting medium Roti-Histokitt II (Carl Roth, Karlsruhe, Germany). When evaluating PAS-stainings semi quantitatively, a minimum of 50 glomerular cross-sections were evaluated as described previously (134). PAS^+ material was scored within glomeruli following a scoring system in which 0 = no PAS^+ material, 1 < 1/3 PAS^+ material, 2 = 1/3 - 2/3 PAS^+ material and 3 > 2/3 PAS^+ material (1). All evaluations of PAS stainings were performed in a blinded manner.

Immunohistochemistry

Kidneys and spleens were frozen in OCT medium and cut into frozen tissue sections (4µm) on a cryomicrotome for immunoperoxidase stainings. On both, kidneys and spleens, a three-layered immunoperoxidase staining technique was performed in order to assess tissue infiltrating and resident cells (1). Slides were stored at -20°C and warmed to room temperature 30 minutes prior to staining. Fixation was done using acetone (4°C) for eight minutes. Slides were then blocked in 20% fetal calf serum, 10% serum of second antibody

species and 200 µl/ml Avidin (Vector Laboratories, Burlingame, CA, USA) for 20 minutes. After two PBS washing steps and one washing step in gelatine, slides were incubated with respective first antibodies dissolved in PBS containing 200 µl/ml Biotin (Vector Laboratories). For kidneys, rat-derived primary antibodies for CD4 (clone YTS191.1; Serotec, Oxford, UK), CD8 (clone KT15, Serotec), CD68 (clone FA-11; Serotec) and an anti-Neutrophil antibody (clone NIMP-R14; Abcam) were used. Primary antibodies for spleen stainings were rat anti-mouse F4/80 (clone CI:A3-1; Serotec) and CD41 (clone MWReg30; BD Pharmingen, San Diego, CA, USA). After one-hour incubation, slides were washed three times in PBS and subsequently incubated with biotinylated goat anti-rat IgG secondary antibody (Jackson ImmunoResearch Laboratories) for 45 minutes. After another three times washing in PBS, slides were immersed in Vectastain ABC HRP kit (Vector Laboratories). Following three washes in PBS and one wash in 0,1M Sodium-acetate buffer, slides were immersed in 3-amino-9-ethylcarbazole substrate-chromogen for 6-8 minutes while careful observation of the reaction was performed under microscopic control. After another three washes in PBS, slides were counterstained in Gill's hematoxylin III (Sigma-Aldrich), and rinsed in tap water and dH_2O. Finally, slides were mounted in Aquatex mounting medium (Merck, Kenilworth, NJ, USA). All evaluations of immunohistochemistry were performed in a blinded manner. Kidney infiltrating $CD4^+$, $CD8^+$ T cells and neutrophils were counted in 6 high-power fields of renal cortex and medulla. For quantification of infiltrating macrophages, a semiquantitative scoring system was used as follows: 0 = 0 to 4 cells stained positive, 1+ = 5 to 10 cells, 2+ = 11 to 50 cells, 3+ = 51 to 200 cells and 4+ >200 cells stained positive per low-power field. Evaluation of F4/80 positive cells, which are indicative for the red pulp and appeared in great numbers in the spleen, was done with Aperio ImageScope software 11.1.2.760 (Leica Biosystems, Nussloch, Germany) and the positive pixel count algorithm (hue value: 0.1; hue width: 0.5; color saturation threshold 0.04). Prior to evaluation with the Aperio ImageScope software, slides were scanned with Aperio ScanScope AT (Leica Biosystems) (1).

Immunofluorescence stainings

Kidneys and spleens were frozen in OCT medium and cut into frozen tissue sections (4

µm) on a cryomicrotome. A minimum of four tissue samples was placed on slides, which were later evaluated for autologous IgG deposition in kidneys. Before staining procedures, slides were warmed to room temperature for a minimum of 30 minutes. Next, kidney and spleen tissue was fixed on slides using acetone (4°C) for 20 minutes. Subsequently, tissues were circled with a hydrophobic pen. Slides were blocked in Avidin (Vector Laboratories) for 15 minutes, followed by three washes in PBS for five minutes. Thereafter, slides were blocked in Biotin (Vector Laboratories) for 15 minutes and again washed three times in PBS. Slides were then blocked in PBS containing 10% donkey serum for 30 minutes. Without further washing, antibodies diluted in PBS were applied to the slides and incubated for 30 minutes. FITC conjugated antibody for $Ter119^+$ cells (clone TER-119; eBioscience, San Diego, CA) was used for the detection of erythroid precursor cells (1). Serial dilutions of FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) were dispensed on kidney sections in order to evaluate autologous IgG deposition in the kidney using direct immunofluorescence titre determination (1). All slides were mounted in mounting medium for fluorescence with DAPI (Vector Laboratories), sealed with nail polish and stored at 4°C.

Assessment of autologous and heterologous antibody responses

96-well plates were coated with 100 µg/ml rabbit IgG (Jackson ImmunoResearch Laboratories). After overnight incubation, plates were washed three times in PBS/0,05% Tween and blocked in PBS/0,05% Tween containing 0,5% BSA for 30 minutes. Plates were then incubated with serial-doubling dilutions of serum from peripheral blood and incubated for two hours. After three washes in PBS/0,05% Tween, HRP-conjugated goat anti-mouse IgG, goat anti-mouse IgG1, IgG2b or IgG3 (all from Jackson ImmunoResearch Laboratories) were used in order to detect mouse anti-rabbit IgG and respective subclasses. HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoRe- search Laboratories) was used when assessing circulating rabbit immunoglobulin (1). After the two-hour incubation time, plates were washed three times in PBS/0,5% Tween. 1 mg 3,3´,5,5´-Tetramethylbenzidine (TMB) completed with TMB-Buffer and 1% H_2O_2 was used as a substrate and the reaction was stopped using 50 µl 2M H_2SO_4. OD was measured at 450 nm. A blank value was subtracted from all values. OD values for each dilution were compared directly between

the groups.

Assessment of serum IFN-γ, Il-6 and TNF-α

IFN-γ, Il-6 and TNF-α levels in serum were determined using commercially available ELISA kits (all from BD, San Jose, CA, USA) (1). Shortly, 96-well plates were coated with respective capture antibody in concentrations suggested by the company and incubated at 4°C overnight. Plates were then washed for a minimum of three times in PBS/0,05% Tween and blocked in PBS containing 10% FCS for one hour at room temperature. Plates were washed in PBS/0,05% Tween again for a minimum of three times and samples and standards as recommended by the company were pipetted into wells. After two hours of incubation at room temperature, plates were washed in PBS/0,05% Tween five times. Working detector containing respective detection antibody and Streptavidin-horseradish peroxidase conjugate were applied to plates and incubated for one hour. Plates were washed in PBS/0,05% Tween for a minimum of seven times with increased soaking time and subsequently substrate solution containing TMB and hydrogen peroxide was pipetted into wells and incubated for 30 minutes at room temperature in the dark. 2M H_2SO_4 was used as stop solution and plates were read at OD 450 nm with wavelength correction (subtraction of absorbance at 570 nm). Concentrations of IFN-γ, Il-6 and TNF-α were determined from standard curves using log-log regression analysis.

RNA isolation, Reverse transcription and Reverse transcription (RT) real-time polymerase chain reaction (PCR)

Total RNA was extracted from spleens and kidneys using TRI Reagent (Sigma-Aldrich) (1). Snap frozen tissue was cut into small pieces and homogenized in 1 ml TRI Reagent using 18G needles and 1 ml syringes. 200 µl Chloroform were added, shook and incubated for eight minutes at room temperature. Suspensions were then centrifuged for 15 minutes at 12000xg at 4°C. After centrifugation, the transparent supernatant was transferred and incubated for eight minutes with isopropanol. After another centrifugation at 12000xg for eight minutes at 4°C, one ml of 70% ethanol was added and samples were centrifuged for

five minutes at 7000xg. Supernatant was discarded and 500 µl of Ethanol were added before a final centrifugation step for five minutes at 7000xg was performed. Ethanol was completely removed and the pellet was dissolved in 20 µl dH_2O before isolated RNA was frozen to -20°C and thawed again. Concentration of RNA was determined photometrically. cDNA was synthesized using Superscript III Transcription Kit (Invitrogen, Carlsbad, CA, USA) (1). 2 µg of total RNA were incubated with 0,5 µl random primers (Invitrogen), 1 µl 10mM dNTPs and dH_2O for five minutes at 65°C. Subsequently, samples were put on ice and 7 µl mastermix containing of 4 µl 5x First-Strand Puffer, 1 µl DTT, 1 µl RNaseOUT recombinant RNase Inhibitor and 1 µl SuperScript III reverse transcriptase as recommended by the company were added. Samples were incubated for five minutes at 25°C, followed by 45 minutes at 50°C and 15 minutes at 70°C in a thermocycler. cDNA was dissolved in 150 µl dH_2O and stored at -20°C. In order to quantify gene expression on mRNA level, real-time PCR was performed in duplicates on a CFX96 Real-Time System (BioRad, Hercules, CA, USA). For quantification of chemokines, TaqMan gene expression assays (Applied Biosystems, Foster City, DA, USA) were used for CXCL12 (Mm00445553_m1), CCL2 (Mm00441242_m1), CCL7 (Mm00443113_m1) and 18S RNA (Mm03928990_g1), which was used as a reference for spleen samples. Erythropoietin was quantified using the TaqMan gene expression assay Epo (Mm00433126_m1) There, 8 µl of TaqMan 2x Universal PCR Master Mix (Applied Biosystems) was mixed with 6,2 µl of cDNA and 0,8 µl of respective TaqMan probe. Hprt served as a reference gene for bone marrow and kidney samples and was assessed using SYBR Green Mastermix (Invitrogen) with 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´. There, 5 µl Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) were used with 0,2 µl of respective primers and 4,6 µl cDNA. Quantification of genes was done using the $\Delta\Delta C_T$ method (1).

Flow cytometry

Spleens, femurs and lymph nodes were harvested and kept in PBS on ice. Spleens and lymph nodes were cut, weighed and mashed through 70 µm cell strainers using ice cold PBS in 50 ml tubes. Femurs were carefully cut open at the ends and flushed with 5 ml PBS. Flushed cells were carefully resuspended using 24G needles and 1 ml syringes. After

centrifugation for 5 minutes at 300xg, cells were mashed through 70 µm cell strainers using ice cold PBS in 50 ml tubes. Cell suspensions were centrifuged at 250xg for 10 minutes and pelleted cells were resuspended in FACS buffer (0,5% FCS, 2 mM EDTA in PBS). 100 µl of cell suspension were transferred to V-bottom plates, centrifuged for 5 minutes at 300xg and stained with antibodies in 100 µl FACS buffer for 30 minutes as follows. Spleens and bone marrow were stained with APC-conjugated anti-human/mouse CD44 (clone IM7; eBioscience), FITC-conjugated anti-mouse Ter119 (clone TER-119; eBioscience) and eFluor450-conjugated anti-mouse CD184 (Cxcr4) (clone 2B11, eBioscience). Both, spleen and lymph node populations, were stained using the following antibodies: APC-conjugated rat anti-mouse CD4 (Clone RM4-5; BD Biosciences, San Jose, CA, USA), FITC-conjugated anti-mouse CD8a (clone 53–6.7; BioLegend, San Diego, CA, USA), PE-conjugated anti-mouse CD69 (clone H1.2F3; BioLegend) PE-conjugated anti-mouse B220 (clone RA3-6B2; BioLegend), and eFluor450-conjugated anti-mouse CD11b (clone M1/70; eBioscience). Fixable viability Dye eFluor 506 (eBioscience) was used in order to exclude dead populations (1). After incubation, plates were centrifuged at 300xg for 5 minutes and washed with 100 µl PBS for three times. Thereafter, stained cells were resuspended in 500 µl FACS buffer and transferred to flow cytometry tubes. 25 µl of Countbright counting beads (Thermo Fisher Scientific, Waltham, MA, USA) were added per tube for quantitative analysis. Samples were analysed on LSRII and FACSCalibur cytometers (both BD Biosciences) (1).

Measurement of haemoglobin

After euthanasia, mice were bled and approximately 50 µl of blood were collected in EDTA precoated collection tubes and analysed on a haematology analyser Melet Schloesing MS9-5 (Diamond Diagnostics, Holliston, MA, USA) on the day of harvesting.

Statistical analyses

GraphPad Prism 6.0 for Macintosh (GraphPad Software, La Jolla, CA, USA) was used for all statistical analyses and results are shown as means ± SEM. Kolmogorov-Smirnov

test with Dallal-Wilkinson-Lillifors correction and the Shapiro-Wilk normality test were used for normal distribution testing. If two groups were compared an unpaired t-test was used according to the distribution. A two-tailed $p<0.05$ was considered statistically significant. If three or more groups were compared, ANOVA or the Kruskal-Wallis test was performed. In case of detected significance, two tailed t-tests or nonparametric Mann-Whitney U test were performed and the Bonferroni method was used to adjust for multiple testing (1).

Ethics statement

All animal experiments were approved by the Committee on the Ethics of Animal Experiments of the Austrian Ministry (BMWF-66.010/o121-II/3b/2014). All animal experiments were conducted under strict adherence to the laws of Austria (BGBl. I Nr. 118/2004). All efforts were made to minimize suffering (1).

Results – Findings

Changes in spleen morphology caused by NTS and immunisation

Secondary lymphoid organs are crucial players in the pathogenesis of NTS (120,121,125). Although the distinct role of the spleen in NTS - compared to the role of the draining lymph nodes - was not elucidated so far, a tremendous increase in spleen size and weight was noticed after induction of NTS (1). This increase was dramatic 14 days after induction of NTS. Interestingly, if mice were only immunized and did not receive nephrotoxic serum injections three days after immunisation, the increase in spleen weight and size was even more evident (Fig. 1) (1).

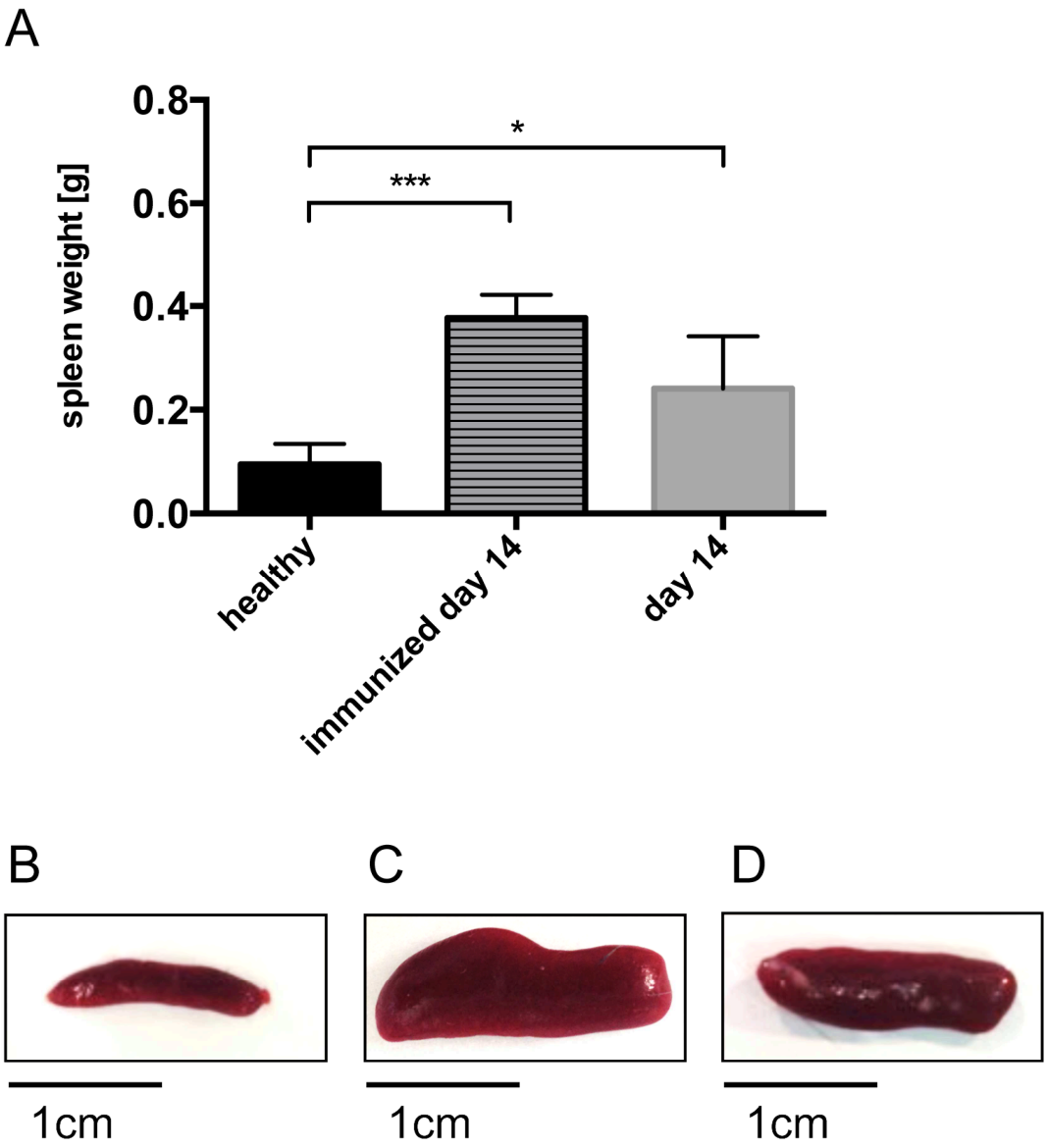

Figure 1 – NTS and immunisation cause increase of spleen size and weight

When compared to healthy mice, mice immunized for 14 days and nephritic mice on day 14 showed increased spleen weight. Healthy mice (n=7) are represented by a black bar, immunized mice (n=5) by a lined bar and nephritic mice on day 14 (n=7) by a grey bar. Volume increase is demonstrated by pictures of spleens from a healthy mouse (B), an immunized mouse (C) and a nephritic mouse on day 14 (D). Data are presented as means ± SEM. *p<0.05; ***p<0.001.

Development of NTS is not reliant on the presence of the spleen

Since NTS seemed to have a big impact on spleen size and weight, the question of a functional contribution to NTS development of this increased mass arose. In order to address this question, C57BL/6J mice were splenectomised or sham operated. After surgery, mice were allowed to recover over a period of two weeks before NTS was induced (1).

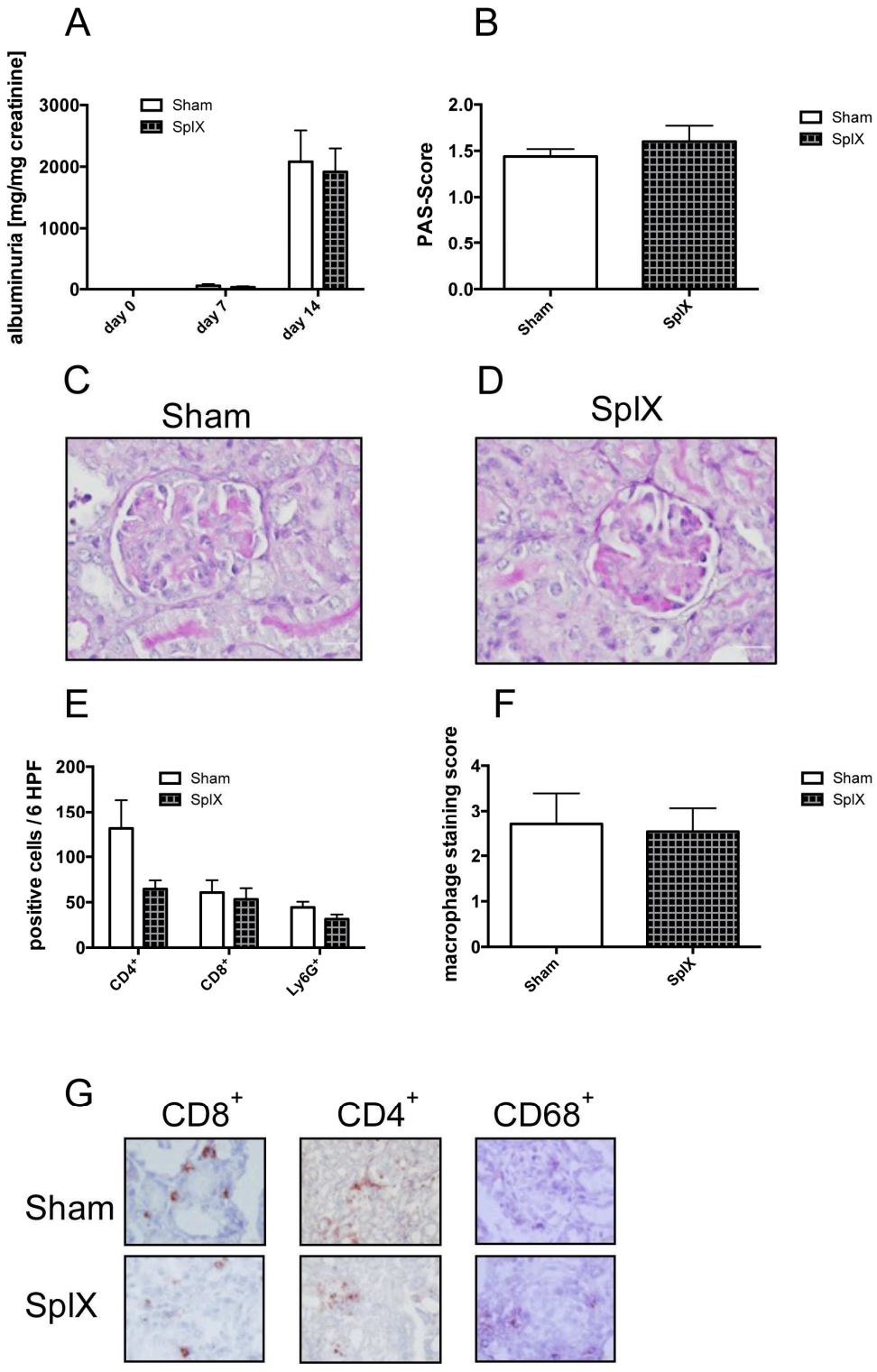

Figure 2 – NTS phenotype is not changed by splenectomy

Mice were either splenectomised as represented with white bars (n=5) or sham-operated as represented with squared bars (n=5). Albuminuria was measured before, 7 and 14 days

after NTS induction in splenectomised and sham operated mice (A). PAS Scores on day 14 of NTS were evaluated (B) and representative pictures of PAS-stained kidney sections from a sham-operated (C) and a splenectomised (D) mouse are shown. Quantification of kidney infiltrating $CD4^+$ and $CD8^+$ T cells (E), as well as $Ly6G^+$ neutrophils (E) and $CD68^+$ macrophages (F) was performed on immunohistochemically stained kidney sections from mice that underwent either sham-operation or splenectomy. Representative pictures of stainings for $CD8^+$ and $CD4^+$ T cells as well as for $CD68^+$ macrophages are shown for sham-operated and splenectomised mice (G). Data are presented as means ± SEM and are representative of three independent experiments with a minimum of n=4 in each group. Magnification for C, D and G is x1000, except for CD68 stainings, where magnification is x600.

While baseline albuminuria was not different between the two groups as expected, also no difference in albuminuria was detected on day 7 and 14 after NTS induction when sham-operated mice were compared to splenectomised mice (Fig. 2A) (1). Glomerulosclerosis as represented by PAS scores (Fig. 2B) did not differ between sham-operated (Fig. 2C) and splenectomised mice (Fig. 2D) (1). Additionally, cells infiltrating kidneys over the course of the disease were evaluated. $CD4^+$ and $CD8^+$ T cells (Fig. 2E, G) were quantified for their presence in nephritic kidneys on day 14, but did not show statistically significant differences between the two groups just as $Ly6G^+$ neutrophils (Fig. 2E) and $CD68^+$ macrophages (Fig. 2F, G) (1). As it is well established that lymph nodes are major places of immune regulation in NTS (120,121,124) and important for disease development, the influence of splenectomy on T cell populations in the draining lymph node was evaluated (Fig. 3).

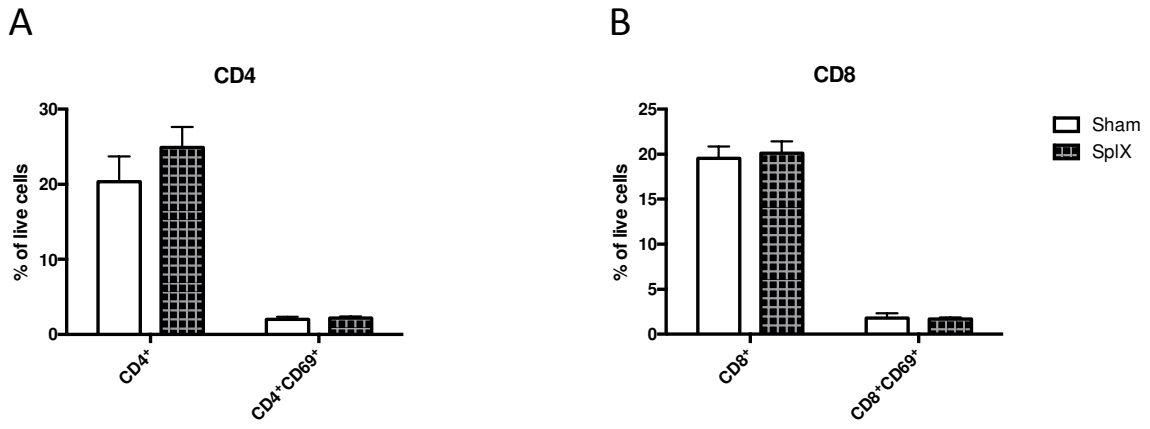

Figure 3 – T cell populations in draining lymph nodes are not changed after splenectomy

Draining lymph nodes were evaluated for their content of T cell populations 14 days after NTS induction in sham-operated (n=5) and splenectomised (n=5) mice by flow cytometry. Lymph nodes from both groups were stained for CD4 (A), CD8 (B) and the activation marker CD69 (A, B). Sham operated mice are represented by white bars, while splenectomised mice are represented by squared bars. All data is given as mean ± SEM.

$CD4^+$ and $CD4^+CD69^+$ T cells did not differ significantly in draining lymph nodes 14 days after NTS induction between sham-operated and splenectomised mice (Fig. 3A). Further, no difference was detected for $CD8^+$ and $CD8^+CD69^+$ T cells in draining lymph nodes between the two groups (Fig. 3B). Thus, neither was an impact of splenectomy on overall kidney pathology detected, nor did splenectomy influence T cell populations in the kidneys and lymph nodes after NTS induction.

B cell responses in NTS after splenectomy

The spleen is known to be involved in antibody production and clearance. Therefore, the impact of splenectomy on the development of NTS was evaluated.

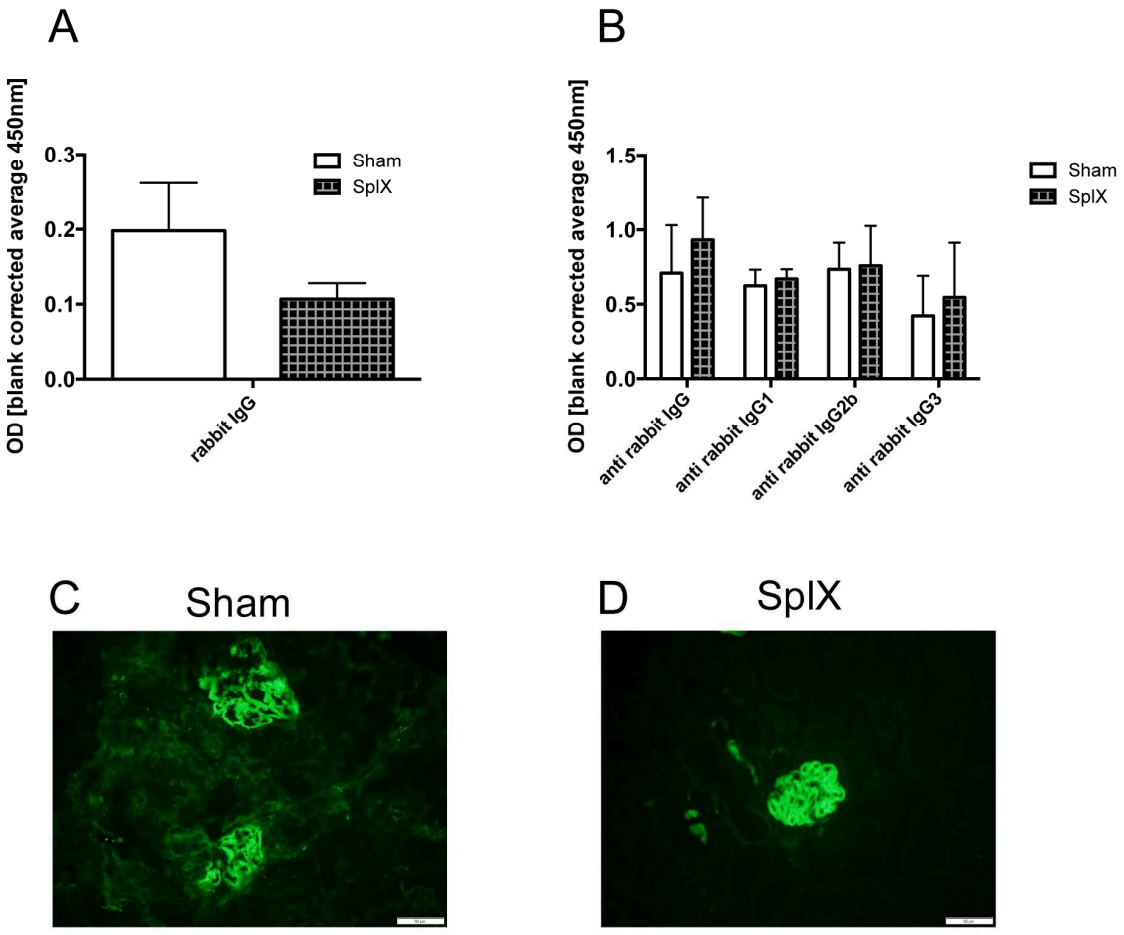

Figure 4 – IgG in serum and kidneys is not altered by splenectomy

Serum of sham-operated (white bars, n=5) and splenectomised mice (squared bars, n=5) was evaluated for circulating rabbit IgG (A), mouse anit-rabbit IgG (B) and mouse anti-rabbit IgG subclasses (B). Autologous mouse anti-rabbit IgG deposition was evaluated using immunofluorescence of mouse anti-rabbit IgG in kidney sections of sham-operated (C) and splenectomised (D) mice 14 days after NTS induction. Two representative pictures are shown (C, D). Data are represented as means ± SEM. Magnification x200.

Although the role of the spleen in antibody production and clearance of immune complexes is well established, there was no difference in rabbit IgG (Fig. 4A) and mouse anti-rabbit IgG and IgG subclass titre (Fig. 4B) in the serum of sham-operated and

splenectomised mice (1). Interestingly, also deposited mouse anti-rabbit IgG in glomeruli of sham-operated (Fig. 4C) and splenectomised (Fig. 4D) mice did not differ between the two groups (1).

Development of anaemia in sham-operated and splenectomised mice

Over time, nephritic mice develop anaemia, as did sham-operated mice on day 28, as expected. Splenectomised mice showed an even more dramatic decrease of haemoglobin levels when compared to healthy and immunized mice on day 14 (Fig. 5) (1).

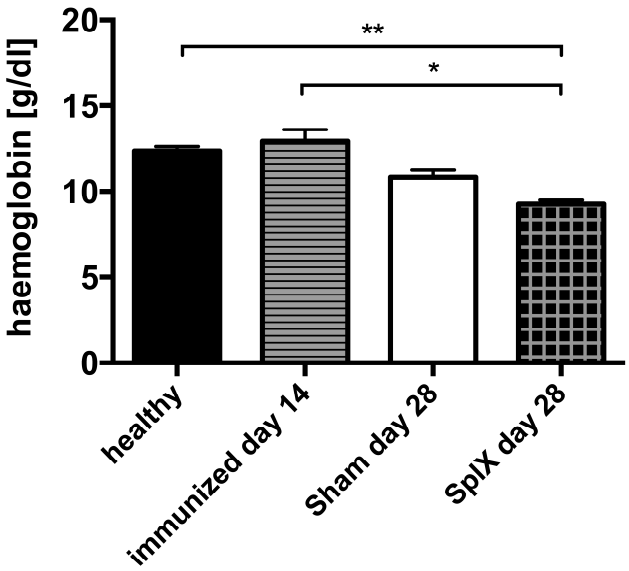

Figure 5 – Anaemia in NTS is aggravated by splenectomy

Bloodsamples from healthy mice (black bar, n=4), immunized mice (lined bar, n=4), sham-operated mice on day 28 after NTS induction (white bar, n=4) and splenectomised mice on day 28 after NTS induction (squared bars, n=4) were evaluated for anaemia. Levels of haemoglobin are given as means ± SEM. *$p<0.05$; **$p<0.01$.

Additionally, gene expression of erythropoietin was evaluated on mRNA level in kidney tissue (Fig. 6), but neither was a significant difference in erythropoietin levels detected between healthy and nephritic mice, nor was a statistically significant difference between sham-operated and splenectomised mice on day 14 and 28 (Fig. 6) found. Therefore, reduced erythropoietin production due to prolonged kidney disease as a cause for anaemia can be excluded in this case.

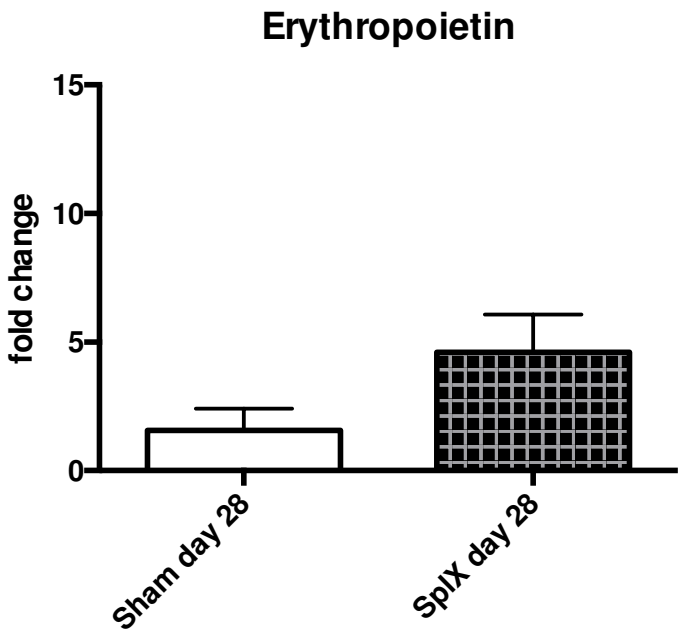

Figure 6 – Erythropoietin levels are not changed significantly due to splenectomy and nephritis

Erythropoietin levels were measured on mRNA level in kidneys of sham-operated (white bars, n=4) and splenectomised mice (squared bars, n=4) on day 28. Data are given as mean ± SEM.

Leukocyte populations in immunized and nephritic spleens

Based on increased spleen size and weight in NTS, the attempt was to identify cell populations responsible for this increase. Therefore, different splenic cell populations were evaluated in nephritic mice using flow cytometry (Fig. 7) (1).

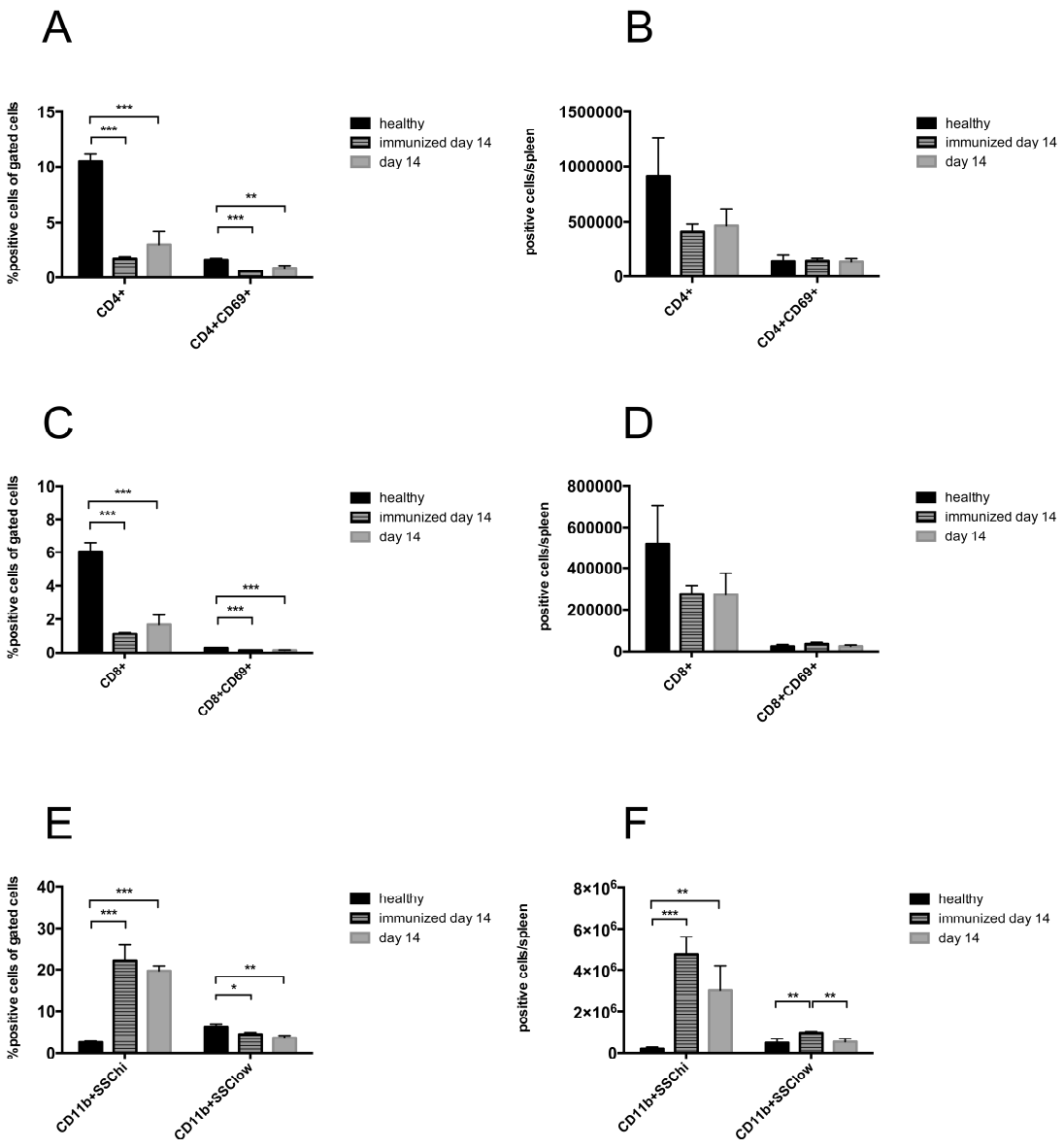

Figure 7 – Splenic leukocyte populations over the course of NTS

Spleens of healthy mice (black bars, n=4), mice immunized for 14 days (lined bars, n=4) and nephritic mice 14 days after NTS induction (grey bars, n=4) were evaluated for the presence of leukocyte populations by flow cytometry. Relative (A, C) and quantitative (B, D) evaluation of $CD4^+CD69^+$ T cells (A, B) and $CD8^+CD69^+$ T cells (C, D) are shown. Relative (E) and quantitative (F) analysis of $CD11b^+SSC^{lo}$ monocytes and $CD11b^+SSC^{hi}$ neutrophils (E, F) are given. Data are represented as means ± SEM. *p<0.05; **p<0.01; ***p<0.001.

First, T cell populations were evaluated in healthy and immunized spleens as well as in spleens of nephritic mice. Relative numbers of $CD4^+$ T cells and activated $CD4^+CD69^+$ T cells as well as $CD8^+$ T cells and activated $CD8^+CD69^+$ T cells decreased significantly when gated on all cells (Fig. 7A, C) (1). Although the decrease did not reach statistical significance, $CD4^+$ and $CD8^+$ T cells did decrease in spleens of immunized and nephritic mice on day 14 in absolute numbers as well (Fig. 7B, D) (1). Compared to spleens of healthy mice, monocytes as reflected by $CD11b^+SSC^{low}$ cells decreased significantly in spleens of immunized and nephritic mice after 14 days of immunisation or NTS induction (Fig. 5E) (1). Interestingly, $CD11b^+SSC^{low}$ cells increased significantly in absolute numbers in spleens of immunized mice only when compared to healthy mice and mice 14 days after NTS induction (Fig. 5F) (1). Neutrophils as reflected by the $CD11b^+SSC^{hi}$ population were the only cell population evaluated which increased in spleens of immunized mice and nephritic mice after 14 days of NTS in relative and quantitative evaluation when compared to healthy controls (Fig. 5E, F) (1). Additionally, no differences in $B220^+$ B cells were found in spleens in relative numbers between healthy mice and nephritic mice on day 14 (1). Next, spleens of healthy, immunized and nephritic mice on day 14 were evaluated histologically for their distribution of red and white pulp areas (Fig. 8).

Extramedullary haematopoiesis develops in spleens of immunized and nephritic mice

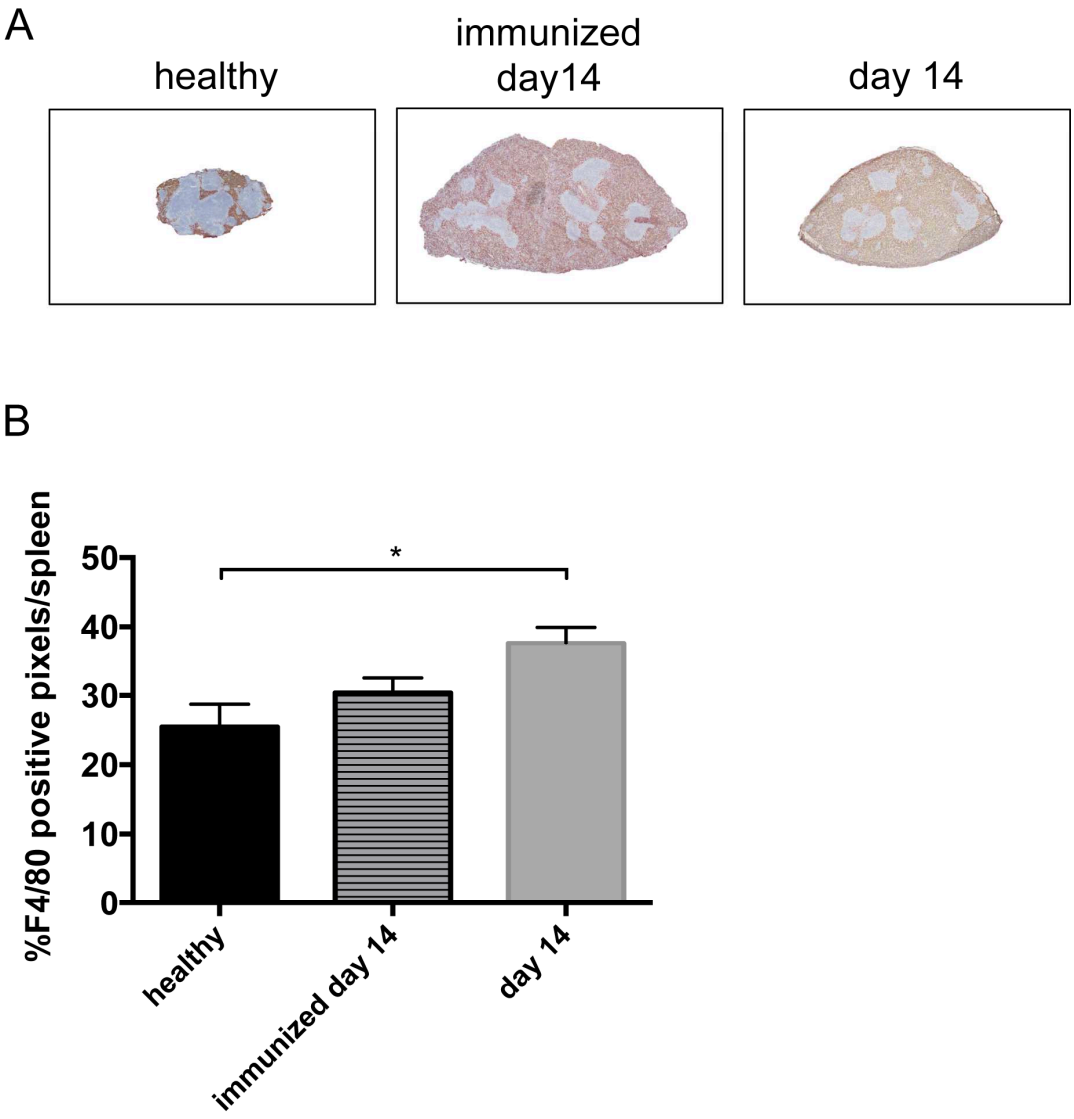

Figure 8 – Red and white pulp distribution in spleens of healthy, immunized and nephritic mice

Healthy mice (black bar, n=5), immunized mice (lined bar, n=5) and nephritic mice on day 14 (grey bar, n=5) were evaluated for their splenic content of the red pulp as indicated by positive staining for F4/80 (A). Representative pictures are shown. Magnification x40 (A).

Red pulp evaluation by means of F4/80 positive cell quantification (B). All data are given as means ± SEM. *p<0.05.

The quantification of the red pulp was done by staining spleens for F40/80, which is highly expressed by red pulp macrophages. However, F4/80 expression on monocytes and monocyte-derived macrophages is low (135). Quantification revealed a dramatic increase of the red pulp of spleens in mice 14 days after NTS induction as compared to healthy mice (Fig. 8A, B) (1). The red pulp of immunized mice increased in comparison to healthy mice as well, but this increase did not reach statistical significance (Fig. 8B).

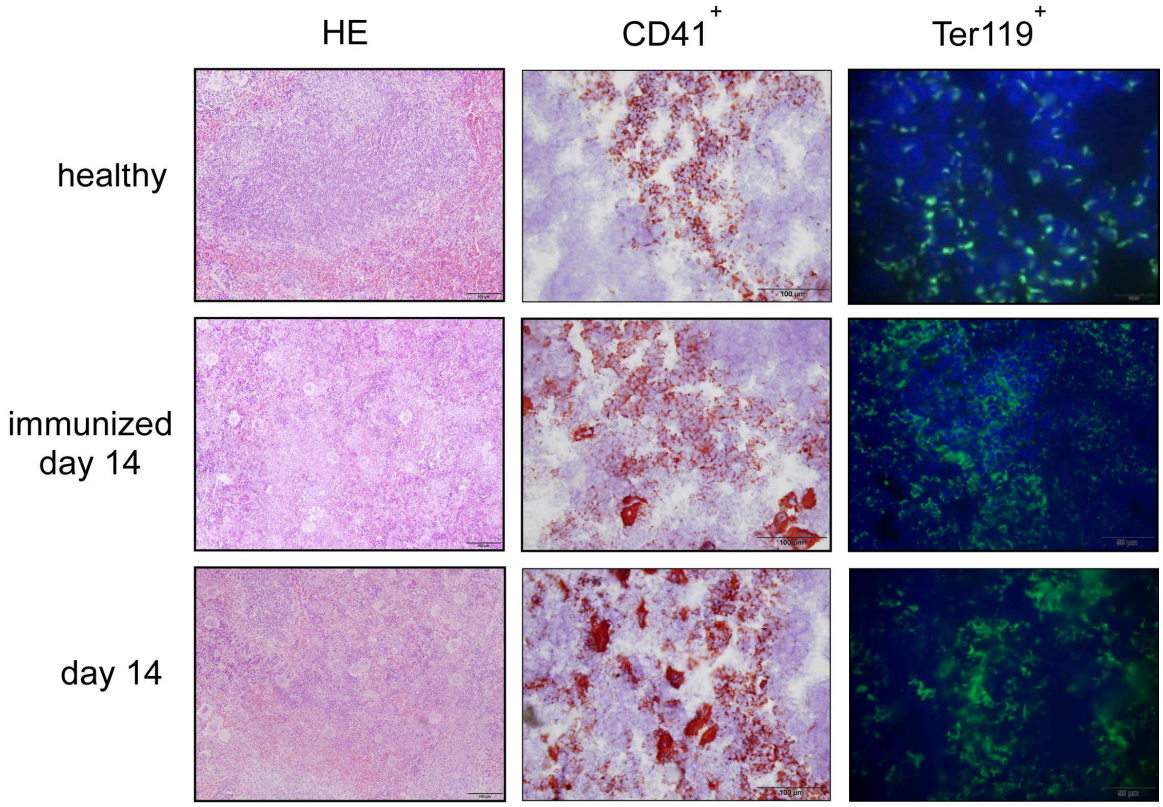

Figure 9 – Extramedullary haematopoiesis in spleens of immunized and nephritic mice

Spleens of healthy mice, immunized mice, and mice with NTS on day 14 stained for HE, CD41 and Ter119 are shown. Representative pictures are shown. Magnification x200 (HE), x400 ($CD41^+$) and x600 ($Ter119^+$).

In spleens of immunized mice and mice with nephritis 14 days after NTS induction, giant cells were detected (Fig. 9). These cells revealed to be positive for CD41, a known marker for megakaryocytes and platelets (Fig. 9) (1). As a next step, Ter119 served to evaluate the erythroid lineage in the spleen. Spleens of immunized and nephritic mice 14 days after NTS induction proved to have a strikingly different Ter119 staining pattern compared to healthy mice. $Ter119^{+}$ cells in spleens of immunized and nephritic mice showed a shift towards a blastic phenotype, which was pronounced when compared to healthy mice (Fig. 9).

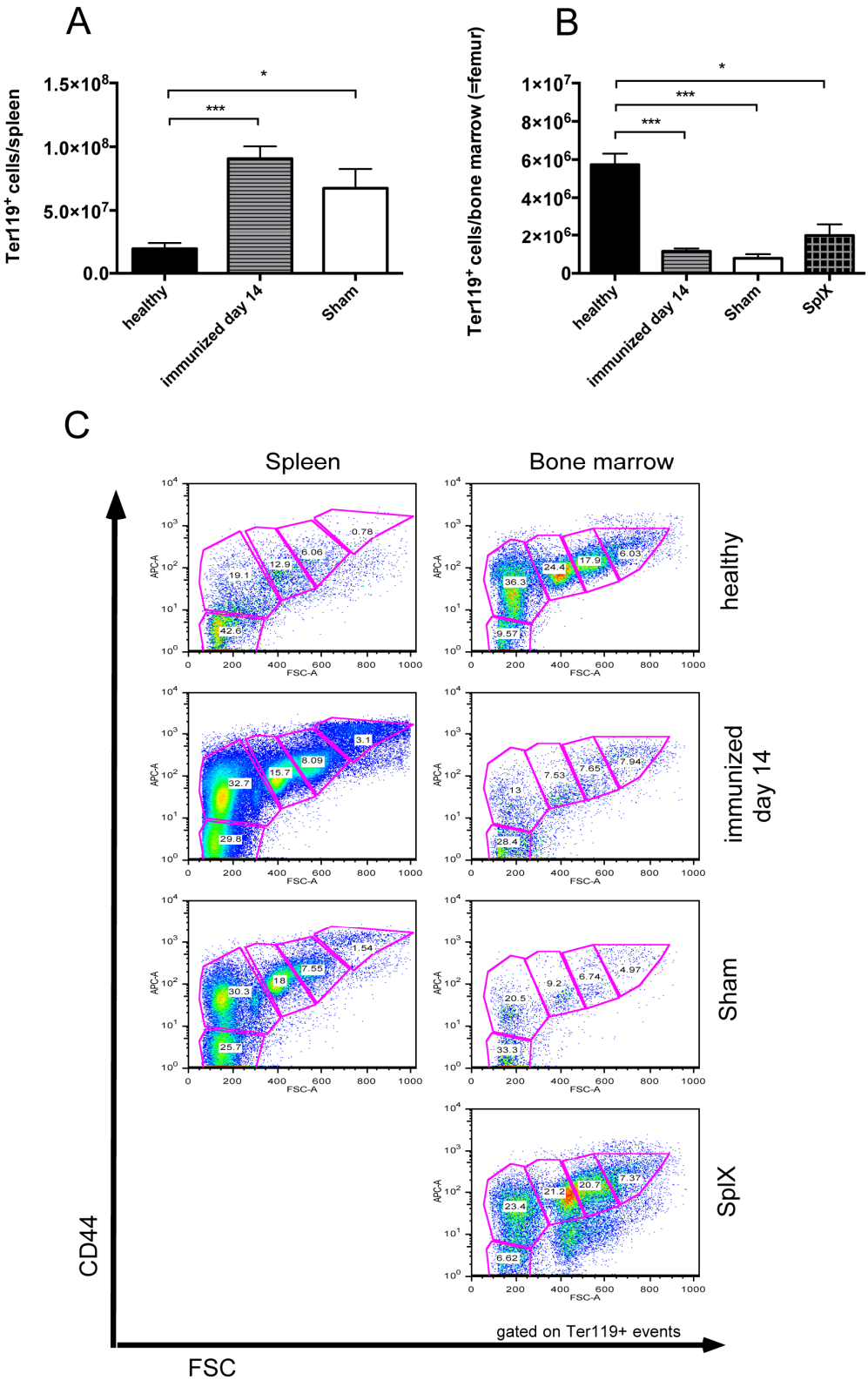

Figure 10 – Decrease of bone marrow haematopoiesis and increase of erythroid precursors in the spleen of immunized mice and mice with NTS

Healthy mice (black bars, n=5), immunized mice on day 14 (lined bars, n=5) and nephritic mice on day 14 that either underwent splenectomy (SplX, squared bar, n=5) or sham-operation (Sham, white bars, n=5) prior to NTS induction are shown. Spleens (A, C) and bone marrow (B, C) were evaluated for erythroid precursors by means of quantitative flow cytometry (A, B). All data are given as means ± SEM. Representative plots are shown (C). *p<0.05; ***p<0.001.

The erythroid lineage in bone marrow and spleens of healthy, immunized and nephritic mice was further evaluated with flow cytometry using markers Ter119 and CD44. Total numbers of erythroid precursors were significantly increased in spleens 14 days after immunisation and NTS induction as compared to healthy mice (Fig. 10A) (1). Meanwhile, the erythroid lineage decreased significantly in the bone marrow of mice with NTS on day 14 as compared to healthy controls (Fig. 10B) (1). Notably, in mice that were only immunized with rabbit IgG and did not receive the anti-GBM serum (Fig. 10A, B) these inverse changes were seen as well (1). Nevertheless, haemoglobin levels of these mice were comparable to haemoglobin levels of healthy control mice (Fig. 5). Although bone marrow erythropoiesis in nephritic splenectomised mice was increased as compared to sham-operated mice with NTS, this increase did not reach statistical significance (Fig. 10B, C) (1).

Systemic inflammatory state in immunized and nephritic mice

As systemic inflammation is known as a major driving force of extramedullary haematopoiesis (68), it was evaluated as a possible cause for the extramedullary haematopoiesis observed in immunized and nephritic mice (1). In order to do so, serum-levels of Il-6, TNF-α and IFN-γ were evaluated in healthy, immunized and nephritic mice on day 14 (Fig. 11).

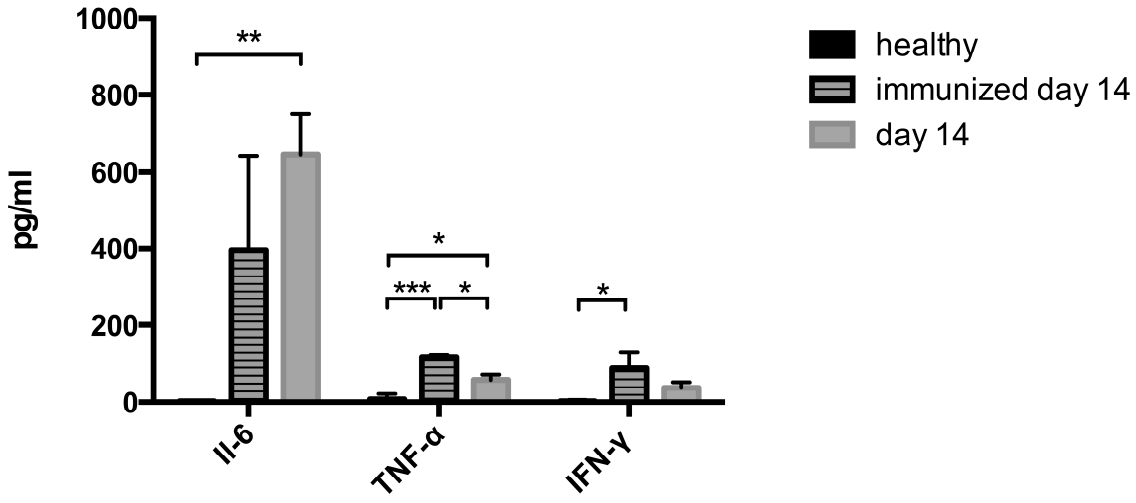

Figure 11 – Increased Il-6, TNF-α and IFN-γ serum levels in immunized and nephritic mice

Serum of healthy mice (black bars, n=4), immunized mice on day 14 (lined bars, n=4) and nephritic mice on day 14 (grey bars, n=4) were evaluated for serum Il-6, TNF-α and IFN-γ. All data are represented as means ± SEM. *p<0.05; **p<0.01; ***p<0.001.

Il-6 and TNF-α serum levels were significantly increased in nephritic mice on day 14 (Fig. 11) (1). Although statistical significance was not reached, IFN-γ levels increased in nephritic mice on day 14 as compared to healthy control mice (Fig. 11) (1). Additionally, increased serum-levels of Il-6, TNF-α and IFN-γ were found in mice that were immunized only and did not receive the GBM antiserum (Fig. 11) (1).

Preferential accumulation of CXCR4 positive erythroblasts in the spleen and their depletion in the bone marrow of immunized and nephritic mice

The CXCR4/CXCL12 axis is known to be involved in the migration of erythroid precursors. In order to evaluate whether the development of extramedullary haematopoiesis in NTS and immunized mice is dependent on this axis, chemokine

expression patterns and the respective receptor, of which all are known to be involved in erythroid precursor migration, were evaluated in the spleen and in the bone marrow (Fig. 12 and Fig. 13) (1).

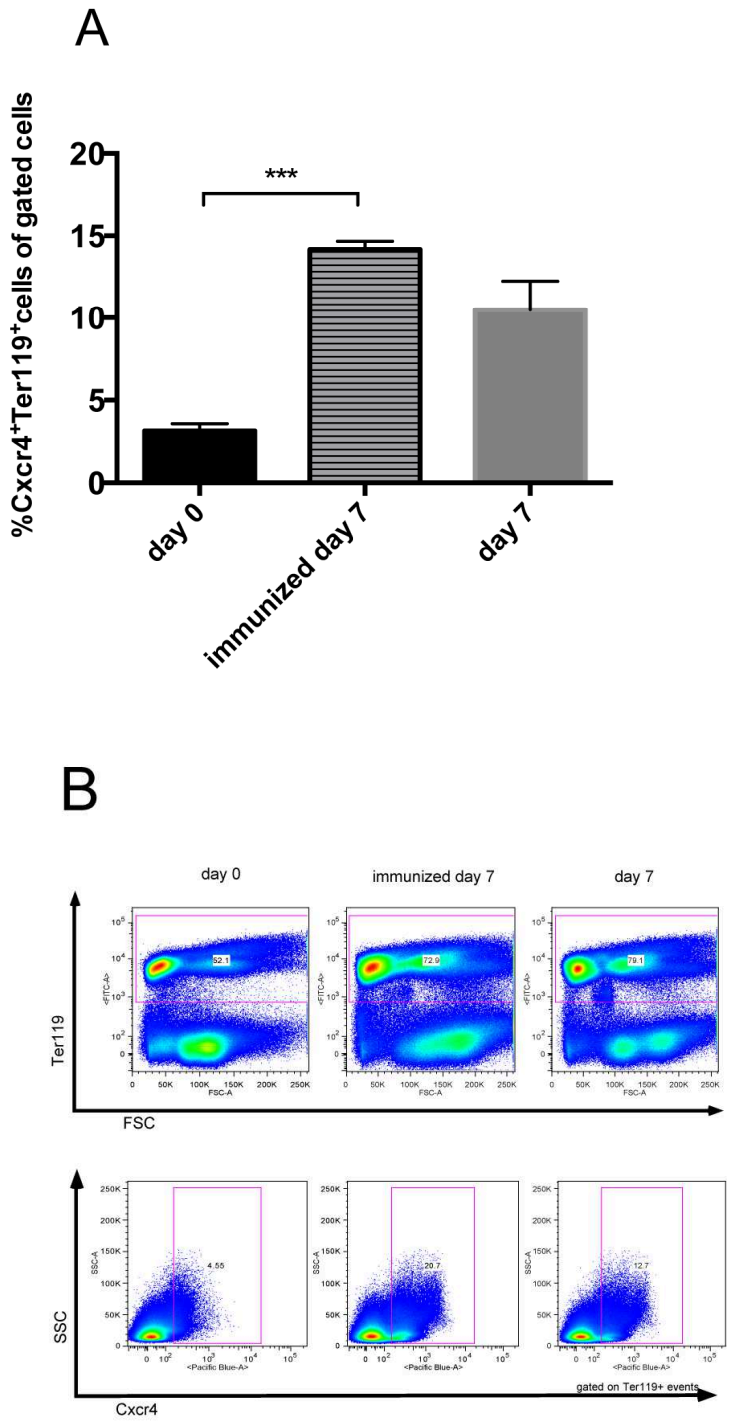

Figure 12 – Proportions of CXCR4$^+$ erythroblasts in the spleen

Relative flow cytometry for Ter119$^+$ cells expressing the chemokine receptor CXCR4 is shown for healthy mice (black bars, n=3), immunized mice (lined bars, n=4) and nephritic mice on day 7 (grey bars, n=4) in spleens (A, B). All data are represented as means ± SEM. ***p<0.001.

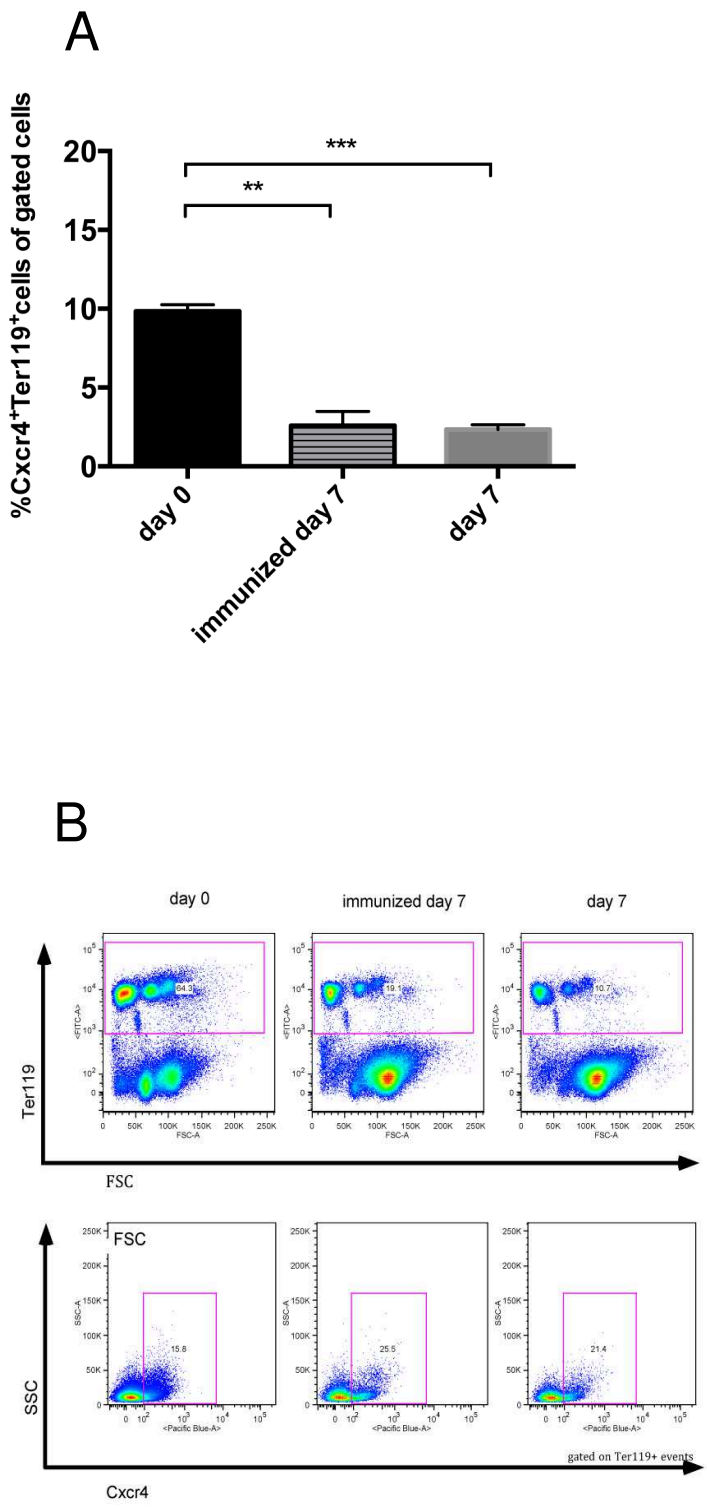

Figure 13 – Proportions of CXCR4$^+$ erythroblasts in the bone marrow

Relative flow cytometry for Ter119$^+$ cells expressing the chemokine receptor CXCR4 is shown for healthy mice (black bars, n=3), immunized mice (lined bars, n=4) and nephritic mice on day 7 (grey bars, n=4) in bone marrow (A,B). All data are represented as means ± SEM. **p<0.01; ***p<0.001.

While less remaining Ter119^{+} bone marrow cells were positive for CXCR4 7 days after NTS induction or immunisation as compared to healthy mice (Fig. 13A, B), more erythroid precursors in the spleen were found to be positive for CXCR4 in immunized and nephritic mice as compared to their healthy controls (Fig. 12A, B).

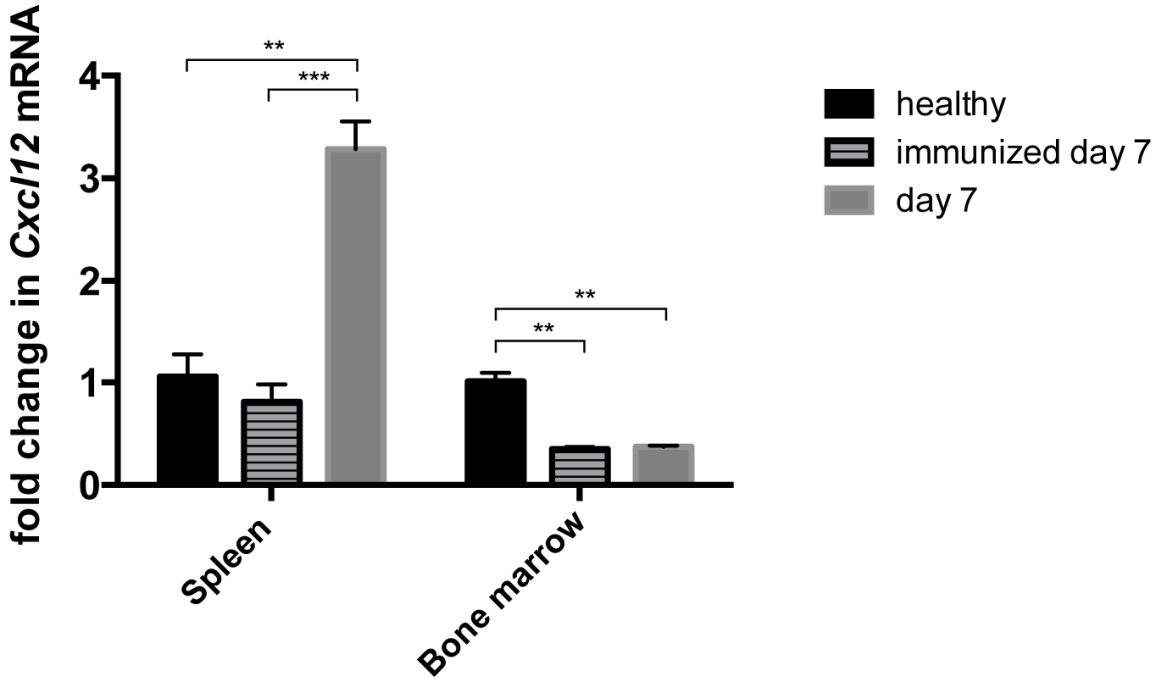

Figure 14 – *Cxcl12* expression in spleens and bone marrow of healthy, immunized and nephritic mice

Cxcl12 mRNA expression from spleens and bone marrow of healthy (black bars, n=3), immunized (lined bars, n=4) and nephritic mice (grey bars, n=4) on day 7 was evaluated by quantitative real-time PCR of total RNA. All data are represented as means ± SEM. **p<0.01; ***p<0.001.

In line with the results of CXCR4^{+}Ter119^{+} cells obtained from bone marrow and spleens (Fig. 12 and Fig. 13), *Cxcl12* mRNA increased in spleens of nephritic mice on day 7, while a decrease in the bone marrow of immunized and mice with NTS on day 7 was observed (Fig. 14) (1). CXCL12 is known as being an important chemokine for homeostasis regulation of haematopoietic stem/progenitor cells (136). Two other chemokines, namely CCL2 and CCL7, have been described to be involved in the

development of extramedullary haematopoiesis as well (137). However, no significant changes of *Ccl2* and *Ccl7* could be detected in spleens over the course of NTS on mRNA level, as these genes showed high cycle thresholds, signifying low or absent expression in this model (1).

Discussion

Over the past decades, research focused on pathological and immunological forces driving GN, as well as on potential therapeutic targets. Still, we lack understanding of full processes leading to GN and more so, optimal therapeutic means are missing. Current therapeutic options are still accompanied by a reduction in life quality and expectancy and are thereby self-limiting. The need for a deeper understanding of GN derives from the fact that GN still counts as an important cause of end-stage renal disease, which leads to a significant proportion of patients who are in need of renal replacement therapy. Renal replacement therapy however goes along with increased comorbidities and reduced quality of life.

In order to address these research needs, the mouse model of NTS was established. This murine model is a model of immune-complex GN and has close related features to human rapid progressive GN (109,120,121,132). The disease is induced by the injection of rabbit anti-mouse GBM serum and requires preceding immunisation against rabbit IgG. It is well known today that the pathogenesis of this disease is dependent on Th1 and Th17 cells, while Tregs contribute to disease limitation (113,120,121,138,139). Importantly, secondary lymphoid organs revealed to be central places of actions of many included cells. There is much evidence for the fact that secondary lymphoid organs such as the draining lymph node and the spleen have important roles in the pathogenesis of this disease, because on the one hand, leukocytes are known to home to these organs (120,121,125). One example of lymph node homing cells are Tregs in NTS (120). On the other hand, however, secondary lymphoid organs such as the spleen are not only involved in antibody presentation, but also in antibody production and clearance of immune complexes. Of note, all of these features are important for the development of NTS. However, the distinct contribution of the spleen to disease development was not known so far. This holds true for human disease as well as for the murine model of NTS. Literature reveals anecdotal case reports about protective, but also potential harmful effects of splenectomy in patients with GN (1). A patient suffering from type II mixed cryoglobulinaemia with typical cryoglobulinaemic glomerulonephritis improved clinically after splenectomy and improvement was also found in repeated renal biopsy (140). This improvement was possibly related to the formation of the type II cryoglobulin IgM component in the spleen

(140). In another case, splenectomy was associated with increased susceptibility to IgA nephropathy as a result of absent immune complex clearance (141). Finally, development of GN and antiphospholipid syndrome was found in a patient after splenectomy with previously diagnosed rheumatoid arthritis and autoimmune hepatitis. The authors suspected a memory B cell redistribution after elective splenectomy to be causal for autoimmune activity (142). Because these case reports do not reveal the role of the spleen in human disease and experimental research in this field was lacking so far, spleen function and morphology in NTS as well as the effect of splenectomy on GN were revealed in detail in this work (1).

Indeed, over the course of GN a significant increase in spleen weight and size was revealed and this increase was due to an increase in the red pulp (1). The exclusion of a white pulp contribution to the increase in spleen mass was highlighted by the fact that most leukocyte populations decreased in the spleen of immunized and nephritic mice, so the increase in spleen weight and size was not attributable to infiltrating leukocytes (1). Increased migration of leukocytes from secondary lymphoid organs to the kidney is a possible explanation for this phenomenon in nephritis. Although neutrophils increased in spleens of immunized and nephritic mice, this cell population was not believed to be responsible for the increase in spleen size alone (1). In order to accurately determine the red pulp area of spleens from healthy, immunized and nephritic mice on day 14 after NTS induction, the macrophage marker F4/80 was used as a determinant for the red pulp (135). Not only did the red pulp as determined by this staining pattern increase. Also, increased numbers of megakaryocytes and proerythroblasts were found in spleens of immunized and nephritic mice, while these cells can be found in lower numbers in healthy mice (1). These cells are precursors of thrombocytes and red blood cells and are therefore indicative of EMH. Accompanying, erythropoiesis in the bone marrow was repressed in immunized and nephritic mice as compared to healthy control mice (1). So far, EMH has been described in a model of systemic lupus erythematosus (143,144). However, there was no documentation of EMH in the model of NTS so far.

Overall processes leading to EMH are not fully understood yet, but pathophysiology seems to be explainable by four theories which all include alterations in the microenvironment of stem cells or stem cells themselves (68). These four theories encompass (1) failure of bone marrow, (2) stimulation of bone marrow, (3) inflammation,

injury and repair of tissue and (4) the production of local or systemic chemokines in an abnormal amount (68). Since nephritic mice had increased levels of serum Il-6, TNF-α and IFN-γ 14 days after NTS induction, the main underlying stimulus for EMH in the model of NTS is most likely systemic inflammation, with undeniable contribution of a chemokine gradient formation as well (1). Regarding systemic inflammation, IFN-γ has been shown to trigger CCL2 and CCL7 chemokine secretion and these chemokines thereby led to splenic CCR2-dependent EMH in acute malaria (137). *Ccl2* and *Ccl7* were evaluated on mRNA level in spleens of healthy, immunized and nephritic mice, but expression levels were too low to assess differences in expression over the timecourse of NTS (1). In a murine model of lupus nephritis, decreased bone marrow haematopoiesis with parallel EMH was described recently (144). There, EMH was dependent on TLR7 and CXCL12 (144). Besides the systemic inflammtory state, *Cxcl12* mRNA was found to be decreased in the bone marrow of immunized mice and nephritic mice, while surprisingly *Cxcl12* mRNA levels increased in the spleen of nephritic mice only (1). Since the increase in *Cxcl12* mRNA in the spleen was not detectable in mice that were only immunized and did not receive the anti GBM serum, it is suitable to speculate about the function of CXCL12. It is probably mainly the releasing effect of the *Cxcl12* decline in the bone marrow on precursor cells rather than the increase of *Cxcl12* as found in the spleen of nephritic mice (1). This observation has also been made by others (70). Also, the lack of *Cxcl12* increase in the spleen of immunized mice possibly reflects a stimulus other than systemic inflammation to be driving the production of this chemokine (1). At least in part however, EMH in NTS seems to develop upon migration of erythroid precursors positive for CXCR4 as this was found in flow cytometry analyses of $Ter119^+$ cells from bone marrow and spleen (1). This increase in $Ter119^+$ cells follows increased *Cxcl12* expression in the spleen, which is evident by a *Cxcl12* decrease in the bone marrow and increase in the spleen (1). However it cannot be ruled out that the increase in $Ter119^+$ cells in the spleen is due to increased survival, proliferation or decreased departure.

Notably, the importance of the CXCR4/CXCL12 axis for formation of extramedullary haematopoiesis in the NTS model is underlined by the increase of $CD11b^{high}$ cells in the spleen. Interestingly, no other leukocyte population but $CD11b^{high}$ cells were found to increase in spleens of immunized and nephritic mice as compared to healthy controls (1). This fact however further strengthens the theory of a CXCR4/CXCL12 axis function in the

NTS model, since the CXCR4/CXCL12 axis was also previously found to affect neutrophil migration (145).

However, a contribution of other yet not known or evaluated chemokines to the pathogenesis of EMH in NTS cannot be excluded and in fact involvement of other chemokines and chemokine receptors is likely (1). In order to fully address the contribution of the CXCR4/CXCL12 axis to EMH in NTS, further experiments are required. These experiments could prove the significance of this axis by means of respective antibody-blockade and models using knockout mice.

Noteworthy, many changes found in this project took place in mice that were only immunized and did not receive the nephrotoxic anti-GMB serum (1). For example, these mice developed signs of systemic inflammation similarly to nephritic mice as evident by Il-6, TNF-α and IFN-γ serum levels (1). Also, mice only immunized without NTS had evidence of $CXCR4^+$ erythroid precursor increase in the spleen as well (1). Interestingly, one big difference in the phenotype between nephritic mice and mice that were immunized only and did not receive the nephrotoxic anti-GBM serum was the absence of anaemia in immunized mice (1).

The development of anaemia in GN is known to happen on the one hand because of decreased erythropoietin production in the kidney and on the other hand because of chronic inflammation, which can have a suppressive effect on the bone marrow (146,147). The impact of anaemia as a stimulus for EMH cannot be ruled out completely. However, the lack of anaemia in mice that were immunized only supports the idea of systemic inflammation as the primary force driving EMH in NTS rather than anaemia due to renal failure (1). Further supporting this hypothesis, erythropoietin levels did not decrease but rather increased slightly in splenectomised mice as compared to their sham-operated controls after 28 days of NTS, which reflects a prolonged follow up. An attempt in compensatory erythropoietin production would very likely fail in case of renal anaemia. In these experiments, splenic EMH compensated reduced bone marrow haematopoiesis at least in part (1). This successful compensation in NTS was evident because of decreased haemoglobin levels in mice that underwent splenectomy as compared to sham-operated control mice (1).

Splenectomy served as a mean to determine whether the spleen plays a central role in the pathogenesis and development of NTS (1). As mentioned earlier, the spleen encompasses a variety of functions. In NTS, functions like CCR7 dependent homing of Tregs mainly takes place in the regional draining lymph nodes, but also in the spleen (120,121). However, in order to determine whether the spleen has functions as important for NTS development as the draining lymph node, splenectomy or sham-operation were performed and subsequently NTS was induced (1). Disease was then followed for 14 or 28 days, in order to adequately evaluate long-term effects. Surprisingly, the renal phenotype was not affected by splenectomy (1). Also levels of autologous and heterologous antibodies in the serum, as well as their deposition on the GBM were unchanged (1). This finding was surprising, as the spleen is known as an important place for antibody production and resolution. Additionally, the fact that kidney infiltrating cells and kidney pathology overall were unchanged between splenectomised and sham-operated mice indicates that the major site of activation and regulation in NTS is represented by the draining lymph node (1).

The primary aims of this study were to (A) characterize the immunologic processes in the spleen in NTS, (B) evaluate the effect of splenectomy on NTS development and thereby (C) to define the extent of contribution of the spleen to the development of NTS. Characterisation of splenic cell populations revealed the presence of EMH in NTS, and splenectomy resulted in aggravated anaemia. However, splenectomy did not cause changes in the kidney phenotype. In conclusion, this experimental data did not show neither beneficial nor harmful effects of splenectomy on the kidney pathology per se and the contribution of the spleen was found not to be essential for the development of NTS (1). Rather, concomitant anaemia was found in NTS, which was due to repressed bone marrow haematopoiesis and accompanied by EMH in the spleen (1). We therefore failed to reject the null hypothesis, which stated that the spleen does not contribute to the pathogenesis of the disease, and that splenectomy does not influence disease development as compared to sham-operated controls.

Future outlook includes antibody-blockade experiments and knock-out models in order to address the contribution of the CXCR4/CXCL12 axis to EMH in NTS. Further, in order to understand the implication in human GN, EMH needs to be evaluated in patients suffering from rapid-progressive GN.

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