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Regulation of Th17 responses in glomerulonephritis by RORγt, Treg subpopulations and the cytokine IL-6

Dissertation

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Table of contents

1 Research objective ... 1

2 Introduction ... 3

2.1 Definition and epidemiology of RPGN ... 3

2.2 Pathogenesis ... 3

2.2.1 General pathogenesis ... 3

2.2.2 The Role of Th17 cells in RPGN ... 4

2.2.3 Role of the regulatory T cells ... 6

2.2.4 Interleukin-6 ... 7

2.3 Established treatment regimens of ANCA-RPGN ... 9

2.4 RORγt-inhibition as a possible therapeutic approach ... 10

3 Materials ... 12

3.1 Compounds, Chemicals, Nucleotides and Sera ... 12

3.2 Buffer and Solutions ... 13

3.3 Antibodies for FACS Surface Staining ... 14

3.4 Antibodies for FACS Intracellular/Intranuclear Staining ... 15

3.5 Antibodies for Immunohistochemistry ... 16

3.6 Commercial Kits ... 17

3.7 Devices and Software ... 18

4 Methods ... 20

4.1 Animal experiments ... 20

4.1.1 Animals ... 20

4.1.2 Animal ethics ... 20

4.1.3 Compounds ... 20

4.1.4 Experimental models and treatment regimes ... 21

4.1.5 Functional studies 23

4.1.6 Morphologic studies 24

4.1.7 Isolation of leukocytes from various tissues 25

4.1.8 In vitro Cytokine production 25

4.1.9 Flow cytometry 25

4.2 Studies on human blood cells 26

4.2.1 Blood samples 26

4.2.2 Isolation of PBMCs 26

4.2.3 Conservation of blood samples and PBMCs 27

4.2.4 Stimulation of PBMCs 27

4.2.5 T cell isolation and Treg suppression assay 27

4.2.6 Flow cytometry of human leucocytes 28

4.3 Statistical analysis 28

5 Results – Part One: RORγt inhibition in different experimental models of inflammation 29

5.1 Effects of RORγt-ASO treatment on T cell responses after sheep IgG immunization 29

5.1.1 The Th17 cell response is not reduced after treatment with RORγt-ASO……. 30

5.1.2 RORγt-ASO treatment does not reduce secretion of pro-inflammatory cytokines 30

5.2 RORγt-ASO treatment of the NTN model of crescentic glomerulonephritis. 30

5.3 GSK805 in the NTN model of crescentic glomerulonephritis 32

5.3.1 Treatment with GSK805 does not ameliorate structural damage or prevent loss of kidney function 32

5.3.2 GSK805 treatment does not reduce secretion of pro-inflammatory cytokines 33

5.4 VTP-44938 in the cisplatin model of acute renal failure 33

5.4.1 VTP-44938 treatment does not ameliorate structural damage or loss of kidney function 33

6 Results – Part Two: Specific IL-6Ra knockout on CD4+ T cells 35

6.1 Effects of specific IL-6Ra knockout on CD4+ T cells on cells infiltrating the kidney in GN 35

6.1.1 IL-6Ra classic signaling in CD4+ T cells mediates renal infiltration of neutrophils in GN 35

6.1.2 IL-6Ra classic signaling in CD4+ T cells mediates renal Treg infiltration 37

7 Results – Part Three: Development of a flow cytometry protocol for analyzing human PBMCs 38

7.1.1 PBMCs are vital after defrosting 38

7.1.2 Establishment of a flow cytometry based protocol for identification of various human peripheral blood T cell subtypes 38

7.1.3 Establishment of a human Treg in vitro suppression assay 41

7.2 Characterization of peripheral blood T cells from patients with ANCA-RPGN.. 42

7.2.1 Blood samples 42

7.2.2 Impaired vitality in PBMCs from ANCA-RPGN patients 43

7.2.3 Th17 expand during ANCA-RPGN 44

7.2.4 Characterization of Regulatory T cells subpopulations in the blood of ANCA-RPGN patients 44

8 Discussion 49

8.1 RORγt inhibitory compounds in the course of experimental glomerulonephritis 49

8.2 Interleukin-6 receptor classic signaling and its effects on Tregs 50
8.3 Characterization of human Treg subtypes 52
9 Summary.. 56
10 Zusammenfassung 57
11 Publication bibliography... 58
12 Danksagung 72
13 Lebenslauf 73
14 Eidesstattliche Versicherung 73

1 Research objective

Rapidly progressive glomerulonephritis (RPGN) is a common cause of end-stage renal disease. End-stage renal disease requires lifelong dialysis treatment or kidney transplantation and is therefore an important individual-health and social-economic factor. A common cause of RPGN are autoimmune diseases, as in particular vasculitis associated with anti-neutrophil cytoplasmic antibodies (ANCA). Many aspects about RPGN are currently unclear, including etiology and pathogenesis, so that specific and individualized therapies are lacking. My dissertation will thus deal with three key aspects of RPGN as outlined below.

1) In the recent years, many studies elucidated the role of pro-inflammatory IL-17 secreting Th17 cells (Th17) in autoimmune diseases. It has become clear, that Th17 cells are among the main drivers of renal inflammation. In animal models knockout of the Th17-defining transcription factor RORγt potently protects from glomerulonephritis. RORγt therefore represents a promising therapeutic target. Hence in a first step, we sought to examine the effects of RORγt-directed therapies in experimental glomerulonephritis. We employed three different RORγt blocking compounds and determined effects on cellular immune responses, as well as on the course of RPGN.

2) Under physiological conditions, Th17 responses are under control of regulatory T cells. Currently, many aspects about Tregs and the way they exert their suppressive functions on inflammatory Th17 cells remain unclear. Furthermore, the signaling pathways orchestrating Treg generation and activation remain largely elusive. The cytokine Interleukin-6 was traditionally thought to empower inflammatory Th17 cell responses and to inhibit regulatory T cells. However, it has recently become clear, that IL-6 effects are more diverse and complex, than previously thought. We thus aimed to study the individual contribution of signaling via the Interleukin-6 receptor to modulation of Th17, as well as regulatory T cell development. For this purpose, we created a mouse strain with IL-6Ra deficiency restricted to different $CD4^+$ T cell populations and induced experimental glomerulonephritis in these mice. In my dissertation, I have analyzed whether

CD4^{+} T cell restricted lack of IL-6Ra affects infiltration of inflammatory and regulatory T cells into the kidney. These findings were linked to other experiments of the author's laboratory group as outlined in the *discussion* section.

3) Regulatory T cells are not a homogenous population. Rather, they can be divided into functionally distinct subtypes that are specialized for the control of particular types of T effector cell responses. It would thus be of interest to understand, whether one of these subtypes is functionally impaired in the course of autoimmune kidney disease. Since there are almost no data available regarding the characterization of human Treg subtypes, I developed a flow cytometry based protocol for their detection and analysis. Using this technique, we provide first data, characterizing Treg subtypes from a small collective of RPGN patients.

2 Introduction

2.1 Definition and epidemiology of RPGN

Rapidly-progressive glomerulonephritis (RPGN) is characterized by severely impaired kidney function due to inflammation induced glomerular injury. While the overall-incidence of RPGN is relatively low [1/100.000 (Veelken and Ditting 2018)], it embodies a main cause of end stage renal disease (United States Renal Data System 2019).

RPGN can be divided into three major types, depending on the etiology. Type I is caused by antibodies directed against the glomerular basement membrane (as in Goodpasture´s Syndrome or anti-GBM disease). Type II RPGN results from glomerular immune complex deposition as e.g. in systemic lupus erythematosus. Type III is comprised of the pauci-immune mediated forms of RPGN usually associated with anti-neutrophil cytoplasmatic antibodies (ANCA), referred to as ANCA associated RPGN. Representing 40 – 50 % of all cases, ANCA-RPGN is the most common single cause of RPGN.

ANCA-associated vasculitis (AAV) itself is as well categorized into four types: (I) microscopic polyangiitis (MPA), (II) granulomatosis with polyangiitis (GPA), (III) eosinophilic granulomatosis with polyangiitis (EGPA) and (IV) renal limited vasculitis (Luqmani et al. 2019).

The incidence of AAV peaks in patients aging 60 – 70 years, but it also occurs in much younger individuals. (Luqmani et al. 2019)

2.2 Pathogenesis

2.2.1 General pathogenesis

Diagnosis of RPGN is proven by renal biopsy. All types of RPGN share the histopathological hallmark of glomerular crescent formation as a consequence of severe capillary wall injury of the glomerular vasculature. Crescents consist of fibrin, cells of the glomerular tuft, cells of the Bowman´s capsule as well as leukocytes. In contrast to the other types of RPGN, ANCA-RPGN shows no or little deposition of immunoglobulins or complement.

Over the last few years, studies have identified the central pro-inflammatory role of T helper cells (Th) of the Th1 and Th17 subtypes in the course of RPGN. Much less, however, is known about regulatory T cells (Treg) as their anti-inflammatory counterparts.

2.2.2 The Role of Th17 cells in RPGN

The permanently growing T helper cell family is currently divided into Th1, Th2, Th17, Th9 and Th22 cells. (Bluestone et al. 2009; Zhu et al. 2010) It has been shown that T cell frequencies in the inflamed tissue vary during the course of experimental glomerulonephritis: While Th17 cell infiltration in the kidney peaks early, around days 5-7 after induction of acute experimental crescentic glomerulonephritis, their percentages decline at lager stages, when Th1 cells and regulatory T cells dominate in the kidney tissue. (Krebs and Panzer 2018)

Under the influence of Interleukin-12, Interleukin-18 and Interferon γ (IFN-γ), naïve $CD4^+$ T cells differentiate into Th1 cells. (Eagar and Miller 2019) Th1 cells normally defend the organism against intracellular bacteria, fungi and viruses. They are programmed by their master transcription factor T-bet (T-box expressed in T cells), which controls production of the key cytokine IFN-γ. (Szabo et al. 2000)

Th17 cells, on the other hand, were found to play an important role in defense against extracellular bacteria and fungi. (Gu et al. 2013) They are defined by their master transcription factors RORγt and STAT3. (Ivanov et al. 2006; Nurieva et al. 2007) Interleukin-1β, Interleukin-6, Interleukin-23 and transforming growth factor β direct T cell differentiation towards Th17 (Yang et al. 2007; Yang et al. 2008b) and initiate the synthesis of the pro-inflammatory cytokines IL-17A, IL-17F, IL-22 (Nurieva et al. 2007; Steinmetz et al. 2011; Riedel et al. 2016), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Codarri et al. 2011) and the CC chemokine receptor 6 (CCR6) (Manel et al. 2008).

Both, Th1 and Th17 cells can independently induce proliferative glomerulonephritis. (Huang et al. 1997; Summers et al. 2009) Th1 cells mediate renal injury via IFN-γ induced macrophage activation. (Ikezumi et al. 2004) The Th17/IL-17 immune response leads to attraction of destructive neutrophils, which results in renal tissue injury. (Disteldorf et al. 2015)

The contribution of Th17 cells to RPGN has been studied in various mouse models. In the T cell-mediated model of nephrotoxic nephritis (NTN) mice lacking IL-17A developed less severe nephritis. (Paust et al. 2009) In the anti-myeloperoxidase (MPO) model, which simulates ANCA-glomerulonephritis, mice deficient in IL-17A showed ameliorated histological injury and preserved kidney function. (Gan et al. 2010) Since Th17 cells do not only produce IL-17A, but also pathogenic IL-17F, TNFα and GM-CSF, further studies concentrated on the Th17 master transcription factor RORγt, which is known to program the Th17 cell phenotype. Indeed, mice deficient for RORγt failed to develop an IL-17A response in the MPO-model (Summers et al. 2011b). In line with that, in the NTN model of crescentic nephritis RORγt$^{-/-}$ mice were strongly protected from disease, as they were not able to develop Th17 immune responses including IL-17A and F production. (Steinmetz et al. 2011)

While the role of Th17 has been documented well in mouse models, data from human studies are, however, scarce. Most studies concentrated on peripheral blood analyses of circulating T cells or cytokine levels in patients with ANCA-associated granulomatosis with polyangiitis. Indeed these studies showed elevated numbers of circulating IL-17 producing T cells, compared to healthy controls. (Abdulahad et al. 2008; Wilde et al. 2012; Szczeklik et al. 2017) After remission, Th17 frequencies in the peripheral blood decrease, but nevertheless stay elevated. (Wilde et al. 2012; Nogueira et al. 2010) Only very few histopathologic studies have examined cellular infiltration in human kidneys from patients with ANCA-associated glomerulonephritis. One study revealed elevated numbers of Th17 cells infiltrating the kidney compared to healthy controls. (Krebs et al. 2016) Interestingly, in this study, frequencies of peripheral Th17 cells were unchanged, possibly indicating preferential Th17 recruitment to the renal tissue. Furthermore, Bitton et al. were able to link the increased presence of RORγt^{+} Th17 cells in the initial kidney biopsy to a higher risk of renal relapse. (Bitton et al. 2020)

Taken together these data suggest a relevant pathogenic role of Th17 cells in ANCA-associated glomerulonephritis, although their exact contribution remains elusive.

2.2.3 Role of the regulatory T cells

Regulatory T cells (Tregs) act as a counterpart to T effector cells. Absence or abnormal function of Tregs, caused by a loss-of-function mutation in the gene encoding Foxp3, causes uncontrolled inflammatory activity, which leads to autoimmune diseases such as IPEX syndrome (IPEX = immunodysregulation, polyendocrinopathy, enteropathy, X linked). (Bennett et al. 2001) Forkhead box P3 (Foxp3) is the master Treg transcription factor controlling cell differentiation from naïve T cells into Tregs. (Hori et al. 2003)

How exactly Tregs exert their suppressive functions on inflammatory T cells is not completely known. Possible mechanisms likely include secretion of key suppressive cytokines, display of surface molecules to impair T cell co-stimulation, competition for substrates and expression of certain chemokine receptors to facilitate co-localization with T effector cells.

In view of the highly diversified T effector immune responses, it is not surprising that recent studies have revealed the existence of distinct Treg subtypes, which are specialized for the control of a particular pro-inflammatory Teff counterpart. T-bet^{+} Foxp3^{+} T cells (Treg1) are able to co-localize with CXCR3^{+} Th1 by also expressing the chemokine receptor CXCR3 and specifically control Th1 immune response in crescentic GN. (Koch et al. 2009; Nosko et al. 2017; Paust et al. 2016) IRF4^{+} FoxP3^{+} T cells (Treg2) have been suggested to control Th2 immune responses but definite evidence is lacking to date. (Zheng et al. 2009) STAT3^{+} FoxP3^{+} T cells (Treg17) are able to co-localize with CCR6^{+} Th17 by also expressing the chemokine receptor CCR6 and specifically control Th17 immune responses in crescentic GN and lupus nephritis. (Chaudhry et al. 2009; Turner et al. 2010b; Kluger et al. 2014; Kluger et al. 2016a)

In addition to these lineage specific effector Tregs, recent research has revealed the existence of another highly activated and potent regulatory T cell lineage. In addition to the Treg hallmark transcription factor Foxp3 these Tregs also depend on activation of the Th17 characteristic transcription factor RORγt. (Kluger et al. 2016b; Sefik et al. 2015; Yang et al. 2016) Unusual for a Treg, they express high levels of IL-17 and the Th17-characteristic chemokine receptor CCR6. (Kluger et

al. 2016b) However, they also secrete high amounts of IL-10 and IL-35, known to exert anti-inflammatory functions. (Kluger et al. 2016b) In depth analyses revealed, that they do not represent a transitory cell type in a trans-differentiation process from Th17 to regulatory T cells but rather constitute a stable and independent Treg subtype. So far, their exact functions remain elusive, but they seem to have both regulatory and reparative, but also pro-inflammatory functions. (Kluger et al. 2016b; Yang et al. 2016) Given their bi-functional character, we proposed to term them ´biTregs´.

To date, almost no data exist about the biology of the above explained different Treg subtypes in the human organism, nor their distinct behavior under pathologic conditions. A better knowledge, however, is much warranted. This is particularly true, since a number of recent breakthrough findings have made it possible to obtain Tregs from patients´ blood. Subsequently, they can be *ex vivo* modulated and expanded to afterwards reinfuse them back into the patient. (Barrett et al. 2015) This technique, originally derived from the field of oncology, has not yet been used as a therapy for GN. However, first promising data have been published regarding regulatory T cell based therapy for kidney transplant recipients. (Harden et al. 2020) It is thus of great interest to achieve more knowledge about the different Treg subtypes involved in protection from renal tissue injury, as in future therapeutic approaches they might be specifically induced or expanded *ex vivo,* or even *in vivo,* to regain control about an overshooting inflammatory T cell response.

2.2.4 Interleukin-6

For a long time, the pleiotropic cytokine Interleukin-6 (IL-6) has been declared as strictly pro-inflammatory. Together with IL-1ß and TGF-β, IL-6 directs differentiation of naïve T cells towards inflammatory Th17 cells. (Bettelli et al. 2006; Veldhoen et al. 2006; Kimura et al. 2007) Furthermore, it was proposed, that during Th17 differentiation IL-6 prevents transcription of the gene encoding Foxp3, hence impairing Treg development. (Bettelli et al. 2006) This paradigm, however, has been challenged recently, as studies revealed also anti-inflammatory effects of IL-6: In T regulatory Type 1 cells IL-6 induces IL-10 synthesis, a highly anti-

inflammatory cytokine. (Jin et al. 2013; Stumhofer et al. 2007) Furthermore, contrary to the previous assumption, IL-6 does not seem to inhibit Treg development, but rather supports T effector cell activation, making them resistant to Treg-mediated suppression. (Nish et al. 2014; Goodman et al. 2009)

IL-6 is produced by numerous different types of leukocytes, as e.g. dendritic cells, Th17 cells and others. (Eagar and Miller 2019; O'Shea et al. 2019) IL-6 signaling is complex and possible *via* three different ways. (1) Classic signaling: IL-6 binds to the membrane bound IL-6R, which consists of the glycoprotein gp130 and the specific IL-6 receptor α subunit. (Schaper and Rose-John 2015) (2) Trans-signaling: IL-6 binds to a soluble IL-6Rα. This IL-6/sIL-6Rα complex then binds to membrane-bound gp130, which induces signal transduction. (Schaper and Rose-John 2015) (3) Cluster-signaling: Dendritic cells trans-present an IL-6/IL-6Rα complex to gp130 on $CD4^+$ T cells. (Heink et al. 2017). The individual contribution of each signaling pathway to the multiple pro- and anti-inflammatory functions of IL-6 has not yet been characterized in detail. In particular, the relevance of Treg modulation by the different IL-6 signaling modalities remains unclear. In this respect, it was recently shown, that IL-6 *in vitro* incubation resulted in RORγt activation in cultured Tregs and they started to produce IL-17. (Lochner et al. 2008; Wang et al. 2015; Xu et al. 2007; Yang et al. 2008a). It is likely to assume, that these $ROR\gamma t^+$ Tregs represent the above described multifunctional biTreg population. Unraveling the functions of IL-6 in detail is rather rewarding, since IL-6 directed interventions have entered clinical practice. In the EU and in the USA the anti-interleukin-6 (IL-6) receptor monoclonal antibody Tocilizumab has been approved for the treatment of numerous autoimmune diseases like rheumatoid arthritis, giant cell arteritis and cytokine release syndrome. (European Medicines Agency 2009; U.S. Food & Drug Administration 5/22/2017) Yet, Tocilizumab has not been successfully used for the treatment of renal autoimmune diseases. The contribution of IL-6 to the development of renal tissue injury, as well as the functional importance of each of the three signaling pathways, still needs to be studied in more detail. (Braun et al. 2016) Data from experimental mouse models revealed differing results (Braun et al. 2016; Luig et al. 2015; Karkar et al. 1997; Sakai et al. 2020) and first data from application to human disease have been

inconclusive. (Holdsworth et al. 2016; Illei et al. 2010; Rovin et al. 2016) Thus it is necessary to further clarify the exact functions of IL-6 signaling in order to develop more precise therapeutic agents.

2.3 Established treatment regimens of ANCA-RPGN

The KDIGO guideline (Kidney Disease: Improving Global Outcomes (KDIGO) Glomerular Diseases Work Group 2021) recommends the use of cyclophosphamide and corticosteroids as initial treatment for ANCA associated GN. Alternatively, cyclophosphamide can be replaced or supplemented by the anti CD20 antibody Rituximab, in particular, if cyclophosphamide has proven to be ineffective or is contraindicated.

In some selected cases, additional plasmapheresis might be considered. In particular patients with highly active disease might profit from this treatment. However, data from available studies do not clearly support this treatment regime.

After remission, a maintenance therapy with azathioprine plus low-dose glucocorticoids is recommended for at least 18 months. As an alternative, recent studies have revealed effectiveness of rituximab in maintenance therapy as well. (Tieu et al. 2020) Rituximab is therefore recommended for maintenance therapy especially for relapsing disease or frail older adults.

However, all recommended treatment regimens are rather unspecific. In order to suppress the pathologic immune response in ANCA-RPGN, they non-selectively affect large parts of the immune system. This causes broad impairment of the defense against pathogens, like bacteria or fungi. An ideal therapeutic agent, in contrast, would precisely block activation of the immunological key players for the respective disease, while maintaining basic functions of the regular immune system. Gaining more knowledge about the individual key players and their particular contribution to the course of renal inflammation is therefore essential.

As highlighted above, Th17 cells are thought to be among the most important mediators for the pathogenesis of GN. In animal experiments, knockout of the Th17-defining transcription factor RORγt leads to strong protection from glomerulonephritis. (Steinmetz et al. 2011) As a consequence, development of

drugs interfering with the RORγt pathway for treatment of GN and possibly other forms of immune mediated diseases seems rather promising.

2.4 RORγt-inhibition as a possible therapeutic approach

As explained above, RORγt is the major transcription factor that induces T cell differentiation towards a Th17 phenotype. (Ivanov et al. 2006) Th17 cells are crucial for renal tissue injury in the course of experimental glomerulonephritis. (Kitching and Holdsworth 2011; Steinmetz et al. 2011; Turner et al. 2010a) Data from human studies suggest a key role in autoimmune renal inflammation as well. (Abdulahad et al. 2008; Krebs et al. 2016; Saito et al. 2009) The pharmaceutical industry has recently developed new agents to interfere with Th17 cell development and/or activation on the level of DNA transcription (RORγt inverse agonists) or mRNA translation (RORγt antisense oligo DNAzymes). As there are no data available regarding application of these compounds in models of experimental glomerulonephritis, this thesis aimed to close this important knowledge gap.

(1) GSK805 and (2) VTP-44938 – Inverse agonists. Under both, *in vitro* and *in vivo* conditions RORγt activation can be can be blocked by inverse agonists. These agonists bind directly and reversibly to the ligand binding domain of RORγt. This binding leads to modulation of the structural conformation of RORγt, inhibiting its transcriptional effects. The inverse agonists have shown to be effective in preventing the development of Th17 cells and to ameliorate the course of experimental autoimmune encephalomyelitis (EAE). (Solt et al. 2011; Xiao et al. 2014) Similar results were published for a mouse colitis model. (Igaki et al. 2019) Finally, cells taken from the blood and inflamed sites of psoriasis patients were treated *ex vivo* with an inverse RORγt agonist, resulting in decreased Th17 activity. (Skepner et al. 2014; Gauld et al. 2019)

(3) RORγt antisense oligonucleotides – DNAzyme. Another novel and unique approach is to inhibit translation of the mRNA encoding for RORγt. Single-stranded DNA molecules, complementary to the mRNA of the target protein have been developed (Santoro and Joyce 1997) and were coupled to catalytic enzymes. This leads to cleavage and degradation of the complementary mRNA

after specific binding. Given the combination of DNA with enzymes these compounds were termed DNAzymes. They have already demonstrated effectiveness in targeting GATA3, the main transcription factor of Th2 cells, in patients suffering from allergic asthma. (Krug et al. 2015) Hence it is imaginable to employ the DNAzyme technique in order to impede translation of the RORγt mRNA. To date, no data have been published in this respect.

3 Materials

3.1 Compounds, Chemicals, Nucleotides and Sera

Name	Source
anti-human CD28	BioLegend; San Diego, CA, USA
anti-human CD3	BD Bioscience; Franklin Lakes, NJ, USA
Aqua (H_2O)	B. Braun Melsungen, Melsungen, Germany
Brefeldin A	Sigma-Aldrich; St. Louis, MO, USA
Cisplatin	Sigma-Aldrich; St. Louis, MO, USA
Collagenase D	Roche; Basel, Switzerland
Complete Freund's Adjuvant (CFA)	Sigma-Aldrich; St. Louis, MO, USA
Deoxyribonuclease I (DNAse I)	Roche; Basel, Switzerland
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich; St. Louis, MO, USA
Fetal Calf Serum (FCS)	Gibco™; Thermo Fisher Scientific; Waltham, MA, USA
Histopaque®-1077	Sigma-Aldrich; St. Louis, MO, USA
Ionomycin	Calbiochem®, Sigma-Aldrich; St. Louis, MO, USA
Nephrotoxic Nephritis Sheep Serum (Batch 11-01)	Seramun Diagnostica; Heidesee, Germany

Penicillin/Streptomycin	Calbiochem®, Sigma-Aldrich; St. Louis, MO, USA
Percoll™ (37%)	Cytiva, formerly GE Healthcare Life Sciences; Marlborough, MA, USA
Phorbol-12-myristat-13-acetat (PMA)	Sigma-Aldrich; St. Louis, MO, USA
RORγt antisense oligonucleotides	Secarna Pharmaceuticals; Munich, Germany
RORγt inhibitor GSK805	GlaxoSmithKline; London, UK
RORγt inhibitor VTP-44938	Vitae Pharmaceuticals; Fort Washington, MD, USA
Sheep Immunoglobulin G (sIgG)	Sigma-Aldrich; St. Louis, MO, USA
β-mercaptoethanol	Calbiochem®, Sigma-Aldrich; St. Louis, MO, USA

Table 1. Compounds, Chemicals, Nucleotides and Sera

3.2 Buffer and Solutions

Name	Source/Content
Erylysis Stock Solution 1	Tris(hydroxymethyl)aminomethane at pH 7.6 in H_2O
Erylysis Stock Solution 2	144mM ammonium chloride in H_2O
Hanks's Balanced Salt Solution (HBSS)	Gibco™; Thermo Fisher Scientific; Waltham, MA, USA
HEPES (1M)	Gibco™; Thermo Fisher Scientific; Waltham, MA, USA

MACS® Buffer	Miltenyi Biotec; Bergisch Gladbach, Germany
Phosphate-Buffered Saline (PBS)	Miltenyi Biotec; Bergisch Gladbach, Germany
RPMI 1640 Medium	Gibco™; Thermo Fisher Scientific; Waltham, MA, USA
X-VIVO™ Medium	Biozym Scientific; Hessisch Oldendorf, Germany

Table 2. Buffer and Solutions

3.3 Antibodies for FACS Surface Staining

Name	Source
anti-human CCR6	BioLegend; San Diego, CA, USA
anti-human CCR8	R&D Systems; Minneapolis, MN, USA
anti-human CD103	BioLegend; San Diego, CA, USA
anti-human CD127	BioLegend; San Diego, CA, USA
anti-human CD161	BioLegend; San Diego, CA, USA
anti-human CD25	BioLegend; San Diego, CA, USA
anti-human CD3	BioLegend; San Diego, CA, USA
anti-human CD4	BioLegend; San Diego, CA, USA
anti-human CD4	BioLegend; San Diego, CA, USA
anti-human CD44	BioLegend; San Diego, CA, USA

anti-human CD45	BioLegend; San Diego, CA, USA
anti-human CD45RA	BioLegend; San Diego, CA, USA
anti-human CD69	BioLegend; San Diego, CA, USA
anti-human CD8	BioLegend; San Diego, CA, USA
anti-human CTLA4	BioLegend; San Diego, CA, USA
anti-human CXCR3	BioLegend; San Diego, CA, USA
anti-human ICOS	BioLegend; San Diego, CA, USA
anti-human IL-1RI	R&D Systems; Minneapolis, MN, USA
anti-human IL-23R	R&D Systems; Minneapolis, MN, USA
anti-human LAP/TGFβ	BioLegend; San Diego, CA, USA
anti-mouse CCR6	BioLegend; San Diego, CA, USA
anti-mouse CD3	BioLegend; San Diego, CA, USA
anti-mouse CD4	BD Bioscience; Franklin Lakes, NJ, USA
anti-mouse CD45	BD Bioscience; Franklin Lakes, NJ, USA
anti-mouse CD8	BioLegend; San Diego, CA, USA
anti-mouse γδTCR	BioLegend; San Diego, CA, USA

Table 3. Antibodies for FACS Surface Staining

3.4 Antibodies for FACS Intracellular/Intranuclear Staining

Name	Source

anti-human Foxp3	BioLegend; San Diego, CA, USA
anti-human Helios	BioLegend; San Diego, CA, USA
anti-human Ki-67	BD Bioscience; Franklin Lakes, NJ, USA
anti-human RORγt	BD Bioscience; Franklin Lakes, NJ, USA
anti-human T-bet	BD Bioscience; Franklin Lakes, NJ, USA
anti-mouse Foxp3	BD Bioscience; Franklin Lakes, NJ, USA
anti-mouse GATA3	BioLegend; San Diego, CA, USA
anti-mouse IFNγ	BioLegend; San Diego, CA, USA
anti-mouse IL-13	BioLegend; San Diego, CA, USA
anti-mouse IL-17A	BioLegend; San Diego, CA, USA
anti-mouse IL-4	BioLegend; San Diego, CA, USA
anti-mouse IL-5	BioLegend; San Diego, CA, USA
anti-mouse RORγt	BD Bioscience; Franklin Lakes, NJ, USA

Table 4. Antibodies for FACS Intracellular/Intranuclear Staining

3.5 Antibodies for Immunohistochemistry

Name	Source
anti-CD3	A0452, Dako; Hamburg, Germany

anti-F4/80	BM8, BMA Biomedicals; Hiddenhausen, Germany
anti-Foxp3	FJK-16s, eBioscience™, Thermo Fischer Scientific; Waltham, MA, USA
anti-GR1	NIMP-R14, Hycult Biotech; Uden, The Netherlands
anti-MAC2	M3/38; Cedarlane-Laboratories, Burlington, Canada

Table 5. Antibodies for Immunohistochemistry

3.6 Commercial Kits

Name	Source
Antibody-alkaline Phosphatase Kit POLAP	Zytomed; Berlin, Germany
FcR Blocking Reagent, Mouse	Miltenyi Biotec; Bergisch Gladbach, Germany
Foxp3 / Transcription Factor Staining Buffer Set	eBioscience™, Thermo Fischer Scientific; Waltham, MA, USA
IFNγ Mouse ELISA Kit	BioLegend; San Diego, CA, USA
IL-13 Mouse ELISA Kit	eBioscience™, Thermo Fischer Scientific; Waltham, MA, USA
IL-17A Human ELISA Kit	BioLegend; San Diego, CA, USA
IL-17A Mouse ELISA Kit	BioLegend; San Diego, CA, USA
IL-4 Mouse ELISA Kit	BioLegend; San Diego, CA, USA

IL-5 Mouse ELISA Kit	BioLegend; San Diego, CA, USA
LIVE/DEAD™ Fixable Dead Cell Stain Kit	Invitrogen Molecular Probes; Eugene, OR, USA
MACS™ CD4+ T Cell Isolation Kit human	Miltenyi Biotec; Bergisch Gladbach, Germany

Table 6. Commercial Kits

3.7 Devices and Software

Name	Source
40 µm Cell Strainer	Falcon™, Thermo Fisher Scientific; Waltham, MA, USA
70 µm Cell Strainer	Falcon™, Thermo Fisher Scientific; Waltham, MA, USA
BD ARIAIII Cytometer	Becton Dickinson; Heidelberg, Germany
BD LSRII Flow Cytometry Cell Analyzer	Becton Dickinson; Heidelberg, Germany
Biometra Thermal Cycler	Biometra, Analytik Jena; Jena, Germany
FlowJo v10	Becton Dickinson; Ashland, OR, USA
gentleMACS™ Dissociator	Miltenyi Biotec; Bergisch Gladbach, Germany
GraphPad Prism v5.01	GraphPad Software; San Diego, CA, USA

Heraeus™ Multifuge™ X3R Centrifuge	Thermo Fisher Scientific; Waltham, MA, USA
K3 EDTA Micro Tube 1.3ml	Sarstedt; Nümbrecht, Germany
Microsoft Excel v13	Microsoft; Redmond, WA, USA
TC20™ Automated Cell Counter	Bio-Rad Laboratories; Hercules, CA, USA
ZEISS Axio Scope.A1	Carl Zeiss Microscopy; Jena, Germany
ZEISS Axiocam HRc	Carl Zeiss Microscopy; Jena, Germany

Table 7. Devices and Software

4 Methods

4.1 Animal experiments

4.1.1 Animals

All utilized age and sex matched male or female mice were 8- to 10-week-old at the beginning of the experiments. LoxP site–flanked IL-6Ra$^{fl/fl}$ mice (B6;SJL-Il6ratm1.1Drew/J) were initially obtained from J. Scheller, Dusseldorf. T cell–specific deletion of the IL-6Ra chain was achieved by crossbreeding with mice expressing CD4-driven Cre recombinase (B6.Cg-Tg[Cd4-cre]1Cwi/BfluJ), commercially available from The Jackson Laboratory. Rag1 knockout and CD45.1 wild type mice initially derived from The Jackson Laboratory as well. All mice were on a C57BL/6 background and were bred in our facility under specific pathogen-free conditions.

4.1.2 Animal ethics

Animal experiments were performed according to national and institutional animal care and ethical guidelines and were approved by local committees. (Approval codes 34/16, 16/062, 62/16, 73/14, 07/15, 20/16, 65/18).

4.1.3 Compounds

To study the effects of RORγt inhibitory therapy on the course of experimental glomerulonephritis we utilized three different compounds.

(1) The RORγt antisense nucleotides (referred to as RORγt-ASO) provided by Secarna Pharmaceuticals (Munich, Germany). These are thought to bind and degrade RORγt mRNA.

(2) GSK805 (commercially available, GlaxoSmithKline, London, UK) and (3) VTP-44938 (provided by Vitae Pharmaceuticals, Fort Washington, USA) are inverse agonists, which are supposed to block transcriptional effects of RORγt by reversibly binding to its ligand binding domain.

4.1.4 Experimental models and treatment regimes

To prove efficacy of RORγt blockade in inflammation we used three well established mouse models.

(1) Sheep IgG Immunization

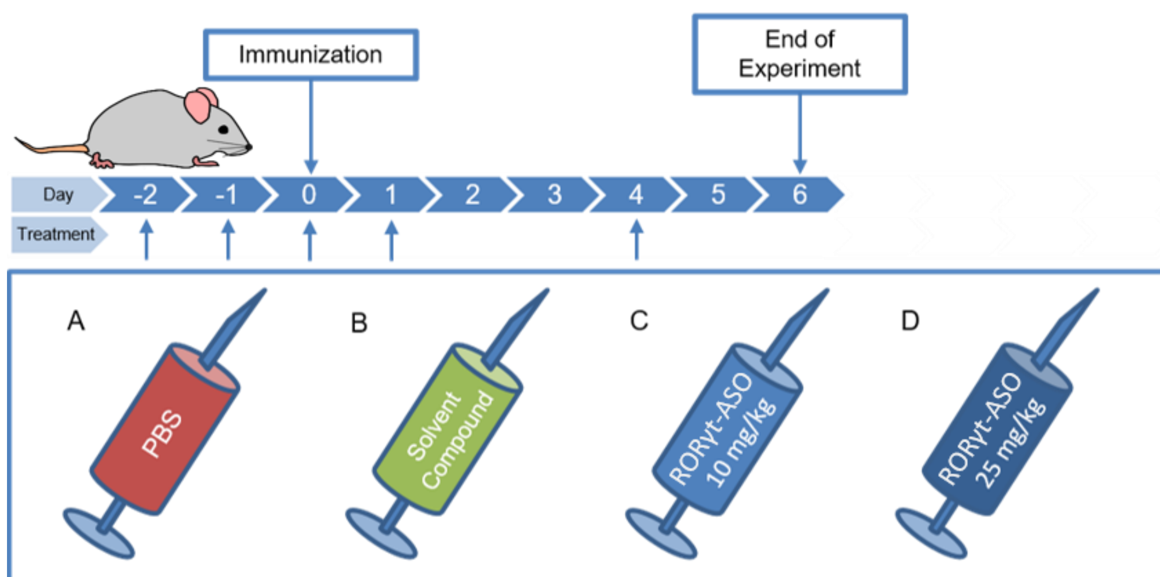

Figure 1. Treatment regime of RORγt-ASO in the immunization model. Mice received either PBS (A), the RORγt-ASO solvent compound (B), or RORγt-ASO at the indicated dosages (C, D).

We first studied efficacy of RORγt-ASO in the sheep IgG immunization model. (Figure 1) To achieve a systemic immune response, we administered 0.5 mg sheep IgG (Sigma-Aldrich; St. Louis, USA) in complete Freund's adjuvant subcutaneously. (Nosko et al. 2017) RORγt-ASO was administered subcutaneously on day -2, day -1, day 0, day 1 and day 4. We used different concentrations: one group of mice received a dosage of 10 mg/kg, another group received 25 mg/kg. We also included a placebo control with PBS and an additional control group with the solvent compound of RORγt-ASO. Endpoints were analyzed on day 6.

(2) Nephrotoxic Nephritis

The nephrotoxic nephritis model (NTN) was induced by single intraperitoneal injection of sheep antibodies against mouse glomerular basement membrane. (Kluger et al. 2016a; Panzer et al. 2007) RORγt-ASO was administered, starting pre-emptively on day -2, day -1, day 0, day 1, day 4, day 6 and day 8. (Figure 2) Dosages used were the same as in the immunization experiments. We again

included controls with application of either PBS or the solvent compound instead of the therapeutic agent. The experiment was terminated on day 10.

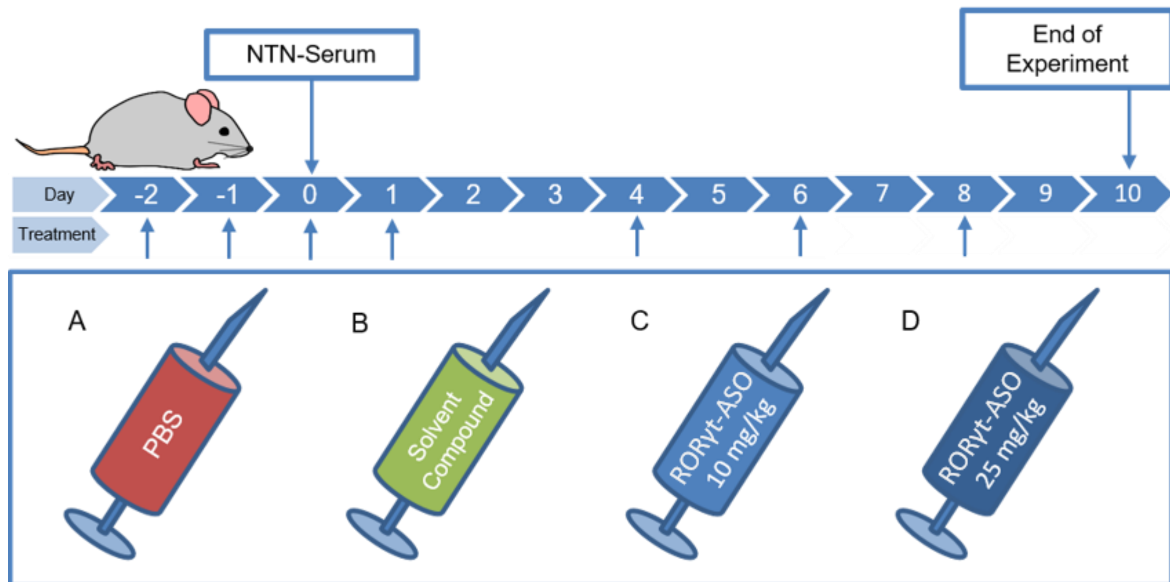

Figure 2. Treatment regime of RORγt-ASO in the NTN model. Mice received either PBS (A), the RORγt-ASO solvent compound (B), or RORγt-ASO at the indicated dosages (C, D).

Likewise, we induced NTN glomerulonephritis and administered GSK805 once daily, starting from day -1. (Figure 3) Mice received GSK805 per gavage at a

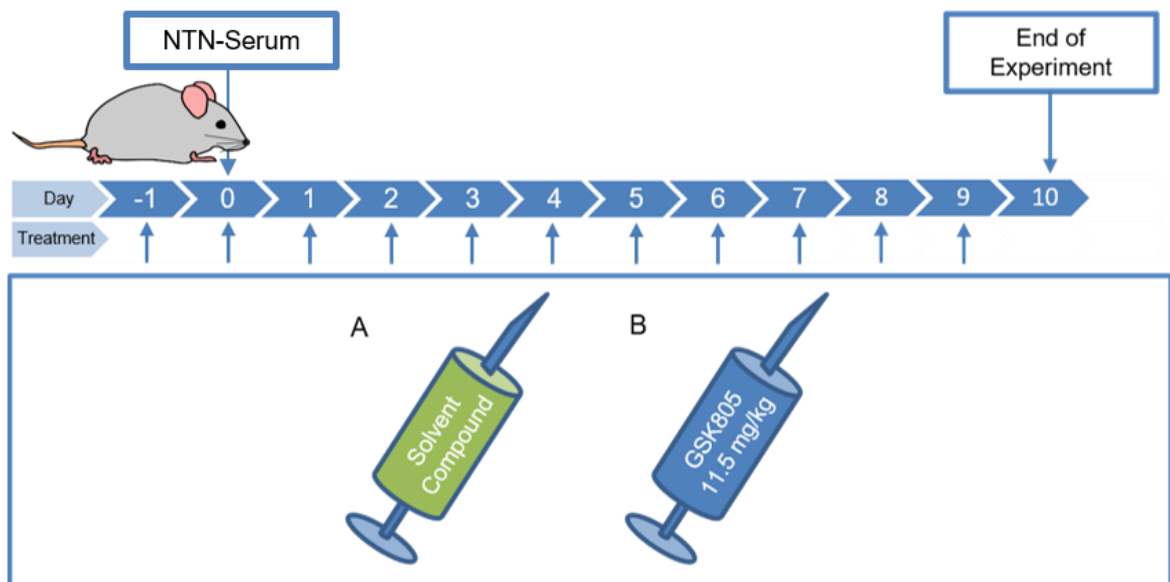

Figure 3. Treatment regime of GSK805 in the NTN model. Mice received either the GSK805 solvent compound (A) or GSK805 (B).

dosage of 11.5 mg/kg, a control group received just the solvent compound. The experiment ended on day 10.

(3) Cisplatin – Acute renal failure

As a model for acute renal failure due to tubular necrosis, we engaged the cisplatin model. (Summers et al. 2011a) Cisplatin was injected intraperitoneally at a dosage of 22 mg/kg. One group of mice received VTP-44938 per gavage at a dosage of 12 mg/kg, a second group received just the solvent compound. Mice were treated daily, starting from day -1. The experiment ended 72 hours after Cisplatin injection.

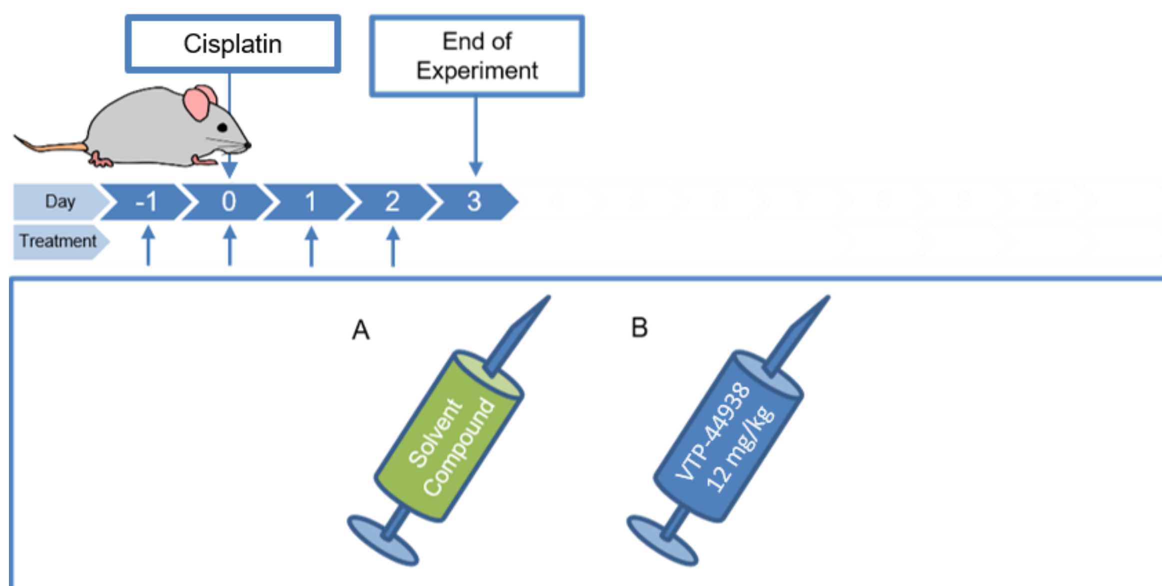

Figure 4. Treatment regime of VTP-44938 in the Cisplatin model. Mice received either the VTP-44938 solvent compound (A) or VTP-44938 (B).

4.1.5 Functional studies

Urine was collected by housing the mice in metabolic cages one day before terminating the experiment. Albumin was determined from the gained urine by using standard ELISA (Bethyl Laboratories, Montgomery, USA). Blood urea nitrogen (BUN) as well as urinary creatinine were measured by standard laboratory methods.

4.1.6 Morphologic studies

In order to compare tissue injury between the different groups, glomerular and tubulointerstitial damage was scored on 2-µm-thin PAS-stained sections. Glomerular crescent formation and necrosis was determined in at least 50 glomeruli per mouse.

To evaluate tubulointerstitial damage, semiquantitative analysis was performed by utilizing a score as published previously. (Phoon et al. 2008) Ten randomly selected cortical areas from each mouse were analyzed at 200-fold magnification. Tubulointerstitial injury was defined as tubular dilation, tubular atrophy, sloughing of tubular epithelial cells, or thickening of the basement membrane, whereas interstitial inflammation was defined according to the degree of leukocyte infiltration in the interstitium. (Phoon et al. 2008) The level of injury was assigned to a scale from 0 to 4: grade 0, no tubulointerstitial injury, no interstitial inflammation; grade 1, less than 25% of the tubulointerstitium injured, minimal tubulointerstitial inflammation; grade 2, 25% to 50% of the tubulointerstitium injured, mild tubulointerstitial inflammation; grade 3, 51% to 75% of the tubulointerstitium injured, moderate tubulointerstitial inflammation; grade 4, more than 75% of the tubulointerstitium injured, diffuse tubulointerstitial inflammation. (Phoon et al. 2008)

To assess the cellular infiltration, the kidney sections were stained with antibodies against CD3 (A0452; Dako, Hamburg, Germany), F4/80 (BM8; BMA, Biomedicals, Hiddenhausen, Germany), MAC2 (M3/38; Cedarlane-Laboratories, Burlington, Canada), Foxp3 (FJK-16s; eBioscience, San Diego, USA), or GR-1 (NIMP-R14; Hycult Biotech, Uden, The Netherlands). Stained sections were developed using a polymer-based secondary antibody alkaline phosphatase kit (POLAP; Zytomed, Berlin, Germany). We counted cell infiltration in 20 glomeruli and 15 tubulointerstitial high power fields per kidney section at 400-fold magnification.

Analyzes were performed in a blinded manner.

4.1.7 Isolation of leukocytes from various tissues

Peripheral blood samples were drawn into EDTA-coated tubes and erylysis was performed.

Spleens were harvested in HBSS (Hanks' Balanced Salt Solution; Thermo Fisher Scientific; Waltham, USA) and passed through 70-µm nylon meshes. After erylysis cells were passed through 40µm nylon meshes.

Kidneys were minced and incubated in digestion medium [RPMI 1640 medium containing 10 % fetal calf serum, 1 % HEPES (all Thermo Fisher), 1 % Penicillin/Streptomycin (Sigma-Aldrich), 8 µg/ml Collagenase D and 0.4 µg/ml DNase (both Roche; Basel, Switzerland)] at 37° C for 45 minutes. In order to achieve a single-cell suspension, kidney tissues underwent a dissociation procedure, using the gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). We next performed Percoll gradient (37 % Percoll; GE Healthcare, Chalfront St Giles, UK) centrifugation at 500 g at room temperature for 20 minutes to further purify cells. Finally, kidney cells also underwent erylysis.

After finishing all isolation steps, cells were washed, counted and prepared for cell culture or FACS staining.

4.1.8 In vitro Cytokine production

In order to examine cytokine production, 4 x 10^6 splenocytes per 1 ml were incubated for 72 hours at 37°C in digestion medium (see above) in the presence of normal sheep IgG (10 µg/ml; Sigma-Aldrich) for antigen specific re-stimulation. Supernatants were harvested and analyzed using commercially available ELISA for IL-13 (eBioscience), IL-4, IL-5, IL-17A and IFNγ (all Biolegend, San Diego, USA).

4.1.9 Flow cytometry

In order to examine cytokine production on the single cell level, isolated leucocytes were activated by incubation in a stimulation medium [X-VIVO™ (Biozym Scientific, Hessisch Oldendorf, Germany) containing 1 µl/ml β-mercaptoethanol, 1

µg/ml Ionomycin (both Calbiochem-Merck, Darmstadt, Germany), 10 µg/ml Brefeldin A and 0,05 µg/ml PMA (both Sigma-Aldrich)] at 37 °C for 150 minutes.

To avoid unspecific antibody-binding, we treated the stimulated cells with a commercial blocking kit (FcR Blocking Reagent, mouse; Miltenyi Biotec), according to the manufacturer's protocol. The cells were then surface stained with fluorochrome-labeled antibodies against CD45, CD4 (both BD Biosciences), CD3, CD8, CCR6 and γδTCR (Biolegend) at 4 °C for 20 minutes. Afterwards cells were washed with PBS to eliminate excess antibodies.

Live/Dead staining (LIVE/DEAD™ Fixable Dead Cell Stain Kit; Invitrogen, Waltham, USA) was used to exclude dead cells. Cells were then permeabilized with a commercial intranuclear staining kit (eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set; Invitrogen).

We performed intracellular and intranuclear staining with fluorochrome-labeled antibodies against Foxp3, RORγt (both BD Biosciences), GATA3, IL-4, IL-5, IL-13, IL-17A and IFNγ (all Biolegend).

4.2 Studies on human blood cells

4.2.1 Blood samples

Human blood samples of ANCA patients were kindly provided by Silke Brix, University Medical Center Hamburg-Eppendorf, Hamburg. She had set up a collective of 112 ANCA patients, according to the guidelines of the local ethics committees (PV3162). Inclusion criteria had been an ANCA detected in the sera and a necrotizing and crescentic GN in the kidney biopsy. (Brix et al. 2018) Healthy controls were achieved from voluntary donors. After informed consent was obtained, peripheral blood was drawn into EDTA-coated tubes.

4.2.2 Isolation of PBMCs

Blood samples were diluted with phosphate buffered saline (PBS) and applied onto the surface of Ficoll (Histopaque®-1077, Sigma-Aldrich). By centrifugation, the blood sample was separated into different phases. PBMCs appeared as a thin layer and were aspirated.

4.2.3 Conservation of blood samples and PBMCs

The solvent dimethyl sulfoxide (DMSO) was added to PBMCs and remaining blood samples. The probes were frozen and stored at – 80 °C.

4.2.4 Stimulation of PBMCs

We performed stimulation assay using only fresh, non-frozen blood cells. 300 ml of a blood sample underwent erylysis (erylysis buffer consists of 1 part Erylysis Stock Solution 1 and 9 parts Erylysis Stock Solution 2). After centrifugation, erythrocyte-free samples were incubated with 1 ml stimulation buffer (see above) at 37 °C for 4 hours. After incubation cells were ready for FACS staining.

4.2.5 T cell isolation and Treg suppression assay

Treg suppression assays were performed using only freshly gained, non-frozen human blood PBMCs. $CD4^+$ T cells were isolated with magnetic-activated cell sorting, following the manufacturer's protocol (MACS $CD4^+$ T Cell Isolation Kit human; Miltenyi Biotec). In order to separate T effector cells from Tregs, the isolated $CD4^+$ T cells were stained with fluorochrome-labeled antibodies against CD4 and CD25 (Biolegend), followed by FACS sorting (performed on a BD ARIAIII Cytometer; BD Biosciences, Franklin Lakes, USA). The isolated CD25 negative T effector cells were incubated alone or as a mixture with CD25 positive Tregs in ascending concentrations as indicated in the results. As controls, Tregs were incubated alone as well. All cells were incubated for 72h at 37°C in culture medium (1 x 10^6 cells/ml in RPMI 1640 medium containing 10 % fetal bovine serum, 1 % Penicillin/Streptomycin, 1 µl/ml β-mercaptoethanol) in anti-CD3 antibody precoated 96-well plates (2 µg/ml; BD Bioscience) with addition of anti-CD28 antibody (2µl/well; BioLegend).

In order to quantify the Treg suppressive capacity, cytokine production was analyzed by ELISA from the supernatants. To assess T effector cell proliferation, we performed FACS analysis of Ki67 expression, using an anti-Ki-67 antibody (BD Biosciences).

4.2.6 Flow cytometry of human leucocytes

Frozen human blood samples were defrosted by adding of warm (37 °C) cell buffer (consisting of 20 % fetal calf serum and 80 % digestion medium). Given the toxicity of DMSO, the solvent was eliminated by centrifugation and resuspension of cells in the above described cell buffer. Cells were then incubated at 37°C for 45 minutes.

To avoid unspecific antibody-binding, we treated the cells with a commercial blocking kit (FcR Blocking Reagent, human; Miltenyi Biotec), according to the manufacturer’s protocol. Surface-staining was performed with fluorochrome-labeled antibodies against CD45, CD3, CD4, CD8, CD25, CCR6, CXCR3, CD45RA, CD44, CD161, CD127, LAP/TGFβ, ICOS, CTLA4, CD103, CD69 (all Biolegend), IL-23R, CCR8 and IL-1RI (R&D Systems; Minneapolis, MN, USA) at 4 °C for 20 minutes. Excess antibodies were washed away by centrifugation and resuspending the cells in PBS. In order to gain ability to differentiate between alive and dead cells, we used a dead cell staining kit (LIVE/DEAD™ Fixable Dead Cell Stain Kit; Invitrogen, Waltham, USA), following the manufacturer’s protocol. For intracellular staining, we engaged a permeabilization kit (eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set; Invitrogen). Permeabilization was performed at room temperature for 45 minutes, according to the manufacturer’s instructions. The permeabilized cells were then washed and stained with antibodies against Foxp3, Helios (both Biolegend), RORγt, T-bet and Ki67 (BD Biosciences) at 4°C for 45 minutes. Excess antibodies were again washed away by centrifugation and resuspending the cells in PBS.

Flow cytometry was performed on a BD LSRII Cytometer (BD Biosciences).

4.3 Statistical analysis

GraphPad Prism v6.01 (GraphPad Software; San Diego, CA, USA) was used for statistical analysis. T tests were performed to compare two groups. To compare more than two groups ANOVA with Tukey post hoc analysis was used. A *P* value < 0.05 was considered to be statistically significant.

5 Results – Part One: RORγt inhibition in different experimental models of inflammation

5.1 Effects of RORγt-ASO treatment on T cell responses after sheep IgG immunization

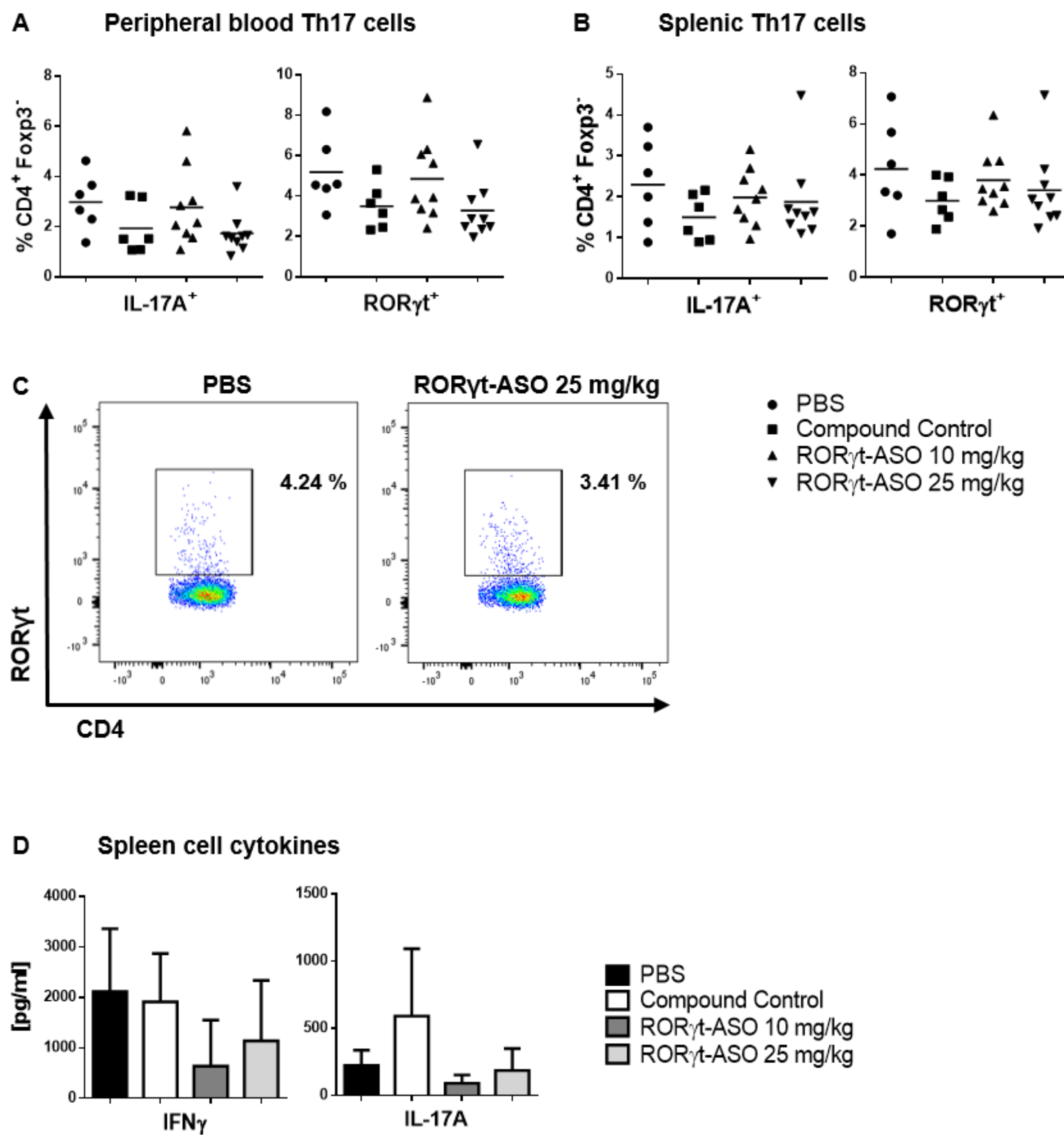

Figure 5. Treatment with RORγt-ASO does not affect Th17 cells in spleen or peripheral blood after sheep IgG immunization. All analyses derive from 8- to 10-week-old mice which were immunized with sheep IgG on day 0 and received the indicated treatment regimens on day -2, -1, 0, 1 and 4. Analyses were conducted at day 6. (A) FACS analysis of peripheral blood T effector cells expressing Th17 cytokine IL-17A or transcription factor RORγt as indicated. (B) FACS analysis of splenic T effector cells expressing Th17 cytokine IL-17A or transcription factor RORγt as indicated. (C) Representative FACS plots of splenic RORγt+ Th17 cells. (D) ELISA analyses of the indicated cytokines from supernatant of sIgG re-stimulated spleen cell cultures from mice of the indicated groups. Symbols represent individual animals, horizontal lines show mean values. Bar graphs show means, and error bars indicate SEM.

5.1.1 The Th17 cell response is not reduced after treatment with RORγt-ASO

In a first step we sought to determine effects of RORγt-ASO on development of Th17 cell responses. We therefore immunized mice with sheep IgG (sIgG) in FCA and administered RORγt-ASO in different concentrations. Two additional groups of mice received either just the solvent compound or PBS. FACS analysis of $CD4^{+}$ T cell subtype frequencies in blood and spleens was performed on day 6. Surprisingly, splenic and blood Th17 cell frequencies and IL-17 production did not differ between RORγt-ASO treated mice and the control groups. (Figure 5 A-C)

5.1.2 RORγt-ASO treatment does not reduce secretion of pro-inflammatory cytokines

In addition, we examined cytokine secretion of cultured sIgG re-stimulated splenocytes from immunized RORγt-ASO treated mice. However, similar to the FACS data, there was no difference in either IL-17 or IFNy levels detected in spleen cell culture supernatants between the groups. (Figure 5 D)

5.2 RORγt-ASO treatment of the NTN model of crescentic glomerulonephritis

We next sought to examine the effectivity of RORγt-ASO in the NTN model of crescentic glomerulonephritis and pre-emptively treated mice either with RORγt-ASO, solvent compound or placebo. First we studied effects of RORγt-ASO on tissue injury. However, no differences with respect to glomerular crescent formation and tubulointerstitial injury were detected (data not shown).

Next, we conducted FACS analyses of renal and systemic T cells. Similar to the results from our immunization studies, we did not detect reductions in either Th17 or Th1 cell responses by RORyt-ASO treatment. (Figure 6)

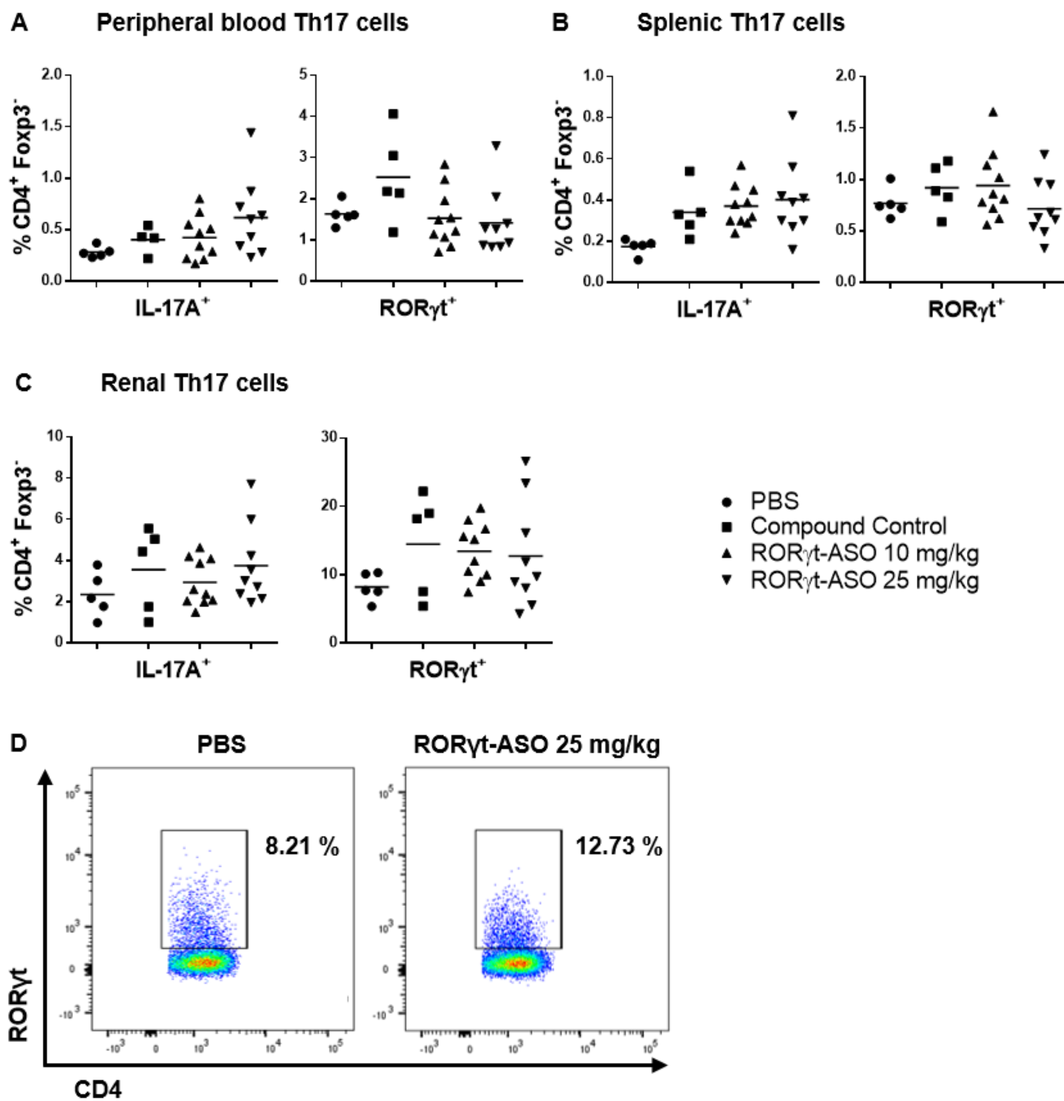

Figure 6. In the NTN model of glomerulonephritis, treatment with RORγt-ASO does not reduce Th17 responses. All analyses derive from 8- to 10-week-old mice which received the indicated treatment regimens on day -2, -1, 0, 1, 4, 6 and 8. Analyses were conducted at day 10. (A) FACS analysis of peripheral blood T effector cells expressing Th17 cytokine IL-17A or transcription factor RORγt as indicated. (B) FACS analysis of splenic T effector cells expressing Th17 cytokine IL-17A or transcription factor RORγt as indicated. (C) FACS analysis of renal T effector cells expressing Th17 cytokine IL-17A or transcription factor RORγt as indicated. (D) Representative FACS plots of renal RORγt+ Th17 cells. Symbols represent individual animals, horizontal lines show mean values.

5.3 GSK805 in the NTN model of crescentic glomerulonephritis

5.3.1 Treatment with GSK805 does not ameliorate structural damage or prevent loss of kidney function

As an alternative RORγt-neutralizing compound, we deployed GSK805 and studied effects on the course of NTN. In a first step we compared histological injury in the kidneys. However, similar to the findings from treatment with the RORγt-ASO, we did not detect relevant changes in crescent formation or tubulointerstitial injury after pre-emptive treatment with GSK805. (Figure 7 A, B)

Next, we aimed to examine functional parameters and determined blood urea nitrogen (BUN) and albuminuria. In congruence with unchanged histological

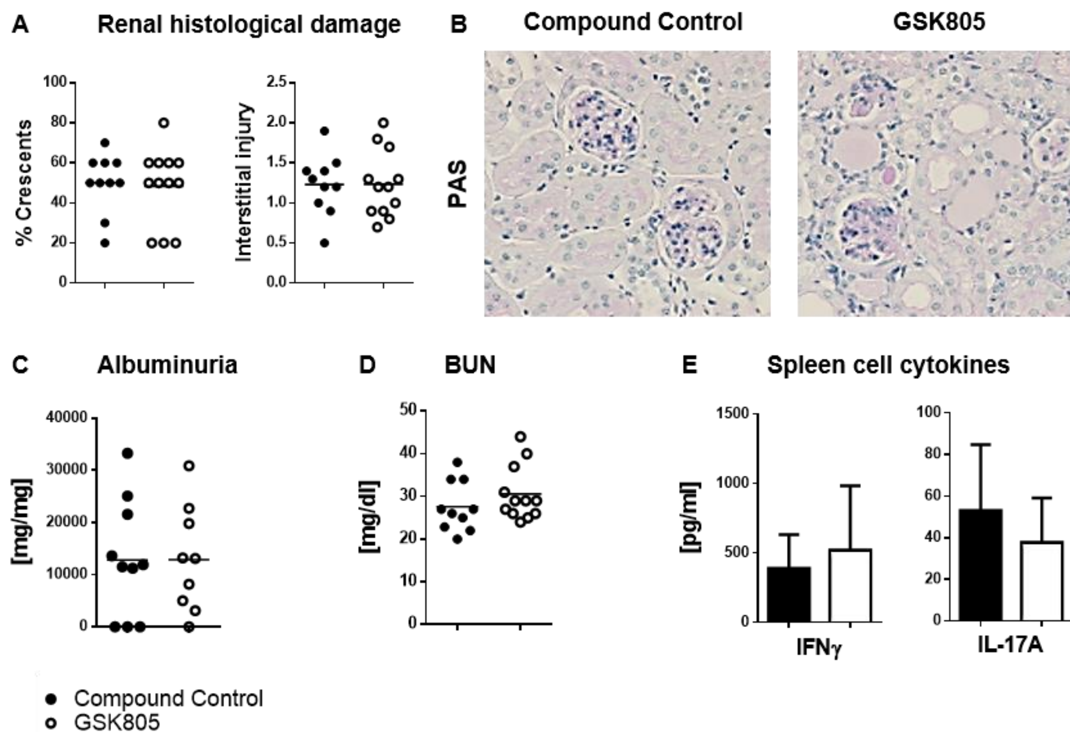

Figure 7. Treatment with GSK805 does not ameliorate renal injury or splenic cytokine secretion in NTN. All analyses derive from 8- to 10-week-old mice, which received the indicated treatment regimens daily, starting from day -1. Analyses were conducted at day 10. Quantification of (A) glomerular crescents (left) and interstitial damage (right). (B) Representative PAS-stained sections of nephritic kidneys. (C) Albuminuria (albumin/creatinine ratio), (D) Blood urea nitrogen (BUN). (E) ELISA analyses of the indicated cytokines from supernatant of anti-sIgG re-stimulated spleen cell cultures. Symbols represent individual animals, horizontal lines show mean values. Bar graphs show means, and error bars indicate SEM.

damage, we did not detect differences in either parameter between the two groups. (Figure 7 C, D)

5.3.2 GSK805 treatment does not reduce secretion of pro-inflammatory cytokines

Next, we studied cytokine secretion by splenocytes obtained from nephritic mice treated either with GSK805 or placebo. Similar to the RORγt-ASO studies, we did not find any significant influence of GSK805 on levels of pro-inflammatory cytokines in cell culture supernatants of re-stimulated splenocytes. (Figure 7 E)

5.4 VTP-44938 in the cisplatin model of acute renal failure

5.4.1 VTP-44938 treatment does not ameliorate structural damage or loss of kidney function

As a third compound interfering with RORγt signaling, we analyzed VTP-44938. As a model, we chose cisplatin induced acute renal failure. Experimental mice were pre-emptively treated either with VTP-44938 or placebo. Similar to our NTN

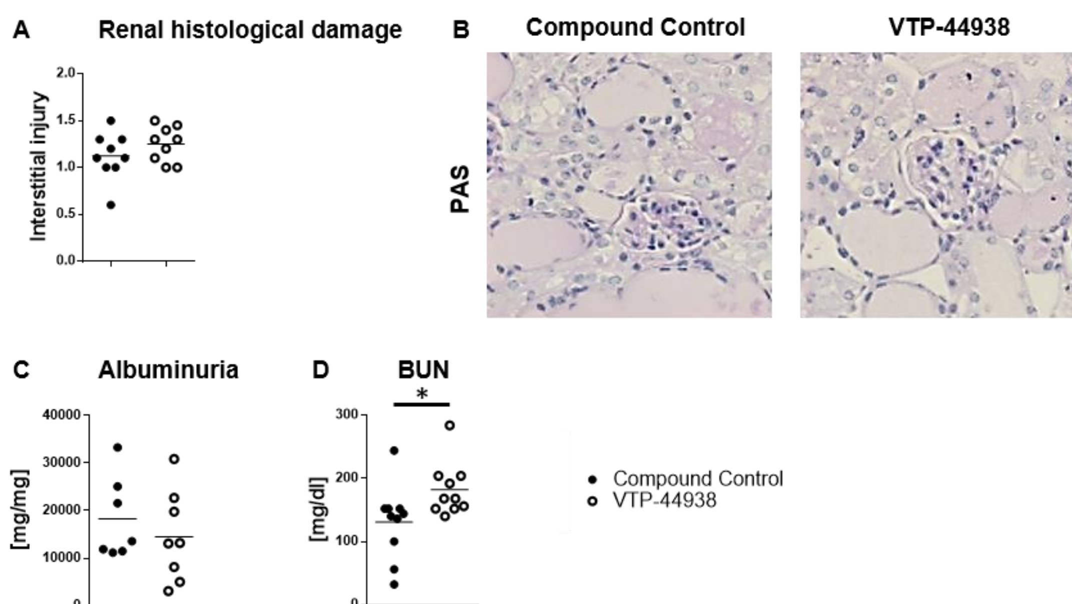

*Figure 8. In the cisplatin model, treatment with VTP-44938 does not ameliorate functional or histological outcome. All analyses derive from 8- to 10-week-old mice which received the indicated treatment regimens daily, starting from day -1. Analyses were conducted at day 3. Quantification of (A) interstitial damage, (B) Representative PAS-stained sections of acutely injured kidneys. (C) Albuminuria (albumin/creatinine) and (D) BUN. Symbols represent individual animals, horizontal lines show mean values. Bar graphs show means, and error bars indicate SEM. * P<0.05.*

experiments, however, we did not find any relevant effects of treatment with VTP-44938 on renal histological damage. (Figure 8 A, B)

In addition, we again examined functional kidney parameters. While albuminuria was similar, BUN levels were even higher in the VTP-44938 treated group than in the controls. (Figure 8 C, D)

6 Results – Part Two: Specific IL-6Ra knockout on CD4$^+$ T cells

As a part of my thesis, I also contributed some analyses to projects, which were mainly managed by other members of our lab group. The results are outlined below and have been included in a published manuscript. (Hagenstein et al. 2019)

6.1 Effects of specific IL-6Ra knockout on CD4$^+$ T cells on cells infiltrating the kidney in GN

6.1.1 IL-6Ra classic signaling in CD4$^+$ T cells mediates renal infiltration of neutrophils in GN

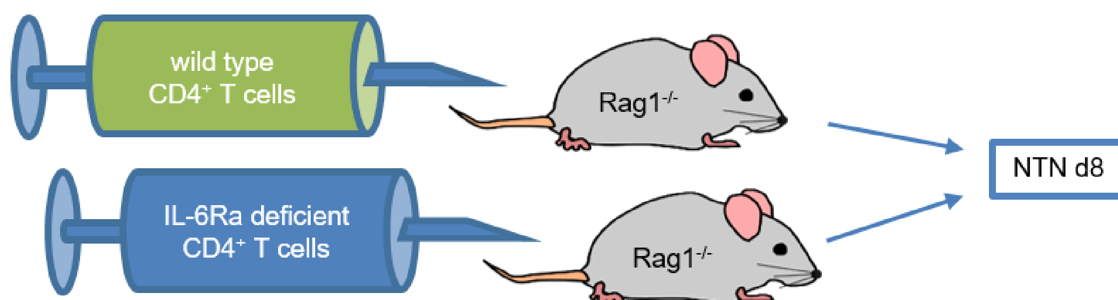

Figure 9. Experimental setup of the cell transfer experiment. Lymphocyte deficient Rag1 $^{-/-}$ mice received either wild type or IL-6Ra $^{-/-}$ CD4 T cells. The experiment ended on day 8 after NTN induction.

Given, that IL-6 is thought to be a central mediator of pathogenic Th17 responses, we aimed to examine whether specific IL-6Ra knockout on CD4$^+$ T cells affects the course of experimental glomerulonephritis. Since in these mice, IL-6R expression is not only abrogated on CD4$^+$ T cells but also on CD8$^+$ T cells, as well as on a subset of dendritic cells, we performed adoptive cell transfer studies. We injected highly purified wild-type or IL-6Ra$^{-/-}$ CD4$^+$ T cells into lymphocyte deficient Rag1$^{-/-}$ mice and induced NTN. (Figure 9)

First we determined renal T cell infiltration by counting CD3 positive cells on day 8 of NTN. No difference was shown between mice that had received wild-type CD4$^+$ T cells and those which had received IL-6Ra$^{-/-}$ CD4$^+$ T cells. (Figure 10 A, B)

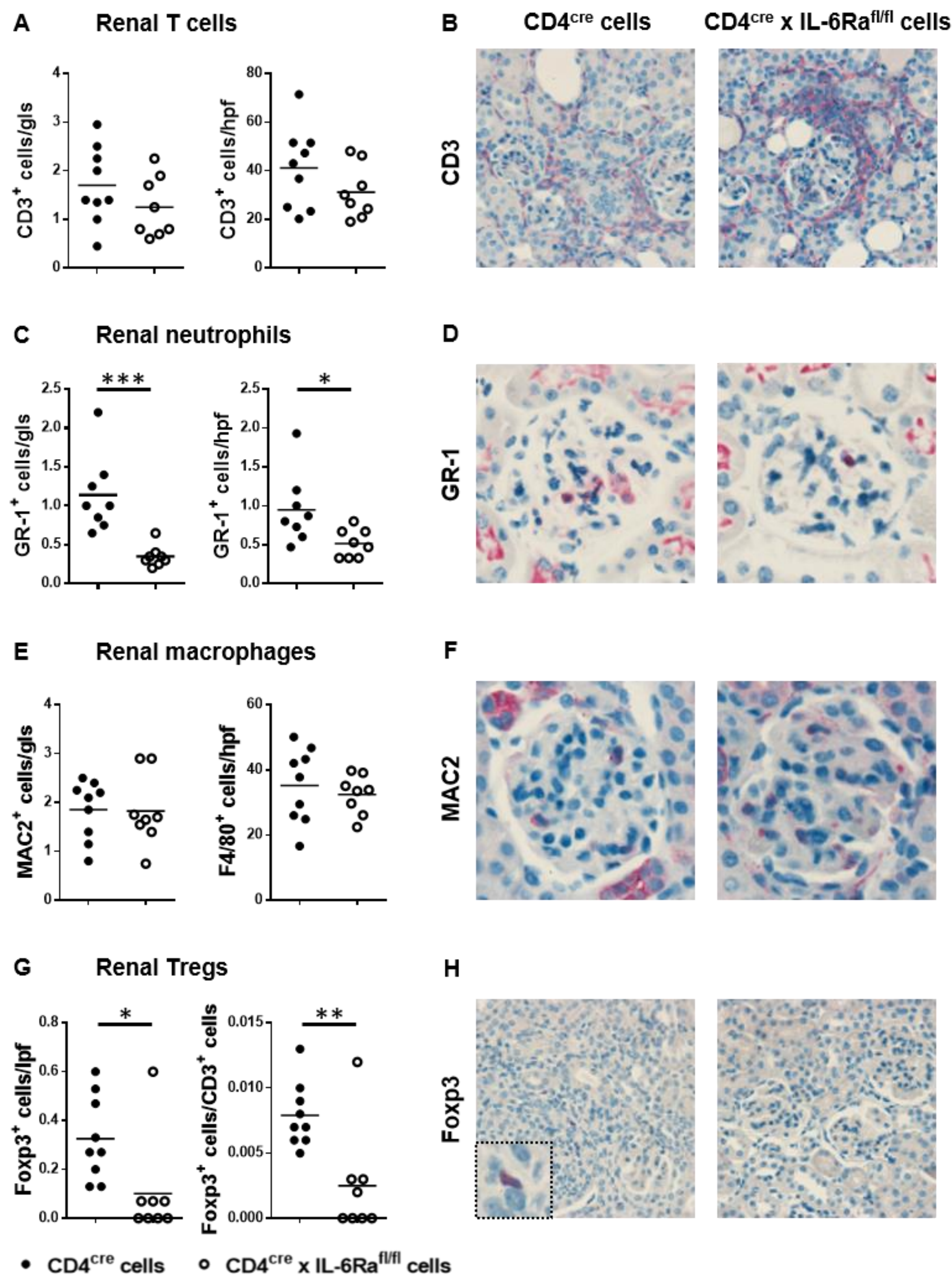

*Figure 10. Renal neutrophil and Treg trafficking is mediated by IL-6Ra signaling in CD4⁺ T cells. Immunohistochemical analysis of renal immune cells. Rag1-/- mice received the indicated donor cells 1 day before induction of NTN. Kidneys were isolated 8 days after NTN induction. (A) Quantification of glomerular (left) and interstitial (right) CD3⁺ T cell infiltration. (B) Representative renal immunohistochemical stainings of CD3 (red). (C) Quantification of glomerular (left) and interstitial (right) GR-1⁺ neutrophil infiltration. (D) Representative renal immunohistochemical stainings of GR-1 (red). (E) Quantification of glomerular (left) MAC-2⁺ and interstitial (right) F4/80⁺ macrophage infiltration. (F) Representative renal immunohistochemical stainings of MAC2 (red). (G) Quantification of interstitial Foxp3⁺ Treg infiltration (left) and the ratio of interstitial Tregs to whole CD3⁺ T cells (right). (H) Representative renal immunohistochemical stainings of Foxp3 (red). Symbols represent individual animals, horizontal lines show mean values. * P<0.05, ** P<0.01, *** P<0.001. gls, glomerulus; hpf, high power field; lpf, low power field.*

Next we analyzed renal infiltration of neutrophils, since they constitute the main effector arm of Th17 responses. Counting GR-1 positive renal cells indeed revealed a significantly diminished glomerular and interstitial infiltration of neutrophils in recipients of IL-6Ra$^{-/-}$ CD4^{+} T cells. (Figure 10 C, D)

In a next step we analyzed renal infiltration of macrophages, which represent the main effector arm of Th1 responses. In order to determine glomerular infiltration we counted MAC2 positive cells, whereas for interstitial infiltration F4/80 positive cells were counted. In both analyses no difference was found between recipients of wild type or IL-6R deficient CD4^{+} T cells. (Figure 10 E, F)

6.1.2 IL-6Ra classic signaling in CD4^{+} T cells mediates renal Treg infiltration

Next we sought to analyze, whether IL6R signaling might not only affect generation and expansion of Th17 cells, but also that of Tregs. Thus, we determined renal Treg infiltration in the aforementioned Rag1$^{-/-}$ recipients of wild type or IL-6R deficient CD4^{+} T cells. Interestingly and somewhat unexpectedly, Treg numbers, as well as percentages were significantly reduced, if IL-6R deficient CD4^{+} T cell were transferred. (Figure 10 G, H)

7 Results – Part Three: Development of a flow cytometry protocol for analyzing human PBMCs

7.1.1 *PBMCs are vital after defrosting*

As the period between withdrawal of human blood samples and their analysis varied from months to years, we initially sought to ensure, that vitality of the cells was still adequate.

We therefore performed dead cell staining on cells that had been frozen for either 1 day or 12 months. These PBMCs did not significantly differ in vitality. (Figure 11)

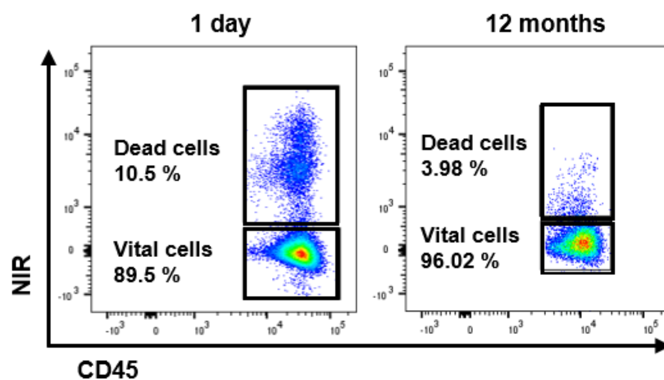

Figure 11. Vitality of defrosted cells is not affected by the time of storage. Representative FACS plots of human CD45⁺ lymphocytes gated on dead/alive cells by NIR-staining after 1 day of freezing (left) or 12 months of freezing (right).

7.1.2 *Establishment of a flow cytometry based protocol for identification of various human peripheral blood T cell subtypes*

Next, we used staining of CD3, CD4 and CD8 to identify T helper and T cytotoxic cell subtypes among PBMCs. Subsequently, we aimed to establish a staining strategy to further differentiate functionally distinct $CD4^+$ T cell subsets.

Th17 and regulatory T cells were differentiated depending on the expression of their nuclear hallmark transcription factors RORγt and Foxp3. $CD4^+$ Tregs coexpressing RORγt and Foxp3 were labelled as biTregs.

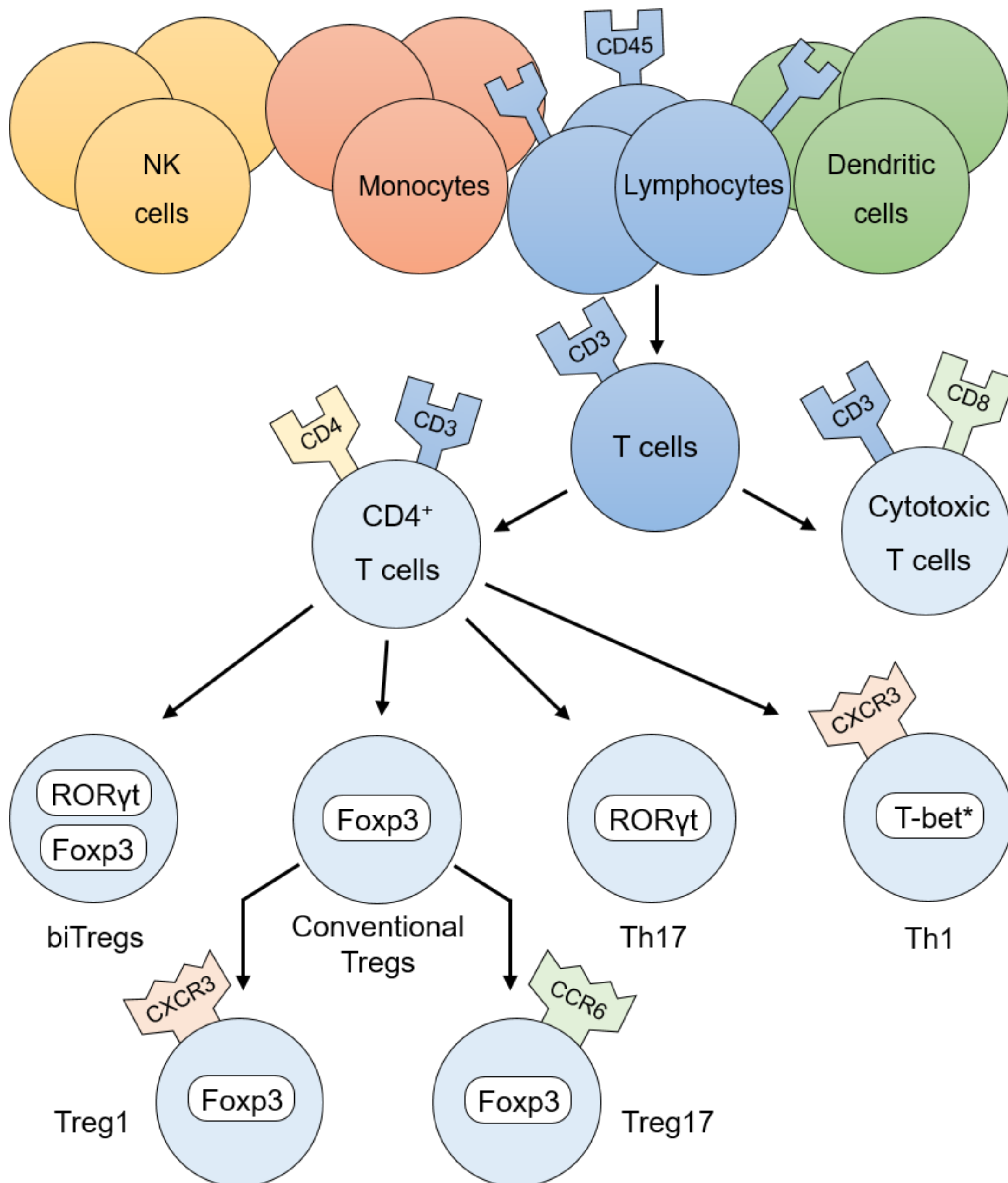

Figure 12. Gating strategy for FACS analysis of human PBMCs. T cells are identified by expression of CD3. T cells can be further divided into $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells. According to differing transcription factor profiles, $CD4^+$ T cells can be further divided into conventional Tregs ($ROR\gamma t^{neg} Foxp3^+$), biTregs ($ROR\gamma t^+$ $Foxp3^+$), Th1 ($CXCR3^+$ $CCR6^{neg}$ $ROR\gamma t^{neg}$ $Foxp3^{neg}$; staining of the Th1 transcription factor T-bet did not work reliably) and Th17 ($ROR\gamma t^+$ $Foxp3^{neg}$) cells. Conventional Tregs can be further separated into $CXCR3^+$ $CCR6^{neg}$ Treg1 and $CXCR3^{neg}$ $CCR6^+$ $ROR\gamma t^{neg}$ Treg17 cells.

In order to identify additional Treg subtypes, we used staining of the chemokine receptors CXCR3 and CCR6. $CXCR3^+$ $Foxp3^+$ $ROR\gamma t^{neg}$ Tregs were referred to as Treg1 cells, whereas $CCR6^+$ $Foxp3^+$ $ROR\gamma t^{neg}$ Tregs were referred to as Treg17 cells in analogy to our classification, which we established in the murine system.

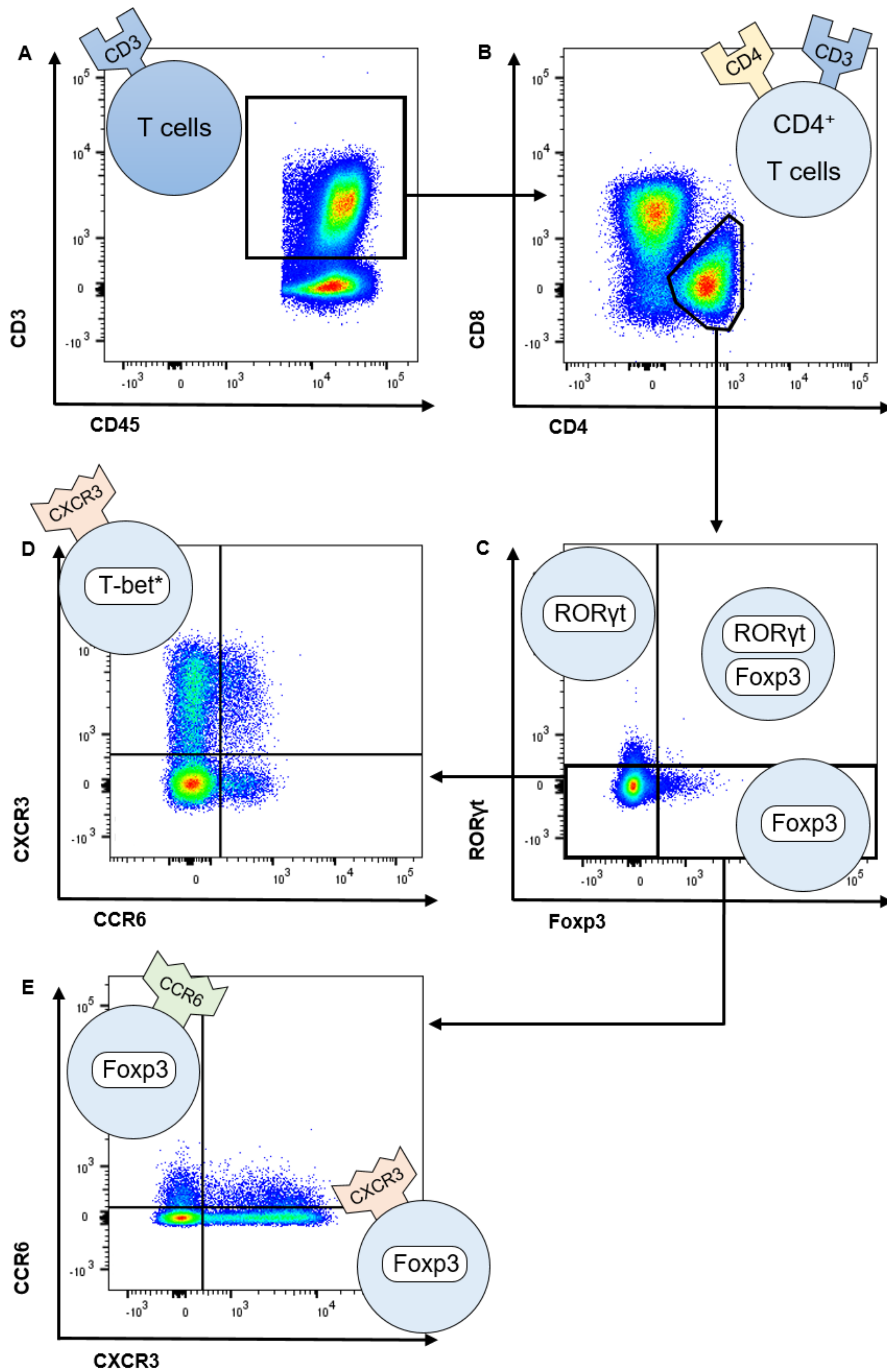

Figure 13. Representative FACS plots of human PBMCs stained and gated according to Figure 12.

Since staining of the Th1 transcription factor T-bet did not work reliably, we classified $CXCR3^{+}$ $CCR6^{neg}$ $ROR\gamma t^{neg}$ $Foxp3^{neg}$ $CD4^{+}$ T helper cells as Th1 cells, knowing that Th1 cells constitutionally express the CXCR3 but not the CCR6. A schematic overview is given in Figure 12, the gating strategy with representative FACS plots is shown in Figure 13.

To gain knowledge about the activation status of the distinct T cell subtypes, we also performed staining of CD45RA and CD44. CD45RA is expressed exclusively on naïve T cells, whereas CD44 is expressed only on activated or memory T cells. (Figure 14) Since these properties exclude each other, we indeed did not find any cells coexpressing these two proteins. (Figure 14)

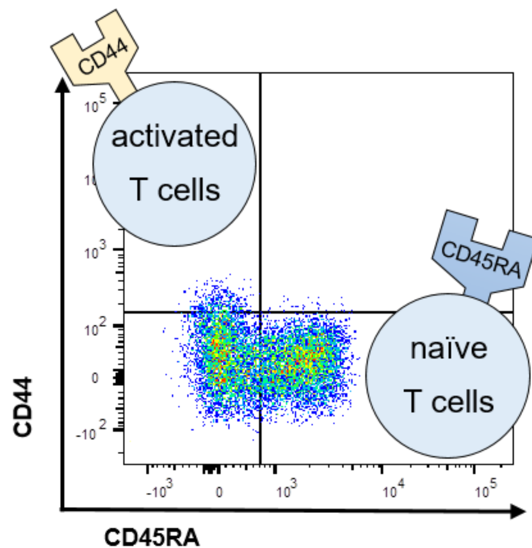

Figure 14. Representative FACS plot of human PBMCs pre-gated on $CD4^{+}$ T cells and stained for CD44 and CD45RA. $CD44^{+}$ $CD45RA^{neg}$ T cells were referred to as activated (or memory) T cells, $CD44^{neg}$ $CD45RA^{+}$ T cells as naïve T cells.

7.1.3 Establishment of a human Treg in vitro suppression assay

Since utilization of Treg suppression assays is a helpful procedure in animal experiments to assess Treg functions, we sought to also establish this method for characterization of human Tregs. We therefore incubated FACS-sorted T effector cells from the blood of healthy human donors with sorted Tregs at the indicated ratios. Proliferation of T effector cells after stimulation was measured by their Ki67

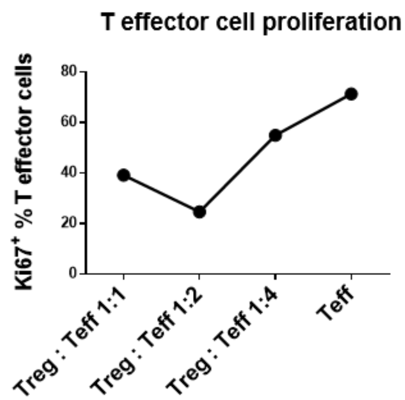

Figure 15. In vitro suppression assays were performed by co-culturing human T effector cells with regulatory T cells at the indicated ratios. T effector cell proliferation was measured by FACS analysis of Ki67 expression in T effector cells.

expression. As expected, increasing Treg concentration resulted in impaired T effector cell proliferation, proving functionality of the assay. (Figure 15)

7.2 Characterization of peripheral blood T cells from patients with ANCA-RPGN

After having established a FACS based protocol to characterize human T cell subpopulations, we wanted to apply this method to PBMCs from ANCA-RPGN patients.

7.2.1 Blood samples

Peripheral blood samples of patients suffering from acute ANCA-RPGN were obtained from our RPGN-study cohort (Brix et al. 2018). Blood withdrawal had taken place after corticosteroid administration but before cyclophosphamide application. 20 blood samples were randomly picked from this cohort. Retrospective linkage to their clinical characteristics was not possible. Blood from healthy controls derived from voluntary donors.

Table 8 displays the main clinical data from voluntary donors and the RPGN-study cohort. (Brix et al. 2018).

	Healthy control	RPGN-study cohort
Patients, *n*	20	112
Age, median years (IQR)	35 (24-39)	66 (54–72)
Male sex, *n* (%)	12 (60)	88 (78.6)
ANCA-type		
Proteinase 3, *n* (%)		58 (51.8)
Myeloperoxidase, *n* (%)		54 (48.2)
Renal function at time of diagnosis		
Creatinine clearance, * median ml/min (IQR)		28 (21-44)
Dialysis dependence, *n* (%)		26 (23.2)

*Table 8. Main clinical characteristics of patients and voluntary donors. 20 randomly chosen blood samples from the RPGN-study cohort were used for further analysis. IQR, interquartile range; * Patients not on dialysis*

7.2.2 Impaired vitality in PBMCs from ANCA-RPGN patients

In order to exclude dead cells from analysis, we performed dead cell staining. Generally, the samples showed a high percentage of live cells, indicating good quality. However, it must be noted, that the fraction of live $CD45^+$ lymphocytes, as

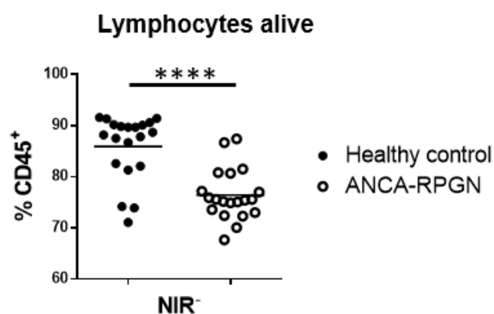

*Figure 16. FACS analysis of lymphocytes. Vital cells were defined as CD45+ lymphocytes, negative for NIR. Symbols represent individual patients, horizontal lines show mean values. **** P< 0.0001.*

indicated by negativity for NIR staining, was slightly but significantly lower in the ANCA-RPGN group. (Figure 16)

7.2.3 Th17 expand during ANCA-RPGN

As a next step we wanted to study frequencies of the different T effector cell subpopulations. Since staining of the Th1 transcription factor T-bet did not work reliably in our hands, we defined Th1 cells as $CD4^+$ T cells negative for RORγt, Foxp3 and the Th17 characteristic CCR6, but positive for CXCR3. (Figure 12, Figure 13) This gating strategy is somewhat limited by the fact, that CXCR3 is probably not expressed on all blood Th1 cells. These limitations in mind, we found reduced frequencies of Th1 cells in the blood of ANCA-RPGN patients. (Figure 17 A, B, E) In contrast, $ROR\gamma t^+$ $Foxp3^-$ Th17 cells were expanded (Figure 17 F, G, J).

By comparing CD44 and CD45RA expression, we sought to compare the activation status of the Th1 and Th17 cells. CD44 expression was indeed higher on Th1 cells from ANCA-RPGN patients (Figure 17 C), indicating a higher activation status. Expression of CD45RA was similar between the groups (Figure 17 D). Looking at Th17 cells, we found no differences regarding CD44 expression (Figure 17 H). In contrast, however, virtually all Th17 cells from ANCA-RPGN patients were negative for CD45RA expression (Figure 17 I), indicating previous activation by antigen.

7.2.4 Characterization of Regulatory T cells subpopulations in the blood of ANCA-RPGN patients

Since currently almost nothing is known about alterations of the Treg compartment in patients with ANCA-RPGN, we next examined Treg frequencies, phenotype, subpopulations and their activation status.

Total Tregs, as well as $Foxp3^+$ $ROR\gamma t^{neg}$ conventional Tregs (cTregs) were significantly expanded in the blood of ANCA-RPGN patients. (Figure 18 A, D) They also had a higher expression level of CD44 and a lower expression level of CD45RA, indicating a higher activation status. (Figure 18 B, C, E, F)

As a distinct effector Treg subtype, we next aimed to characterize $Foxp3^+$ $ROR\gamma t^+$ biTregs in the peripheral blood of ANCA-RPGN patients. However, their numbers

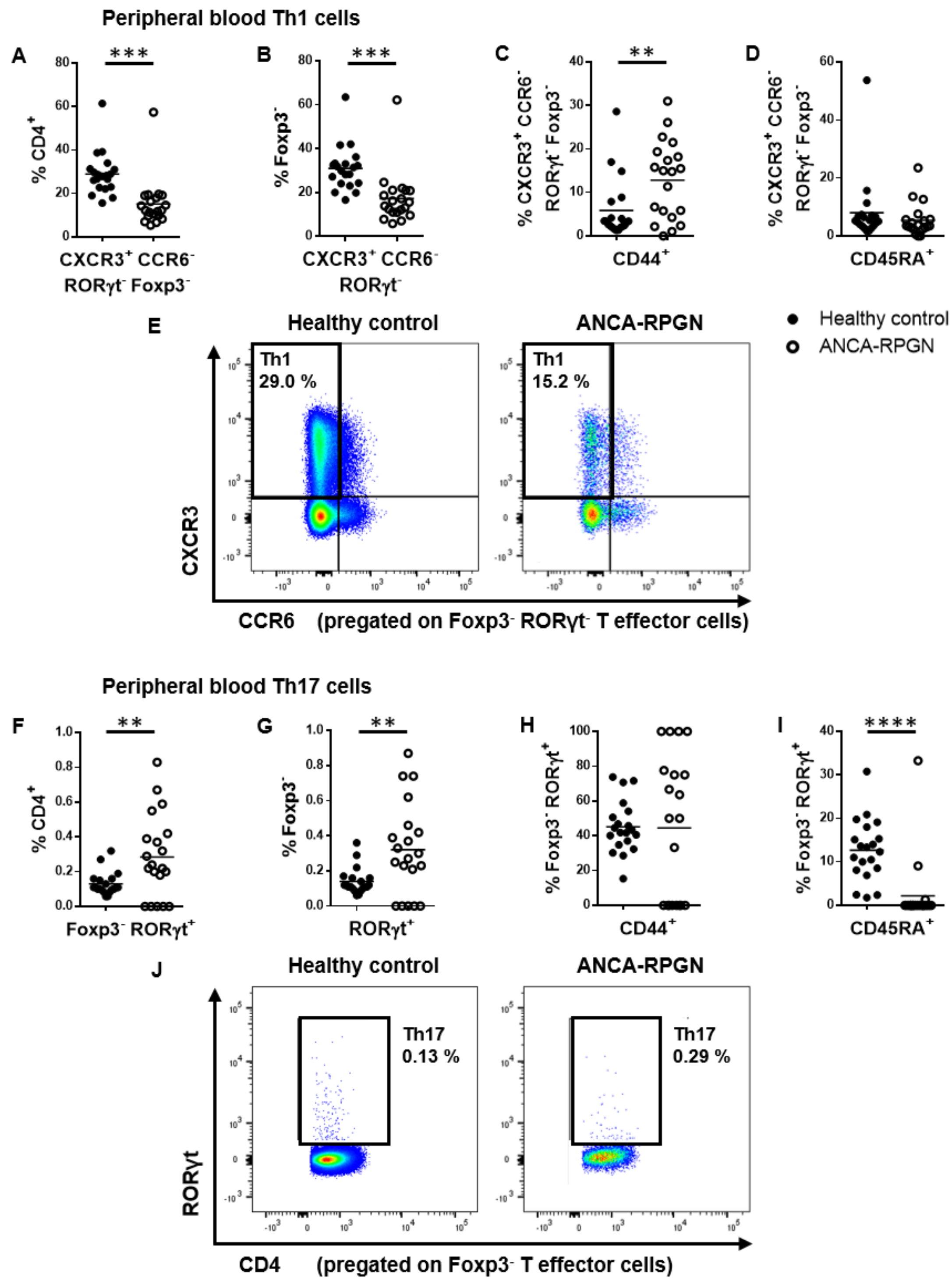

*Figure 17. Th17 cells expand during ANCA-RPGN, while Th1 frequencies decrease. Frequencies and representative FACS plots of Th1 (A, B, E) and Th17 cells (F, G, J) among CD4+ T cells and their distinct expression levels of CD44 (C, H) and CD45RA (D, I). Symbols represent individual patients, horizontal lines show mean values. ** P<0.01, *** P<0.001, **** P< 0.0001.*

were rather low and we did not detect differences between the groups with respect to their frequencies. (Figure 18 G) Measurement of the activation status of biTregs was not possible due to their low frequencies.

An overview of the gating strategy is shown in Figure 18 H, I.

Next, we went on to study Treg1 and Treg 17 cells.

Treg1 cell frequencies among total blood $CD4^+$ T cells were unchanged (Figure 19 A), while their frequencies among blood total Tregs were significantly lower in the ANCA-RPGN group (Figure 19 B). Frequencies of $CD44^+$ Treg1 cells were similar between the groups (Figure 19C). In contrast, CD45RA expression on Treg1 cells was reduced in the ANCA-RPGN group, indicating higher antigen activation. (Figure 19 D)

Treg17 frequencies among $CD4^+$ T cells were somewhat higher in the ANCA-RPGN group (Figure 19 E), but did not differ substantially among total Tregs (Figure 19 F). Treg17 cell activation was significantly enhanced in the ANCA-RPGN group, as shown by higher CD44 (Figure 19 G) and lower CD45RA expression (Figure 19 H). An overview of the gating strategy is given in Figure 19 I, J.

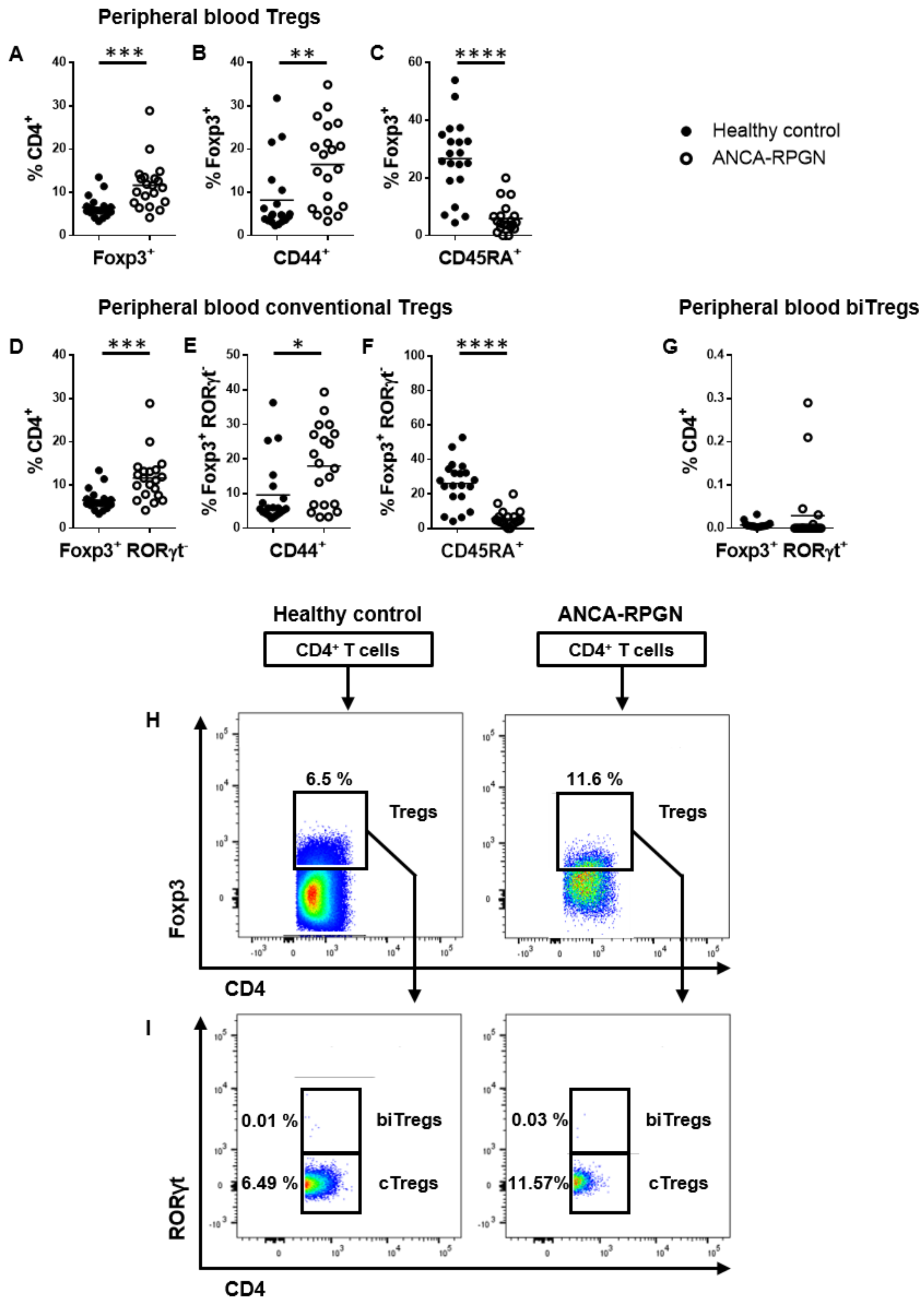

*Figure 18. Total Tregs as well as cTregs expand during ANCA-RPGN. (A-C) Frequencies of total Tregs and their expression levels of CD44 and CD45RA. (D-F) Frequencies of conventional Tregs and their expression levels of CD44 and CD45RA. (G) Frequencies of biTregs. (H, I) Representative FACS plots showing the gating strategy. Symbols represent individual patients, horizontal lines show mean values. * P<0.05, ** P<0.01, *** P<0.001, **** P< 0.0001.*

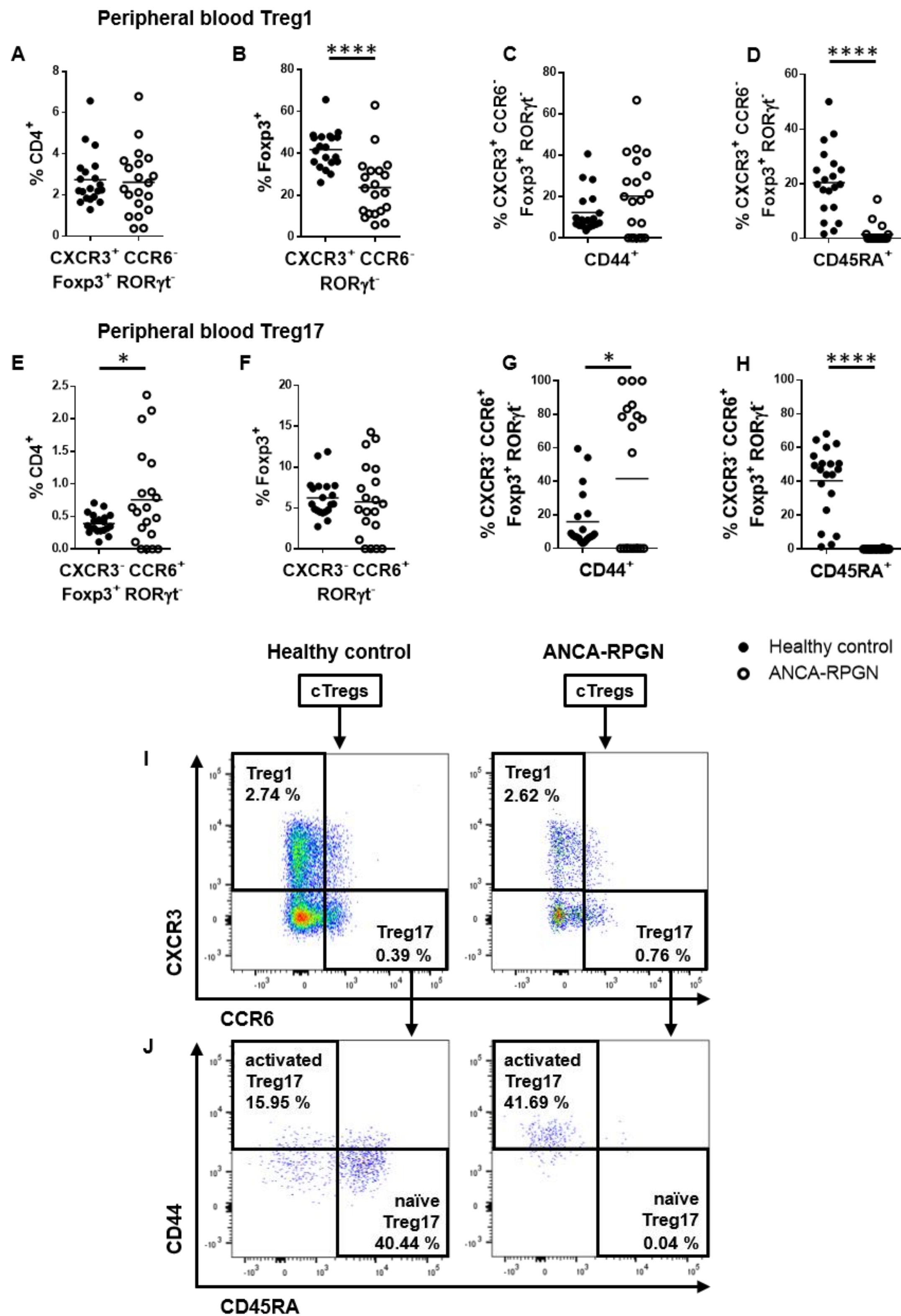

*Figure 19. Treg1 frequencies decrease, while Treg17 cells slightly expand in patients with ANCA-RPGN. In the ANCA-RPGN group, both Treg subtypes show a more activated phenotype. Frequencies of Treg1 (A, B) and Treg17 (E, F) cells and their distinct CD44 and CD45RA expression levels (C, D; G, H). (I, J) Representative FACS plots showing the gating strategy. Symbols represent individual patients, horizontal lines show mean values. * P<0.05, **** P< 0.0001.*

8 Discussion

8.1 RORγt inhibitory compounds in the course of experimental glomerulonephritis

Th17 cells are major contributors to kidney injury in RPGN (Krebs et al. 2017) and knockout of the Th17-defining master transcription factor RORγt leads to significant amelioration of renal injury in experimental models (Steinmetz et al. 2011). Since therapeutic regimes for RPGN are rather unspecific and toxic, we wanted to study effects of a target specific intervention directed against RORγt. We employed three different compounds neutralizing RORγt at various stages. One of these compounds (GSK805) had already proven efficacy in experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis. (Xiao et al. 2014). A second compound (RORγt-ASO) belongs to the group of DNAzymes. This group of agents had previously been shown to effectively inhibit GATA3, the Th2 master transcription factor. (Krug et al. 2015) The third compound (VTP-44938) had even passed phase 2a trials in patients with psoriasis, another Th17-mediated disease.

First we determined the effects of the compounds on immune responses in mice that were immunized with the model antigen sheep IgG. Surprisingly, none of the engaged compounds was able to impair splenic Th17 frequencies or IL-17 cytokine production. We next sought to study effects of a RORγt-inhibitory therapy on the course of experimental GN. We subjected the mice to the NTN model of RPGN and treated them either with a RORγt-directed compound or with placebo controls. Unfortunately, none of the compounds efficiently impaired development of Th17 immune responses or renal tissue injury. The same negative results were obtained in the treatment of cisplatin-induced acute kidney injury. In summary, our data suggest, that none of the three different compounds, we characterized was efficient to block pathogenic Th17 responses.

In contrast to conventional antibody treatment directed against targets on the cell surface, targeting intranuclear targets, like RORγt, brings some methodical obstacles. Blocking agents need to cross the cell membrane and reach the

nucleus. However, in other experimental models, the principle of blocking intranuclear targets – as mentioned above – had proven efficacy. Although none of the compounds had been applied in experimental glomerulonephritis, the experimental model of EAE and the psoriasis disease share the central pathogenic role of Th17 cells, which in GN is also a main driver of pathologic autoimmune responses. Therefore, our negative results remain somehow incomprehensible to us.

One possible explanation might be the discovery by us and others, that the transcription factor RORγt is not only present in Th17 cells, but also in a particularly active and highly immunosuppressive subset of RORγt^{+} Foxp3^{+} Tregs, which we previously termed biTregs. (Kluger et al. 2016b; Yang et al. 2016) Blockade of RORγt in biTregs might lead to an impairment of their immunosuppressive capacity. Hence, a simultaneous blockade of RORγt in pro-inflammatory Th17 cells and anti-inflammatory biTregs could cause opposing effects, leading to an unchanged inflammatory activity as observed in our experiments.

Finally, it remains to be mentioned, that previous studies had shown that RORγt knockout mice die from lymphoma within the first months of their life. (Ueda et al. 2002) Since it has become obvious during later studies by other groups, that also temporary blockade of RORγt bears a high risk of lymphoma development (Guntermann et al. 2017; Haggerty et al. 2021), most pharmaceutical companies have terminated development of further reagents directed against RORγt.

8.2 Interleukin-6 receptor classic signaling and its effects on Tregs

Another molecule important for generation of Th17 cell responses is IL-6. Signaling of IL-6 is complex and includes classic-, trans- and potentially cluster-signaling. Therapies simultaneously targeting all three IL-6 signaling pathways by blocking the IL-6 receptor have been established for the treatment of certain autoimmune diseases, like in particular rheumatoid arthritis (Rubbert-Roth et al. 2018). However, it was recently speculated, that in addition to Th17 cell generation, IL-6 has potent but yet ill characterized anti-inflammatory effects. We

thus wanted to elucidate the potentially differing effects of IL-6 classic signaling on Th17 and Treg development.

Our work showed significantly reduced Th17 cell frequencies in the kidneys of mice with IL-6Ra deficient T cells. This alone was an important finding, since it contradicts the recent hypothesis, that Th17 responses are induced by IL-6 cluster signaling. (Heink et al. 2017) In line with reduced Th17 cell infiltration, we found impaired renal infiltration of neutrophils, which are the main effectors of Th17 responses. Surprisingly, although Th17 responses and neutrophil infiltration was reduced, histological injury and functional impairment did not differ between mice lacking the IL-6Ra on $CD4^+$ T cells and mice with preserved IL-6Ra expression. Therefore, we decided to study Tregs in more detail, since we suspected IL-6 to also affect Treg biology and functions. Indeed, our work showed diminished $Foxp3^+$ Treg numbers in nephritic kidney of mice with IL-6Ra deficient T cells. In line with these results, further investigation by our group showed, that IL-6 is required to induce the aforementioned $ROR\gamma t^+$ $Foxp3^+$ biTregs. Using adoptive cell transfer studies, we co-injected Tregs, taken either from IL-6Ra deficient or sufficient control mice together with wild-type T effector cells and studied the course of NTN. Indeed, renal injury was much aggravated in recipients of Tregs lacking the IL-6 receptor. In conclusion, our data confirmed, that IL-6 classic signaling has pro-inflammatory properties by induction of Th17 responses. However, we also found that IL-6 has additional anti-inflammatory properties by expansion of strongly protective $ROR\gamma t^+$ $Foxp3^+$ biTregs. These data were published recently (Hagenstein et al. 2019) and suggest caution in the use of IL-6 directed therapies for Treg dependent diseases as e.g. ANCA-RPGN. One potential solution would be to selectively interfere with IL-6 trans-signaling and leave IL-6 classic signaling unimpaired. Since IL-6 trans- signaling has been reported to be pathogenic in experimental GN (Braun et al. 2016), a possible future therapeutic approach would be to precisely interfere with this IL-6 alternative signaling pathway.

Another future direction for the treatment of RPGN might be to *ex vivo* incubate sorted Tregs with IL-6 to enhance generation of highly immunosuppressive biTregs and subsequently reinfuse them back into the patient in analogy to a

recently established protocol for the treatment of renal transplant rejection (Chandran et al. 2017).

8.3 Characterization of human Treg subtypes

Multiple studies from the past decade have revealed, that Tregs are by no means a uniform population. Rather, the existence of specialized Treg subtypes that are tailor made for the control of distinct T effector cells has been proven. (Koch et al. 2009; Zheng et al. 2009; Chaudhry et al. 2009; Turner et al. 2010b) Importantly, this concept of T cell control by lineage specific Tregs was shown to be conserved in humans. (Sacramento et al. 2018; Kluger et al. 2014; Paust et al. 2016) So far no comprehensive data have been published about frequencies of the different Treg subtypes in humans in health and disease. This, however, would be of great interest, since specialized Tregs, involved in specific T effector cell control might represent novel therapeutic targets. As there is currently no established flow cytometry protocol for the identification of Treg subsets in humans, we decided to fill this gap and established a diagnostic panel, which was one of the main goals of my thesis. We then analyzed PBMCs from a cohort of ANCA-RPGN patients as well as PBCMs from a cohort of healthy individuals.

As somewhat expected, we found increased Th17 responses in PBMCs of ANCA-RPGN patients. These data are in line with previously published studies, which could demonstrate elevated Th17 frequencies in patients with ANCA-associated autoimmune disease. (Abdulahad et al. 2008; Saito et al. 2009; Nogueira et al. 2010) Th1 frequencies on the other hand, were significantly reduced. This was somehow surprising, as the few studies that examined Th1 cells in the peripheral blood of ANCA patients have documented no difference between ANCA patients and healthy controls. (Abdulahad et al. 2008; Szczeklik et al. 2017)

However, cell frequencies alone are not the only relevant parameter to assess the implication of the different Th cell responses. Indeed, further and more detailed analyses showed a higher activation status of both Th1 and Th17 cells in the ANCA-RPGN group.

Next, we aimed to analyze the Treg compartment. As mentioned above, we established a novel FACS panel for this purpose, which allowed to differentiate the

main effector Treg subtypes. Total Tregs, as well as the RORγt negative Treg population were found to be expanded in the blood of ANCA-RPGN patients. Similar to the T effector cells, patient Tregs were also more activated. This was somehow predictable, as physiologically an expansion of pro-inflammatory T cells always causes a rise of counteracting regulatory T cells in order to prevent an overshooting inflammatory reaction. Hence, a lack of counteracting Tregs in total nor lack of Treg activation seem to be the reason for autoimmunity in ANCA-RPGN.

Thus, it was important to take a closer look at the distinct Treg subtypes. Since RORγt^{+} biTregs are suspected to play a role in the pathogenesis of many autoimmune diseases, including RPGN (Bovenschen et al. 2011; Hovhannisyan et al. 2011; Kluger et al. 2017; Kluger et al. 2016b; Okui et al. 2012), frequencies of this bifunctional Treg subtype were of special interest. However, their abundance in the peripheral blood was generally low. This was somewhat expected, since biTregs are thought to be fast acting effector-Tregs, which are rapidly induced upon inflammation and infiltrate away from the blood stream into the affected tissues. (Kluger et al. 2016b)

Nevertheless, some individuals from the ANCA-RPGN group exhibited increased blood biTreg frequencies compared to healthy controls. This was, however, not consistent throughout all patients and we did not find significant differences if all individuals were included in the analysis.

In the subsequent analysis of the other Treg subtypes, we found Treg1 frequencies in ANCA-RPGN patients unchanged among total CD4^{+} T cells and even somewhat reduced among total FoxP3^{+} Tregs. Thus, in contrast to the observed upregulated Treg response in total, Treg1 cells did not expand. As Treg1 cells are thought to specifically control Th1 immune responses, one could have expected an overshooting Th1 immunity in ANCA patients. But, as outlined above, Th1 cell frequencies in the peripheral blood were reduced, too. This might be explained by the fact, that even though the Treg1 population was diminished, Treg1 cells showed a more activated phenotype in the RPGN group.

For future studies, it would be of great interest to evaluate, whether Treg1 cells are diminished in both compartments, peripheral blood *and* kidney, or whether Treg1 cells maybe ‘follow’ Th1 cells into the inflamed renal tissue and are therefore less present in the peripheral blood.

Since the Th17 population was expanded in the blood of ANCA-RPGN patients, it was of great interest for us, whether Treg17 cells as their regulatory counterpart, might be impaired in terms of frequency or activation status.

However, in contrast to our expectations, we did not find reduced Treg17 frequencies among ANCA-RPGN patients. Furthermore, similar to the other Treg populations, Treg17 cells also showed a more activated phenotype in the disease group. Hence, the pathologic Th17 cell expansion does not seem to result from reduced numbers or generally decreased activation of Treg17 cells. It would thus be of interest to further investigate, whether the suppressive capacity of Treg17 cells might be somehow impaired. In order to address such questions, in vitro assays, allowing to study the function of the different Treg subtypes need to be established. Ongoing experiments in our lab currently aim to establish this goal.

Taken together, the techniques which I developed in my thesis provide a first approach to characterization of human Treg subtypes in the peripheral blood and their adaptation to inflammatory disease. Even after years of freezing, our FACS panel could reliably identify T effector cells, as well as several different Treg subtypes among our patients’ PBMCs. This is important, since ANCA-RPGN is a rare disease and it takes time to create a cohort that is large enough to perform robust analyses.

Future research work needs to improve flow cytometric staining techniques, to characterize Treg subtypes and their particular contribution to the course of autoimmune kidney disease in more detail. As a particularly important future perspective, flow cytometry will have to be extended to renal biopsy tissue, in order to compare cells from the blood and the inflamed tissue with healthy controls by surface staining, including cite-seq, as well as by single cell RNAseq techniques. This technique has recently been established in our lab and analysis of first results is ongoing.

Overall, a more detailed characterization of the Treg compartment might result in a better understanding of ANCA pathogenesis and could help to develop new and more precise therapeutic strategies.

9 Summary

Rapidly progressive glomerulonephritis (RPGN) is a common cause of end-stage renal disease. End-stage renal disease is linked with high individual and social-economic burden. ANCA-associated vasculitis is one important cause of RPGN. We therefore decided to study three aspects of RPGN.

1) Since Th17 cells are key players in the pathogenesis of ANCA-RPGN, a Th17 directed therapy seemed to be a promising therapeutic option. We engaged three compounds that block the Th17 master transcription factor RORγt at various stages. We studied these compounds in different models of acute glomerulonephritis. However, none of the compounds was able to effectively block Th17 responses and to ameliorate the course of glomerulonephritis. Based on currently available data, further investigation of a RORγt-directed therapy does not seem to be worthy.

2) The inflammatory process of RPGN underlies the influence of cytokines. In this regard we focused on the effects of Interleukin-6 classic signaling on both Th17 cells and regulatory T cells. In conclusion, our data demonstrate that IL-6 has pro-inflammatory effects by inducing Th17 immune responses but also anti-inflammatory effects by expansion of immunosuppressive RORγt^{+} FoxP3^{+} biTregs. Hence, IL-6 directed therapies need to be applied carefully in Treg-dependent diseases, as e.g. ANCA-RPGN.

3) In mice and humans, specialized Treg subtypes are thought to exist, which predominantly control their respective pro-inflammatory T helper cell counterpart. Since not much is known about these effector Treg subtypes in human diseases, we developed a novel FACS panel, which allowed us to sub-differentiate the main human T helper cell and Treg subsets. This method was applied to a collective of ANCA-RPGN patients, which showed an expansion of peripheral Th17 cells. Treg17 cells, as the regulatory counterpart of Th17, however, were not diminished or less activated, leaving the reason for the enhanced Th17 responses elusive. Further and more detailed research into Treg mediated regulation of immune responses in humans is therefore warranted.

10 Zusammenfassung

Die Rapid-progressive Glomerulonephritis (RPGN) ist eine wichtige Ursache der terminalen Niereninsuffizienz. Diese geht mit hoher individueller und sozio-ökonomischer Belastung einher. Eine wichtige Ursache der RPGN ist die ANCA-assoziierte Vaskulitis, welche in dieser Arbeit aus drei Blickwinkeln untersucht wurde.

1) Da Th17 Zellen zu den wichtigsten Faktoren in der Pathogenese der ANCA-RPGN gehören, erschien eine Neutralisierung der Th17-Immunantwort als interessanter Therapieansatz. Drei verschiedene Wirkstoffe, welche den Th17-Schlüssel-Transkriptionsfaktor RORγt auf unterschiedliche Weise blockieren, wurden in Tiermodellen der akuten Glomerulonephritis getestet. Leider konnte keiner der Wirkstoffe das renale Outcome verbessern, sodass eine weitere Untersuchung RORγt blockierender Therapeutika aktuell nicht sinnvoll erscheint.

2) Der Entzündungsprozess bei der RPGN wird maßgeblich durch Zytokine beeinflusst. Diesbezüglich haben wir uns mit den Auswirkungen des klassischen Interleukin-6 Signalweges auf Th17 und regulatorischen T-Zellen beschäftigt. Unsere Daten zeigten, dass IL-6 pro-inflammatorische Effekte besitzt über eine Induktion der Th17 Antwort, darüber hinaus jedoch auch anti-inflammatorische Effekte über eine Expansion immunsuppressiver $RORγt^{+}$ $FoxP3^{+}$ biTregs. Daher sollten gegen IL-6 gerichtete Therapien nur mit Vorsicht angewendet werden.

3) Die unterschiedlichen T-Helfer-Zellreihen werden in der Maus und im Menschen durch spezialisierte Treg Subtypen kontrolliert. Da zurzeit nur wenig über die Rolle dieser Linien-spezifischen Effektor-Tregs bei humanen Erkrankungen bekannt ist, entwickelten wir ein neues FACS Panel, um T-Helfer-Zell sowie Treg Subtypen im menschlichen Blut weiter differenzieren zu können. In der Anwendung dieses Panels auf ein Kollektiv von ANCA-RPGN Patienten konnten wir vermehrt Th17 Zellen nachweisen. Treg17 Zellen, als deren regulatorischer Gegenpart, waren jedoch nicht vermindert oder weniger aktiv. Die Ursache für die verstärkte Th17 Antwort bleibt damit unklar. Weitere und tiefergehende Forschungsarbeit im Bereich der Treg-vermittelten Regulierung humaner Entzündungsprozesse ist daher notwendig.

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13 Lebenslauf

entfällt aus datenschutzrechtlichen Gründen

14 Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: ....