Analysis of TH17-cell plasticity into regulatory fates during crescentic glomerulonephritis in a mouse model

Dissertation with the aim of achieving a doctoral degree at the Faculty of Mathematics, Informatics and Natural Sciences

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Submitted by

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Hamburg, April 2020

This study was performed between September 2016 and April 2020 at the University Medical Center Hamburg-Eppendorf in the laboratory of Prof. Dr. Samuel Huber.

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Date of disputation 05.06.2020

Acknowledgment

Undertaking this PhD has been a truly unique experience for me and it would not have been possible to do without the encouragement, support and guidance that I received from many people.

Firstly, I would like to express my sincere gratitude to my supervisor, Prof. Samuel Huber who gave me the opportunity to work in his laboratory and has challenged and encouraged me to never be satisfied with the status quo. I am truly thankful for the inspiring discussions and the excellent example he has provided to me for the last years. Without his guidance, constant feedback and continuous support this dissertation would not have been achievable.
Besides my supervisor, I would like to thank Prof. Christian Lohr who kindly offered to review my dissertation. In this regard, I would also like to thank Prof. Wolfgang Streit and Prof. Dietmar Kuhl, who offered to complete my defence committee.

I would especially like to thank Prof. Nicola Gagliani for his constant motivation, his immense knowledge and his unconditional support for my research. Our very constructive discussions helped me develop as a scientist. Furthermore, I thank PD. Christian Krebs, for sharing his knowledge and experience as a nephrologist and scientist. I am very thankful to all members and former members of the AG Huber: Dr. Penelope Pelczar, Dr. Laura Garcia Perez, Dr. Tanja Bedke, Dr. Franziska Muscate, Dr. Babett Steglich, MD Anastasios Giannou, Dr. Can Ergen-Behr, Friederike Stuhlmann, Franziska Bertram, Morsal Sabihi, Mikolaj Nawrocki, Beibei Liu, Jöran Lücke, Jan Kempski, Mustafa Shiri, Sandra Wende, Cathleen Haueis, Marie Gaulrapp, Jaana Helmuth, Francis Huber, Dr. Leonie Brockmann and AG Krebs: Alina Borchers and Leon Enk. I would like to thank you for the great team spirit and the constant support over the past years. All of you contributed to both my scientific and personal growth. It has been an honor to be part of the team and I will always keep you in my mind as collogues and friends.

Acknowledgment

Most importantly I would like to thank my friends and my family: My parents Dorothea and Charles, who laid the foundation for my scientific career early on, sparking my interest in biology and nature. My siblings, Cedi, Cebio and Ena who will always be part of my heart and my life and who were always there for me. Last but not least, I thank you, Till, for your love, your unlimited backing, the amazing antipasti you prepare on a plate and the way you make me laugh when I struggle. I am looking forward to what comes next.

All of you have made me the brave woman and curious scientist that I am today. There are no words to express the unrestricted love I feel for you and I could never have gone down that road without you.

Summary

Originally, CD4 positive T-cell subsets were thought to be stable and homogenous after differentiation. Recent data however, have shown that CD4 positive T cells, and TH17 cells in particular, have higher plasticity and heterogeneity than previously expected. Thus, TH17 cells can convert into pro-inflammatory TH1 cells, but also into regulatory IL-10 producing cells, referred to as $TR1^{exTH17}$. However, the role of this process in extra-intestinal diseases, e.g. in the kidney, was unclear.

In the first part of the thesis, we could show the existence of $TR1^{exTH17}$ cells in the kidneys of mice in a mouse model of glomerulonephritis. The emergence of these cells was independent of IL-10 signaling. Interestingly, IL-10 production by TH17 and exTH17 cells was dispensable for the outcome of glomerulonephritis. On the basis of these data we hypothesized that the lack of IL-10 production by TH17 and exTH17 cells could be compensated by another source. Thus, in the second part of the thesis, we did a broader analysis of $Foxp3^-$ IL-10 producing CD4 positive T cells on a molecular and functional level in the kidney in glomerulonephritis. We found a low frequency of $Foxp3^-$ IL-10 producing CD4 positive T cells expressing the surface markers CD49b and LAG3, which have previously been reported to be expressed by IL-10 producing T cells with a regulatory function, referred to as TR1 cells. However, next generation sequencing analysis of the transcriptome of single cells, revealed that about 30 % of the $Foxp3^-$ IL-10 producing CD4 positive T cell displayed intermediate to high expression of the transcriptional signature of regulatory cells. In order to clarify this apparently discrepant finding we assessed the suppressive the function of these cells *in vitro*. We found, that $Foxp3^-$ IL-10 producing CD4 positive T cells, which had been isolated from the inflamed kidney, are suppressive *in vitro*, indicating that these are indeed TR1 cells. Additionally, we performed functional experiments with *in vitro* generated TR1 cells, showing that TR1 cells in principle can sufficiently suppress TH17-cell driven glomerulonephritis *in vivo*.

Overall, we demonstrated the existence of TR1^{exTH17} cells in glomerulonephritis. The functional relevancy of this subgroup of cells remained however unclear. But we were able to identify a functional relevance of TR1 cells during glomerulonephritis.

Zusammenfassung

Ursprünglich dachte man, dass CD4 positive T-Zellsubtypen nach der Differenzierung stabil und homogen sind. Jüngste Daten zeigten jedoch, dass CD4 positive T-Zellen, und insbesondere TH17-Zellen, eine höhere Plastizität und Heterogenität aufweisen als bisher erwartet. So können TH17-Zellen sich in pro-inflammatorische TH1-Zellen, aber auch in regulatorische IL-10-produzierende Zellen, die als $TR1^{exTH17}$ bezeichnet werden, umwandeln. Die Rolle dieses Prozesses bei extra-intestinalen Erkrankungen, z.B. in der Niere, war jedoch unklar.

In dem ersten Teil dieser Arbeit, konnten wir die Existenz von $TR1^{exTH17}$-Zellen auch in der Niere in einem Mausmodell der Glomerulonephritis zeigen. Die Entstehung dieser Zellen war unabhängig von einem intaktem IL-10-Signalweg. Interessanterweise war die IL-10-Produktion durch TH17- und exTH17-Zellen für den Ausgang der Glomerulonephritis entbehrlich. Auf der Grundlage dieser Daten stellten wir die Hypothese auf, dass die fehlende IL-10-Produktion durch TH17- und exTH17-Zellen durch eine andere Quelle kompensiert werden könnte. Daher führten wir in einem zweiten Teil der Arbeit auf molekularer und funktioneller Ebene eine umfassendere Analyse von $Foxp3^-$ IL-10 produzierenden CD4 positive T-Zellen in der Niere bei Glomerulonephritis durch. Wir fanden eine geringe Häufigkeit von $Foxp3^-$ IL-10 produzierenden CD4 positiven T-Zellen, die die Oberflächenmarker CD49b und LAG3 exprimieren, von denen berichtet wurde, dass sie von IL-10 produzierenden T-Zellen mit regulatorischer Funktion, die als TR1-Zellen bezeichnet werden, exprimiert werden. Die anschließend durchgeführte “Next-Generation”-Sequenzierungsanalyse des Transkriptoms einzelner Zellen ergab jedoch, dass etwa 30 % der $Foxp3^-$ IL-10-produzierenden CD4-positiven T-Zellen eine mittlere bis hohe Expression der transkriptionellen Signatur von regulatorischen Zellen aufwiesen. Zur Klärung dieses scheinbar diskrepanten Befundes untersuchten wir die unterdrückende Funktion dieser Zellen *in vitro*. Wir fanden heraus, dass $Foxp3^-$ IL-10 produzierende, CD4-positive T-Zellen, die aus der entzündeten

Niere isoliert worden waren, *in vitro* suppressiv wirken, was darauf hindeutet, dass es sich tatsächlich um TR1-Zellen handelt. Darüber hinaus zeigten funktionelle Experimente mit *in vitro* erzeugten TR1-Zellen, dass TR1-Zellen im Prinzip eine TH17-getriebene Glomerulonephritis *in vivo* unterdrücken können.
Insgesamt konnten wir die Existenz von $TR1^{exTH17}$-Zellen bei Glomerulonephritis nachweisen. Die funktionelle Relevanz dieser Untergruppe von Zellen blieb unklar. Wir konnten jedoch eine funktionelle Relevanz von TR1-Zellen bei Glomerulonephritis identifizieren.

Contents

1. Introduction

The kidneys play a central role in detoxifying the body. They filter the blood and produce primary urine [1]. This primary urine is cleared from toxins that are concentrated in the secondary urine to be released from the organism. Furthermore, the kidneys are important for the water balance and the electrolyte metabolism of the body [2]. Autoimmune or chronic inflammatory diseases such as Rapid Progressing Glomerulonephritis (RPGN) can result in kidney dysfunction. Immune mediated inflammatory diseases (IMIDs) are often a result of an imbalance in the T-cell repertoire. Cluster of differentiation 4 (CD4) positive thymocyte cell (T cells), in particular, T helper (TH)17 and TH1 cells have a strong impact on the development of glomerulonephritis [3-5].

Interestingly, pro-inflammatory TH17 cells can upregulate Interleukin (IL) 10 (IL-10) (hereafter referred to as physiological TH17) and yet others can convert into Forkhead box p3 (Foxp3) negative (-) T regulatory Type 1 (TR1) cells (hereafter referred to as $TR1^{exTH17}$) [6, 7]. These plastic effector TH17 cells can change their phenotype under certain circumstances. However, it is still unclear whether described differences in TH17 cell fate are dependent on the organ, where the conversion takes place, or determined only by the stimulus that actually drives T-cell conversion.

One part of this project was to determine whether the kidneys of nephritic mice harbor plastic TH17 cells that have the potential to convert to $TR1^{exTH17}$ cells. Furthermore, the role of IL-10 production and IL-10 signaling in TH17 cells during glomerulonephritis will be part of the investigation.

The second part of the project addresses TR1 cells that are described as a heterogeneous cell subset with different cell functions [8]. To get an estimation of the heterogeneity and function of $Foxp3^-$ IL-10 producing CD4 positive T cells

derived from the kidneys, single cell Ribonucleic acid (RNA) sequencing and functional analysis has been performed.

1.1 The immune system in chronic inflammatory disease

The immune system plays a key role in IMIDs, such as Rheumatoid Arthritis, Multiples Sclerosis (MS), Diabetes, Inflammatory Bowel Disease (IBD) or RPGN. IMIDs can affect several organs like the central nervous system (CNS), the gut, and also the kidneys. Until now, it is still not fully understood what drives the development of such diseases. Genetic predisposition, spontaneous mutations, but also the microbiome and environmental factors are described to influence disease initiation and progression affecting patients [9-13], in some cases, already at a young age.

Particularly, barrier organs such as the intestine, skin and lungs are strongly influenced by direct exposure to antigens and toxins from the air, ingested food or bacteria [14]. Because of this, those organs need to be able to distinguish between food and foreign antigens. Therefore, a well-regulated immune system is essential. The immune system contains immune cells such as B cells, Monocytes, Natural Killer (NK) cells, as well as $CD8^+$ and CD4 positive T cells [15]. For the welfare of every organism it is important for the immune system to be maintained at homeostasis [16, 17]. Generally, when the immune system is imbalanced, changes in immune cell compositions can appear. These changes can be caused by dysbiosis and furthermore result in inflammatory processes [18]. In order to sufficiently treat patients that suffer from IMIDs, further investigations studying the elicitors of such diseases have to be carried out. Moreover, it is important to understand these inflammatory processes.

1.2 Rapidly progressive glomerulonephritis

The most important organ from the urogenital tract is the kidney. The kidney is a paired created organ, each located on the right and the left side of the retroperitoneal space. The kidneys are of great importance for blood filtration and urine concentration, however one healthy kidney is sufficient. Macroscopically, it

is separated into regions of the cortex, mark layer, as well as the kidney chalices and the kidney basin [19]. Each unit contains further small structural subunits which carry different functions during urine processing. The whole renal system is of great importance for maintaining water balance and detoxification of the whole mammalian body [20].

Chronic inflammatory kidney diseases affect around 10 % of the human population [21]. One of those is RPGN. There is a classification of three types of RPGN which all mediate in the Bowman's capsule the typical deformation of the glomeruli defined as ‘crescent’. Type I is driven by antibodies against the basal membrane, Type II RPGN is mediated through the accumulation of immune complexes, which include activated T cells [4]. Type III, the most common source for RPGN, is induced by anti-neutrophil cytoplasmic autoantibodies (ANCA) [22]. All of these types of RPGN cause extra capillary proliferation within the majority of glomeruli. Crescentic glomerulonephritis, a clinical picture of RPGN is described with tubular and glomerular damage [23]. It is driven by the infiltration of effector T lymphocytes TH1 and TH17 [5].
Thereby TH1 cells, producing high levels of Interferon-gamma (IFN-ɣ), activate macrophages whereas IL-17A producing TH17 cells mainly secrete attractants and recruit neutrophils [24]. Furthermore, also strong proliferation of T-box transcription factor TBX21 (T-bet) expressing TH1 and retinoid acid receptor-related orphan receptor-gamma t (ROR-ɣt) positive TH17 cells alone or in combination is described to enhance tissue injury during glomerulonephritis [3, 24-27].
The two cell types drive the pathology of glomerulonephritis at different time points of disease development. TH17 cells are responsible for recruiting TH1 through the expression of CXCL9 in the kidneys [5]. This drives the early damage. Subsequently, infiltrating TH1 cells strongly increase in numbers and drive later stages of disease [5]. In Tbet knock out mice, which lack the transcription of TH1 cells, increased IL-17A levels during glomerulonephritis are observed. This suggests that TH17 cells seem to compensate for the absence TH1 cells [25]. Similar effects are demonstrated in mice lacking the TH17-cell specific

transcription factor ROR-ɣt. In those mice, only a mild crescentic glomerulonephritis can be observed while IFN-ɣ levels are increased [26]. Nonetheless, independent from the TH subset that drives inflammation, the kidneys execute insufficient glomerular filtration rate (GFR). This results among other things in insufficient clearance of the blood from toxins. Furthermore, dysfunctional kidneys can negatively affect many organs, such as the lung or the intestine, the heart or the liver [28-30].

1.3 Current therapies of rapid progressing glomerulonephritis

Patients diagnosed with RPGN immediately receive a common induction therapy. In order to restrict local inflammation, immune suppressive medications are applied. These contain glucocorticoids in the form of Cortisone. Furthermore, those are combined with either Cyclophosphamide or Rituximab [31]. After 6 months on this treatment, patients undergo a maintenance therapy. This involves for example Rituximab or Azathioprine [31]. Until today not many drugs have been developed to treat these patients. Thus, available drugs are commonly used for patients with all kinds of RPGN. Unfortunately, they are not specific for each particular kidney failure. This results in some patients that do not respond to any treatment. Most of the patients with end-stage kidney injury need to undergo dialysis, in order to clean the blood mechanically. In some patients, kidneys recover their normal function after a few months of dialysis, whereas others do not recover but require urgent kidney transplantation [32]. Since treatments for efficient inhibition of disease progression are limited, new therapy strategies have to be developed. After kidney transplantation, the use of T-cell based therapy has already become one possibility to increase acceptance of the grafts. One therapy approach is the use of donor specific TR1 cells, which are transferred to patients that have undergone kidney transplantation [33].

1.4 CD4 positive T-cell subsets

As a part of the adaptive immune system, T lymphocytes play an important role in host defense and clearance of pathogens. Derived from the thymus, lymphocytes express T-cell receptors (TCR). While the majority of T cells express TCRs with an α- and β- chain, others carry TCRs with a ɣ- and a δ chain.
Generally, T cells are separated into two groups which are defined by either CD8 or CD4 expression. $CD8^+$ T cells, also referred to as cytotoxic T cells, respond to antigens of the MHC-I group, which are expressed on nucleated cells. They are mainly involved in cancer cell and virus infected cell defense. CD4 positive T cells are activated in response to antigens on antigen presenting cells (APCs) such as dendritic cells, which carry MHC-II molecules on their surface.
This activation gives rise to cell differentiation into various CD4 positive T-cell subsets that orchestrate the immune response by secreting cytokines and other factors. These subsets exhibit both pro- and anti-inflammatory functions. The first two of TH subsets were described by Mosmann and Coffmann in 1986 [34]. For the sake of convenience, these cells were named TH1 and TH2.

TH1 cells are defined by the expression of the transcription factor T-bet, and by the production of the cytokine IL-12, the Tumor Necrosis Factor-alpha (TNF-α) and IFN-ɣ [35, 36]. During TH1 cell differentiation, binding of the cytokine IL-12 to its receptor plays a fundamental role [37]. The binding of IL-12 to its receptor causes an activation of the Signal Transducer and Activator of Transcription-4 (STAT4) and is required for the expression of IL-10 by TH1 cells [38]. Through this the master transcription factor T-bet can transcribe the gene *IFN-ɣ [39]*. Together IFN-ɣ and Tbet drive the activation of STAT1 which is important for the maintenance of TH1 cells by upregulating the IL-12Rβ2 [37, 40]. The secretion of IFN-ɣ even can be enhanced by IL-12 expression together with IL-18 [41]. The appearance of TH1 cells is mostly associated with infections by intracellular pathogens. In particular, when the organism is infected with mycobacteria such as *Mycobacterium tuberculosis* or *Mycobacterium lepromatosis*, TH1 cells play a major role for host defense. Through the activation of phagocytes by TH1 cells, infected cells can then be eliminated and support the antimicrobial response [42].

The signature cytokines produced by TH2 cells are IL-4, IL-5, IL-9 and IL-13. Furthermore, the TH2 cells are able secrete IL-10 [43]. Through the upregulation of IL-10, TH2 cells have the capacity to inhibit TH1 cells by dampening IFN-ɣ secretion [43]. Nonetheless, the cytokines IL-4 together with IL-2 are mandatory for the differentiation of TH2 cells [44]. The binding of IL-4 to its receptor, results in an activation of the STAT6. Furthermore, STAT6 is important for the expression of the subset specific trans-acting T-cell specific transcription factor Gata3 [45, 46].
Generally, TH2 cells play a fundamental role during infections with extracellular parasites like *Nippostrongylus brasiliensis* [47] or *Schistosoma mansoni* [43]. The release of IL-5 and IL-13 by TH2 cells can induce eosinophils which result in protection by pushing infected cells into an apoptotic state [48]. Beside these protective effects, TH2 cells are also involved in airway inflammation [49]. Many subtypes of asthma are associated with the abundance of TH2 cells in the lungs.

Since this initial description of TH1 and TH2 cells, further TH subsets have been identified. TH9 is the major subset that produces the cytokine IL-9. This subset is described to fulfill similar functions as TH2 cells. Generally, TH9 cells are mainly described to be involved in allergic inflammation [50].

Another proposed T-cell subset is the TH22 subset. These cells mainly produce the cytokine IL-22, IL-13 and TNF-α [51]. IL-22 is known for its role during host defense driven by its tissue protective effect against Gram negative bacteria mainly at mucosal sides such as the intestine [52, 53].

Furthermore, other populations of CD4 positive T cells are known to play a fundamental role in the immune system. Foxp3$^-$ TH17 cells describe an effector-cell population distinct from TH1 and TH2 [54]. These cells express the transcription factor ROR-ɣt and secrete high levels of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), as well as their signature cytokines, IL-17A and F [55, 56]. TH17 cells are described to drive inflammation in the CNS, the skin, the lungs but also in the gut and the kidneys [3, 57-60]. Nonetheless, in many

conditions, TH17-cell proliferation and effector cytokine production can be controlled by Foxp3^{+} and Foxp3^{-} regulatory T cells (Tregs cells) [61, 62].

Since TH17 cells and regulatory T cells are of great importance during glomerulonephritis, the main focus of the next sections will be on those T-cell subsets.

1.5 TH17 cells

1.5.1 Induction and generation of TH17 cells

Microbes are strong inducers of TH17 cell differentiation [63]. Those microbes can be divided into two groups; commensals and pathogens. Generally, commensals are symbiotic bacteria whose presence is beneficial for the hosts. They provide a barrier on top of the epithelia cells and are strongly involved in food processing [64].
In contrast, pathogens describe bacteria, fungi, viruses and other foreign microorganisms. As reviewed by Gomez *et al.* they can induce host defense mechanisms followed by a cascade of inflammatory processes [65].
Commensals, such as segmented filamentous bacteria (SFB) or *Candida albicans*, induce beneficial TH17 cells which are indispensable for gut homeostasis and the integrity of the mucosal barrier [66, 67]. TH17 cells elicited by SFB abundance were described to not migrate or contribute to inflammation [68]. Instead, they support the structure of the epithelial barrier to protect the gut from infiltrating pathogens. In particular, the appearance of SFBs can directly prevent from overgrowth and colonization of pathogens in the gut lumen [66]. The importance of this host defense has been shown when mice were treated with broad-spectrum antibiotics. These animals developed severe colitis [66]. Furthermore, germ-free mice display an underdeveloped repertoire of immune cells [69].

TH17 cells can be induced *in vivo*, as well as *in vitro* [55]. In mouse and human, IL-6 and transforming growth factor beta (TGF-β) are described to be the main

drivers for the generation of TH17 cells [70-72]. Although IL-23 is not the main driver of TH17-cell differentiation, it plays an important role in their proliferation [70, 71]. Today, the combination of IL-6 and TGF-β is described to actually induce non-pathogenic TH17 cells which can produce IL-10 [73]. This IL-10 secretion under TH17 polarizing conditions is regulated by c-musculoaponeurotic fibrosarcoma (c-Maf) [74], which is in turn induced by IL-6 and TGF-β [74]. Interestingly, ROR-γt, the master transcription factor of TH17 cells also represses IL-10 production in TH17 cells in order to sustain their effector function during IBD [75].

In contrast to this, pathogenic TH17 cells, are known to be induced by IL-6, IL-1β and IL-23 [57, 76]. Some cells polarized in the presence of IL-1β and IL-23 resulted in TH17 cells that produce high levels of IL-22 [77]. Recently, it was described that pathogenic IL-22 producing TH17 cells produce high levels of IFN-γ. These TH17 cells display a TH1 like phenotype and fulfil characteristics of a pathogenic TH17 cell that strongly contributes to inflammation [68].

Besides cytokine expression levels, differences between the TH17 populations on transcriptional level can also be observed [76]. The gene signature of non-pathogenic or physiological TH17 cells is described to be highly enriched for genes such as *Maf*, *Ahr* and *IL-10* [76]. Instead, pathogenic TH17 cells express high levels of *Csf2*, *Tbx21* and *Gzmb* [76].

The populations of TH17 cells can be separated according to their physiological and pathogenic function. This classification is determined by the executed function of the cell.

1.5.2 Function of TH17 cells

1.5.2.1 Physiological TH17 cells

IL-17A is described as a cytokine that is important for the fortification of the epithelial barrier in order to protect the host from pathogen invasion [78]. Thereby, it has a crucial role for the activation of the innate immune system such as the recruitment of neutrophils [79].
Furthermore, TH17 cells that co-produce IL-17A and IL-22 cells produce β-defensin, which is important for the secretion of antimicrobial peptides by epithelia cells [80]. These antimicrobial peptides are important for host defense against bacterial infections. The bacterium *Citrobacter* rodentium is a well described intestinal pathogen which causes tissue damage driven by over proliferation of TH17 cells with inflammatory potential [68]. TH17 cells display a protective role against Citrobacter infections. Although it is not the main cytokine that drives TH17 cell differentiation, IL-23 strongly drives TH17 function to fight against *Citrobacter rodentium* infection [81].
Generally, the natural presence of TH17 cells in the small intestine, allows a fast TH17-cell response after infection.

1.5.2.2 Pathogenic TH17 cells

Although physiological TH17 cells are important for the maintenance of gut homeostasis and barrier integrity, the immune system also possess pathogenic TH17 cells. After induction, pathogenic TH17 cells strongly contribute to inflammation in different diseases. They can drive antigen specific autoimmunity in experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis [57], but also colitis described by strong inflammation in the gut [82].
During glomerulonephritis, the development of the disease strongly depends on the generation of the effector cells TH1 together with TH17 [26, 27]. Some of these TH17 cells are shown to be generated in the gut [3]. In the gut, the expression of the chemokine receptor (CCR)-6 on TH17 cells is important for their potential to migrate into the small intestine [60]. Likewise, gut derived TH17 cells

migrate in a CCR6 dependent manner through the blood stream towards the inflammatory site and increase kidney injury [3]. When cell migration is blocked during glomerulonephritis, kidney injury appears milder [3] due to the eliminated trafficking of TH1 and TH17 cells. In many inflammatory conditions, including rheumatoid arthritis, it has been shown that TH17 cells often need to migrate in order to contribute to inflammation [83].
As described, pathogenic TH17 cells exhibit plasticity by which they can upregulate IFN-ɣ. This upregulation induces a stronger effector phenotype in TH17 cells.

The line between beneficial and effector function can be very thin. TH1, TH2 and TH17 cells contribute to gut homeostasis and defense against pathogens [84]. They are all involved in maintaining the integrity of the mucosal barrier. Nonetheless, uncontrolled expansion of these cell types can result in their contribution to inflammatory processes and induction of tissue damage.
Besides effector subsets, the immune system also comprises of CD4 positive Foxp3$^+$ Tregs and TR1 cells. These cells function as regulatory cells by suppressing effector cell proliferation, and thereby, restoring immune homeostasis. An important cytokine produced by most of these cell types is IL-10.

1.6 Regulatory T cells

Regulatory T cells are mandatory to control immune response. There are two big subsets, that show strong capacity of effector-cell suppression. In both IL-10 is an immune-regulatory cytokine that plays a major role in cell maintenance and thereby, sustaining homeostasis [85]. In the gut, Foxp3$^+$ Tregs and TR1 have the potential to suppress TH17 and TH1 cells directly via IL-10 [61].

1.6.1 Foxp3$^+$ Tregs

Within the group of Treg cells, one subset is majorly described by the expression of its master transcription factor Foxp3 [86]. Foxp3 expression is essential for the differentiation and maintenance of this suppressive cell subset. On their surface,

Foxp3$^+$ Tregs cells express the IL-2 receptor α chain [87]. They can be separated in natural occurring thymus derived tTregs (or nTregs) and induced in the periphery called pTregs (or iTregs). For the generation of both types the activation of Foxp3 depends on IL-2 and TGF-β signaling [88, 89]. These two cytokines are described in mice and humans as well as for the generation of Foxp3$^+$ Treg cells *in vitro*. The important role of Foxp3$^+$ Tregs cells is shown when patients lack the gene, Foxp3, which results in the development of a fatal autoimmune disease [90]. Furthermore, both mice and humans with a defect in Foxp3 develop severe colitis [90].
Nonetheless, during inflammatory conditions, functional Foxp3$^+$ Treg cells exhibit a strong suppressive potential to inhibit TH17 and TH1 cells during colitis and glomerulonephritis respectively [61, 91, 92]. Furthermore, in mice and humans, Foxp3$^+$ Treg cells were identified to sufficiently reduce kidney damage as well as to support acceptance of a transplant kidney by the host [93, 94]. Endogenous IL-10 and IL-10 derived from Foxp3$^+$ Tregs cells is described to ameliorate crescent formation, by modulating TH1 and TH17 cell response [92, 95].

Nonetheless, the role of Foxp3$^-$ TR1 cells and the inhibitory effect of produced IL-10 on TH17 cells remains poorly understood in the context of glomerulonephritis.

1.6.2 T regulatory Type 1 cells

TR1 cells are induced in the periphery and are described to be potent suppressor cells of the immune system. They were originally defined based on the lack of Foxp3 expression and high expression of their signature cytokine, IL-10 [96]. Furthermore, TR1 cells produce moderate levels of TGF-β and IFN-ɣ. However, the cytokines of IL-4, IL-2 and IL-17A/F are expressed at very low levels on TR1 cells [97]. Originally, IL-10 was assumed to majorly induce TR1 cell generation. Until now, the role of IL-10 was shown during the regulation of stability and continuous IL-10 production by TR1 cells [85]. However, IL-27 has been identified to strongly induce the generation of TR1 cells by the interaction of aryl hydrocarbon receptor (Ahr) and c-Maf [98]. Furthermore, *in vitro*, IL-27 and TGF-β sufficiently induce high numbers of TR1 cells [98]. Although IL-10 is not

mandatory for the generation of TR1 cells [99], when peripheral blood mononuclear cells (PBMCs) are differentiated in the presence of dendritic cells (DC)-10, sufficient induction of IL-10 producing TR1 cells can be observed [100].

Different to other T-cell subsets, there has not been a single transcription factor identified to be responsible for TR1-cell differentiation. Nonetheless, various transcription factors such as Eomesodermin (Eomes) [101], liver X receptor (LXR) [8] or early growth response 2 (Egr-2) [102], as well as c-Maf and PR domain zinc finger protein 1 (Blimp-1) [103], are discussed as being strongly involved in TR1 generation.

As described previously, TR1 cells can originate from naïve CD4 positive T cells. Interestingly, recent investigations show that they can have their origin also in former effector T cells [6]. This generation is observed mainly in the small intestine, when mice are injected with an anti-CD3 specific antibody, that induces high numbers of suppressive TR cells [104]. *In vivo*, this antibody is generally used to induce a transient inflammation of the small intestine of mice. Similar to differentiation of TR1 cells, the generation of TR1 cells from TH17 cells is dependent on TGF-β and Ahr [6].
The administration of the anti-CD3 specific antibody leads to a strong induction of IL-17A in all parts of the small intestine [60]. Furthermore, high numbers of regulatory cells are induced in order to inhibit TH17 over proliferation [61, 105]. TR1 cells play an important role in retaining homeostasis during gut inflammation. With the first description in 1997, they have already been described to be antigen specific and successfully prevent colitis from forming [106]. The potency of TR1 cells depends on the possibility to produce and to respond to IL-10 [61, 85, 107]. IL-10 is a cytokine mostly associated with suppressive function. Regulation of proinflammatory cell types via IL-10 has been shown to be beneficial in healthy patients under homeostatic conditions. Also, in patients suffering from colitis or RPGN, treatment with IL-10 or TR1 cell enriched CD4 positive T-cell cocktail has strong potential to inhibit inflammatory responses [33, 108-110]. Generally, in

mice and humans that lack TR1 cells, the development of spontaneous colitis can be observed [11, 111].
Furthermore, when colitogenic mice are treated with IL-10, decreased levels of TH17 cells and IFN-γ^+ TH17 cells can be observed [61]. Also, the inhibitory role of endogenous IL-10 on TH1 cells has been described during glomerulonephritis [95].

1.7 Co-inhibitory receptors

T-cell subsets were described to be heterogeneous already shortly after the first identification of TH subsets [34]. Nonetheless, until recently, TR1 cells were considered a homogeneous T-cell subset. Furthermore, all Foxp3$^-$ IL-10 producing CD4 positive T cells were defined as suppressive TR1 cells with a common function. In recent years, this assumption has been challenged. Interestingly, IL-10 expression alone is insufficient to conclude regulatory functions. Indeed, the Foxp3$^-$ IL-10 producing CD4 positive T-cell population from different organs show clear molecular differences on RNA and Protein level resulting in different functions [8].
Those differences are caused by variable frequencies of a defined suppressive profile. First markers of this profile have been identified already in 2013 [97]. Until now, it is mainly defined by a combination of lymphocyte-activation gene 3 (LAG3), CD49b, T-cell immunoglobulin and ITIM domain (TIGIT) and the transmembrane protein TIM-3 (TIM3), programmed cell death protein 1 (PD-1) and CCR5 [8]. Furthermore, by this expression pattern non-suppressive cells can be sufficiently separated from the suppressive ones [8, 97]. Hence, different frequencies between different organs are the main cause for divergent observations. What drives this distribution is still not known. Interestingly, gating on CD49b and LAG3 double positive Foxp3$^-$ IL-10 producing CD4 positive T cells is sufficient to include most of the cells, being positive for all five markers [8]. As described for the generation of TR1 cells, many transcription factors are discussed to drive the expression of the regulatory profile. The knockout of the transcription factor, LXRα, reduces the expression of IL-10 and the suppressive profile [8]. Furthermore, the double knock out of the transcription factors Blimp-1

and c-Maf strongly eliminated the expression of co-inhibitory receptors [103]. The suppressive marker expression has been detected on different CD4 and CD8 positive T cells [112]. The role of these co-inhibitory markers continues to be discussed and remains a controversial topic. While some scientists define them to function as markers of exhaustion, others attribute them to direct regulatory functions [103, 113, 114].

As a consequence of these findings, the nomenclature of TR1 for all Foxp3$^-$ IL-10 producing CD4 positive T cells is obsolete. By now, clear differences between Foxp3$^-$ IL-10 producing CD4 positive T cells and actual suppressive TR1 cells can be made and should be considered when the term TR1 is used.

1.8 T-cell plasticity

Originally, every T-helper subset was assumed to be stable after differentiation. Specific transcription factors and cytokines were dedicated to one T-cell subset [34]. Interestingly, T cells indeed display a much higher flexibility than initially thought. At this present time, T-cell plasticity is generally accepted in the field of immunology. Cells displaying a plastic phenotype, can actually change the expression of transcription factors and cytokines within one lifecycle [6, 115]. This change can be transient when cells co-express cytokines. Interestingly, the mechanism of full cytokine switch is named ‘conversion’. It is not restricted to only a few cell subsets but is described in both regulatory and effector CD4 positive T cells [116]. The change can happen spontaneously, but it can also be influenced *in vitro*, as well as *in vivo*. T-cell priming, as well as the environment, can modulate T-cell plasticity [68, 117]. Nonetheless, the whole network that drives T-cell plasticity is still unknown. Scientists hypothesize that in some cases, T-cell conversion is part of the differentiation from naïve CD4 positive T cell, to its final T-cell subset. The idea is that some cells naturally pass through different characteristics of T-cell subsets. TR1 cells, for example, are described to display a progressive adaptation to the tissue on the way of differentiation, which results in different TR1 profiles [118]. While some cells acquire a regulatory phenotype, others switch to effector cells.

T-cell conversion has been described to be multidimensional in terms of cell fate. TH17-cell conversion can result in diverse functions. During EAE, some IFN-ɣ producing cells are described to originate from TH17 cells (named exTH17 cells) [6, 119]. T-cell conversion has been observed to promote the pathogenic properties of the cells within the host [119, 120]. Likewise, *Citrobacter* induced TH17 cells are highly plastic and mainly drive inflammation by expressing additional IFN-ɣ [68]. In contrast, TH17 cells in the small intestine tend to upregulate IL-10 during transient gut inflammation [6]. This upregulation is described to be driven by the transcription factor c-Maf that supports IL-27 driven IL-10 production in TH17 cells [121].

Besides IL-10 and IL-17A co-expression, some TH17 cells become TR1 cells. Those cells are called TR1^{exTH17} cells. In this case, TGF-β and Ahr are described to be the main drivers of conversion [6]. These cells mainly display a strong regulatory phenotype [6]. Many factors favoring T-cell plasticity have been described. T-cell priming, as well as the environment can drive T-cell conversion [68]. T-cell conversion from effector cells expressing IL-10 has been described in a context of self-limitation [38]. Converted cells keep some characteristics of the effector cells they originated from, such as mild expression of the CCR6 that enabled cells to migrate [6]. Furthermore, low levels of ROR-ɣt can be detected in converted TH17 cells [6]. Although cells express chemokine receptors and transcription factors to a lesser extent than TH17 cells, the origin of the cell still potentially influences cell function. Nonetheless, during functional *in vivo* experiments, in comparison to conventional TR1 cells that originate from naïve CD4 positive T cells, both cells exhibit the same capacity to regulate colitis development [6]. Nonetheless, in some organs, the phenotype of effector TH17 cells is less flexible compared to others [7]. Interestingly, TH17 cells in the kidneys of nephritic mice seem to have a more stable phenotype compared to TH17 cells in the inflamed CNS [7]. When TH17 cells are either transferred into immunocompromised mice and glomerulonephritis was induced, or disease is induced straight in IL-17A^{CRE} x Rosa26^{YFP} fate reporter mice, only around 30 % of the cells are unstable in its IL-17A production and even less switch to a TH1 or an TH2 like phenotype [7]. Interestingly, in nephritic, immunocompetent mice,

additional challenge with CD3-specific antibodies resulted in an induction of IL-10 production in TH17. Furthermore, these mice showed less glomerular, as well as lower tubulointerstitial damage [7].
Nonetheless, TH17-cell plasticity is described to be related to different TH17 cell fates. Whereas some TH17 cells acquire a physiological or even TR1 phenotype, others take up TH1 characteristics by upregulating IFN-ɣ [6, 119].

1.8.1 Interleukin-10 and IL-10 receptor signaling

IL-10 is a cytokine with anti-inflammatory properties. Within CD4 positive T cells, different subsets produce IL-10, either alone or in combination with other signature cytokines. Besides effector T cells such as TH1 [122, 123] and TH2 [124, 125] to TH17 cells, $Foxp3^+$ as well as $Foxp3^-$ regulatory cells represent an important source for IL-10. Since IL-10 is produced by various cells that induce different functions, it is a cytokine always described for its dual functions. IL-10 produced by TH2 cells has been shown to inhibit cytokine production in TH1 cells [126]. Nonetheless, when IL-10 is produced by TH1 cells, it is associated with intestinal homeostasis [84]. Mostly, its functions are involved in gut homeostasis and cell survival [84].
IL-10 is described to be a multifactorial cytokine. When the important role of IL-10 for gut integrity is absent, mice develop spontaneous colitis with age [127]. Furthermore, mice deficient for IL-10, display less abundance of probiotic bacteria species such as *Lactobacillus* which is described to correlate with induce colitis development. Interestingly, when IL-10 knock out animals are treated with *Lactobacillus*, colitis progression can be corrected and the gut integrity can be restored [128].
Furthermore, IL-10 strongly supports the stability of regulatory T cells [61]. IL-10 produced by TR1 and $Foxp3^+$ Treg cells controls TH17 cells and pathogenic TH17 cells that co-produce IL-17A and IFN-ɣ [61]. However, it is not mandatory for their induction, but for their maintenance [129]. Especially, for the maintenance of IL-10 production in regulatory T cells, for which functional IL-10 receptor signaling is mandatory [85, 130].

The receptor for IL-10 is a heterotetramer, which is expressed on the surface of CD4 positive T cells. When IL-10 binds to its receptor, the activation of Janus kinase 1 (JAK1) and Tyrosine kinase 2 (TYK2) takes place [131]. After this activation, the phosphorylation of STAT3 is initiated followed by a translocation of STAT3 dimers to the nucleus [132]. By this, an anti-inflammatory transcriptional program and other physiological processes are activated [133, 134].

The IL-10 receptor is expressed on both effector and regulatory T cells [61, 85]. It has been shown in TR1 cells, as well as $Foxp3^+$ Treg cells, that STAT3 expression together with IL-10 signaling are responsible for the suppression of TH17 cells [85, 135]. Thus, in IL-10 knock out or STAT3 deficient mice, Treg cells were incapable of TH17 cell suppression [135]. Similar to this observation, the necessity of IL-10 and IL-10 receptor abundance is also known in human patients suffering from IBD. In those patients, defects in the IL-10 receptor have been identified [9].

Furthermore, the effect of IL-10 can be direct or indirect. Whereas TR1 cells are able to inhibit TH17 proliferation in an IL-10 dependent manner in the intestine [61], IL-10 itself has the capacity to act on APCs, and thereby inhibit cytokine production in TH1 cells [136].

1.9 Aims

Within the human western society, 5-7 % suffer from immune mediated inflammatory diseases [137]. In European hospitals around 19.3 % to 25.2 % of the stationed patients are affected by acute kidney injury [138]. As for inflammatory kidney diseases, these account for 5-12 % of chronic kidney disease [139, 140].

The complex mechanisms how T cells drive inflammation, have not yet been fully understood. Nonetheless, major milestones identifying TH17 and TH1 cells to be strong drivers of kidney injury, provide the opportunity of developing and improving therapeutic interventions. In this regard targeting TH17-cell plasticity and enticing them into regulatory fates would allow us to diminish pro-inflammatory cells and at the same time to expand regulatory cells. These regulatory cells would have the same antigen specificity, and thus, would be potentially highly efficient in blocking inflammation. Therefore, we aimed to understand whether the kidneys harbor plastic TH17 cells that can convert to $TR1^{exTH17}$ cells. To that end, the following questions will be addressed in the first part of this thesis:

1. Do the kidneys of nephritic mice contain plastic TH17 cells which can convert into TR1 cells?
2. What is the role of IL-10 produced by those cells?
3. Do these cells require functional IL-10 signaling in order to be suppressed or to acquire suppressive function?

The second part of this project addresses a molecular and functional analysis of $Foxp3^-$ IL-10 producing CD4 positive T cells in general in the kidneys. In this part we aimed to decipher the role of $Foxp3^-$ IL-10 producing CD4 positive T cells in general during glomerulonephritis.

Investigations of this T-cell subset in the small intestine, spleen and liver have revealed a strong heterogeneity. To what extent $Foxp3^-$ IL-10 producing CD4 positive T cells infiltrate the kidneys during glomerulonephritis, and whether they

have regulatory function is unknown. Thus, the following questions will be addressed in the second part of this thesis:

1. Are the parameters that define regulatory TR1 cells in the gut expressed by Foxp3$^-$ IL-10 producing CD4 positive T cells that emerge in the kidneys during glomerulonephritis?
2. Do Foxp3$^-$ IL-10 producing CD4 positive T cells fulfill suppressive function during glomerulonephritis?
3. What is the transcriptional signature of Foxp3$^-$ IL-10 producing CD4 positive T cells infiltrating the kidneys during glomerulonephritis?

2. Material & Methods

2.1 Material

2.1.1 Consumables

Table 1 Consumables

Productname	Company
Cannula	Braun
C tubes	Miltenyi
Cell strainer 100µm	Greiner
Cell strainer 40µm	Greiner
Cell culture plate 12well	Sarstedt
Cell culture plate 24well	Sarstedt
Cell culture plate 48well	Sarstedt
Cell culture plate 6well	Sarstedt
Cell culture plate 96well flat bottom	Sarstedt
Cell culture plate 96well round bottom	Sarstedt
Cell culture plate 96well v-bottom	Sarstedt
Cosmetic tissue	Van Merhagen
EDTA tube	Sarstedt
Embedding cassette	Kabe
Eppi 0,5mL	Sarstedt
Eppi 1,5mL	Sarstedt
Eppi 1,5mL RNase-free	Sarstedt
Eppi 2,0mL	Sarstedt
FACS-tube with lid	BD
FACS-tube without lid	Sarstedt
Falcons 15mL	Greiner
Falcons 50mL	Greiner
Filter tip 10µL	Sarstedt
Filter tip 1250µL	Sarstedt
Filter tip 200µL	Sarstedt
Filter tip 20µL	Sarstedt
Gentle MACS C-Tubes	Miltenyi
Gloves Gr.S	Ansell
Gloves Nitril purple extra-long Gr. S	Halyard
Lidchain for PCR-tubes	Sarstedt
MACS Separation Column	Miltenyi

PCR-tubes with lid	Sarstedt
PCR-tubes without lid	Sarstedt
Petridish	Sarstedt
Pipetttip 10µL StackPack	Sarstedt
Pipetttip 1250µL	Sarstedt
Pipetttip 200µL StackPack	Sarstedt
Serological pipette 10mL	Greiner
Serological pipette 25mL	Greiner
Serological pipette 5mL	Greiner
Serological pipette50mL	Greiner
Syringe 1,0mL	BD
Syringe 1,0mL	Braun
Syringe 10mL	BD
Syringe 5mL	BD
Syringe 0,5mL	BD
Syringe 20mL	Braun
Syringe filter 0,22µm	Sarstedt
Syringe filter 0,45µm	Sarstedt

2.1.2 Equipment

Table 2 Equipment

Productname	Company
10X Chromium Controller	10X Genomics, San Francisco
Axio Cam MRc	Carl Zeiss Microscopy GmbH, Hamburg
Axio Scopre. A1	Carl Zeiss Microscopy GmbH, Hamburg
Centrifuge 5424 R	Eppendorf AG, Hamburg
Centrifuge 5427R	Eppendorf AG, Hamburg
Centrifuge 5810/5427R	Eppendorf AG, Hamburg
Dissecting set	Fine Science Tools, Heidelberg
EL 808 Ultra Microplate Reader	Biotec-Instruments Inc., USA
Embedding cassettes	Lonza, Köln
Embedding System Tissue-Tek® TEC	Sakura Finetek, Netherlands
Flow cytometer AriaIIIu	BD Biosciences, Heidelberg
Flow cytometer LSRII Fortessa	BD Biosciences, Heidelberg
Freezer MedLine (-20°C)	Liebherr, Stuttgart
gentleMACS™ Octo Dissociator	Miltenyi Biotec, Bergisch Gladbach
Hera Safe Clean bench	Heraeus Deutschland GmbH & Co. KG, Hanau
Hertherm Incubator	ThermoFischer Scientific, Waltham USA
i-sensys MF419x Printer	Canon, Amsterdam
Labor fume hood TEC-Onomic	C+P Möbelsystem GmbH, Breidenbach

Microscope DMIL LED	Leica Biosystem, Nußloch
Microtome CUT 5062	SLEE Medical, Mainz
Neubauer chamber (0.0025mm2)	Superior Marienfeld, Lauda Königshofen
Perfect Blue Gelsystem Maxi	Peqlab Biotechnologie GmbH, Erlangen
Pipettes 10µL, 20µL, 200µL, 1000µL	Eppendorf AG, Hamburg
Practum224-1S	Sartorius, Göttingen
Refrigerator KG KSVV30A	Siemens, München
Sanyo CO2 Incubator	Ewald Innovationstechnik GmbH, Rodenberg
Shandon Citadel 1000	Thermo Electron Corporation, USA
Spectrophotometer: Nano-drop	ThermoFischer Scientific, Waltham USA
Thermal cycler C1000	Bio-Rad Laboraties, München
Thermomixer comfort	Eppendorf AG, Hamburg
Ultra-Low Temperature Freezer MDF-U5386S	Panasonic Healthcare Co., Ltd., Biomedical, Japan
UV trans-illuminator Gel Doc TM XR+	Bio-Rad, Berkeley USA
Vortex Genie 2	Scientific Industries Inc. New York, USA
Water bath WNB	Memmert, Schwabach

2.1.3 Reagents

Table 3 Reagents for genotyping

Reagent	Company	Registered Office
Agarose LE	Biozym	Hessisch Oldendorf, GER
dNTP Mix	Thermo Fisher Scientific Inc.	Schwerte, GER
DreamTaq DNA Polymerase	Thermo Fisher Scientific Inc.	Schwerte, GER
DreamTaq TM Green Buffer (10X)	Thermo Fisher Scientific Inc.	Schwerte, GER
Ethidium bromide	AppliChem	Darmstadt, GER
Gene Ruler DNA ladder Mix	Thermo Fisher Scientific Inc.	Schwerte, GER
Isopropanol	Th. Geyer	Hamburg, GER
Proteinase K recombinant	Roche	Basel, CH

Table 4 Primer sequences for PCR

Gene	Primer sequence
17AyfpCreR	GCA GCA GGG TGT AGG CAA TGC
17AyfpF	CAA GTG CAC CCA GCA CCA GCT GAT C
17AyfpRwt	CTT AGT GGG TTA GTT TCA TCA CAG C
FIR1	CAA AAC CAA GAA AAG GTG GGC
FIR2	GGA ATG CTC GTC AAG AAG ACA GG
FIR3	CAT CTT GGA GAG TCG GTG TG

GFP-3	AAG TCG TGC TGC TTC ATG TG
GFP-5	ACG TAA ACG GCC ACA AGT TC
GFP-3 IRES	GGACGTGGTTTTCCTTTGAA
GFP-5 IRES	GAACTTCAGGGTCAGCTTGC
IL-10Rα floxYAK236	GTG AGC GGA GAT TTT AAC AG
IL-10Rα wt YAK235	ACT GCT GTA TCC CCT CAT CT
IL-17A IRES	ACC GGC CTT ATT CCA AGC
IL-17A KI anti sense	ACA AAC ACG AAG CAG TTT GG
IL-17A KI sense	CAC CAG CGC TGT GTC AAT
IL10fl 932	CCA GCA TAG AGA GCT TGC ATT ACA
IL10fl 933	GAG TCG GTT AGC AGT ATG TTG TCC AG
IL10KOF	GTG TGT ATT GAG TCT GCT GGA C
IL10KOR1	GTG TGG CCA GCC TTA GAA TAG
IL10KOR2	GGT TGC CTT GAC CAT CGA TG
Rag1MF	TGG ATG TGG AAT GTG TGC GAG
Rag1R	CCG GAC AAG TTT TTC ATC GT
Rag1WTF	GAG GTT CCG CTA CGA CTC TG
Rosa 26 Fwd 1	AAA GTC GCT CTG AGT TGT TAT
Rosa 26 RevMut	GCG AAG AGT TTG TCC TCA ACC
Rosa 26 RevWt	GGA GCG GGA GAA ATG GAT ATG

Table 5 Reagents for cell culture and *in vitro* assays

Reagent	**Company**
Biotin anti mouse CD25 (1:400)	Biolegend
Biotin anti mouse CD3 (1.200)	Biolegend
Biotin anti mouse CD44 (1:200)	Biolegend
CD4 microbeads, mouse (1:10)	Miltenyi Biotech
CellTrace Violet dye Proliferation kit (1:500)	ThermoFisher Scientific
Click's Medium	Irvine Scientific
Collagenase D	Roche
Collagenase IV (100 U), from Clostridium histolyticum	Sigma-Aldrich
Dimethylsuloxide (DMSO)	Merck
Dithiothreitol (DTT)	Aplichem
DNAse I	BD Bioscience
DNAse I	Roche
Dulbecco's Phosphate Buffered Saline (PBS) (1X)	PAA
Ethylenediaminetetraacetic acid (EDTA)	Fluka

Fetal bovine serum (FBS)	PAA
Hanks‘ balanced salt solution (HBSS) (10X)	Gibco
HEPES kidney	Invitrogen
L-Glutamine	Invitrogen
Lympholyte M	Cedarlane
Penicillin/Streptomycin, 10,000 units/ml	Invitrogen
Percoll TM	GE Healthcare
RPMI medium	Gibco
Streptavidin microbeads	Miltenyi Biotec
Trypan blue solution, 0.4 %	Sigma-Aldrich
Trypan blue solution, 0.4 %	Sigma-Aldrich
β-Mercaptoethanol	Gibco

Table 6 Reagents for Albumin-ELISA

Reagent	**Company**	**Registered Office**
Carbonat-Bicarbonat buffer capsule pH 9,6	Sigma Aldrich	Missouri, USA
H_2SO_4 2M	Merck	Darmstadt, GER
HRP conjugated secundary antibody	Sigma Aldrich	Missouri, USA
Multistix (10SG) reagents strips (Urea sticks)	Bayer HealthCare	Leverkusen, GER
TMB peroxidase substrate	AVIVA Systems Biology	San Diego, USA
Tris buffered saline with 0,05 % Tween 50mM pH 8,0	Sigma Aldrich	Missouri, USA
Tris buffered saline with 0,1 % BSA, pH 8,0	Sigma Aldrich	Missouri, USA
Tween 20 10 %	Bethyl Laboratories Inc.	Montgomery, USA

Table 7 Antibodies for surface and intracellular staining

Specificity	Fluorochrome	Clone	Dilution	Company
CD11b	PE-Cy7	M1/70	[1:400]	BioLegend
CD11c	PE-Cy7	N418	[1:400]	BioLegend
CD195 (CCR5)	PE/Cy7	HM-CCR5	[1:400]	BioLegend
CD223 (LAG3)	APC	C9B7W	[1:100]	BioLegend
CD25	BV650	PC61	[1:100]	BioLegend
CD3	BUV379	17A2	[1:200]	BD
CD4	Pac Blue	RM4-5	[1:600]	BioLegend
CD4	BV650	RM4-5	[1:400]	BioLegend
CD45	BV785	30-F11	[1:800]	BioLegend
CD45 RB	AF 647	C363-16A	[1:600]	BioLegend
CD45.1	APC	A20	[1:400]	BioLegend
CD45.2	PE Cy7	104	[1:400]	BioLegend
CD49b	PE	HMa2	[1:100]	BioLegend
CD8α	PE-Cy7	53-6,7	[1:400]	BioLegend
Foxp3	APC	FJK-16s	[1:80]	eBioscience
Foxp3	PE	NRRF-30	[1:80]	eBioscience
IFN-γ	BV785	XMG1.2	[1:100]	BioLegend
IFN-γ	APC	XMG1.2	[1:100]	Biolegend
IL-10	PE-Dazzle	JES5-16E3	[1:100]	BioLegend
IL-17A	BV 421	TC11-18H10.1	[1:100]	Biolegend
NK1.1	PE-Cy7	PK136	[1:400]	BioLegend
PD1	BV 605	29F.1A12	[1:400]	BioLegend
TCR-γδ	PE-Cy7	GL3	[1:400]	BioLegend
TIGIT	PerCPCy5.5	GIGD7	[1:400]	eBioscience
TIM-3	BV 421	RMT3-23	[1:400]	BioLegend

Table 8 Reagents for flow cytometry

Reagents	Company	Registered Office
Ionomycin (1:1000)	Sigma Aldrich	Missouri, USA
Monensin A (1:1000)	BioLegend	London, UK
Nonidet P40 (NP40) (1:10.000)	Sigma Aldrich	Missouri, USA
Pacific OrangeTM Succinimidyl Ester (1:1000)	Life technologies	Darmstadt, Germany
Phorbol-12-myristat-13-acetat (PMA) (1:20.000)	Merck (Darmstadt)	Darmstadt, Germany

2.1.4 Buffers and Solutions

Table 9 Buffers and solutions for genotyping

Buffer	Components
Proteinase K buffer	12.1 g Tris, 10 ml 0.5 M EDTA, 11.7 g NaCl, 5 ml SDS (from 20 % Stock), fill up with distilled H2O to final volume of 1L
TBE buffer	108 g Tris, 55 g Boric acid, 40 ml 0.5 M EDTA, fill up with distilled H2O to final volume of 1L

Table 10 Buffers and solutions for cell isolation

Buffer	Components
ACK Buffer (10X)	20.05 g NH4Cl, 2.5 g KHCD3, 0.093 g EDTA, ad 250 ml distilled H2O
Collagenase Solution	500 ml RPMI, 55 ml FBS, 5,5 ml 100X HGPG, 1 ml 0,5 M of CaCl2, 1 ml of 0,5 M MgCl2, 100 U/ml collagenase
DTT Solution	50 ml 10X HBSS, 50 ml HEPES-bicarbonate buffer, 50 ml FBS, 350 ml dH2O, 15,4 mg/100 ml DTT
FACS Buffer	25 ml FBS, 0,03 % Sodium acid, 975 ml 1X PBS
Cell culture medium	Click's medium supplemented with 10% FBS, 1% l-glutamine, 1% penicillin/streptomycin and 1:1000 β-Mercaptoethanol
Hepes	23,8 g HEPES (100 mM final), 21 g sodium bicarbonate (250 mM final), dH2O to 1 liter, adjust pH to 7.2 with HCl
HGPG (100X)	59,6 g HEPES, 14,6g L-glutamine, 1x106 U penicillin, 1 g streptomycin, 2,5 mg gentamicin, RPMI to 500 ml, adjust pH to 7,5 using HCl
Kidney digestion medium	500 ml RPMI, 1 % FBS, 1 % HEPES, 1 % penicillin/streptomycin
MACS Buffer	500 ml 1X PBS, 2 mM EDTA, 1 % FBS
Percoll buffer	90 ml Percoll, 10 ml 10X PBS
Perm buffer	0.1 % NP40 in MACS buffer
PBS (10X)	NaCl (1,37 M), KCl (26,8 mM), Na2HPO4 x 2 H_2O (64,6 mM), KH2PO4 (14,7 mM) adjust pH TO 7,4 with either HCl or NaOH

Table 11 Buffers and solutions for cell culture and *in vitro* assays

Buffer	Components
Cell culture medium	Click's medium supplemented with 10 % FBS, 1 % l-glutamine, 1 % penicillin/streptomycin and 1:1000 β-Mercaptoethanol
Hepes	23,8 g HEPES (100 mM final), 21 g sodium bicarbonate (250 mM final), dH2O to 1 liter, adjust pH to 7.2 with HCl
HGPG (100X)	59,6 g HEPES, 14,6g L-glutamine, 1x106 U penicillin, 1 g streptomycin, 2,5 mg gentamicin, RPMI to 500 ml, adjust pH to 7,5 using HCl

Table 12 Buffers and solutions for Albumin-ELISA

Buffer	Components
Coating buffer	0,05 mol/l Carbonat-Bicarbonat pH 9,6, fill up with distilled H_2O to final volume of 1L
Post-coat buffer	50 mmol/l Tris pH 8,0, 0,1 % BSA, fill up with distilled H_2O to final volume of 1L
Sample Diluent	Tween 20 bring to 0,05 % solution by dissolving in 200 ml Post-coat Puffer
Washing buffer	Dissolve 50 mmol/l Tris (0,05 % Tween 20) in 1L distilled H_2O

Table 13 Buffers and solutions for flow cytometry

Buffer	Composition
Fix buffer	Prepare from stock (3,65 %) formaldehyde solution using PBS/FBS
Perm buffer	0,1 % NP40 solution using MACS-buffer (10µl/10ml)
FACS buffer	25 ml FBS, 0,03 % Sodium acid, 975 ml PBS (1X)

2.1.5 Animals

Mice were kept under specific pathogen free conditions in the animal research facility of the University Medical Center Hamburg-Eppendorf (UKE). Food and water were provided *ad libitum*. *Rag1*$^{-/-}$ were obtained from the Jackson Laboratory. *Il17a*^{Cre} *Il10Rα*$^{flox\ flox}$, *Il17a*^{Cre} *Il10*$^{flox\ flox}$ transgenic mice, *Foxp3*^{mRFP}, *Il17a*^{eGFP}, *Il17a*^{FP635}, *Il10*^{eGFP} reporter mice and *Il17a*^{Cre}, *Rosa26*^{YFP} are described elsewhere [60, 62, 97, 104, 119]. Age and sex matched littermates between 8-12 weeks were used. All animals were cared for in accordance with the institutional review board 'Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz' (Hamburg, Germany).

2.1.6 Cytokines

Table 14 Cytokines for *in vitro* assays

Cytokine	Company	Registered Office
hTGF-β1	R&D Systems	Minneapolis, USA
IL-2	Biolegend	San Diego, USA
Interleukin-1β	Biolegend	San Diego, USA
Interleukin-23	BioLegend	San Diego, USA
Interleukin-27	BioLegend	San Diego, USA
Interleukin-6	BioLegend	San Diego, USA

2.1.7 Antibodies for animal experiments, cell culture and *in vitro* assays

Table 15 Antibodies for animal experiments, cell culture and *in vitro* assays

Antigen	Clone	Company	Registered Office
Mouse anti-CD3	2C11	BioLegend	San Diego, USA
Mouse anti-CD28	37.51	BioLegend	San Diego, USA
Mouse anti-IFN-ɣ	XMG1.2	BioLegend	San Diego, USA
Mouse anti IL-4	11B11	BioLegend	San Diego, USA
Mouse anti GMB	--------	provided by Prof. Ulf Panzer	

Table 16 Amount of injected anti-GMB serum per mouse

Serum code	ml injected/ mouse
SS 14-7	0,3
SS 14-8	0,2
SS 14-9	0,45
Seramin 11	0,2

2.1.8 Kits

Table 17 Kits

Product name	Company	Registered Office
Chromium TM Single Cell 3' v2	10X Genomics	Pleasanton, USA
Creatinin Jaffe Kinetisch	Hengler analytik	Steinbach, Germany

2.1.9 Software

Table 18 Software

Software	Company	Registered Office
Image Lab 5.2.1	Bio-Rad	Hercules, USA
Rstudio	Rstudio Inc.	Massachusetts, USA
Cytobank	Cytobank, Inc.	Santa Clara, USA
EndNote x6	Thomson Reuters	New York, USA
FACS Diva v.6.1.3	BD Pharmingen	Heidelberg, GER
FlowJo_V10	FlowJo LLC	Ashland, USA
Graphics	Apple Inc.	Cupertino, USA
GraphPad Prism 7	GraphPad Software, Inc.	San Diego, USA
KC Junior	BioTek instruments Inc.	Winooski, USA
macOSMojave 10.14.6	Apple Inc.	Cupertino, USA
Microsoft Office 365	Microsoft Corporation	Redmond, USA
Tbase	Abase	Gundelfingen, GER
Windows XP	Microsoft Corporation	Redmond, USA

2.2 Methods

2.2.1 Genotyping

2.2.1.1 Digestion of biopsies

Tail biopsies were taken by animal technicians and stored at -20°C. Biopsies were digested in a water bath (55°C, overnight) using 80 µl Proteinase K buffer with freshly added 3 µl Proteinase K. The enzyme was heat-inactivated for 15 minutes at 95°C. Next, 300 µl distilled water was added. From this solution, PCR was performed (DNA).

2.2.1.2 Polymerase-chain-reactions (PCR) protocols

All reagents used for genotyping are listed in Table 3,4 and 9. To determine the genotype of genetically modified mice, DNA amplification using PCR was used. The master mix for each reaction contained 3 µl 10X DreamTaq master mix buffer, which already contained already green loading dye, 0,22 µl DreamTaq polymerase, 1,5 µl dNTPs (10 mM), 0,9 µl of each primer (10 mM), which were either sets of two or three primer. With H_20, the total volume of the master mix was brought to 26 µl. 2 µl of DNA (2.2.1.1) was added to the PCR master mix. For all PCR reactions, the PCR thermocycler ran the program listed in table 19.

Table 19 Sequence of PCR Program

Step	Temp. [°C]	Time (sec.)
1	94	180
2	94	40
3	65	40
4	72	40
5	Repeat from step 2 for 35 times	
6	72	300
7	15	∞

In order to genotype Foxp3^{mRFP} reporter mice, three primers, FIR1, FIR2 and FIR3 were used. Wild type band showed a band size of 692 bp, knock in band was 470bp.

IL-10^{eGFP} reporter mice were genotyped using two PCR reactions. One PCR was performed using a three-primer set of IL10KOF, IL10KOR1 and IL10KOR2. The wild type PCR amplicon resulted in a size of 340 bp. The amplification of the knock out resulting in a size of 550 bp was not efficient. Therefore, a second PCR was used in order to amplify the inserted *Gfp* or *Gfp IRES*. Two primer GFP 3' and GFP 5' or GFP 3' IRES and GFP 5' IRES reactions have been used. Both amplicons were 200 bp.

IL-17A^{eGFP} and IL-17A^{FP653} were detected using the same three primer IL-17A KI sense, IL-17A KI anti sense and IL-17A KI IRES. Wild type amplicon showed size of 370 bp, the knock in amplicon was 300 bp.

The fate reporter, Rosa26^{YFP}, was detected in two PCR reactions. The PCR for the wild type amplicon with a band size of 600 bp was performed with the primers Rosa26 Seq1 and Rosa26 Seq3, and the knock in gene was amplified using Rosa26 Seq1 and Rosa26 Seq2. The amplicon was detected at 350 bp.
For detection of IL-17ACre, three primers 17AyfpF, 17AyfpRwt and 17AyfpCreR were used. The PCR for IL-17ACre resulted in a wild type amplicon of 304 bp and a knock in amplicon of 597 bp.

IL-10 flox sites were detected using a two primer PCR IL10fl 932 and IL10fl 933. The wild type amplicon showed a size of 480 bp, whereas the knock in amplicon was detected at 514 bp.

The IL-10Rα gene was detected with the primer YAK235 and YAK236. Wildtype amplicon showed a bandsize of 160 bp and the knock in a bandsize of 190 bp.

2.2.1.3 Fragmentation of DNA

For DNA fragmentation, gel matrix with 1,5 % agarose was made using 0,5X TBE buffer and ethidium bromide. PCR products were loaded on the gel and separated by electrophoresis running for 30 minutes at 400 Volt. Ethidium bromide in the gel matrix intercalated with the DNA and bands were visualized using an Ultraviolet transilluminator.

2.2.2 Mouse disease models

2.2.2.1 Anti-glomerular basement model

For the preparation of sheep serum, sheep were immunized with murine glomeruli. The animals developed antibodies against cortical components of the murine kidneys. Sheep were sacrificed and antiserum, containing the IgG against the glomerular basement membrane, was purified. This purification was performed by the technical assistant of AG Panzer Ms. Anett Peters. For the induction of the glomerulonephritis 8 to 12 weeks old mice were injected intraperitoneally with according to Table 16. indicated volume of anti-mouse IgG. For the following three days mouse cages were held on warm mats and fed additional soft food. After three days, protein concentration was determined in the urine by using urea sticks from Bayer Healthcare.

2.2.2.2 CD4 positive T-cell transfer model

For CD4 positive T-cell transfer, TH17, TR1 and Foxp3^{+} Treg cells were generated *in vitro* according to the protocols specified in 2.2.9. TH17 cells were FACS-sorted (2.2.7) by negative expression of Foxp3^{mRFP} and positive expression for IL-17A^{eGFP}. Foxp3^{-} TR1 cells were sorted for high expression of IL-10^{eGFP}. Foxp3^{+} Tregs cells were sorted according to Foxp3^{mRFP} expression. 1x10^{5} TH17 cells were either transferred alone or in combination with 2,5x10^{3} Foxp3^{+} Treg cells or 5x10^{5} TR1 cells.

2.2.2.3 Anti-CD3 specific antibody mouse model

Mice were injected intraperitoneally with 15 µg anti-CD3 specific antibody (Clone 2C11) dissolved in PBS. Injections were performed either day 6, 8 and 10 or day 8 and 10 after induction of glomerulonephritis. Independent from the number of injections as indicated, all mice were sacrificed four hours after the last injection was applied.

2.2.3 Isolation and preparation of organs, blood and urine samples

On the day of analysis, mice were first anesthetized with a combination of CO_2 (80 %) and O_2 (20 %). Afterwards, the O_2 supply was stopped and mice were sacrificed by exposure to 100 % CO_2. Quickly after the last breath of air, the fur was sprayed with 70 % ethanol. The thorax was opened, and a small syringe was used to take blood from the right ventricle. For isolation of the spleen, kidneys and small intestine, the abdomen was opened. Spleen was exempt from fat. The right and the left kidney were isolated, and the capsules were removed. A cross section from the middle of one kidney was transferred into a cassette and put in 4 % paraformaldehyde (PFA) for later histological analysis. The small intestine was taken from the end of the pylorus to the beginning of the caecum. It was opened longitudinally with an intestinal scissor. The content was removed, and the tissue washed out twice with PBS. All organs were kept in 1X PBS on ice until cell isolation.

2.2.3.1 Isolation of lymphocytes from fresh kidneys

Kidneys were reduced to a pulp, transferred into C Tubes and incubated at 37 °C for 45 minutes in kidney digestion medium, containing 200 µg/ml DNAse I and 2 mg/ml Collagenase D. After incubation, the tubes were run on the program mSpleen 1.01 followed by mLung 2.01 in the GentleMACS machine. During this step, digested kidney tissue was mechanically homogenized to a cell suspension. Cells were pelleted for 8 minutes at 300 G. The supernatant was discarded, and the pellet re-suspended in 5 ml 37 % Percoll and ran in the centrifuge for 10 min at 500 G (ACC:9 DEC:4). The upper band was drained, and the pellet washed

with 2 ml PBS/1 %FBS buffer. Cells were pelleted for 7 minutes at 300 G. Lastly, remaining erythrocytes from blood filtrated in the kidneys were lysed via ACK-lysis (2.2.3.4).

2.2.3.2 Isolation of lymphocytes small intestine

For the isolation of intraepithelial lymphocytes (IELs), small intestines were cut in 1 cm long pieces and transferred into a 15 ml Falcon tube. 10 ml DTE solution with freshly added 1,5mg DTT/10 ml were added. Tissues were incubated for 20 minutes at 37°C shaking horizontally in an incubator. Next the content was poured over a metal strainer and washed with 35 ml PBS/1 %FBS. The cloudy liquid containing the intraepithelial lymphocytes was pelleted (7 minutes at 350 G, 4°C) and the pellet was kept on ice. For the isolation of lamina propria lymphocytes (LPLs), tissue was cut to a pulp and digested in 6 ml Collagenase solution containing collagenase (100U/ml) and DNAse I (5000U/ml) for 45 minutes at 37°C. Contents were poured over a metal strainer and smashed through completely while being washed with PBS/1 %FBS. Lamina propria lymphocytes were pelleted for 7 minutes at 350 G, 4°C and pooled with intraepithelial lymphocytes, which were then resuspended in 40 % Percoll solution (3 ml). 60 % Percoll solution (4 ml) was added in a 15 ml Falcon tube. The 40 % Percoll containing both cell fractions was carefully added on top. The gradient was run at room temperature (400 G, 20 minutes, ACC:1 DEC:1). The formed interphase was taken out and washed in PBS/1 %FBS. Cells were pelleted by centrifugation for 7 minutes at 350 G and erythrocytes lysed according to protocol 2.2.3.4.

2.2.3.3 Isolation of lymphocytes from spleen and lymph nodes

Spleens and lymph nodes were smashed through 100 µm cell strainer and washed with PBS/1 %FBS followed by pelleting the cells for 7 minutes at 350 G, 4°C. Spleens were further processed for erythrocyte lysis (2.2.3.4).

2.2.3.4 Erythrocyte lysis

For the lysis of erythrocytes, 10X Ammonium-Chloride-Potassium- (ACK) buffer was diluted with H_2O to 1X concentration. Pelleted cells were resuspended in 1 ml 1X ACK buffer and incubated for 5 minutes at room temperature. After this incubation, lysis was stopped by adding 15 ml PBS 1 %FBS buffer. Lastly cells were pelleted for 7 minutes at 350 G, 4°C.

2.2.3.5 Extraction of blood serum

Fresh whole blood was transferred into EDTA tubes and stored on ice. Next, tubes were centrifuged at 3000 rpm for 12 minutes at 4°C. The upper clear phase was transferred into RNAse free 1,5 ml Eppendorf tube and stored at -20°C until analysis.

2.2.3.6 Isolation of urine

One day before the experimental endpoint, urine was collected. For this, one single mouse was put on a 96-well plate of which three wells contained drinking water. Furthermore, one food-pellet was added. A plastic box pervious to air was put over the mouse. In order to collect the secreted urine in the wells, the mouse was held on for 4-5 hours until a minimum volume of 200 µl urine was reached. Urine was transferred into 1,5 ml Eppendorf tube and stored at -20°C until analysis.

2.2.4 Urine- and blood diagnostics

2.2.4.1 Analysis of urea nitrogen in bloodserum

Blood serum was analyzed for the concentration of urea nitrogen using a cobas® analyzer. Measurements were performed by Carsten Rothkegel, a technical assistant from AG Tiegs.

2.2.4.2 Analysis of albumin and creatinine in urine samples

All reagents and preparation of buffers used for the detection of albumin in mouse urine are listed in Table 6 and 12. Solutions were brought to room temperature before use.

Mouse urine was tested for protein concentration using urea sticks. Depending on the concentration a color turnabout could be observed. To adjust urine samples to similar protein concentration, dilutions were prepared according to Table 20 and 21.

Table 20 Urine dilution for the Albumin-ELISA according to the protein content

Result	Trace or +	++	+++	++++
Dilution	1:1000	1:20.000	1:50.000	1:100.000

Table 21 Preparation of working concentration urine sample

Final Dilution	**Diluted stock I**		**Diluted stock II**		**Diluted stock III**	
	Stock	S/C D	Stock I	S/C D	Stock II	S/C D
1 : 100	10 µl	+ 90 µl	50 µl	+ 450 µl		
1 : 500	10 µl	+ 90 µl	10 µl	+ 490 µl		
1 : 1000	10 µ	+ 90 µl	5 µl	+ 495 µl		
1 : 20.000	10 µl	+ 90 µl	10 µl	+ 90 µl	5 µl	+ 495 µl
1 : 10.000	10 µl	+ 90 µl	10 µl	+ 190 µl	5 µl	+ 495 µl
1 : 50.000	10 µl	+ 90 µl	10 µl	+ 490 µl	5 µl	+ 495 µl
1 : 100.000	10 µl	+ 90 µl	5 µl	+ 495 µl	5 µl	+ 495 µl
1 : 200.000	10 µl	+ 90 µl	5 µl	+ 995 µl	5 µl	+ 495 µl

Stock = Urine

S/C D = Sample/Conjugate Diluent

First, a 96-well-plate was coated with anti-mouse albumin antibody. 100 µl were transferred to each well and the plate was sealed with a foil. The plate was incubated overnight at 4°C. On the next day, the plate was washed three times with washing solution and the remaining liquid was removed. To block open binding sites, 200 µl/well Postcoat buffer was transferred on the plate. After sealing the plate, the Postcoat buffer was incubated for 30 minutes at room temperature with moderate shaking. After incubation, the plate was washed three times with washing solution and the remaining liquid was removed. Next, protein

standard solutions (Concentrations listed in Table 22) were prepared using a descending series of dilutions.

Table 22 Working concentrations of protein standards for Albumin-ELISA

Standard	Final concentration
S0	10.000 ng/ml
S1	1000 ng/ml
S2	500 ng/ml
S3	250 ng/ml
S4	125 ng/ml
S5	62,5 ng/ml
S6	31,25 ng/ml
S7	15,625 ng/ml
S8	7,8 ng/ml

100 µl of standard solutions S1 to S8 and samples were pipetted twice to the 96-well plate. In two wells, only 100 µl of the sample/conjugate was used as a blank. The plate was sealed and incubated in the dark for one hour at room temperature with moderate shaking. After one hour of incubation time, the plate was washed six times with washing solution and the remaining liquid was removed. For protein detection, an HPR conjugated secondary antibody was used in a dilution of 1:50.000 using 50 ml sample/conjugate diluent. Each well of the 96-well plate was covered with 100 µl of antibody solution and incubated in the dark for one hour at room temperature with moderate shaking. The plate was washed six times with washing solution and the remaining liquid was removed. 100 µl substrate solution was pipetted per well and incubated without exposure to light for 15 minutes. After half the incubation time has passed, a color turnabout to blue could already be observed. During incubation, the ELISA-reader was turned on, in order to warm up the light source and to enter the dilutions of the urine samples. Incubation was stopped, either once the samples showed darker coloring than S1 or until S8 showed a mild color turnabout. The substrate reaction was stopped by adding 100 µl H_2SO_4 (2M) to each well, substrate reaction was stopped. Within 10 minutes, of stopping the reaction, the color intensity was measured at 650 nm using an ELISA-reader.

Urine samples were tested for creatinine concentration using the detection kit Jaffee' Kinetics. Measurements were performed according to the corresponding protocol of Hengler Analytik.

2.2.5 Magnetic activated cell sorting (MACS)

- The whole procedure of MACS separation was performed under the clean bench.
- Washing of MACS columns was performed three times using each three ml MACS buffer.
- For each mouse, 300 µl of MACS buffer was used.
- Cell pelleting was executed by centrifugation at 350G for 7 minutes at 4°C.
- Used dilutions of antibodies and beads are listed in Table 5.

Isolation of lymphocytes and APCs from spleens and lymph nodes

Isolated spleens and lymph nodes were exempt from fat and excessive tissue. Using MACS buffer as washing solution, organs were smashed through 100 µm sterile cell strainer. Total cells were pelleted. For TH17 and $Foxp3^+$ Treg cell condition (2.2.9) cells were differentiated from naïve CD4 positive T cells. To this end, cells were first incubated with biotinylated antibodies against CD25 (1:400) and CD44 (1:200) in MACS buffer for 15 minutes at 4°C. The TR1 condition requires the use of memory CD4 positive T cells, which is why cells were incubated with CD25 antibody only, for 15 minutes at 4°C. Cells were washed with 5 ml MACS buffer and pelleted. Next, cells were incubated for 30 minutes at 4°C with magnetic Streptavidin beads (40 µl/ml MACS buffer), which enabled specific binding to biotinylated primary antibodies. MACS column was placed in a magnet and calibrated with 1 ml MACS buffer. The cell suspension was filtered through 100 µm sterile cell strainer and transfered into the column. The column was washed and the flow through was collected in a 15 ml Falcon tube. Cells were pelleted and incubated for 15 minutes in MACS buffer with CD4 positive T-cell selecting magnetic beads (1:10). A new MACS column was put on a magnet and calibrated as mentioned above. Column was loaded with the cell suspension and washed. The flow through, containing $CD4^-$ cells, was collected in a 15 ml

Falcon tube. The column was removed from the magnet and placed on an empty 15 ml Falcon tube. 5 ml MACS buffer was added into the column and quickly pressed through with a stamp. The flow through solution containing CD4 positive T cells were determined for cell number using a Neubauer chamber. Cells were pelleted and resuspended in Click's full medium and kept cold until they were plated. $CD4^-$ cells were incubated with biotinylated primary antibody against CD3 for 15 minutes at 4°C. Cells were washed and pelleted. For 30 minutes at 4°C, cells were then incubated with magnetic Streptavidin beads. A MACS column was put on a magnet and calibrated. The CD3 negative fraction containing APCs was collected with the flow through. To avoid proliferation during *in vitro* culture, APCs were irradiated with 30 Gy.

2.2.6 Flow cytometry („FACS")

The following washing steps were performed with 1 ml MACS buffer.
Cell pelleting was executed by centrifugation at 350G, 7 minutes at 4°C.

2.2.6.1 Identification of dead cells

To discriminate dead from living cells, isolated cells were pelleted and stained with a fluorochrome labeled violet DNA dye that has the capacity to pass through the cell membrane, enter the cell and thereby stain dead cells. Living cells with an intact cell membrane are unaffected by the dye. Staining was performed in PBS for 30 minutes at 4°C using in Table 8 listed dilution. Cells were washed and pelleted.

2.2.6.2 Surface staining

After cell isolation, fluorochrome labeled antibodies were resuspended in MACS buffer, using listed dilutions according to Table 7. Surface staining was performed for 15 to 20 minutes at 4°C in the dark. CD49b and LAG3 staining was performed for 30 minutes at 37°C. Cells were washed and pelleted. Next, cells were either re-suspended in 150 µl MACS buffer or further processed for intracellular staining.

2.2.6.3 Intracellular staining

To enable intracellular staining, cells were stimulated for 3 hours at 37°C in Click's full-medium containing PMA (50ng/ml) and Ionomycin (1mM) and Monensin A. After stimulation, cells were washed and pelleted. Firstly, surface staining was performed according to 2.2.6.2. After staining of the extracellular markers, cells were fixed with 100 µl Fix-buffer for 20 minutes at room temperature without exposure to light. Another washing step was performed, and cells were pelleted. Next, cells were resuspended with 100 µl Perm-buffer, and the cell membranes were permeabilized for 4 minutes at room temperature without exposure to light. Cells were washed and pelleted. Lastly, a staining cocktail with fluorochrome labeled antibodies for intracellular staining was added to the cells using the appropriate dilutions, listed in Table 7. Staining was performed either for one hour at room temperature or overnight at 4°C both in the dark.

Fluorochrome detection was performed on an LSR II flow cytometer using FACS Diva software. For analysis, data were exported from the FACS Diva to FlowJo vX software for MAC or Windows.

2.2.7 Fluorescent activated cell sorting (FACS-sorting)

Cells were stained with extracellular surface markers according to 2.2.6.2. To avoid cell clogging in the fine sorter nozzle with a diameter of 70 µm, the cell suspension was first filtered through a 100 µm cell strainer. Cell sorting was performed on a BD FACS Aria Illu or AriaFusion.

2.2.8 Histology

2.2.8.1 Fixation of tissue samples

Cross sections of kidneys were kept at 4 % paraformaldehyde overnight. Paraformaldehyde was removed by washing three times the tissue for 10 minutes each with fresh PBS on a shaker at room temperature.

2.2.8.2 Histological sample preparation

Next, the tissues were kept in 50 % (v/v) Ethanol at room temperature for three hours. This was the first step followed by an automatically controlled sequence. In this, samples were first transferred in a specific order into an increasing content of ethanol (60 %, 70 %, 96 %, 100 % EtOH). This process served the purpose of dehydrating the tissue. The last steps required transferring the tissues, first in Xylol, and last in paraffin. The whole process took approximately 15 hours. Afterwards, tissue samples were prepared for final embedding in paraffin.

2.2.8.3 Fabrication and treatment of samples slices

To facilitate the fabrication of tissue intersections, paraffin blocks were kept at -20°C until being processed. Using a microtome, thin tissue sections of 1 µm were cut. Before tissues were stained with PAS reagents, first paraffin was first removed (Table 23 Step 1-3) followed by hydration of the tissues (Table 23 Step 4-12) and washing of the glass slides with distilled water (Table 23 Step 13-15).

Table 23 Preparation of kidney tissues before PAS-staining

No. of step	Component	Gehalt (%)	Duration (min.)
1	Xylol	100	5
2	Xylol	100	5
3	Xylol	100	5
4	EtOH	100	5
5	EtOH	100	5
6	EtOH	100	5
7	EtOH	96	5
8	EtOH	96	5
9	EtOH	96	5
10	EtOH	70	5
11	EtOH	70	5
12	EtOH	70	5
13	dH_2O	100	5
14	dH_2O	100	5
15	dH_2O	100	5

2.2.8.4 Periodic acid-Schiff reaction (PAS)

The whole staining-procedure was performed at room temperature. Before use, all solutions were brought to their corresponding appropriate temperatures. De-paraffinized and hydrated tissues were transferred into 0,5 % periodic acid solution and incubated for 15 minutes. The solution was washed off first under running tap water for three minutes and then with distilled water. Next, glass slides were moved into a cuvette, covered with Schiff's reagent, and stained for 40 minutes. Schiff's reagent was removed under warm running tap water followed by a wash step with distilled water. The incubation step in Hematoxylin solution for one minute, colored nuclei, mitochondria and the cell membrane. Staining was checked microscopically and if needed, glass slides were again stained for another minute in Hematoxylin solution. After efficient staining, glass slides were washed for three minutes under warm running tap water and dipped for a few times in hydrochloric acid (HCl/EtOH) solution. Glass slides were washed with cold running tap water for three minutes followed by one washing step with distilled water. Glass slides were dried under the hood until no remaining water was left. Carefully, tissues were coated with mounting medium and sealed with a cover glass. The staining procedure was performed with the help of Alina Borchers the technical assistant of AG Krebs.

2.2.9 T-cell *in vitro* differentiation

2.2.9.1 TH17 differentiation cocktail

For TH17 cell proliferation, 24 well plates were coated with PBS containing 2 µg/ml anti-CD3 specific antibody. Plates were incubated overnight at 4°C or for 3 hours at 37°C. $1x10^6$ naïve CD4 positive T cells/ml isolated from reporter mice according to 2.2.6 were cultured in Click's full-medium that contained $4x10^6$ irradiated APCs/ml, anti-CD3 specific antibody (3µg/ml) anti-CD28 (2µg/ml) and TGF-β (0,5ng/ml), IL-6 (10ng/ml), IL-23 (20ng/ml), anti-IFN-ɣ (10µg/ml) and anti-IL-4 (10µg/ml). Cells were cultured for 5 days at 37°C with $C0_2$. The expression of $Foxp3^{mRFP}$ and $IL\text{-}17^{eGFP}$ was determined using flow cytometry.

2.2.9.2 TR1 differentiation cocktail

For TR1 cell proliferation, 24 well plates were coated with PBS containing 2 µg/ml anti-CD3 specific antibody. Plates incubated overnight at 4°C or for 3 hours at 37°C. Prior culture, coating solution was removed completely. $1x10^6$ CD25 depleted memory cells CD4 positive T cells/ml (2.2.5) from reporter mice were resuspended in Click's full-medium that contained anti-CD28 (2µg/ml) and IL-27 (30ng/ml). Cells were cultured for 5 days at 37°C with CO_2. The expression of $Foxp3^{mRFP}$ and $IL\text{-}10^{eGFP}$ was determined using flow cytometry.

2.2.9.3 Foxp3$^+$ Tregs differentiation cocktail

For iTreg cell proliferation, 24 well plates were coated with PBS containing 2 µg/ml anti-CD3 specific antibody. Plates incubated overnight at 4°C or for 3 hours at 37°C. Prior culture, coating solution was removed completely. To induce Foxp3$^+$ Treg cells, $1x10^6$ naïve CD4 positive T cells/ml from reporter mice (2.2.5) were resuspended in Click's full-medium that contained anti-CD28 (2µg/ml) and IL-2 (50U/ml). Cells were cultured for 5 days at 37°C with CO_2. $Foxp3^{mRFP}$ expression was determined using flow cytometry.

2.2.10 CD4 positive T-cell proliferation assay

To test the suppressive capacity of T-cell subsets, spleens of wild type mice were isolated. With a biotinylated antibody, first CD25$^+$ T cells were removed from cell suspension (2.2.5). Next CD4 positive T cells were separated from CD4 negative cells using CD4 enrichment kit (2.2.5). CD4 negative cells were further processed until irradiation of APCs (2.2.5). CD4 positive T cells (named here Responder cells) were next labeled with proliferation dye (2.2.10.1).

2.2.10.1 Labeling of CD4 positive responder T cells with proliferation dye

Isolated CD4 positive T cells were washed with PBS and pelleted (7 minutes at 350 G, 4°C). During this time, 500 µl PBS were pre-warmed at 37°C. In order to generate a stock solution of 5 mM violet dye was dissolved in DMSO. Next 1 µl

violet dye was mixed with warm PBS and added to approximately $2x10^6$ cells. Cells were stained for 7 minutes at 37°C. The reaction was stopped by adding 3 ml FBS to the cocktail. Cells were pelleted (7 minutes at 350 G, 4°C) and washed again with 3 ml FBS. After another centrifugation step, cell number was determined using a Neubauer-chamber.

2.2.10.2 Scheme of pipetting *in vitro* proliferation assay

Per required well, 100 µl Click's full-medium with $1,5x10^4$ responder cells together with $7,5x10^4$ APCs were plated in a 96-well plate round bottom. Additional soluble anti-CD3 specific antibody (1.5 mg/ml final volume/well) affected further cell stimulation. Next, $1x10^4$ Treg cells/well/100 µl Click's full-medium were added to the responder-APC-mix (total volume 200 µl/well). As a neutral control, the Treg cells were omitted and the volume was adjusted with pure Click's full-medium. As negative control, $1x10^4$ responder were added. The *in vitro* assay ran for 96 hours at 37°C in a $C0_2$ incubator. The proliferation of responder cells was determined via FACS by detecting the intensity of violet dye per cell.

2.2.11 RNA isolation from sorted kidney cells to perform 10X single cell sequencing

After sorting of $6x10^4$ $Foxp3^-$ IL-10 producing CD4 positive T cells from nephritic kidneys, the sample was processed with Chromium TM Single Cell 3' v2 kit according to the corresponding protocol of 10X Genomics™.

2.2.12 Data analysis of single cell sequencing

Single cell sequencing has been performed by Novogene. Data alignment and analysis was carried out by Dr. Can Ergen-Behr be using the Seurat package for Rstudio software.

2.2.13 Statistical analysis

Statistical evaluations were performed using the GraphPad Prism software. Unpaired, nonparametric Mann-Whitney t-test or One-way ANOVA using Tukey's

multiple comparisons-test were used for evaluation of statistical significance. A p-value of <0,05 was used to define significance.

3. Results

TH1 and TH17 cells are both reported to participate in inciting tissue damage in the kidneys during inflammation [3, 5, 27]. The immune system has safeguard mechanisms by which effector T-cell proliferation can be kept in check. One such mechanism is the suppression of effector cells by regulatory T cells. Literature referring to copious inflammatory diseases describe the potential of Foxp3^{+} Tregs cells [91] and Foxp3^{-} TR1 cells in dampening TH1 or TH17 driven tissue damage [61]. Second, TH17 cells have the potential to self-limit by undergoing a mechanism called conversion [6]. This is a mechanism whereby a cell can change its identity by becoming plastic and switching their transcriptional program and cytokine expression, e.g. from IL-17A to IL-10 [6]. IL-10 is a cytokine which is produced by pro-and anti-inflammatory T cells [43, 121, 141]. Moreover, the gut harbors effector TH17 cells, that convert to Foxp3^{-} TR1 cells, so called TR1^{exTH17} cells. During gut inflammation, these converted cells have been identified, not to contribute to inflammation, but to balance gut homeostasis. Cell conversion has also been observed in the lungs of *N. brasiliensis* infected mice and in the brain of mice after EAE induction [6]. The role of Foxp3^{+} Tregs in kidney inflammation has been well described [91, 142, 143]. However, whether TR1 and TR1^{exTH17} cells emergence in the inflamed kidney plays a role in controlling disease was unknown and this was the aim of this study.

3.1 Emergence of IL-10 producing cells in the kidney

During ongoing kidney inflammation, IL-10 producing Foxp3^{+} Treg cells are present and increase in numbers during disease progression [144]. Furthermore, these cells are described to be very potent inhibitors of CD4 positive T-cell driven inflammation in the kidneys and brain [145, 146]. Particularly, in the gut, Foxp3^{-} TR1 cells sufficiently protect from colitis development [61]. We therefore questioned whether TR1 cells and other Foxp3^{-} IL-10 producing CD4 positive T cells are represented in the kidney in steady state and inflammation.

3.1.1 Foxp3^{+} and Foxp3^{-} CD4 positive T cells produce IL-10 during glomerulonephritis

At first, we assessed the different time points when spontaneous induction of IL-10 producing CD4 positive T cells in the kidneys could occur during progressive inflammation in the kidneys. To avoid the need to restimulate the cells, glomerulonephritis was induced in *Foxp3^{mRFP} Il17a^{Katushka} Il10^{eGFP}* reporter mice. It was previously shown that the frequency of effector TH17 cell appear at day 3 after disease induction, increase until day 7 and decrease thereafter [3]. Therefore, we decided on three time points to check for IL-17A and IL-10 producing CD4 positive T cells in the kidneys.

Cells were isolated from kidneys at day 3, 7 and 10 after disease induction (Figure 1A). As a control, we analyzed the frequency of these cells under steady state conditions. Gated on living CD4 positive, CD45 positive cells, the frequency of IL-17A expression was displayed within Foxp3^{-} CD4 positive T cells (Figure 1B + C). Low frequencies of IL-17A single positive T cells were present under steady state conditions, but increased constantly from day 3 onwards until day 10 after disease induction (Figure 1C). The use of triple acute reporter mice enabled us to display co-production of effector and regulatory cytokines. A fraction of IL-17A positive cells co-produced IL-10 (Figure 1C). IL-17A positive T cells, which co-produce IL-10 and have regulatory function, have been described in the gut and are referred to as regulatory (rTH17) cells [147]. However, the regulatory function of these cells has not been assessed in the kidney so far, which is why we refer to these cells as IL-10 producing TH17 cells. During disease progression, IL-10 producing TH17 cells increased in frequencies, but were not found in as high levels as conventional TH17 cells. IL-10 single production was assessed in both Foxp3^{+} and Foxp3^{-} cells (Figure 1C). Over time, increasing IL-10 production in Foxp3^{+} and Foxp3^{-} CD4 positive T cell was detected in the kidneys of nephritic mice (Figure 1C). Under steady state conditions hardly any Foxp3^{-} IL-10 producing CD4 positive T cells were present in the kidneys. However, Foxp3^{+} cells showed high frequencies of IL-10 production (Figure 1C). In Foxp3^{-} and Foxp3^{+} regulatory populations, IL-10 expressing cells were constantly increasing

until day 7. After this peak, the production of IL-10 was reduced at day 10 post disease induction (Figure 1C).

In summary, during the progression of glomerulonephritis, the frequency of IL-10 positive cells, infiltrating the kidneys, increased. This increase could be observed in all analyzed T-cell subsets.

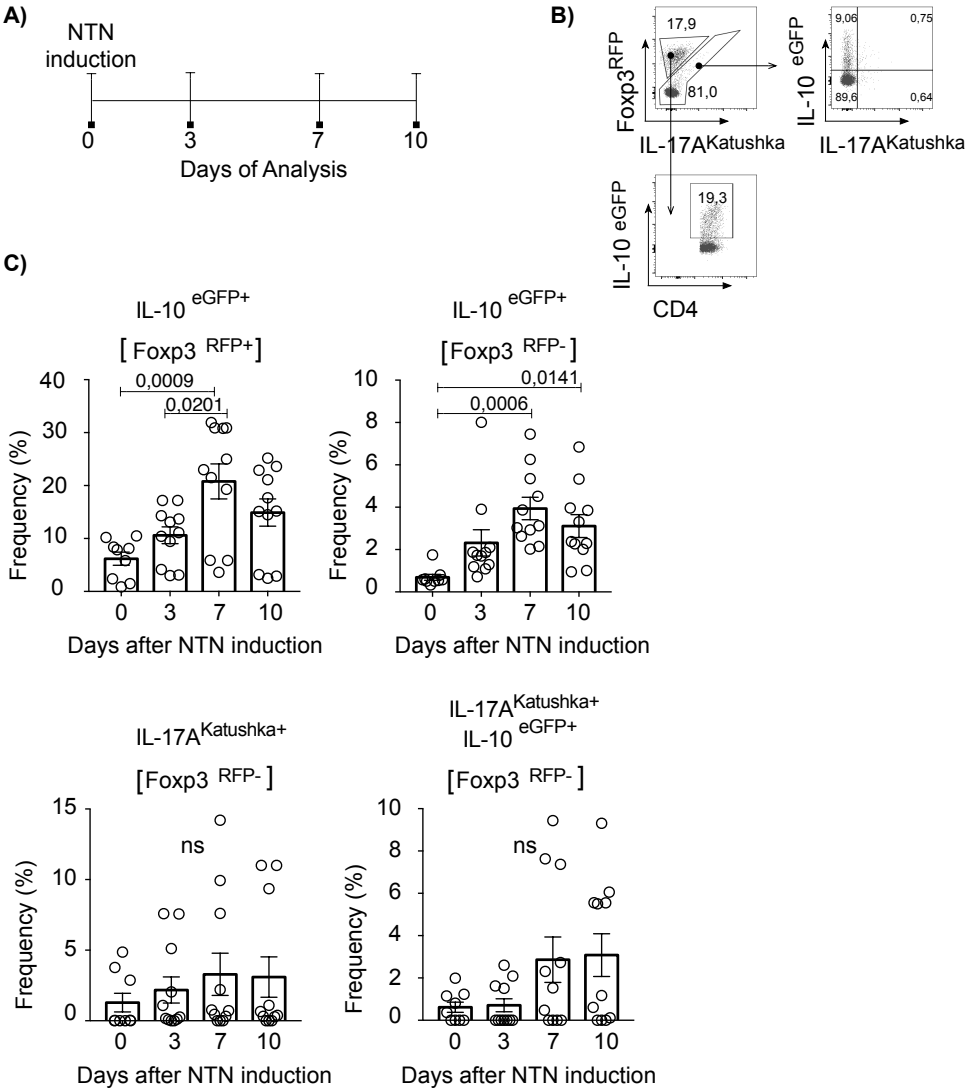

Figure 1: Cytokine production in Foxp3^{+} and Foxp3^{-} CD4 positive T cells
Foxp3^{mRFP} Il10^{eGFP} Il17a^{Katushka} mice were injected with nephrotoxic nephritis serum and sacrificed under steady state conditions as well as day 3, 7 and 10 days after disease induction. Cells were isolated from kidneys. **A)** Experimental setup. **B)** Representative gating strategy and **C)** scatter plots are shown (lines indicate mean ± SEM). FACS data are cumulative of three independent experiments. Day 0 n=9, day 3 n=11, day 7 n=11, day 10 n=11, One-way ANOVA; Turkey's multiple comparisons test ($p<0,05$).

Next, the disease phenotype was analyzed in fixed kidney tissue samples from the same mice. It is published that increasing TH17 cell frequencies involve progressive tissue damage and crescent formation [3]. This effect was corroborated by our results. Whereas no crescents were formed under steady state, up to 5 % of analyzed glomeruli were deformed at day three (Figure 2A + B). With ongoing disease and increasing frequencies of infiltrating TH17 cells, numbers of crescents had doubled until day 7. This percentage of crescent formation further aggravated to almost 50 % at day 10 post disease induction (Figure 2B).

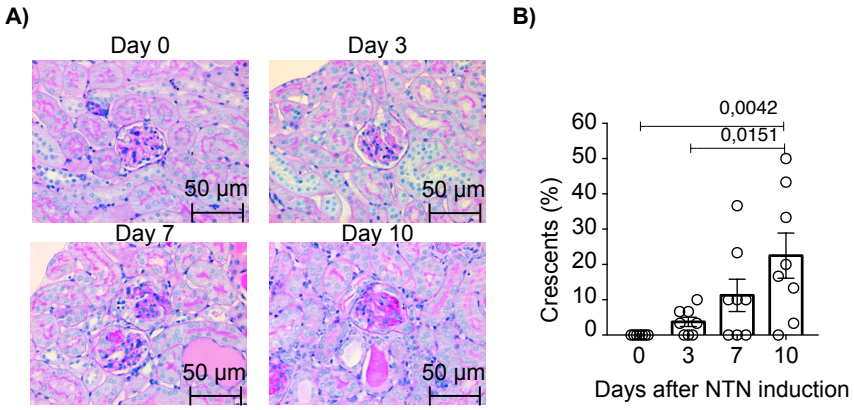

Figure 2: Development of crescents during the progression of glomerulonephritis
Foxp3^{mRFP} *Il10*^{eGFP} *Il17a*^{Katushka} mice were injected with nephrotoxic nephritis serum and sacrificed under steady state conditions as well as day 3, 7 and 10 days after disease induction. Crescents were analyzed from fixed kidneys tissue samples. **A)** Representative histology pictures **B)** Statistical analysis. Data are representative of two independent experiments One-way ANOVA; Turkey's multiple comparisons test ($p<0,05$). Day 0 n=7, day 3 n=8, day 7 n=8, day 10 n=8.

Taken together, increasing frequencies of IL-17A positive cells were observed during glomerulonephritis until day 7. Likewise, IL-10 producing cells were increasing during this time frame. IL-10 production was observed in Foxp3^{+} cells as well as in Foxp3^{-} CD4 positive T cells, including TH17 cells. Nonetheless, despite the infiltration of IL-10 producing CD4 positive T cells, the mice developed kidney disease.

3.1.2 Anti-CD3 specific antibody treatment induces the generation of Foxp3^{-} IL-10 producing CD4 positive T cells that originated from TH17 cells (TR1^{exTH17}) in the kidney

The above-mentioned experiments identified Foxp3^{-} TH17 cells co-producing IL-17A and IL-10 in the kidney. In the gut, these regulatory TH17 cells do not contribute to inflammation [60]. In fact, they represent a population of the immune system that inhibits CD4 positive T-cell proliferation *in vitro*, which indicates suppressive function of these cells [60]. Moreover, studies using Fate^{+} reporter mice have revealed the capacity of TH17 cell to become a TR1 cell [6]. For the generation of these fate mapping mice, acute reporters for Foxp3^{mRFP}, IL-17A^{Katushka} and IL-10^{eGFP} [104] were crossed with IL-17A fate reporter mice (*Il17a^{Cre} Rosa26 STOP $^{flox\ flox\ YFP}$*) [97, 119]. Using these mice, it was shown that TH17 cells can downregulate IL-17A expression and produce only IL-10 [6]. These cells were referred to as TR1^{exTH17} cells. Of note, the environment seems to impact TH17 cell plasticity. Accordingly, TH17 cells found in the kidneys have been reported to have a more stable phenotype [7], with regards of acquiring IFN-ɣ production, compared to TH17 cells in the intestine or CNS during colitis or autoimmune experimental encephalomyelitis, respectively. As mentioned above TH17 cells can also convert into TR1 cells in the intestine. On the basis of these data, we wondered whether TR1^{exTH17} that derived from TH17 cells could also emerge in the kidney.

To address this question, Fate^{+} mice were treated with nephrotoxic nephritis serum. One half received additionally an anti-CD3 specific antibody at day 8 and 10 post disease induction. Ten days after disease induction, mice were sacrificed. CD4 positive CD45^{+} T cells were isolated from kidneys. Treatment with the anti-CD3 specific antibody was shown to be very efficient in inducing IL-10 production in cells from the small intestine [61]. Therefore, we used the small intestine as a control for the presence of IL-17A and IL-10 producing CD4 positive T cells. Expression levels of Rosa26^{YFP} IL-17A^{Katushka} IL-10^{eGFP} were analyzed in Foxp3^{-} CD4 positive T cells.

Our results showed that the ratio between YFP^+ and YFP^- CD4 positive T cells was slightly increased by the treatment with the anti-CD3 specific antibody. In the kidneys, the IL-10^{eGFP} expression, within YFP^- cells, was almost 10-fold higher after this treatment (Figure 3A + B). Those IL-10 producing cells have not produced IL-17A at any time point. Furthermore, when identified in the gut, their regulatory function had been previously shown in *in vivo* experiments [6]. Thus, we referred to these cells as to conventional TR1, which had originated from non-TH17 cells. Within the YFP^+ population, cells were further separated in acute IL-$17A^{Katushka}$ positive, defined as TH17 cells, and negative cells, named exTH17 cells (Figure 3A small red and black box). IL-10^{eGFP} IL-$17A^{Katushka}$ double positive cells were present after NTN induction (Figure 3A + B).
Indeed, the analysis of IL-10 expression in exTH17 cells revealed the existence of so-called $TR1^{exTH17}$ cells in the kidneys after glomerulonephritis (Figure 3A + B) green box). Interestingly, treatment with anti-CD3 specific antibody did not only increase expression of IL-10 in $Foxp3^-$ CD4 positive T cells including TH17 cells, but also showed higher frequencies of conversion from TH17 cells to $TR1^{exTH17}$ cells (Figure 3A right panel + 3B). The strong upregulation of IL-10 production in mice treated with anti-CD3 specific antibody could also be observed in $Foxp3^+$ Tregs cells (Figure 4B). As expected, the control samples from small intestine showed strong production of IL-10 when anti-CD3 specific antibody was administered in all cell populations (Figure 3C + 4C).

In summary, we found that the inflamed kidney contains some plastic TH17 cells. Furthermore, those plastic TH17 cells were capable of acquiring IL-10 expression and stop producing IL-17A. The populations of IL-17A negative IL-10 producing CD4 positive T cells consisted of both cells that had and had not emerged from TH17 cells. Overall, in every tested cell subset, including $Foxp3^+$ Treg, the *in vivo* administration of an anti-CD3 specific antibody resulted in increased IL-10 production.

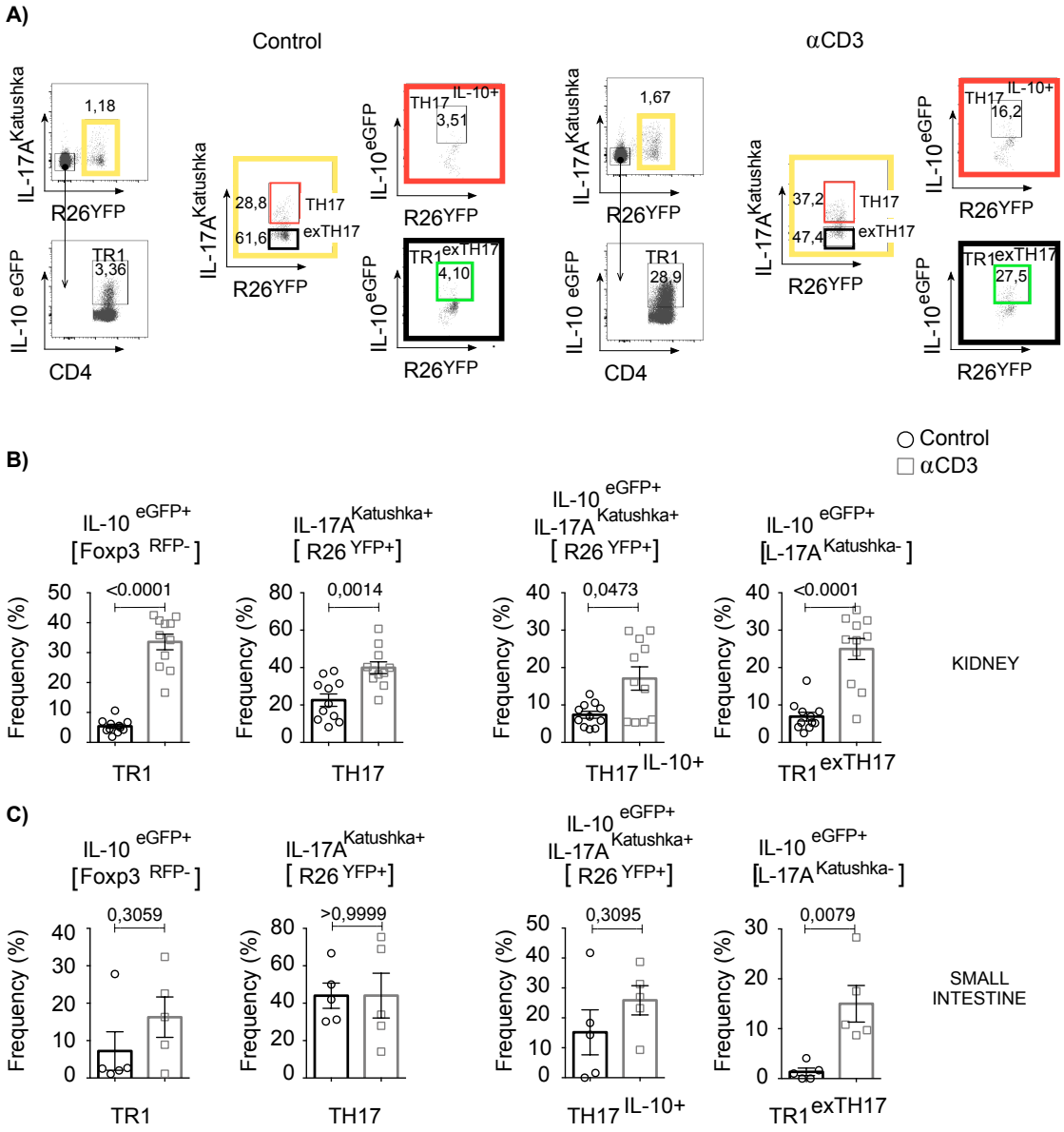

Figure 3: Spontaneous and induced induction of TH17-cell conversion

Foxp3^{mRFP} *Il10*^{eGFP} *Il17a*^{Katushka} *Il17a*^{Cre} *Rosa26*^{YFP} mice were separated in two groups and injected with nephrotoxic nephritis serum. One group received additional 15 µg of anti-CD3 specific antibody on days 8 and 10 post disease induction. Mice were sacrificed 4 hours after the last antibody injection. Control group received PBS instead. Cells were isolated from kidneys and small intestine. Foxp3^{-}, IL-17A positive and IL-10 positive cells were measured by flow cytometry. **A)** Representative dot plots and scatter plots are shown (lines indicate mean ± SEM). **B)** Kidney data are cumulative of four independent experiments. Mann Whitney test p<0,05. Control n=11 anti-CD3 n=11. **C)** Small intestine data are cumulative of three independent experiments. Control n=5 anti-CD3 n=5

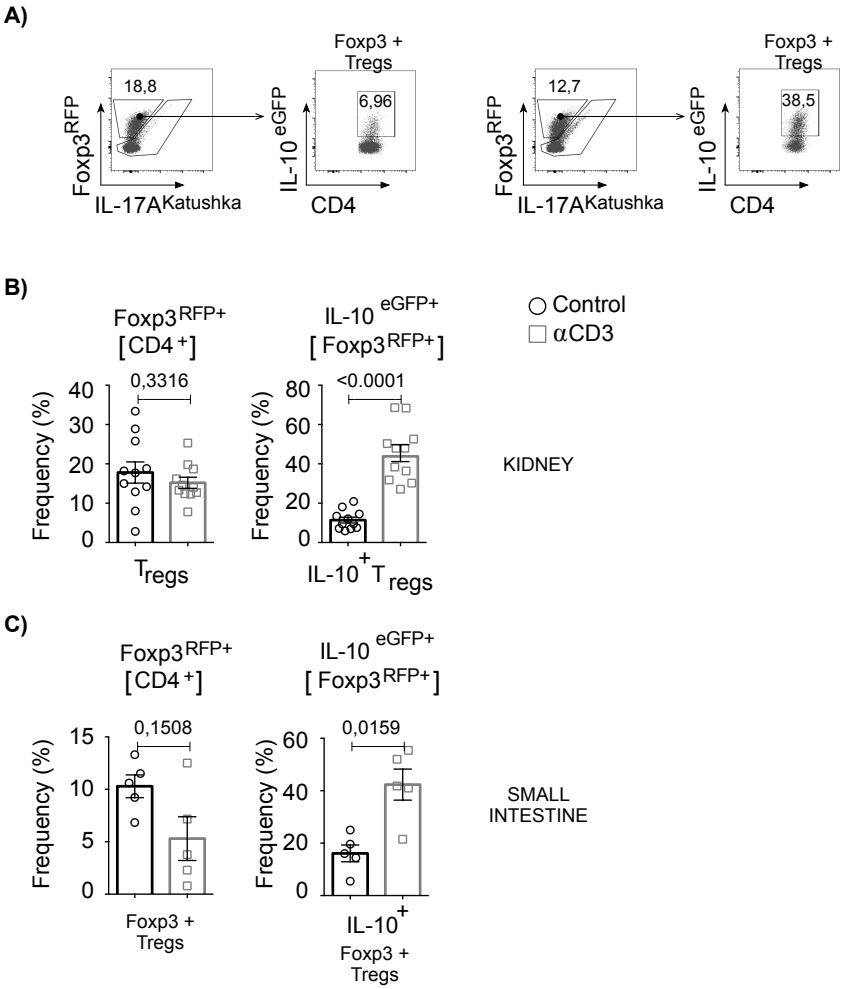

Figure 4: IL-10 positive cells within the Foxp3⁺ Treg population during glomerulonephritis

Foxp3^{mRFP} Il10^{eGFP} Il17a^{Katushka} Il17a^{Cre} Rosa26^{YFP} mice were separated in two groups and injected with nephrotoxic nephritis serum. One group received additional 15 µg of anti-CD3 specific antibody on day 8 and 10 post disease induction. Mice were sacrificed four hours after the last antibody injection. Control group received PBS instead. Cells were isolated from kidneys and small intestine. Foxp3⁺ IL-10 producing CD4 positive T cells were measured by flow cytometry. **A)** Representative dot plots and scatter plots are shown (lines indicate mean ± SEM). **B)** Kidney data are cumulative of four independent experiments. Mann Whitney test p<0,05. Control n=11 anti-CD3 n=11. **C)** Small intestine data are cumulative of three independent experiments. Control n=5 anti-CD3 n=5

3.1.3 Late treatment with anti-CD3 specific antibody does not ameliorate glomerulonephritis

In previous studies, it was shown that treatment of nephritic mice with an anti-CD3 specific antibody reduced tissue damage and revealed a milder disease outcome and protection from strong crescent formation [7]. In these experiments the mice were injected on day 6 and 8 after glomerulonephritis induction. Since our aim was to study the effect of anti-CD3 specific antibody on already differentiated TH17 cells, we injected the mice later, namely on days 8 and 10 after glomerulonephritis induction. To measure disease activity urea nitrogen levels in the sera, as well as albumin and creatinine in the urine were analyzed. Mice treated with anti-CD3 specific antibody did not display lower levels of urea nitrogen in their blood serum (Figure 5A). Also, the ratio between albumin and creatinine did not result in differences between the mice (Figure 5B). Lastly, disease severity was assessed by the formations of crescents (Figure 5C). Unaffected by the treatment with an anti-CD3 specific antibody, both mouse groups developed around 25 % crescents within their glomeruli (Figure 5D).

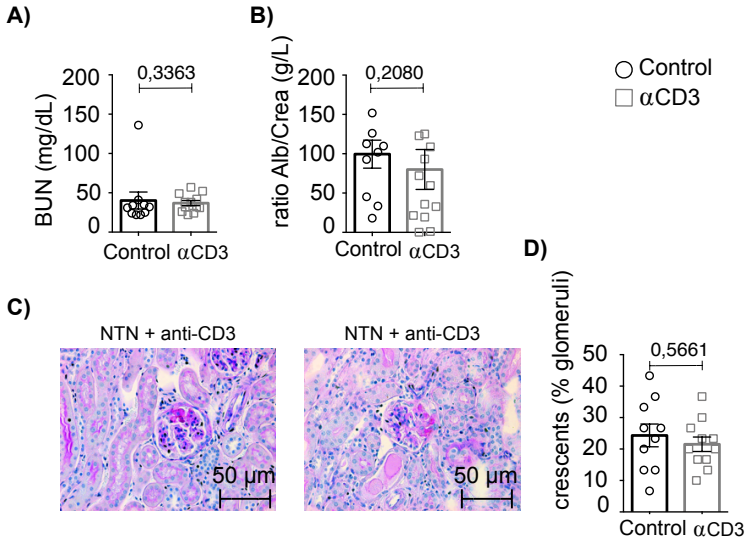

Figure 5: Treatment with an anti-CD3 specific antibody did not reduce disease outcome

Foxp3^{mRFP} *Il10*^{eGFP} *Il17a*^{Katushka} *Il17a*^{Cre} *Rosa26*^{YFP} mice were separated in two groups and injected with nephrotoxic nephritis serum. One group received additional 15 µg of an anti-CD3 specific antibody on day 8 and 10 post disease induction. Mice were sacrificed four hours after the last antibody injection. Control group received PBS instead. Blood was isolated at day 10 and serum was purified. Urine was collected day 9 post disease induction. 30 glomeruli were analyzed for crescent formation in fixed kidney tissues. Scatter plots are shown (lines indicate mean ± SEM). Data are representative of four independent experiments. Mann Whitney test p<0,05. **A)** Blood urea nitrogen (BUN) Control n=10 anti-CD3 n=12; **B)** Ratio between albumin and creatinine (Alb/Crea) Control n=10 anti-CD3 n=13; **C+D)** Crescents Control n=10 anti-CD3 n=11

Considering all clinical parameters, the application of the anti-CD3 specific antibody at later stages of disease development did not improve clinical outcome of treated mice significantly.

3.1.4 Blockade of IL-10 receptor signaling in TH17 cells does not impact glomerulonephritis

During anti-CD3 specific antibody treatment, impaired IL-10 signaling in CD4 positive T cells leads to more sever intestinal inflammation and causes significant mortality [61, 85]. Furthermore, increased proliferation of TH17 cells has been observed when CD4 positive T cells could not respond to IL-10 [61]. This over proliferation was a consequence of dysfunctional TR1 and $Foxp3^+$ Treg cells, whose suppressive capacity was dependent on functional IL-10 signaling in the responding cell population [61]. However, the role of IL-10 signaling in TH17 cells in inflammatory conditions of the kidneys was unknown. To address this, *Il17a*^{Cre} *Il10Rα*$^{flox\ flox}$ mice were used. In these mice, the IL-10 receptor is not expressed only on cells in which the CRE recombinase was active [62]. To test our hypothesis, glomerulonephritis was induced in *Il17a*^{Cre} *Il10Rα*$^{flox\ flox}$ mice and wild type littermate controls. At day 10 post disease induction, T cells were isolated from the kidneys. Expression levels of the transcription factor Foxp3 and proinflammatory cytokines were determined.

The cellular analysis indicated similar expression levels of Foxp3 within wild type and knock out mice (Figure 6B). Furthermore, gating on $Foxp3^-$ CD4 positive T cells, expression levels of IL-17A and IFN-ɣ were analyzed (Figure 6A). Both effector cytokines were not altered in knock out mice compared to wild type counterparts (Figure 6B).

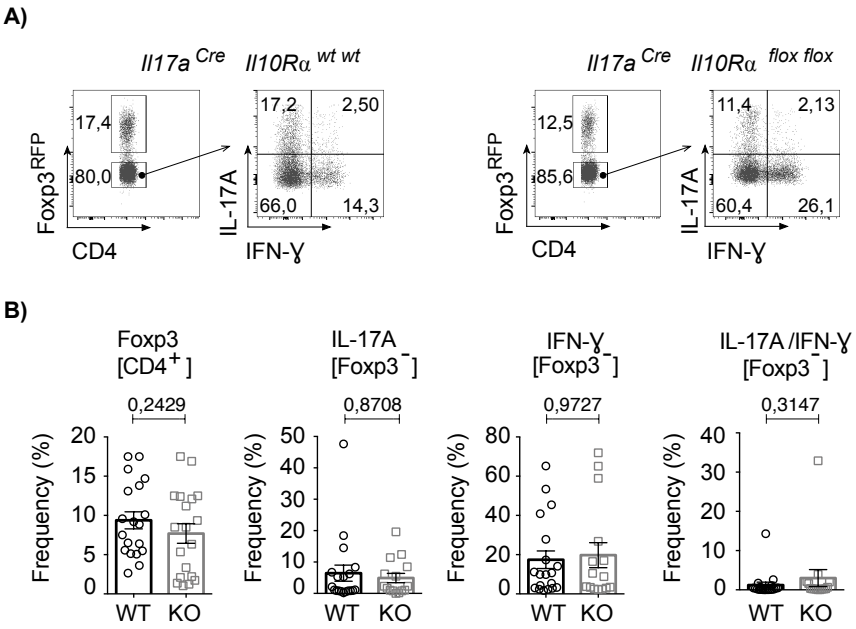

Figure 6: Functional IL-10 receptor signaling on TH17 cells is dispensable during glomerulonephritis

Il17a^{Cre} *Il10Rα*$^{flox\ flox}$ (KO) and wild type controls (WT) were injected with nephrotoxic nephritis serum. At day 10 cells were isolated from kidneys. Foxp3$^+$, Foxp3$^-$, IL-17A positive and IFN-γ positive cells were measured by flow cytometry. **A)** Representative dot plots and **B)** scatter plots are shown (lines indicate mean ± SEM). Data are cumulative of four independent experiments. Mann Whitney test p<0,05. Wild type n=19, Knock out n=19

The frequency of Foxp3 and effector cytokines was not affected when IL-10 signaling was impaired in TH17 cells.

To test, whether the absence of the IL-10 receptor on TH17 cells resulted in a change in disease severity, clinical parameters and histology were analyzed. To this end, urine was collected from the mice one day before they were sacrificed and was tested for creatinine and albumin. Furthermore, on the day of euthanization, kidney tissue samples and blood were isolated. Blood urea nitrogen levels in the blood of the mice were comparable between wild type and knock out mice (Figure 7A). Furthermore, analysis of urine did not indicate differences in the ratio of albumin and creatinine between the genotypes (Figure 7B). Histological parameters of infiltrating T cells and damage of the glomerular

basement membrane (crescents) were analyzed (Figure 7C). Both groups developed similar severities of glomeruli deformation (Figure 7D).

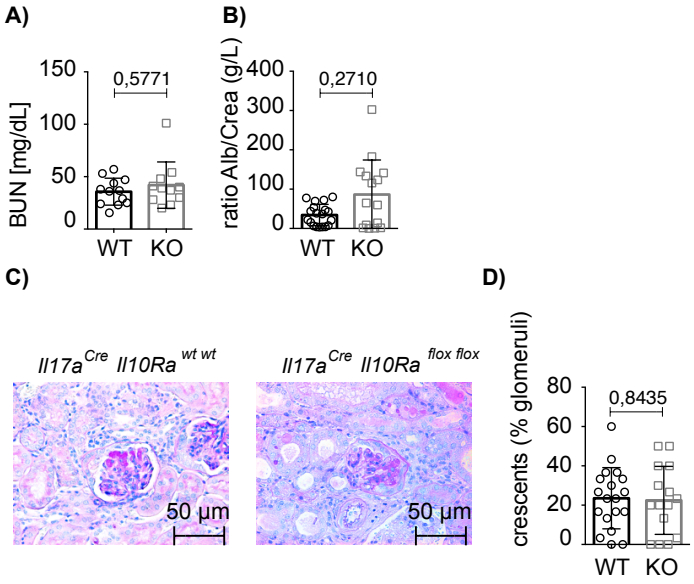

Figure 7: Clinical and histological analysis of nephritic wild type and IL-17A specific IL-10Rα deficient mice

Final blood withdrawal and collection of tissue samples were performed at day 10 post disease induction from *Il17a*^{Cre} *Il10Rα*$^{flox\ flox}$ (KO) and wild type controls (WT). Blood serum was purified. Urine was collected day 9 post disease induction. **A)** Blood urea nitrogen (BUN); **B)** Ratio between albumin and creatinine (Alb/Crea); 30 glomeruli were analyzed for crescent formation in fixed kidney tissues. Scatter plots are shown (lines indicate mean ± SEM). BUN data are representative of three independent experiments. Mann Whitney test $p<0,05$. Wild type n=12 Knock out n=11. Scatter plots are shown (lines indicate mean ± SEM) Alb/Crea and **C)** Representative histology picture. **D)** (%) crescents data are representative of four independent experiments. Wild type n=19 Knock out n=15.

In summary, the deletion of the IL-10 receptor on TH17 cells did not alter the composition of IFN-ɣ and IL-17A producing CD4 positive T cells infiltrating the kidneys during glomerulonephritis. Furthermore, the disease severity in knock out compared to wild type mice was comparable. Therefore, deletion of IL-10 receptor signaling in TH17 cells does not seem to impact the progression of glomerulonephritis.

3.1.5 IL-10 produced by TH17 or exTH17 cells does not impact glomerulonephritis outcome

On the basis of the above-mentioned data, we next aimed to assess the role of $TR1^{exTH17}$ cells and IL-10 produced by TH17 cells in glomerulonephritis. To that end, *Il17a*^{Cre} *Il10*$^{flox\ flox}$ *Rosa26*^{YFP} mice were used. In these mice, the expression of IL-10 is silenced in all cells once they produce high amounts of IL-17A [61]. Cre expression is sufficient in high IL-17A producing cells, as shown by Hirota *et al.* using *Il17a*^{Cre} *Rosa26*^{YFP} mice [119]. Glomerulonephritis was induced in wild type and knock out littermate controls. To further increase the frequency of these cells, the mice were treated with anti-CD3-specific antibody.

At day 10 post disease induction, cells were isolated from the kidneys. The fate reporter, as well as the transcription factor and cytokine analysis were performed on CD4 positive, CD3 positive, CD45 positive T cells. In both wild type and knock out mice, YFP^+ cells were observed (Figure 8A yellow box). In those cells, $IL\text{-}17A^{CRE}$ recombinase had been activated and IL-10 expression was silenced. In Figure 8A, sufficient inhibition of IL-10 production in YFP^+ cells could be noted in knock out, but not wild type mice (Figure 8B). Foxp3 expression was comparable between both genotypes (Figure 8A + B). Also, the frequencies of IL-10 producing cells was not altered in $Foxp3^-$ CD4 positive T cells (Figure 8B). Within YFP^+ cells, IL-17A expression was not altered after IL-10 silencing (Figure 8A + C). In the population of YFP^-, as well as YFP^+ CD4 T cells, IL-17A and IFN-ɣ producing cells were analyzed. Expression levels of both effector cytokines were comparable in both groups between YFP^+ and YFP^- cell population (Figure 8A + C).

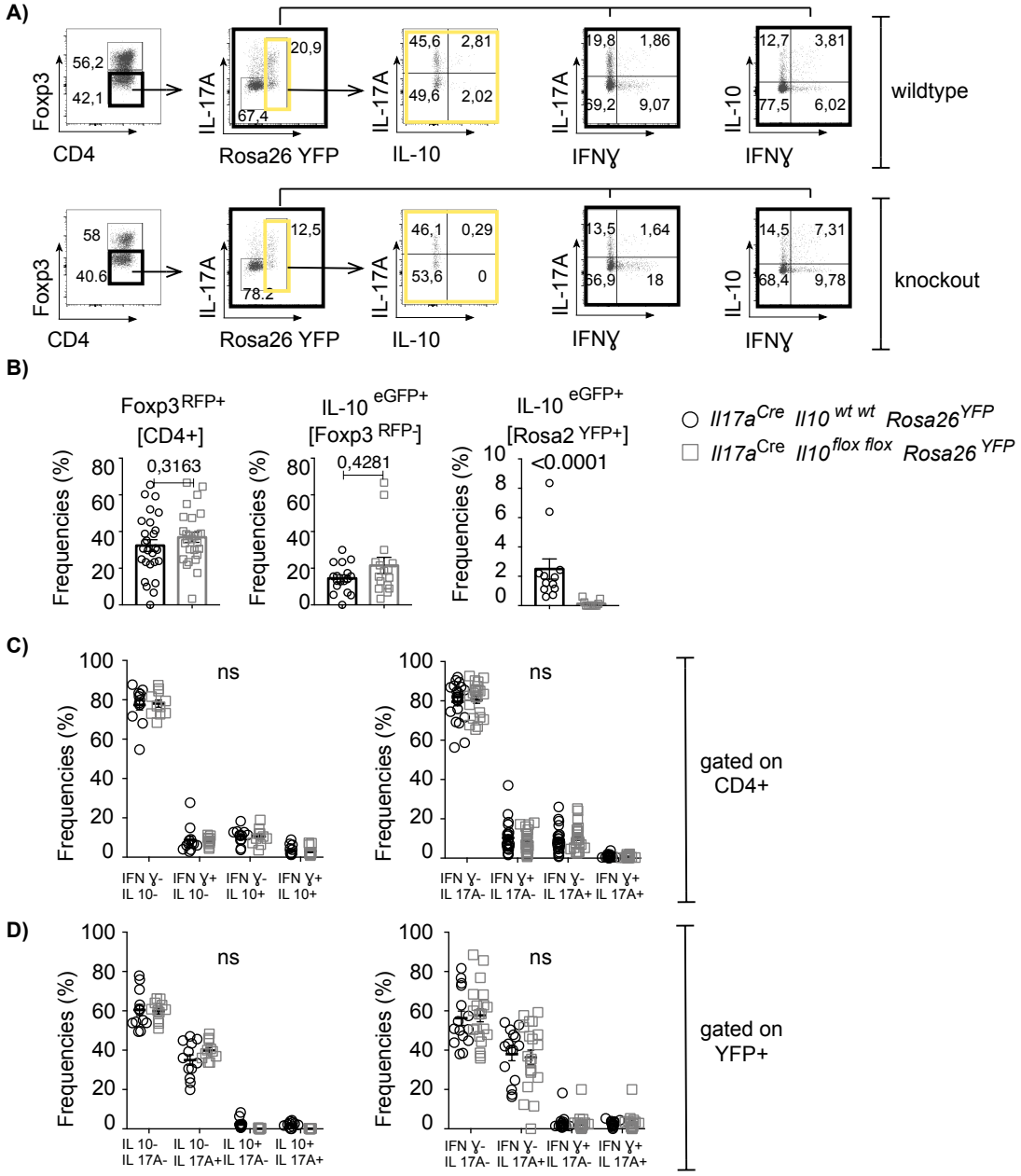

Figure 8: Flow cytometry analysis of nephritic wild type and *Il17a^{Cre} Il10$^{flox\ flox}$ Rosa26^{YFP}* mice

Il17a^{Cre} Il10$^{flox\ flox}$ Rosa26^{YFP} (knock out) and wild type littermate controls were injected with nephrotoxic nephritis serum and three doses of anti-CD3 specific antibody. At day 10 cells were isolated from kidneys. Foxp3^{+}, Foxp3^{-}, YFP^{+}, IL-17A^{+} and IFN- ɣ positive and IL-10 positive cells were measured by flow cytometry. **A)** Representative dot plots **B) +C**) Flow cytometry analysis of indicated cytokines. Scatter plots are shown (lines indicate mean ± SEM). Data are cumulative of four independent experiments. Mann Whitney test p<0,05. Wild type n=19, Knock out n=15 (Values for IL-17Asingle, IFN-ɣ single and IL-17A/IFN-ɣ double positive cells); Wild type n=19, Knock out n=19)s

Besides the cellular phenotype, in which no changes in cytokine expression levels between wild type and knock out mice could be observed (Figure 8), disease development was also analyzed.

During the ten days of experiments, knock out mice suffered more from glomerulonephritis, indicated by higher disease scores. Those animals had to be euthanized ahead of schedule (Figure 9A). Overall, knock out mice showed by trend less survival during glomerulonephritis compared to their wild type littermate controls (Figure 9A). Furthermore, blood urea nitrogen was measured and in the urine the ratio between albumin and creatinine was calculated. Both clinical parameters showed trends towards higher values for knock out mice compared to their wild type littermates (Figure 9B + C). Nonetheless, no statistically significant differences between the groups were observed in any of the clinical scoring (Figure 9B + C). In a final analysis, formation of crescents was determined in kidney samples (Figure 9D), where no significant difference was found between the genotypes (Figure 9E).

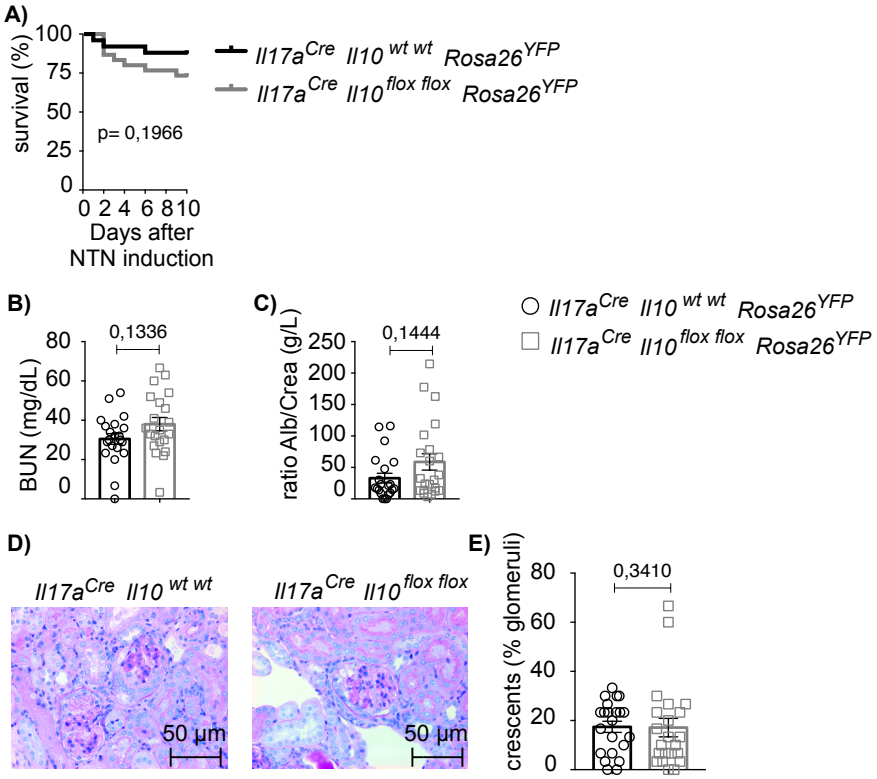

Figure 9: Survival, clinical and histological analysis of nephritic wild type and *Il17a^{Cre} Il10$^{flox\ flox}$ Rosa26^{YFP}* mice

Survival of mice was monitored throughout the experiment. Final blood withdrawal and collection of tissue samples were performed at day 10 post disease induction. Blood serum was purified. Urine was collected day 9 post disease induction. Blood urea nitrogen (BUN); Ratio between albumin and creatinine (Alb/Crea); 30 glomeruli were analyzed for crescent formation in fixed kidney tissues. Scatter plots are shown (lines indicate mean ± SEM). **A)** Survival data of four independent experiments. Statistic for survival curve: Comparison of survival curves; Log-rank (Mantel-Cox) test $p<0,05$. Statistic for BUN and Alb/Crea: Mann Whitney test $p<0,05$. **B)** BUN data are representative of three independent experiments. Wild type n=21 Knock out n=21. Scatter plots are shown (lines indicate mean ± SEM) **C)** Alb/Crea data are representative of three independent experiments. Wild type n=21 Knock out n=21. **D)** Representative histology picture **E)** (%) crescents data are representative of three independent experiments. Wild type n=22 Knock out n=22.

In summary, silencing of IL-10 in TH17 cells, and exTH17 cells did not alter the composition of IFN-ɣ and IL-17A producing CD4 positive T cells infiltrating the kidneys during glomerulonephritis. It also did not change the clinical disease

parameters significantly compared to control mice. Of note, total deletion of IL-10 aggravates glomerulonephritis [92, 95]. Therefore, these data indicate, that loss of IL-10 production from TH17 cells and exTH17 cells can be compensated by other sources during glomerulonephritis. Therefore, we aimed to do a broader characterization of Foxp3^{-} IL-10 producing CD4 positive T cells in glomerulonephritis next.

3.2 Analysis of Foxp3^{-} IL-10 producing CD4 positive T cells

3.2.1 The majority of Foxp3^{-} IL-10 producing CD4 positive T cells in the kidneys does not express co-inhibitory receptors

We previously reported that IL-10 producing T cells are a heterogeneous population, and that only the ones rich in the expression of co-inhibitory receptors have suppressive capacity [8]. However, we did not analyze the kidney in this study. Thus, we next aimed to characterize the Foxp3^{-} IL-10 producing CD4 positive T cells in glomerulonephritis in more detail. For this, Foxp3^{mRFP} IL-10^{eGFP} IL-17A^{Katushka} were injected with nephrotoxic nephritis serum. Mice were separated into two groups based on which received additional treatment with an anti-CD3 specific antibody at day 8 and 10 post disease induction.

After ten days, lymphocytes from both groups were isolated from the kidneys. Within the population of CD4 positive T cells, the expression of Foxp3^{mRFP} and IL-10^{eGFP} was monitored. Whereas glomerulonephritis alone induced only 5 % of CD4 positive T cells in the kidneys that produced IL-10, anti-CD3 specific antibody treatment strongly promoted cells expressing IL-10 (Figure 10B + C). Indeed, frequencies of IL-10 were six times higher when mice were treated additionally with anti-CD3 specific antibody (Figure 10B). The majority of IL-10 producing CD4 positive T cells in the control group was negative for the expression of analyzed surface marker CD49b, LAG3, TIM3 and TIGIT (Figure 10E). In detail, less than 1 % of analyzed cells expressed the previously defined marker combinations, CD49b and LAG3, for suppressive TR1 cells (Figure 10E). A big proportion of 59 % showed only single marker expression or in combination

with one or two others (Figure 10E). The remaining 40 % of IL-10 positive cells did not express either CD49b, LAG3, TIM3 or TIGIT (Figure 10E). Anti-CD3 specific antibody administration significantly increased the frequency of cells expressing at least CD49b and LAG3 within the IL-10 positive CD4 positive T cells to about 8 % (Figure 10E), which however is still much lower than the frequencies previously observed in the intestine [8]. Furthermore, more than 74 % of the IL-10 producing CD4 positive T cells displayed other markers than the mentioned marker combinations in Figure 10E, whereupon numbers of cells expressing two or three markers strongly increased. Finally, after treatment with the anti-CD3 specific antibody, only around 18 % did not express either CD49b, LAG3, TIM3 or TIGIT (Figure 10E).

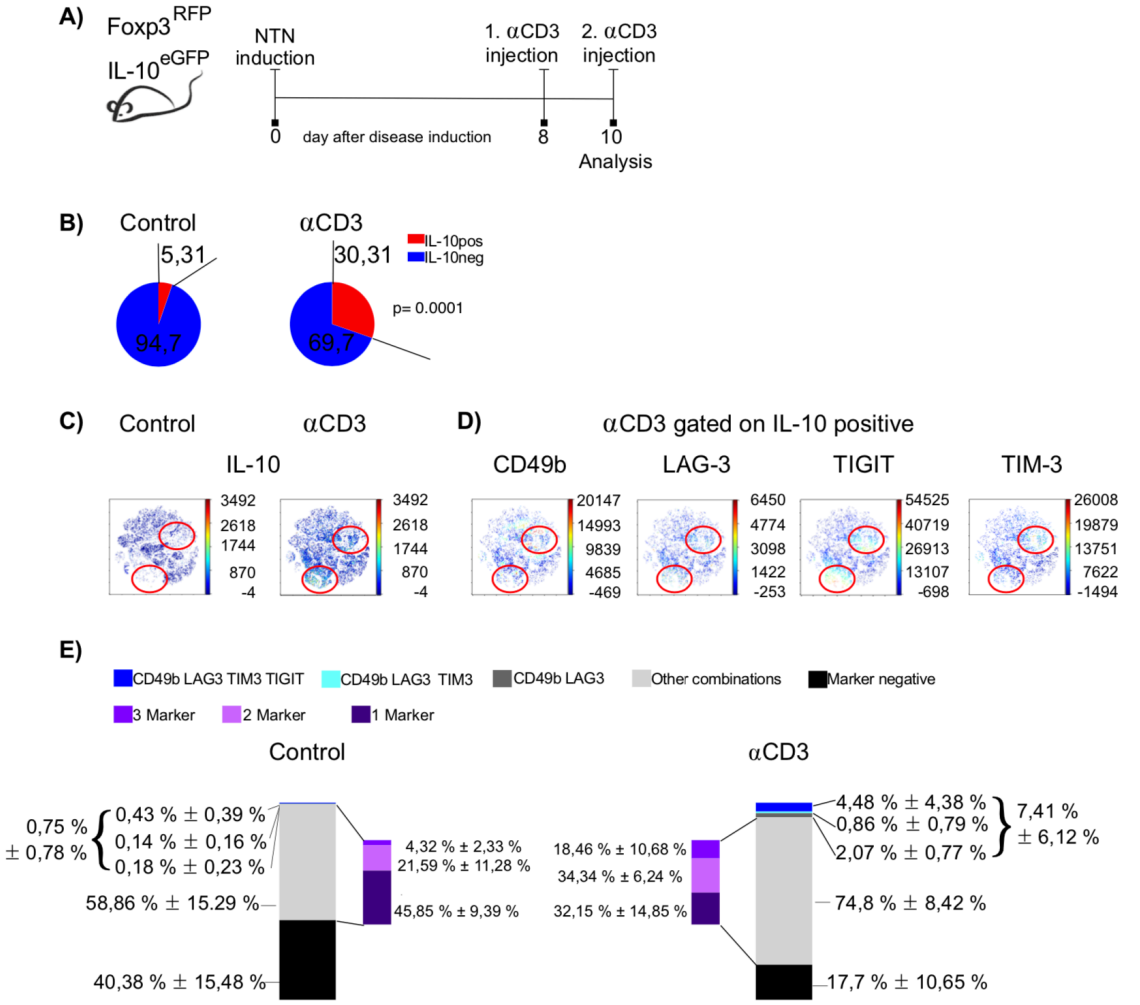

Figure 10: IL-10 expression and upregulation of Co-stimulatory profile of suppressive TR1 cells by anti-CD3 specific antibody treatment

A) Experimental setup. Glomerulonephritis was induced in Foxp3^{mRFP} IL-10^{eGFP} IL-17A^{Katushka} mice. Cells were isolated ten days after disease induction from the kidneys of nephritic mice. Analysis of IL-10 and co-stimulatory profile expression (CD49b, LAG3, TIM3, TIGIT) in Foxp3^{-} CD4 T cells after glomerulonephritis with and without anti-CD3 specific antibody treatment was performed. **B)** Pie chart and **C)** tSNE graphs show expression of IL-10 gated on Foxp3^{-} CD4 positive T cells. **D)** tSNE plots show representative single expression of co-stimulatory surface markers gated on Foxp3^{-} IL-10 producing CD4 positive T cells in nephritic mice treated with anti-CD3 specific antibody. **E)** Bar charts show distributions of various marker combinations gated on Foxp3^{-} IL-10 producing cells. Data are representative of three independent experiments. Mann Whitney test $p<0,05$. Control n= 11, anti-CD3 (αCD3) n=6

In summary, the population of Foxp3$^-$ IL-10 producing CD4 positive T cells in the kidneys displayed a low expression of IL-10, CD49b, LAG3, TIM3 and TIGIT when only glomerulonephritis was induced in the mice. The additional treatment of nephritic mice with an anti-CD3 specific antibody, which is known to induce tolerance [105, 148, 149], increased the frequencies of IL-10 producing cells infiltrating the kidneys. However, within this Foxp3$^-$ IL-10 producing CD4 positive T-cell population, also upon CD3-specific antibody treatment only a small fraction of around 8 % of the cells expressed CD49b and LAG3, the markers which have previously been reported to identify suppressive cells in the gut [8]. Therefore, these data suggest that the majority of Foxp3$^-$ IL-10 producing T cells in the inflamed kidney might not have a regulatory function.

3.2.2 Single cell sequencing analysis of Foxp3$^-$ IL-10 producing CD4 positive T cells from the kidneys reveals a significant heterogeneity of these cells

Originally, Foxp3$^-$ IL-10 producing CD4 positive T cells were thought to be a homogeneous cell population with regulatory function. However, recent publications have questioned this assumption [8]. Accordingly, T-cell heterogeneity is now observed within most T-cell subsets [8, 68, 150].
As outlined above, the majority of Foxp3$^-$ IL-10 producing CD4 positive T cells did not express CD49b and LAG3, the markers which have been shown to identify TR1 cells. Here, to further analyze these cells in an unbiased approached, we analyzed the transcriptome of the total cell population on single cell level. Furthermore, we questioned whether kidney derived Foxp3$^-$ IL-10 producing CD4 positive T cells showed a similar transcriptional profile as already described in detail in the gut and spleen. To address this, Fate$^+$ mice were injected with nephrotoxic nephritis serum. After ten days, CD4 positive T cells were isolated from the kidneys (Figure 11A), pooled together and 40.000 Foxp3$^-$ IL-10 producing cells were FACS-sorted (Figure 11B). To that end, next generation single cell sequencing analysis could be performed with a total of 4.592 single cells.

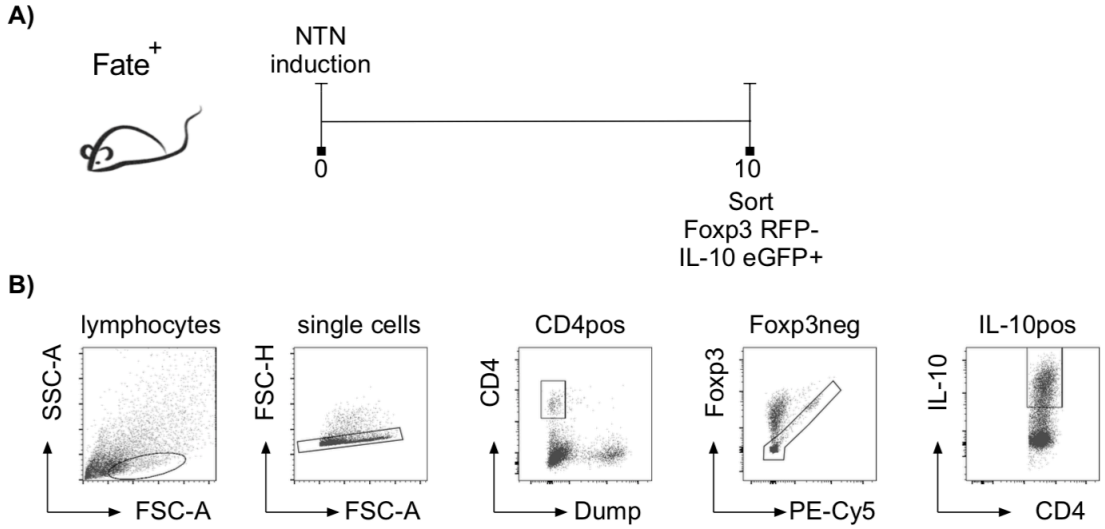

Figure 11: Experimental setup and sorting strategy for RNA single cell sequencing
A) Experimental setup Foxp3^{mRFP} IL-10^{eGFP} IL-17A^{Katushka} IL-17A^{CRE} Rosa26^{YFP} mice were injected with nephrotoxic nephritis serum. Ten days after injection, kidneys of 25 male mice were pooled. **B)** 40.000 Foxp3^{-} IL-10 producing CD4 positive T cells were sorted accordingly to the gating strategy.

First, similarities between IL-10 producing CD4 positive T cells derived from different organs were closely examined. Previously, a suppressive cluster was identified in small intestine and spleen derived IL-10 producing CD4 positive T cells [8]. Furthermore, we wondered whether a tSNE cell clustering would result in an overlap between similar cells from the different organs. Therefore, tSNE analysis of all cells from three different organs was performed. For this tSNE analysis of the spleen and the small intestine, the raw single cell sequencing data, generated by Brockmann *et al.*, were used [8]. This clustering revealed three big clusters, whereas each cluster mainly represented one organ (Figure 12A). Only a small subpopulation of the small intestine sample clustered apart (Figure 12A). Another small subset of splenic IL-10 producing CD4 positive T cells clustered closer to the kidney cluster (Figure 12A). Nevertheless, the strongest differences on which clustering was based were actually determined by the organs of which the cells originally derived.

Next, when each organ was analyzed individually, analysis showed distinct sub-clustering in all three organs (Figure 12B + C). The kidney showed 5 different clusters (C0-C4) (Figure 12B), same as the small intestine, that separated in 5

clusters (C0-C4) (Figure 12C). The highest number of 7 clusters (C0-C6) was found in IL-10 producing CD4 positive T cells isolated from the spleen (Figure 12C).

Next, via Spearman correlation, every cluster of each organ was cross-compared to the others.
Previously, one cluster of small intestine and spleen were identified to be highly suppressive. These cells are in the presented analysis in splenic cluster C4 and the cluster C0 from the intestine, although the majority of cells in the small intestine had suppressive function at different levels [8]. The cross-comparison between the clusters from the kidney and the small intestine revealed no similarities (Figure 12D white to blue colors). However, some clusters from the kidneys correlated with some of the spleen (Figure 12D pink to red colors). There was a higher similarity between kidney cluster 2 and spleen clusters 5 and 1 (Figure 12D). Furthermore, kidney cluster 4 showed high similarities with spleen cluster 3. Lastly, kidney cluster 1 displayed milder similarities with spleen cluster 2 (Figure 12D).

Nonetheless, spleen cluster 4 and small intestine cluster 0, which were previously described to be the highly suppressive clusters didn't show similarities with any of the clusters found in the kidney. As expected, the highly suppressive spleen cluster 4 displayed high similarities with all small intestine clusters except cluster 3. Thus, it seems that the heterogeneous Foxp3$^-$ IL-10 producing CD4 positive T-cell population from the inflamed kidney is, on a transcriptional level, distinctly different from the ones observed in the intestine and spleen. However, this does not exclude the possibility that some of the clusters in the kidney might have a transcriptional signature of regulatory TR1 cells, which might be still overall distinct from the once in spleen and intestine.

Therefore, we next aimed to assess, whether Foxp3$^-$ IL-10 producing CD4 positive T cells would express a regulatory transcriptional signature of TR1 cells. Previously, bulk sequencing was performed with IL-10 positive CD49b$^+$ LAG3$^+$,

IL-10 positive but not CD49b^{+} LAG3^{+} and IL-10 negative, CD49b^{-} LAG3^{-} CD4 positive T cells from the spleens of anti-CD3 specific antibody treated mice. This analysis was used to identify the transcriptional signature profile of TR1 cells [8]. The score included the expression of cytokines, transcription factors, receptors and other markers such as integrins and chemokine receptors [8]. To test whether the kidneys contains Foxp3^{-} IL-10 producing CD4 positive T cells positive for this signature, the signature was overlaid on single cell data from the kidney. This result was compared with an overlay of the TR1 transcriptional signature on top of splenic and small intestine derived IL-10 producing CD4 positive T cells. Indeed, we had previously shown that cells within this profile are mainly in small intestine cluster 0 (30 %) and spleen cluster 4 (8 %) [8]. This observation could be confirmed also with the new clustering (Figure 12E).

Interestingly, around 30-40 % of the kidney derived Foxp3^{-} IL-10 producing CD4 positive T cells from nephritic mice displayed a transcriptional signature similar to the one of a TR1 cell (Figure 12E). Most of the cells in clusters 3 and 4 showed intermediate to high expression for the previously defined TR1 signature (Figure 12E light dots). Cluster 0 showed a very heterogeneous cluster regarding the TR1 signature. Nonetheless, a small portion of Cluster 0 displayed also intermediate to high expression of the TR1 transcriptional signature. Cluster 1 and 2 showed a low TR1 transcriptional signature expression (Figure 12E dark dots).

Taken together, the population of Foxp3^{-} IL-10 producing CD4 positive T cells from different organs did not show similarities between the kidney compared to the small intestine and spleen. Nonetheless, 30 % of Foxp3^{-} IL-10 producing CD4 positive T cells in the kidney displayed the transcriptional signature of a TR1 cell.

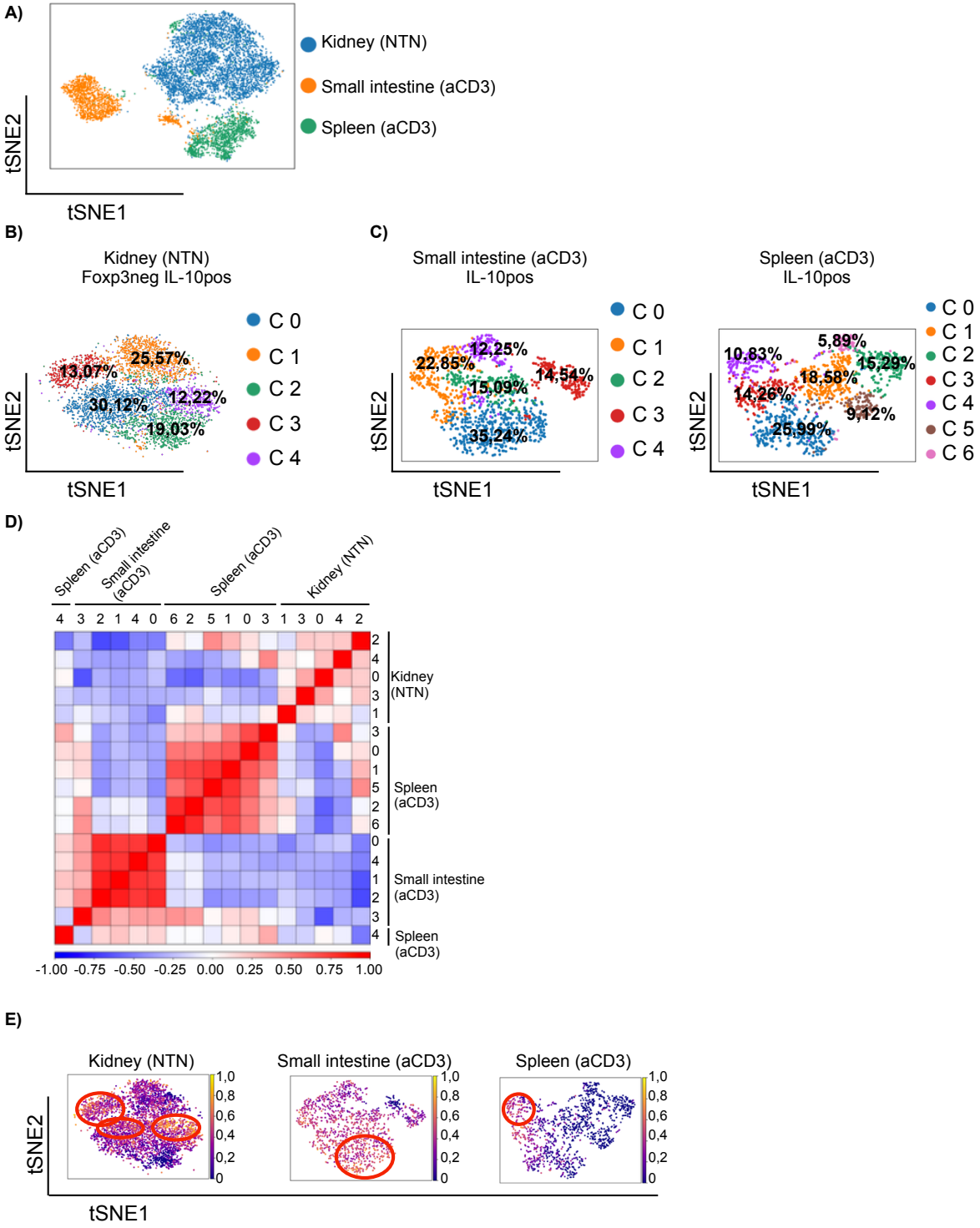

Figure 12: Score of cells with transcriptional TR1 gene signature
A) Clustering of IL-10 producing CD4 positive T cells from small intestine and spleen of anti-CD3 specific antibody treated mice together with Foxp3^{-} IL-10 producing CD4 positive T cells from nephritic kidneys. **B) + C)** clustering of in A mentioned IL-10 producing single cells **D)** Correlation between clusters formed in B) +C). **E)** Expression levels of cells positive for “TR1”-gene signature based on previous Bulk RNA-sequencing data

Next, we wanted to analyze in more detailed which of the clusters expressed genes associated with regulatory or effector function respectively. Therefore, expression levels of single genes in the different clusters of kidney-derived Foxp3$^-$ IL-10 producing CD4 positive T cells were deciphered. IL-10 cytokine expression, as well as expression levels of surface molecules indicating a suppressive profile were first analyzed. Co-inhibitory receptors such as LAG3, TIM3, TIGIT and PD-1 as well as integrin CD49b, or the chemokine receptor CCR5 are described to be expressed on highly suppressive TR1 cells [8, 97, 112].

Interestingly, as for IL-10 expression, cluster 0, 3 and cluster 4 showed a high, and cluster 1 and 2 displayed low *IL-10* gene expression (Figure 13B). The expression of *Itga2*, the gene encoding for the integrin CD49b was low in all cells (Figure 13B). *LAG3*, *Pdcd1*, *CCR5* and *Ctla4* showed similar expression patterns as *IL-10*. The highest expression was detected in cluster 0, 3 and cluster 4 (Figure 13B. The lowest expression was seen in cluster 1 and 2 (Figure 13B). *Havcr2*, the gene encoding for TIM3 was mainly expressed in cluster 0 and 4. However, *Tigit* showed an overall low expression in all clusters (Figure 13B).

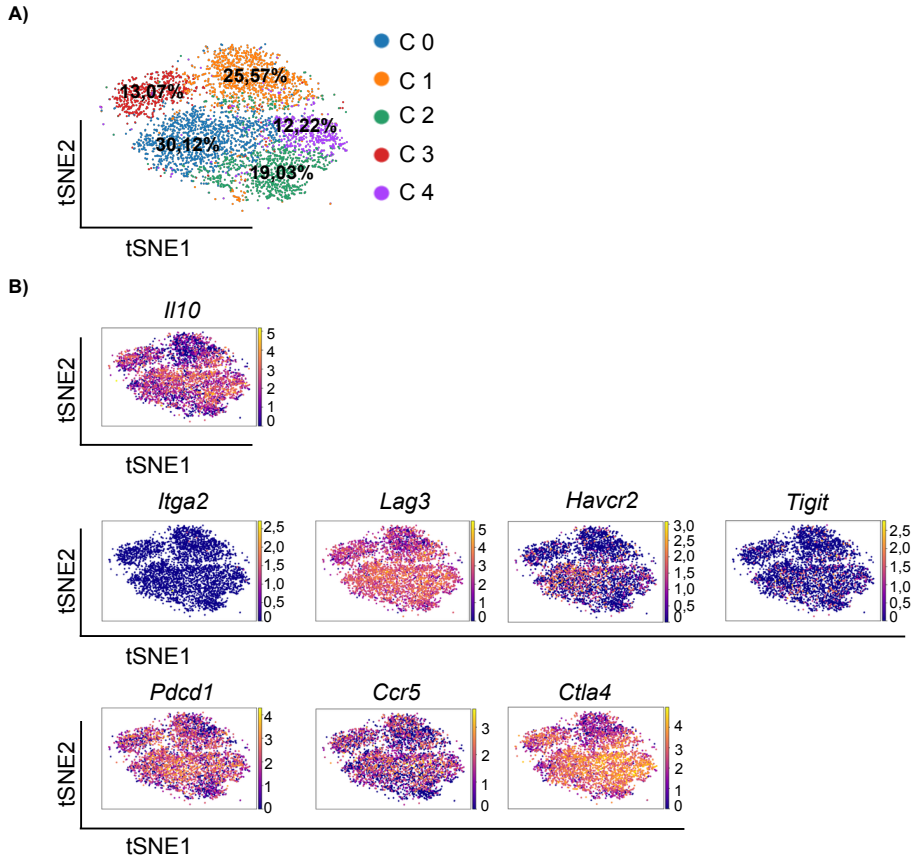

Figure 13: Clustering and gene expression levels of Foxp3$^-$ IL-10 producing CD4 positive single cells
A) tSNE analysis of single cell clustering of 4592 Foxp3$^-$ IL-10 positive cells isolated from the kidneys of nephritic mice **B)** Expression levels of indicated genes in tSNE-analysis

In the next part of the analysis, effector cytokines and related transcription factors were analyzed. Cluster C0 and C3 showed the highest expression of the transcription factor *IRF8* (Figure 14). *Maf*, the gene encoding for c-Maf was highly expressed within all four clusters. C-Maf is known for its important role during IL-27 induced TR1 differentiation [98]. *Prdm1*, the gene encoding for Blimp-1 displayed overall low, but homogenous expression in all clusters (Figure 14). Blimp-1 was described to be important during effector T helper-cell conversion upregulating IL-10 [38]. A small fraction within cluster 1 and 2 showed expression of the TH17 specific signature cytokine *Il17a* and *Rorc*, the gene encoding for the corresponding transcription factor ROR-ɣt (Figure 14). Next, gene expression levels of *Ifng*, the signature cytokine of TH1 cells and *Tbx21* (Tbet), the transcription factor for TH1 cells were evaluated. *Ifng* expression was

predominantly expressed in cluster 0 and 3 (Figure 14). The expression pattern for *Tbx21* was homogeneusly expressed at low levels in all five clusters (Figure 14). Lastly, TH2 related cytokines were investigated. Surprisingly, exclusively cluster C3 showed high expression of *Il4* (Figure 14). *Il13* was not expressed in any of the clusters (Figure 14). Furthermore, the TH2 related transcription factor *Gata3* showed low gene expression in all clusters except cluster 0 (Figure 14).

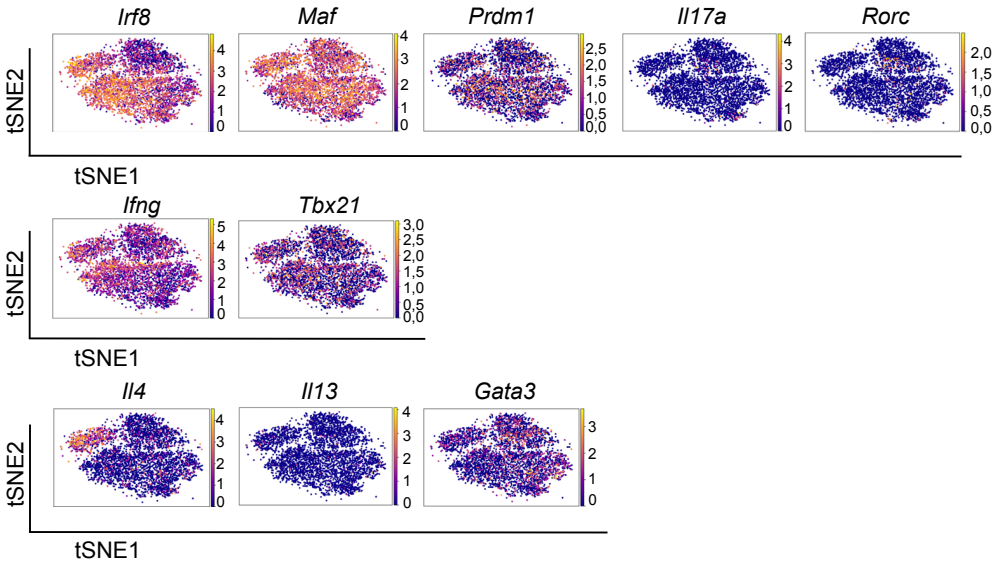

Figure 14: Gene expression levels of Foxp3$^-$ IL-10 producing CD4 positive single cells
Gene expression levels of indicated genes in tSNE-analysis

In conclusion, cluster 0 in the kidney revealed to be the cluster with the highest expression of transcripts, which are typical expressed by a suppressive TR1 cell population. With the lowest *IL-10* gene expression, cluster 1 furthermore displayed genes like *Il17a*, *Rorc*, *Ifng*, *Tbx21* or *Gata3,* associated with effector like phenotype. In addition, it showed low expression of the suppressive profile defined by *Itga2*, *Lag3*, *Havcr2*, *Tigit*, *Pdcd1*, *Ccr5* and *Ctla4*. Taken together, the analysis of Foxp3$^-$ IL-10 positive CD4 positive single T cells from the kidneys revealed a heterogeneous cell population. Nonetheless, the genes encoding for the transcriptional TR1 profile were expressed in about 30-40 % of the cells,

indicating that these cells, despite the low protein expression of CD49b and LAG3, might be regulatory.

3.2.3 *In vivo* generated Foxp3$^-$ IL-10 producing CD4 positive T cells from nephritic kidneys are suppressive *in vitro*

Thus, we next aimed, to evaluate the suppressive capacity of kidney derived Foxp3$^-$ IL-10 producing CD4 positive T cells. To this end, we first used an *in vitro* assay.

To generate Foxp3$^-$ IL-10 producing CD4 positive T cells ("TR1") suppressor cells, glomerulonephritis was induced in Foxp3^{mRFP} IL-10^{eGFP} IL-17A^{Katushka} mice. An anti-CD3 specific antibody was administered 8 and 10 days after disease induction. Four hours after the second injection, CD4 positive T cells were isolated from the kidneys and Foxp3$^-$ IL-10 producing CD4 positive T cells were FACS-sorted. Foxp3$^+$ Treg cells are known for their highly suppressive *in vitro* activity [151]. For this reason, beside the population of interest, they were also FACS-sorted from the same mice and were used as positive controls. In order to remove CD25$^+$ Foxp3$^+$ Treg cells, only CD25 negative CD4 positive T cells were purified from spleens of wild type mice (Responder cells). To track their proliferative capacity during the five days of *in vitro* culture, they were stained with a violet proliferation dye right after isolation. During cell division, the intensity of the dye is halved to both daughter cells [152]. Together, responder cells incubated for 5 days either alone or in combination with one subset of regulatory cells (Figure 15A). The dye was measured after five days using flow cytometry (Figure 15B). Whereas non-proliferating T cells showed bright intensity of the dye, indicating that they did not divide, cells that passed through more division steps displayed reduced dye intensity. Cells were grouped depending on the divisions they have passed during the five days of *in vitro* culture. As a negative control, regulatory T cells were exchanged with the same numbers of non-suppressive CD4 positive responder cells (Figure 15B). This condition did not show any suppression but rather enhanced proliferation of responder cells (Figure 15C). Addition of kidney derived Foxp3$^-$ IL-10 producing CD4 positive T

cells inhibited proliferation of almost 85 % of the responder cells (Figure 15C). Even stronger suppression was seen in conditions with Foxp3$^+$ Tregs, which suppressed more than 95 % of the cells (Figure 15C).

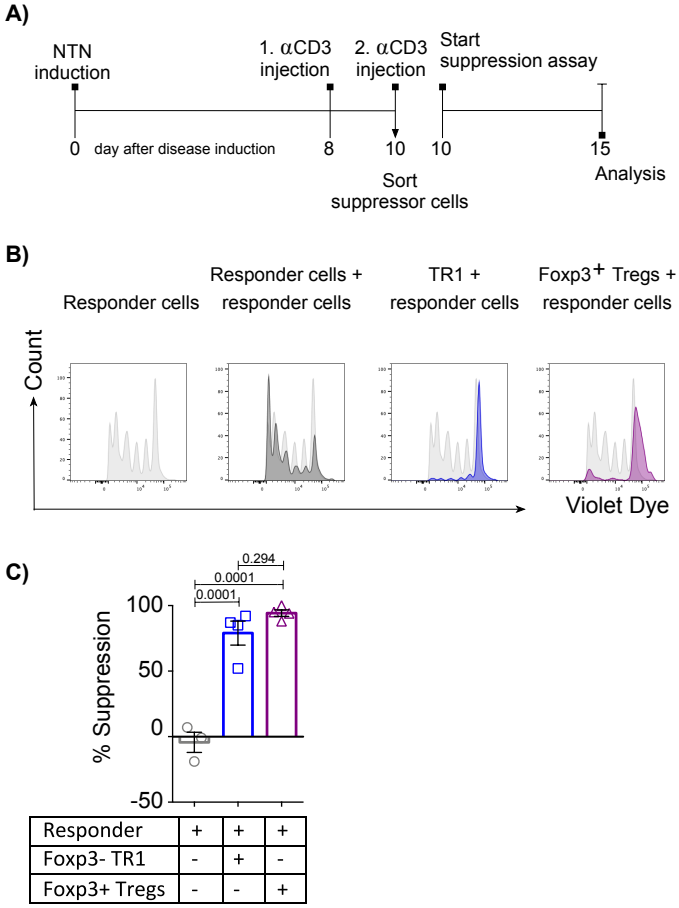

Figure 15: *In vitro* assay of CD4 T-cell proliferation and inhibition by regulatory T cells isolated from nephritic kidneys

A) Experimental setup. **B)** Histograms and **C)** statistical analysis of violet dye intensity in CD4 positive responder cells after 5 days *in vitro* culture. 1,5 x 10^4 responder cells were plated per well. Additional 7,5 x 10^4 irradiated APCs served the purpose of cell activation. 1x10^4 suppressor cells were add to each well. Violet dye intensity was measured via flow cytometry. Data are representative of three independent experiments. One-way ANOVA; Turkey's multiple comparisons test ($p<0,05$). Responder + responder n=3; Responder + Foxp3$^-$ TR1 n=5; responder + Foxp3$^+$ Tregs n=5

Taken together, sufficient numbers of *in vivo* generated Foxp3$^-$ IL-10 producing CD4 positive T cells could be sorted from the kidneys of nephritic mice. It was shown, that *in vivo* generated Foxp3$^-$ IL-10 producing CD4 positive T cells from the kidney could suppress the proliferation of CD4 positive T cells *in vitro*. Thus, Foxp3$^-$ IL-10 producing CD4 positive T cells, isolated from the kidneys of nephritic mice, are capable of suppression *ex-vivo*.

3.2.4 *In vitro* generated Foxp3$^-$ IL-10 producing CD4 positive T cells can improve survival and diminish crescent formation during glomerulonephritis

On the basis of these data, we next aimed to assess the function of Foxp3$^-$ IL-10 producing CD4 positive T cells during glomerulonephritis. Of note, TH17 cells have been shown to further enhance glomerulonephritis when transferred in *Rag1*$^{-/-}$ mice [3]. In order to test the suppressive capacity of TR cells to inhibit TH17 cell driven glomerulonephritis, co-transfer experiments were performed. Such transfer experiments require high numbers of cells in order to induce disease. Therefore, effector TH17 and regulatory cells were first generated *in vitro* using specific proliferation cocktails for each condition (Figure 16A). After five days, *in vitro* cultured cells were FACS-sorted. Foxp3$^+$ Treg cells were used as a positive control. The population of interest was defined as Foxp3$^-$ IL-10 producing CD4 positive T cells. After sorting, TH17 cells were transferred into immune incompetent mice alone or in combination with either Foxp3$^+$ Tregs or Foxp3$^-$ IL-10 producing CD4 positive T cells (Figure 16B). 24 hours after, glomerulonephritis was induced in the mice (Figure 16B). Survival rate was monitored.

These data showed that mice receiving only TH17 cells before disease induction showed increased mortality (Figure 16C). During the experiment, at day 10, 50 % of the mice had reached a high score. According to the criteria of animals' welfare mice had to be sacrificed (Figure 16C). Despite the difference in survival, clinical parameter of blood urea nitrogen and albumin creatinine in the urine displayed no significant differences between mice that received a TH17-cell transfer alone or together with additional regulatory T cells (Figure 16D + E).

Histological analysis of kidney intersections revealed the highest crescent formation in mice that received TH17 cells alone (Figure 16G). Both mouse groups that received additional $Foxp3^-$ IL-10 producing CD4 positive T cells or $Foxp3^+$ Tregs cells displayed less crescent formation (Figure 16G). The strongest difference was seen between TH17 only and the group where $Foxp3^+$ Tregs were transferred (Figure 16G). Nonetheless, the transfer of additional *in vitro* generated $Foxp3^-$ IL-10 producing CD4 positive T cells also attenuated disease severity (Figure 16G).

In conclusion, *in vitro* differentiated $Foxp3^-$ IL-10 producing CD4 positive T cells promote the survival of mice during TH17-cell mediated glomerulonephritis.

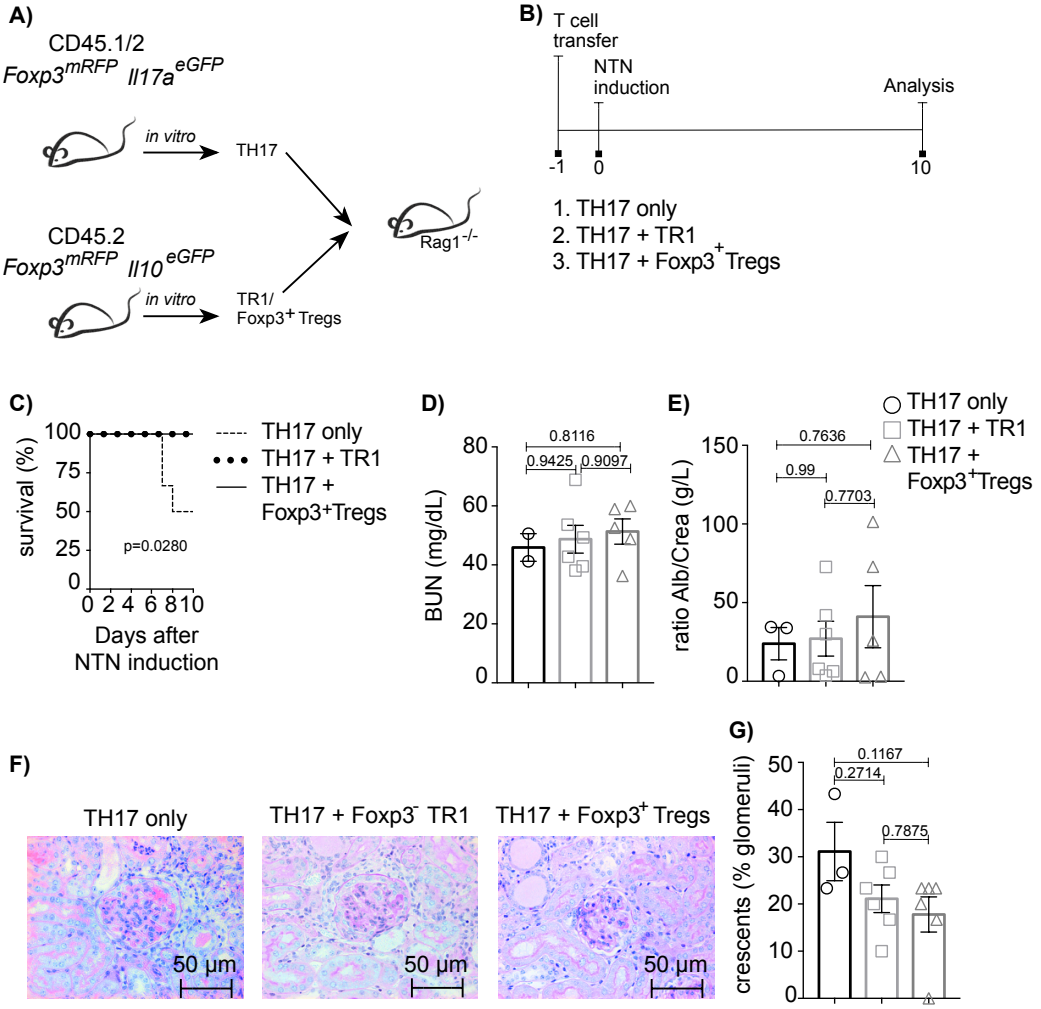

Figure 16: *In vivo* suppressive function of regulatory CD4 T cells on TH17-cell driven tissue damage during kidney inflammation

A+B) Experimental setup. **C)** Survival data show overall survival of two independent experiments. TH17 only n=6 TH17 + TR1 n=6 TH17 + Foxp3⁺ Tregs n= 5. Statistics for survival curve: Comparison of survival curves; Log-rank (Mantel-Cox) test for trend p<0,05. **D)** BUN data are representative of two independent experiments. TH17 only n=2 TH17 + TR1 n=6 TH17 + Foxp3⁺ Tregs n= 5. **E)** Alb/Crea data are representative of two independent experiments. TH17 only n=3 TH17 + TR1 n=6 TH17 + Foxp3⁺ Tregs n= 5. **F)** Representative histology pictures of crescent formation **G)** (%) crescents data are representative of two independent experiments. TH17 only n=3 TH17 + TR1 n=6 TH17 + Foxp3⁺ Tregs n= 6. Scatter plots are shown (lines indicate mean ± SEM) Statistic for crescents, BUN, Alb/Crea: One-way ANOVA; Turkey's multiple comparisons test (p<0,05).

4. Discussion

In the last few decades, the incidence of chronic inflammatory and autoimmune diseases has been increasing [153]. The underlying reason for most of these diseases is unclear, but is seems that an imbalance between pro- and anti-inflammatory CD4 positive TH-cell responses plays a key role [55, 154-156]. Furthermore, it seems that part of this imbalance is driven by T-cell plasticity [16]. Contrary to original findings, differentiated T cells are plastic in their phenotype [6, 157]. Therefore, the opinion on T-cell commitment has changed over the last few years and there are observations on plastic T cells switching their cytokine expression. This observation is referred to as 'T-cell conversion' and is a phenomenon which can occur in various cell types. Within CD4 positive T cells, T-cell conversion is described in regulatory T cells becoming effector cells, and vice versa [116, 158]. Interestingly, this effect seems to be influenced by the micromilieu. Data from the last few years suggest that TH17 are more plastic in the inflamed CNS and intestine, and less plastic in the inflamed kidney. Accordingly, a large fraction of TH17 cells converted to TH1 cells in the CNS and intestine, whereas this was less the case in the kidney [6, 7]. Interestingly, TH17 cells can also acquire regulatory properties and start producing IL-10 in the intestine. These cells are referred to as TR1^{exTH17} cells. Likewise, it was shown that anti-CD3 specific antibody treatment, which is known to induce tolerance, can promote IL-10 production by TH17 cells [7]. However, before this study it was unclear whether TH17 cells can convert into TR1^{exTH17} cells in the kidney. More importantly it was unknown, whether TR1^{exTH17} cells can suppress inflammation in the kidney. Thus, the first goal of this thesis was to identify whether TH17 cell conversion to TR1^{exTH17} cells also appears in the inflamed kidney, and whether this conversion would be important to control inflammation. To that end, we specifically aimed to study the role of IL-10 production and IL- 10 receptor signaling in TH17 and TR1^{exTH17} cells.

Foxp3$^+$ Treg cells have been characterized to decrease kidney injury by suppressing TH17-cell mediated pathology via IL-10 [143]. Their power to stabilize immune homeostasis was also found to be common for different organs and diseases [61]. Besides Foxp3$^+$ Treg cells, TR1 cells can also inhibit TH17-cell driven proliferation in the intestine [159]. Of note, Foxp3$^-$ IL-10 producing CD4 positive T cells are described to be a heterogeneous cell population on a molecular and functional basis, and only a proportion of those have a regulatory function [8]. This subset is then referred to TR1 cells. Nonetheless, the heterogeneity and function of Foxp3$^-$ IL-10 producing CD4 positive T cells in the kidneys during glomerulonephritis has not been addressed so far. Thus, the second goal was to specifically assess the heterogeneity and function of Foxp3$^-$ IL-10 producing CD4 positive T cells in the kidneys during glomerulonephritis.

Taken together, I found that TH17 cells can convert to TR1^{exTH17} cells during glomerulonephritis. Nonetheless, their appearance was very low in numbers and frequencies. Accordingly, functional experiments silencing the IL-10 production and IL-10 receptor signaling specifically in IL-17A positive cells did not result in aggravated kidney injury. Furthermore, I described the molecular and functional heterogeneity of Foxp3$^-$ IL-10 producing CD4 positive T cells in the kidney. Indeed, I found that besides Foxp3$^+$ Treg cells, some Foxp3$^-$ IL-10 producing CD4 positive T cells, which were isolated form the inflamed kidney, fulfilled the criteria defining TR1 cells. Furthermore, *in vitro* differentiated TR1 cells were also able to ameliorate glomerulonephritis *in vivo*.

4.1 IL-10 producing CD4 positive T cells increase in the inflamed kidney during glomerulonephritis

IL-10 is a cytokine with potent anti-inflammatory functions. It is produced by regulatory T cells such as TR1 cells and Foxp3$^+$ Tregs cells. Besides T cells, B cells, dendritic cells, eosinophils and monocytes are also capable of producing IL-10 [160-162]. In the kidneys, Foxp3$^+$ IL-10 producing T cells are described to be able to control TH17 cells [91]. However, in organs such as the gut, not only Foxp3$^+$ Tregs but also TR1 cells have the potential to suppress effector cells and

thereby restore immune homeostasis. Furthermore, they can sufficiently prevent colitis development [106].

Therefore, we aimed to investigate whether in the kidneys, also TR1 cells might play a regulatory role. Before testing their function, the abundance of Foxp3$^-$ IL-10 producing cells was analyzed in steady state conditions and during kidney inflammation.

Under steady state conditions, only few IL-10 producing CD4 positive T cells could be detected. This data fit to previous observations, for example in the gut, in which only low numbers of TR1 cells are present in uninflamed tissue [99]. In the uninflamed kidneys, Foxp3$^+$ Treg cells were the main IL-10 producing T-cell subset. As the disease progressed, frequencies of IL-10 strongly increased in both Foxp3$^+$ and Foxp3$^-$ CD4 positive T cells. This increase seemed to correlate to increasing infiltration of effector cells, which is described already in the kidneys as a response mechanism [7, 144, 163].

Furthermore, as described in previous publications [3], Figure 1C confirmed that increasing frequencies of infiltrating IL-17A positive CD4 positive T cells coincide with increasing crescent formation of the glomeruli [3]. However, it should be mentioned, that in one out of three experiments TH17 cell numbers were very low (data not shown). Between the two displayed experiments, also a high variability in TH17 cells was observed. One possible reason is the use of different sera for the induction of glomerulonephritis. Indeed, each batch of serum originated from a different sheep. Consequently, during the course of this project, different sera have been used. As this effect was to be expected, each serum was titrated in terms of disease severity. However, despite this, we observed relatively high variability in terms of TH17 cell response. Thus, in the future, the cellular read out via flow cytometry should be taken into consideration when titrating the serum. However, also other environmental factors, such as the microbiome play a key role in modulating the TH17 cell response. Thus, a relatively high variability in TH17 cell frequencies is observed also in most experiments studying diseases in

other organs, and as a consequence higher mouse numbers are required to obtain a sufficient statistical power. Interestingly, it seems, that when TH17 cells were low in frequency, the frequencies of IL-10 in Foxp3^{+} Treg cells were also reduced. In contrast, the expression of IL-10 in Foxp3^{-} cells was similar between the experiments independent of IL-17A expression. Therefore, one might speculate that *in vivo*, the IL-10 production by Foxp3^{+} Tregs cells might be influenced by the same factor, which are important for the differentiation of TH17 cells. Since this effect was not the same with IL-10 derived from Foxp3^{-} cells, one might speculate that IL-10 production by Foxp3^{-} CD4 positive T cells during glomerulonephritis is modulated by different factors. Further experiments will be essential to clarify these hypotheses.

4.2 Analysis of TH17-cell stability during glomerulonephritis

As mentioned above, I found that Foxp3^{-} IL-10 producing CD4 positive T cells increase in the kidneys over the course of glomerulonephritis. Interestingly, TH17 cells numbers preceded the emergence of Foxp3^{-} IL-10 producing CD4 positive T cells. Thus, we next wanted to assess, whether TH17 cells would convert into TR1^{exTH17} during glomerulonephritis.

Interestingly, during transient inflammation of the small intestine, TH17 cells can convert into TR1^{exTH17} cells [6]. But whether this would also be the case in the kidney during glomerulonephritis was unknown. Of note, the micromilieu seems to have a big impact on TH17 cell plasticity. Indeed, during EAE, TH17 cells can acquire IFN-ɣ production and convert to TH1 cells [119]. Likewise, TH17 cells can convert into TH1 cells in the intestine during colitis. However, in the kidney, TH17 cells have been described to have a higher stability and only fewer TH17 cells converted in TH1 cells compared to the data described in EAE and colitis [6, 7, 119]. However, so far, the experiments of Hirota *et al.* and Krebs *et al.* have been performed with YFP fate reporter mice that did not have an acute reporter for IL-17A^{Katushka} expression [7, 119]. Thus, it was required to restimulate the cells *in vitro* and to perform intracellular cytokine staining to assess IL-17A production. To overcome this technical boundary, we used Fate+ mice (Foxp3^{mRFP} x IL-17A^{CRE} x Rosa26^{YFP} x IL-17A^{Katushka} x IL-10^{eGFP}) [6]. Thus, we were able to assess

TH17 cells plasticity into TR1 cells avoiding the restimulation of the cells upon isolation.

In our data, *in vivo* experiments indicated that more than 60 % of the YFP^+ cells did not produce IL-17A, on average, on the day of analysis (Figure 3), indicating that they are either resting or converted into other TH subsets. This is a relative high frequency when compared to the data obtained by other groups before (Table 24) [6, 7, 119]. Of course, it is difficult to directly compare my data with the data of these above-mentioned publications, since these experiments were performed by different groups at different times. Furthermore, there is an important technical difference: the cellular read-out of these experiments was performed with isolated cells that were stimulated with PMA/Ionomycin *in vitro* for 4 hours. This stimulation-cocktails reactivates the cells and results in strong production of cytokines [164]. It should be mentioned, that this activation allows one to test, whether a cell is per se able to produce one cytokine. However, it does not necessarily mean that this cytokine was indeed produced *in vivo* at the time of isolation. Thus, YFP^+ cells in previous publications might not have produced IL-17A *in vivo* at the time of isolation and before the *in vitro* restimulation.

One further difference is the sheep serum used between the above-mentioned experiments assessing TH17 cell plasticity in the kidney. With the serum used in this study, overall, the kidney injury was milder compared to previous experiments. This overall milder disease could have also impacted TH17 cell plasticity.

Table 24 Frequency of YFP positive IL-17A negative CD4 T cells

Scientist, organ, treatment	(%)	YFP+ IL17A-
Hirota TH17 *in vitro* culture	16	Fate reporter
Hirota dLN naive mice	20	Fate reporter
Hirota dLN (EAE day 6)	28	Fate reporter
Hirota dLN (EAE day 12)	36	Fate reporter
Hirota spinal cord (EAE day 15)	51	Fate reporter
Gagliani small intestine (Steady state)	42	Fate+ reporter
Gagliani small intestine (aCD3)	14	Fate+ reporter
Gagliani small intestine EAE (aCD3) MOG positive cells	14	Fate+ reporter
Gagliani small intestine EAE (aCD3) MOG negative cells	19	Fate+ reporter
Gagliani small intestine (*S. aureus*)	50	Fate+ reporter
Krebs kidney (NTN)	27	Fate reporter
Krebs kidney (Lupus nephritis)	20	Fate reporter
Krebs kidney (NTN + aCD3)	33	Fate reporter
Current study kidney (NTN)	60	Fate+ reporter

Fate reporter = IL-17A^{CRE} x Rosa26^{YFP}
Fate+ reporter = Foxp3^{mRFP} x IL-10^{eGFP} x IL-17A^{Katushka} x IL-17A^{CRE} x Rosa26^{YFP}

Taken together, our data indicate that some TH17 cells are also plastic in the inflamed kidney, and thus might have the capacity to become TR1^{exTH17} cells. Future side-by-side experiments would be critical to assess and compare, TH17-cell plasticity in different organs and diseases to assess the role of micromilieu in modulating TH17 cells plasticity.

4.3 TH17 cells can convert into TR1^{exTH17} cells during glomerulonephritis

As discussed above TH17 cells in the kidney have the ability to convert into other TH-cell subsets. Thus, we next aimed to assess, whether TH17 cells can convert into TR1^{exTH17} cells during glomerulonephritis.

The monolithic view of stable T-cell lineages has been challenged in the last decade. Whereas originally, every T-cell subset was thought to be stable after differentiation, publications have indicated that this is not always the case [6, 119, 165].

In the intestine, observations of TH17 cells converting to IL-10 producing TR1^{exTH17} cells have been described. Furthermore, the regulatory function of these cells was shown in a subsequently performed *in vivo* transfer colitis model [6]. Accordingly, we wanted to test, whether the observed plasticity of TH17 cells in the kidneys also resulted in a conversion into TR1^{exTH17} cells.

To address this question, Fate^{+} mice were used [6]. These mice harbor acute reporters as well as a fate reporter for IL-17A^{+} cells. This has the advantage over conventional acute reporter, that the course of an IL-17A^{+} cell can be followed [119]. To check induction of TH17 cell conversion, we first induced again glomerulonephritis and treated then half of the mice with anti-CD3 specific antibody.

As mentioned in the previous section, a big fraction of TH17 cells did not produce IL-17A anymore. Nonetheless, a few cells switched the cytokine expression from IL-17A to IL-10. This fraction was very low in frequency and cell number. With this, we could prove that the kidneys contain some TH17 cells that have the potential to become a TR1 cell. As expected, treatment with an anti-CD3 specific antibody increased IL-10 producing cell frequencies in Foxp3^{+} and Foxp3^{-} T-cell subsets. This antibody is known to induce tolerance, which in part is mediated via IL-10 induction [61, 105]. Furthermore, in the gut, anti-CD3 specific antibody

treatment is able to induce TH17 cells conversion into IL-10 producing TR1^{exTH17} cells [6]. Likewise, I found here that TR1^{exTH17} cells can also emerge in the inflamed kidney. TGF-β1 has been shown *in vitro* to promote the conversion of TH17 cells into TR1^{exTH17} cells. This is in line with data showing that TH17 cells react to environmental changes very quickly [117]. Thus, the increased infiltration of regulatory T cells, which are known to produce TGF-β1, into the kidneys might have pushed the cells, to adapt by converting into TR1^{exTH17} cells. However, further experiments will be essential to address this hypothesis.
To test the suppressive function of TR1^{exTH17} generated in the kidneys, cells first needed to be induced *in vivo* using Fate^{+} mice. Ideally, in case sufficient numbers can be reached, *in vivo* transfer experiments should be performed. Unfortunately, only very few TR1^{exTH17} cells can be isolated from the kidneys. Thus, I could not do the proposed *in vivo* suppression experiment. Another possibility would be to assess their function *in vitro*, which requires less cells. However, we could also not obtain the cell numbers required for those experiments, and we therefore decided to assess the *in vivo* function of TR1^{exTH17} by deleting IL-10 production in those cells as mentioned in the following section.

Taken together, we could identify the existence of TR1^{exTH17} cells in the inflamed kidney during glomerulonephritis. However, despite the emergence of these cells, the mice still developed disease. Whether the lack of protection in mice was due to the impaired suppressive function of the generated T cells remained elusive.

4.4 Late anti-CD3 specific antibody treatment does not ameliorate glomerulonephritis

Previously, it was shown that anti-CD3 specific antibody treatment ameliorates crescent formation and tubular damage during glomerulonephritis, when administrated early after disease induction [7]. As described above, we administered anti-CD3 specific antibody treatment later in order to assess the effect on already differentiated TH17 cells. Regardless, we aimed to test, whether late onset treatment with anti-CD3 specific antibody treatment, would also improve kidney disease. As mentioned above, anti-CD3 specific antibody

treatment did increase IL-10 production in all analyzed T-cell subsets. However, no differences in disease phenotype could be observed between treated and untreated mice.

Of note, the effect of CD3-specific antibody treatment has been extensively characterized before. CD3-specific antibody treatment induces a rapid activation induced cell death, which is also accompanied by a cytokine storm [166]. $Foxp3^+$ regulatory T cells and TH17 cells are also being activated. However, these cells are more resistant to activation induced cell death [166-168]. Furthermore, TGF-β1 and IL-6, which are produced by phagocytes upon phagocytosis of the dead cells, further promote the expansion of the $Foxp3^+$ Treg and TH17 cells [70, 71, 169-171]. Finally, TH17 cells, which are CCR6 positive, are recruited via IL-17A induced CCL20 production by intestinal epithelial cells to the small intestine. Interestingly, $Foxp3^+$ and $Foxp3^-$ IL-10 producing T cells are likewise recruited to the small intestine, and thereby limit intestinal inflammation and tissue damage [61, 104]. Accordingly, in the small intestine, the treatment induces only a transient inflammation [172]. In line with these data I found that increased frequencies of IL-10 producing CD4 positive T cells and increased frequencies of TH17 cells in the kidney upon CD3-specific antibody treatment. Of note there was no difference in disease severity between treated and untreated animals, thereby suggesting that the expansion of the regulatory T-cells pool was able to balance the expansion of the TH17 cell pool, but was not able to completely control them. Of note, a protective effect of anti-CD3-specific antibody treatment was observed when mice were treated day 6 and 8 after glomerulonephritis induction [7]. However, in our experiments, mice were treated at days 8 and 10 after disease induction. Therefore, one could hypothesize that the protective effect of the anti-CD3 specific antibody was observed, only when it was administered in an early phase of disease. To be precise, treatment with the anti-CD3 specific antibody only protected the mice when administered before the majority of the T cells infiltrate into the kidneys [7]. In the current study, the antibody was administered after the first effector T cells infiltrated the kidneys. Thus, the kidney injury could potentially occur before anti-CD3 specific antibody was even injected.

In line with the above-mentioned data, previous publications have described SFB induced TH17 cells to be tissue resident and not to contribute to inflammation [68]. In contrast, pathogen induced TH17 cells were highly plastic. In the gut, the latter actually exhibited high plasticity to IFN-ɣ producing TH17 cells and were metabolically more active [68]. Other studies have also described that TH17 cells with high metabolic activity were capable of conversion to TH1 [173]. In our experiments, only around 30 % of the exTH17 cells actually produced IL-10 after anti-CD3 specific antibody treatment. Due to the use of the acute reporter, there was no necessity to re-activate the cells *in vitro* in order to stain for cytokine expression. Nonetheless, our mice did not include a reporter gene for IFN-ɣ. Therefore, IFN-ɣ production in TH17 cells and their plasticity towards TH1 cells could not be assessed. It might be possible that during glomerulonephritis, a fraction of exTH17 cells actually converted to TH1 cells. Those cells might have additionally outranked the possible protective effect of the anti-CD3 specific antibody and thus no protection could be detected.

In conclusion, anti-CD3 specific antibody treatment promoted the conversion of TH17 cells into TR1^{exTH17}. However, it did not ameliorate disease severity, when administered at later stages.

4.5 TH17 and TR1^{exTH17} cell derived IL-10 does not play an essential role during glomerulonephritis

As shown before, in the kidneys, effector TH17 cells have the capacity to upregulate IL-10 [7]. Furthermore, I found that TH17 cells can even convert into TR1^{exTH17} cells. Of note, it was shown previously that IL-10 production by Foxp3^{+} Treg plays an essential and protective role in glomerulonephritis [92, 144, 150, 163]. In conclusion, IL-10 not only drives but also supports their suppressive function. On the basis of these data, we wanted to test the role of IL-10 produced by TH17 and exTH17 cells. This would potentially also allow us then to conclude the functional relevancy of TR1^{exTH17} cells as discussed above. To this end, we used transgenic IL-17A^{CRE} IL-10$^{flox/flox}$ Rosa26^{YFP} mice. Indeed, I could show that IL-10 is deleted from all YFP expressing CD4 positive T cells. However, during

glomerulonephritis, wild type and knock out mice did not exhibit any differences, neither on cellular nor on disease level. Both IFN-ɣ and IL-17A expression was comparable between wild type and knock out mice. One caveat of this experiment is that some IL-17A positive cells did not express YFP. Something that was also found before in experiments by other groups [6, 119]. Indeed, it is known that in order to activate the IL-17A^{CRE} recombinase, a strong signal above a certain IL-17A threshold is necessary [119]. Consequently, when the cells did not reach this threshold, no activation of the IL-17A^{CRE} recombinase took place. Thus, those TH17 cells were still able to produce IL-10.

Deletion of Foxp3 Treg derived IL-10 aggravated crescent formation during glomerulonephritis and was associated with an increased TH1 and TH17 driven immune response [92]. Of note, Foxp3^{+} Treg would still be able to produce IL-10 in 17A^{CRE} IL-10$^{flox/flox}$ Rosa26^{YFP} mice. Additionally, in the majority of TR1 cells, which did not emerge from TH17 cells, IL-10 expression is not deleted in 17A^{CRE} IL-10$^{flox/flox}$ Rosa26^{YFP} mice. Also, it has to be mentioned that only very few cells were found to produce IL-17A after glomerulonephritis. Consequently, the general number of IL-17A positive cells that were silenced in their IL-10 expression was very low. Thus, the remaining IL-10 producing TH17 cells and TR1^{exTH17} cells as well as the IL-10 produced by Foxp3^{+} Treg and TR1, which did not derive from TH17 cells, might have been enough to compensate for the loss of IL-10 production by the cells with high IL-17A expression. Furthermore, it should be mentioned that TR1 cells can produce several other inhibitory factors besides IL-10 [6, 8]. Thus, a final proof about the suppressive potential of TR1^{exTH17} cells *ex-vivo* is missing. The function of these cells, could be tested by a similar approach to that used by Gagliani *et al.*; he assessed the suppressive activity of *in vivo* induced TR1^{exTH17}, which had been isolated from the small intestine using an *in vivo* transfer colitis model [6]. Similar experiments would be necessary to identify whether the cells found in the kidneys are, in principle, able to perform a suppressive function.

Taken together and considering the efficiency of the IL-17A^{CRE} IL-10$^{flox/flox}$ Rosa26^{YFP}mice, deletion of IL-10 in high IL-17A expressing CD4 positive T cells

did not significantly impact clinical parameters and mouse survival in glomerulonephritis. Thus, suggesting that IL-10 produced by TH17 cells and exTH17 cells is dispensable for the outcome of glomerulonephritis.

4.6 IL-10 receptor signaling in TH17 cells during glomerulonephritis is dispensable

As mentioned above the production of IL-10 by TH17 and exTH17 cells seems to be redundant during glomerulonephritis. However, many other cells including TR1 and Foxp3$^+$ Treg cell can produce IL-10. Furthermore, as for TR1 and Foxp3$^+$ Treg cells it is known that IL-10 signaling in themselves plays a key role for their suppressive function [62, 85]. Consequently, we wondered what effect the deletion of the IL-10 receptor on TH17 cells would have during glomerulonephritis.

To address this question, I used *Il17a*^{Cre} *Il10Rα*$^{flox\ flox}$ mice. Our data show that specific deletion of IL-10 receptor-alpha in TH17 cells did not aggravate the outcome of glomerulonephritis. Between wild type and knock out mice, no differences between the cellular phenotype were detected. Both IL-17A and IFN-ɣ effector cytokine levels were unaffected when TH17 cells could not respond to IL-10. Also, the clinical parameters were comparable between wild type and knock out mice.

Previous experiments using a mouse model with T-cell specific impaired IL-10 signaling performed in inflammatory gut models identified higher expression levels of IL-17A and IFN-ɣ when regulatory cells or effector cells could not respond to IL-10 [61, 85]. This upregulation was a consequence of both impaired suppressive function of the regulatory T cells, and increased proliferation of effector TH17 cells. Furthermore, also in infantile onset IBD, a deficiency in the IL-10 receptor in all hematopoietic cells is detrimental and results in a strong TH17 polarization [174]. This dependency is also proven in regulatory cells in other organs. Thus, also in the kidneys, Foxp3$^+$ Treg cells require functional IL-10 receptor signaling in order to suppress TH17 cells [62].

Of note in the above-mentioned experiments by Huber *et al*. a transgenic mouse model with impaired IL-10 signaling specifically in all CD4 positive and CD8+ T cells was used. In this model it was shown, that TH17 cells in the gut need to respond to IL-10 in order to be suppressed by $Foxp3^{+}$ and $Foxp3^{-}$ regulatory T cells [61]. In contrast I used *Il17a*^{Cre} *Il10Rα*$^{flox\ flox}$ in which IL-10 signaling is completely and selectively used in all cells with high IL-17A expression. Furthermore, I assessed the role of IL-17 signaling in glomerulonephritis, while previous experiments assessed the role in colitis. Thus, differences in the transgenic mouse model and organs might account for the different outcome. However further experiments will be critical to test these hypotheses.

One additional caveat of my experiments is that, the total number of IL-17A producing cells was very low in performed glomerulonephritis models. Even after stimulation for three hours with PMA Ionomycin, which should result in strong cytokine re-activation, an average of less than 10 % produced IL-17A [164]. From this, it can be assumed that under *in vivo* conditions, even less than displayed cells actually produced IL-17A. On the one hand, this stays in correlation with the low numbers of TH17 cells that were affected by the CRE recombinase. But again, we faced the problem about generally low numbers of effector cells producing IL-17A during glomerulonephritis. Furthermore, because of this lack in TH17 cells, the overall kidney injury was very low. Even if the knock out mice would have slightly aggravated kidney injury, the chances to detect this were assumedly low.

Lastly, but referring to the same context of low IL-17A expression, the next point has already been described for the IL-17A^{CRE} IL-10$^{flox/flox}$ Rosa26^{YFP}mouse. The CRE recombinase needs strong activation by high IL-17A expression in order to silence the IL-10 receptor [119].

In conclusion, my data indicate that IL-10 signaling in TH17 cells does not play an essential role in glomerulonephritis. Further experiments will be essential to understand, whether this is specific for kidney inflammation.

To summarize the first part of my thesis, I found that TH17 cells can convert into $TR1^{exTH17}$ cells during glomerulonephritis. However, IL-10 production by and IL-10 signaling in TH17 and exTH17 did not seem to play a pivotal role for the outcome of glomerulonephritis. However, I also found that $Foxp3^-$ IL-10 producing CD4 positive T cells emerge in the kidneys of nephritic mice. Of note, it has been previously shown that $Foxp3^-$ IL-10 producing CD4 positive T cells in the intestine are a heterogeneous population on a molecular and functional level, and we thus aimed to study these cells in more detail in glomerulonephritis in the second part of this thesis.

4.7 Molecular heterogeneity of $Foxp3^-$ IL-10 producing CD4 positive T cells in glomerulonephritis

In the last few years, the definition of TR1 cells has been refined. Originally, $Foxp3^-$ CD4 positive T cells, producing IL-10 and having immunosuppressive function were named TR1 cells [141]. More recently, the identification of CD49b and LAG3 as surface marker of TR1 cells has helped to further define these cells [97]. Thereafter, more co-inhibitory and chemokine receptors such as PD-1, TIM-3, TIGIT or CCR5 have been shown to be particularly expressed by TR1 cells and to help to separate those from $Foxp3^-$ IL-10 producing CD4 positive T cells, which do not have a regulatory function [103, 175, 176]. Various biomarker combinations have been accepted up until now [175, 177]. However, we need to stress the point, that none of the identified marker combinations is perfect and the definitive criterion to identify TR1 cells is still to test their suppressive function. Nevertheless, I aimed in a first step to measure the expression level of the above-mentioned surface markers in $Foxp3^-$ IL-10 producing CD4 positive T cells. Indeed, I found that some cells showed the expression of select markers. However, only a minority of $Foxp3^-$ IL-10 producing CD4 positive T cells co-expressed CD49b and LAG3, which have previously been shown to help to enrich

suppressive TR1 cells within Foxp3$^-$ IL-10 producing CD4 positive T cells [8]. Even fewer cells expressed 3 or more of the above-mentioned markers.
Anti-CD3 specific antibody treatment has been identified to be a good model to induce high frequencies of TR1 cells including the ones displaying a suppressive profile [8, 61]. Interestingly, when nephritic mice were additionally treated with anti-CD3 specific antibody, the frequencies of Foxp3$^-$ IL-10 producing CD4 positive T cells increased. However, only a small fraction also acquired a regulatory expression profile, meaning that they expressed at least CD49b and LAG3. Comparing these data to the ones obtained in previous publications analyzing the intestine of anti-CD3 specific antibody treated mice, the frequency in the kidneys was around 4-5 times lower.

4.7.1 Heterogeneity of Foxp3$^-$ IL-10 producing CD4 positive cells in the kidneys

To characterize Foxp3$^-$ IL-10 producing CD4 positive T cells in an unbiased way, we performed single cell sequencing. Published data show that comparisons of IL-10 producing CD4 positive T cells in different organs resulted in varying percentages of cells within the suppressive cluster, which is based on the transcriptional signature profile of bona fide TR1 cells [8]. Indeed, in the model of CD3-specific antibody treatment 40 % IL-10 producing CD4 positive T cells of the small intestine were in this suppressive cluster, while this was only the case for 8 % of the IL-10 producing CD4 positive T cells from the spleen. These data resulted from RNA sequencing data in which total IL-10 producing CD4 positive T cells from the different organs were analyzed on single cell level. On the basis of these data we analyzed Foxp3$^-$ IL-10 producing CD4 positive T cells which had been isolated from the kidney of mice with glomerulonephritis. The clustering of the total cells displayed a heterogeneous cell population, with different clusters. While some clusters revealed regulatory like gene expression pattern (30-40 %), others displayed gene expression levels associated with effector cells.
Interestingly, cluster 3, which expressed high levels of IL-4 represented 13 % of total Foxp3$^-$ IL-10 producing CD4 positive T cells found in the kidneys. This is interesting, since in humans TR1 cells have been described to express high

levels of IL-10, low levels of IL-2, and no IL-4 [106]. Interestingly, IL-4 has been described to support the survival and immunosuppressive function of Foxp3$^+$ Treg cells [178]. Nonetheless, analyzed cells were sorted for negative Foxp3 expression. Whether IL-4 might have a protective effect on TR1 cells is not known. Nonetheless IL-4 is described to abolish TH17 cells by IL-23 silencing, and by this, is able to indirectly disturb the maintenance of TH17 cells [179]. Maybe this IL-10, IL-4 co-producing subpopulation has regulatory functions in terms of TH17 cell elimination. Lastly, there were observations in rats in which IL-4 ameliorated crescentic glomerulonephritis [180]. However, further experiments will be essential to test this hypothesis.

Furthermore, we analyzed the whole transcriptome of Foxp3$^-$ IL-10 producing CD4 positive T cells, which had been isolated from the kidneys. Interestingly, we found that some cells expressed *Tbet* and *Gata3*. However, we sorted all Foxp3$^-$ IL-10 producing CD4 positive T cells, and we did not deplete IFN-ɣ or IL-4 producing CD4 positive T cells. Therefore, it is not surprising, that some cells express transcriptions factors like Tbet or Gata3, which are known to be important for TH1 or TH2 generation [35, 46]. Nonetheless, those results are not in conflict to what is already published. IL-10 can be produced by many CD4 positive T-cells subsets [43, 73, 124]. Furthermore, numerous T-cell subsets infiltrate the kidney during glomerulonephritis [3, 24, 181]. TR1 cells have been proposed to also originate from different progenitor cells [6, 38]. Beside TH17 cells, TH1 cells have been proposed to be potential progenitor cells of TR1 cells. Nevertheless, a final proof that TR1 cells originate from TH1 cells is missing. However, in previous publications, Tbet expression was shown to play an important role for early TR1 differentiation [101].

4.7.2 Discrepancy between the regulatory fraction based on RNA expression versus expression of extra cellular markers

On RNA level, the population of Foxp3$^-$ IL-10 producing CD4 positive T cells, isolated from the kidneys displayed to be a heterogeneous cell population. In the small intestine and the spleen, similar observations about a strong heterogeneity

were observed [8]. Nonetheless, the gene expression on RNA level from the small intestine and the spleen did reflect the actual expression of markers such as LAG3, TIM3 or TIGIT on protein levels. However, in the kidney only 8 % of Foxp3$^-$ IL-10 producing CD4 T cells expressed CD49b, LAG3, TIM3 and TIGIT by protein. Surprisingly, 30-40 % of kidney-derived Foxp3$^-$ IL-10 producing CD4 T cells kidney-derived cells had a medium to high expression profile of the transcriptomic signature of a TR1 cell when analyzed by RNA single cell sequencing. One reason for this discrepant finding in the expression levels of CD49b, LAG3, TIM3 and TIGIT is, that one analysis describes RNA and the other one protein. We use the RNA single cell sequencing approach because we assume that RNA expression mirrors what the cell should be able to express on protein. That this is not always the case is shown with the here presented data. Nonetheless, the flow cytometry staining to which the expression on protein level refers to did not include markers such as PD1, CCR5 or CTLA-4 which are described to drive suppressive function of TR1 cells. We previously found that the use of CD49b and LAG3 gives the best trade of between sensitivity and specificity to identify TR1 cells in the intestine, spleen and liver. However, this marker combination might not be the best to identify these cells in the kidney. Further experiments assessing the expression of several other putative TR1 markers will be critical to answer this point.

4.7.3 Heterogeneity of one T-cell subset between different organs

Furthermore, when single clusters of IL-10 producing CD4 positive T cells were compared between different tissues, this revealed a variable heterogeneity within one but also among the compared organs. The heterogeneity of effector and regulatory T cells depends on many factors, such as the surrounding environment and the stimulus that induces the cell [68]. Thus, it is actually not surprising to see such variations in the profile of IL-10 producing CD4 positive cells when analyzed in such different organs. Since each organ is exposed to a different environment and some interact closer than others, the infiltrated organs can strongly shape the T-cell repertoire. Whereas the kidneys, under healthy conditions, represent an organ with higher sterility [182-184], the gut is influenced

by a constant flush of microbiota [185]. Here, T cells are constantly shaped by food components, commensals and pathogens. For the generation and function of T cells, those parameters can directly modulate cytokine expression and thereby shape T-cell abundance [186]. For example, TH17 cells are present in the lamina propria and even under physiological conditions, they are induced by natural occurring SFBs. Nonetheless, TH17 cells induced under physiological conditions are functionally distinct from those induced by pathogens. Some of those cells have the capacity to migrate and contribute to inflammation whereas others don't [68].
Also, in the kidneys, numbers of infiltrating TH17 cells were dependent on the abundance of gut microbiota [3]. The appearance of gut TR1 cells has also been described to be shaped by the gut microbiota [187]. This might also be the case for the abundance and expression patterns of regulatory TR1 cells in the kidneys. Without commensals, the immune system is primed insufficiently. Its dependency was shown using germfree mice, in which the mice failed to generate a normal T-cell repertoire. Furthermore, the effects can also be described vice versa, as T cells can shape the microbiome [114]. Thus, considering the kidney as a highly sterile organ, a lower expressed T-cell repertoire on protein level compared to the gut is not very surprising.

With regards to our analysis, we hypothesize a transcriptional network in which many transcription factors interact and collectively generate TR1 cells. In this network, every factor acts at a different time point during TR1-cell differentiation [188]. Comparable to a hierarchical system, we hypothesize a scheme in which some factors will be identified as being responsible for the first activation, whereas others will support TR1-cell generation from different progenitor cells. Supported by our findings, we assume that specific transcription factors might drive the gene expression of IL-10, whereas others are responsible for the expression of CD49b and LAG3. Furthermore, another group of transcription factors might be able to support the actual capacity of the cell to finally transcribe and translate the RNA into a protein. Lastly, activation of certain transcription factors will support maintenance of suppressive function. With the help of single

cell sequencing and trajectory analysis, several approaches can be taken to decipher the development from naïve CD4 positive T cells into functional suppressive TR1 cells. So far, single-cell-sequencing approaches enable us among other things, to get an estimation about differences in gene expression between single cells of one or more cell populations. Differences resulting from this, need be tested *in vitro* and *in vivo*. Here, this approach enabled us to decipher a heterogeneous cell population in the kidneys of nephritic mice. Nonetheless, these classifications were made according to known transcription factors, cytokines or chemokine receptor expression described in the gut or spleen.

In the same context, many transcription factors such as c-Maf, Blimp-1, Eomes, or LXR have been discussed as being responsible for the production of IL-10 or the expression of CD49b and LAG3, PD1 and others that define a suppressive TR1 profile [103, 189, 190]. Nonetheless, until now, scientists have not been able to identify a hierarchy within the transcription factor which is exclusively responsible for the generation of functional TR1 cells. Various transcription factors have been suggested to drive IL-10 production and marker expression [103, 190]. Some of those were expressed on analyzed cells from the kidneys. Nonetheless, it is not known whether, always the same transcription factors are responsible for the expression of a certain TR1 profile, independent from the organ.

In conclusion, a relatively big fraction of Foxp3$^-$ IL-10 producing CD4 positive T cells from the kidney showed the transcriptional signature of a TR1 cell, but most of them did not co-express CD49b, LAG3, TIM3 and TIGIT by protein. To clarify this contradictive finding, we tested the function of these cells *in vitro*.

4.8 Suppressive capacity of TR1 cells *in vitro*

On the basis of the above-mentioned molecular data, we next aimed to test, whether Foxp3$^-$ IL-10 producing CD4 positive T cells would fulfil the ultimate criterion defining TR1 cells, which is regulatory activity. Indeed, TR1 cells have

the potential to efficiently suppress effector T cells. In order to address this question, we performed *in vitro*-suppression assays with Foxp3$^-$ IL-10 producing CD4 positive T cells from the kidneys of nephritic mice. However, the induction of glomerulonephritis did not induce high numbers of Foxp3$^-$ IL-10 producing CD4 positive T cells in the kidneys. Thus, the anti-CD3 specific antibody was applied to promote the emergence of IL-10 producing T cells. Our results show that during glomerulonephritis generated Foxp3$^-$ IL-10 producing CD4 positive T cells suppress CD4 T-cell proliferation *in vitro*. Thus, these cells can be referred to as TR1 cells.

Regarding the 8 % Foxp3$^-$ IL-10 producing CD4 positive T cells in the kidney that produced CD49b and LAG3 TIM3 and TIGIT after induction of glomerulonephritis, these cells might still be highly suppressive compared to the rest of IL-10 positive cells that are negative for the expression of CD49b and LAG3. Potentially these suppressive cells found in the kidney express the same marker combination as known for the gut because they originally generated in the gut and then migrated into the kidneys as some effector TH17 cells do [3]. In order to test this hypothesis, the origin of highly suppressive Foxp3$^-$ IL-10 positive CD49b, LAG3 positive cells, following approaches could be performed in the future.
Firstly, KAEDE mice could be used. In those mice, every cell expresses a green fluorescent protein on its surface [191]. By exposure to blue light, the green fluorescent color is photoconverted to red. Notably, only specific areas can be converted. To investigate the above-mentioned hypothesis about the gut as the origin of IL-10 positive CD49b and LAG3 positive TR1 cells, firstly, the small intestine could be photo-converted. A few days later, the abundance of Kaede red and Kaede green cells expressing the CD49b and LAG3 could then be analyzed. In a successful experiment, a cell separation could be made by Kaede green and Kaede red cells. This approach might reveal that the cells, which confirm the regulatory profile originated in the small intestine.

Another approach to test the origin of the few Foxp3$^-$ IL-10 producing cells expressing CD49b and LAG3 in the kidneys would be the application of a

chemical that blocks cell migration between organs through the bloodstream. With Fingomolid 2-Amino-2-(2-(4-octylphenyl)ethyl)propan-1,3-diol (FTY720) cells are restrained in the lymph nodes and cell trafficking is restricted [192]. Thus, if regulatory cells could still be found in the kidneys, it might indicate that the cells originated directly in the kidney.

Regardless of the origin of TR1 cells found in the kidneys of mice treated with NTN serum only, we can conclude that even when the expression of CD49b and LAG3 is low on those Foxp3$^-$ IL-10 producing CD4 positive T cells, these cells are almost as suppressive as Treg cells from the same mice *in vitro*.
After first proof of *in vitro* suppression, we wanted to go further and determine the suppressive capacity of TR1 cells, in an *in vivo* TH17-cell transfer model for glomerulonephritis [3].

4.9 TR1 cells are able to suppress TH17-cell mediated glomerulonephritis

As mentioned above, we could demonstrate the suppressive function of kidney derived TR1 cells *in vitro.* However, due to technical limitations we were not able to assess their function *in vivo*. Therefore, in order to test, whether TR1 cells in general would be able to suppress TH17-cell mediated glomerulonephritis, we performed an *in vivo* transfer experiment of TH17 cells alone or together with TR1 cells into *Rag1*$^{-/-}$ prior to induction of glomerulonephritis. To this end, regulatory T cells had to be first generated *in vitro*.
After several experiments, in which a 1:1 ratio between TH17 cells and TR1 was used, had failed, *in vivo* ratios after glomerulonephritis were closely examined (Figure 3). In these experiments, frequencies of TR1 cells displayed to be five times higher than those of TH17 cells. This observation led to adjustments of cell numbers for *in vivo* cell transfer experiments. Finally, successful *in vivo* suppression by TR1 cells could be observed, when used at a ratio of 5x 10^5 TR1 + 1x 10^5 TH17 cells. Our data showed for the first time, that *in vitro* induced TR1 cells were capable of suppressing TH17-cell mediated kidney inflammation. The positive control using Foxp3$^+$ Tregs exhibit even stronger suppression.

Interestingly, only a quarter of Foxp3^{+} Treg cells was necessary to result in a suppression of TH17 cell mediated kidney disease. Nonetheless, the observed data are still preliminary, as they are based on a limited number of mice. Thus, further experiments should be performed.

In conclusion, our preliminary data indicate that *in vitro* differentiated TR1 cells are capable of suppressing TH17 cell mediated glomerulonephritis. Of note, TR1 cells were already used to support organ acceptance after kidney transplantation [33]. Nonetheless, to our knowledge TR1 cells are not used during T-cell based therapy to treat patients suffering from RPGN. However, our data, if reproducible, could be the basis to establish a TR1 based T-cell therapy for RPGN, similar to what is already done in IBD patients [108, 193].

The second part of this discussion covered the topic about the molecular and functional heterogeneity of Foxp3^{-} IL-10 producing CD4 positive T cells found in the kidneys and possible reasons for the observed findings. Foxp3^{-} IL-10 producing CD4 positive T cells emerge spontaneously during glomerulonephritis. However, despite their appearance the mice develop glomerulonephritis. It is unclear, whether the mice would be even sicker in the absence of these cells. Indeed, our experiments suggest that Foxp3^{-} IL-10 producing CD4 positive T cells from the kidney are suppressive and might contribute to suppression of disease. However, given that Foxp3^{-} IL-10 producing CD4 positive T cells in the kidney are a heterogeneous population, further analysis of different subsets will be critical to finally answer this point. This is particularly important, since we noticed that treatment with anti-CD3 specific antibody does not only expand Foxp3^{-} IL-10 producing CD4 positive T cells, but also impacts the composition of these cells, possibly expanding the regulatory cell fraction. However, it is unclear whether anti-CD3 specific antibody simply activated Foxp3^{-} IL-10 producing CD4 positive T cells in the kidney, which might have been resting. Alternatively, the Foxp3^{-} IL-10 producing CD4 positive T-cell pool could have been expanded due to recruitment of these cells to the kidney or the conversion of other cells, such as TH1 cells. Further experiments will be critical to clarify these points.

5. Appendix

5.1 References

1. Berliner, R.W., et al., *Dilution and concentration of the urine and the action of antidiuretic hormone.* Am J Med, 1958. **24**(5): p. 730-44.
2. Bland, J.H., *Role of the kidneys in regulation of fluid and electrolyte balance.* Am J Clin Pathol, 1953. **23**(11): p. 1070-81.
3. Krebs, C.F., et al., *Autoimmune Renal Disease Is Exacerbated by S1P-Receptor-1-Dependent Intestinal Th17 Cell Migration to the Kidney.* Immunity, 2016. **45**(5): p. 1078-1092.
4. Tipping, P.G. and S.R. Holdsworth, *T cells in crescentic glomerulonephritis.* J Am Soc Nephrol, 2006. **17**(5): p. 1253-63.
5. Paust, H.J., et al., *Chemokines play a critical role in the cross-regulation of Th1 and Th17 immune responses in murine crescentic glomerulonephritis.* Kidney Int, 2012. **82**(1): p. 72-83.
6. Gagliani, N., et al., *Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation.* Nature, 2015. **523**(7559): p. 221-5.
7. Krebs, C.F., et al., *Plasticity of Th17 Cells in Autoimmune Kidney Diseases.* J Immunol, 2016. **197**(2): p. 449-57.
8. Brockmann, L., et al., *Molecular and functional heterogeneity of IL-10-producing CD4(+) T cells.* Nat Commun, 2018. **9**(1): p. 5457.
9. Glocker, E.O., et al., *Infant colitis--it's in the genes.* Lancet, 2010. **376**(9748): p. 1272.
10. Xue, C. and C.L. Mei, *Polycystic Kidney Disease and Renal Fibrosis.* Adv Exp Med Biol, 2019. **1165**: p. 81-100.
11. Engelhardt, K.R. and B. Grimbacher, *IL-10 in humans: lessons from the gut, IL-10/IL-10 receptor deficiencies, and IL-10 polymorphisms.* Curr Top Microbiol Immunol, 2014. **380**: p. 1-18.
12. Zhu, L., et al., *IL-10 and IL-10 Receptor Mutations in Very Early Onset Inflammatory Bowel Disease.* Gastroenterology Res, 2017. **10**(2): p. 65-69.
13. Begue, B., et al., *Defective IL10 signaling defining a subgroup of patients with inflammatory bowel disease.* Am J Gastroenterol, 2011. **106**(8): p. 1544-55.
14. Kalinkovich, A. and G. Livshits, *A cross talk between dysbiosis and gut-associated immune system governs the development of inflammatory arthropathies.* Semin Arthritis Rheum, 2019. **49**(3): p. 474-484.
15. Brodin, P. and M.M. Davis, *Human immune system variation.* Nat Rev Immunol, 2017. **17**(1): p. 21-29.
16. Harrison, O.J., et al., *Commensal-specific T cell plasticity promotes rapid tissue adaptation to injury.* Science, 2019. **363**(6422).
17. Foussat, A., et al., *A comparative study between T regulatory type 1 and CD4+CD25+ T cells in the control of inflammation.* J Immunol, 2003. **171**(10): p. 5018-26.

18. Lin, L. and J. Zhang, *Role of intestinal microbiota and metabolites on gut homeostasis and human diseases.* BMC Immunol, 2017. **18**(1): p. 2.
19. Kriz, W. and H. Koepsell, *The structural organization of the mouse kidney.* Z Anat Entwicklungsgesch, 1974. **144**(2): p. 137-63.
20. Wirz, H., B. Hargitay, and W. Kuhn, *[Localization of the concentration process in the kidney by direct kryoscopy].* Helv Physiol Pharmacol Acta, 1951. **9**(2): p. 196-207.
21. Disease, G.B.D., I. Injury, and C. Prevalence, *Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016.* Lancet, 2017. **390**(10100): p. 1211-1259.
22. Couser, W.G., *Basic and translational concepts of immune-mediated glomerular diseases.* J Am Soc Nephrol, 2012. **23**(3): p. 381-99.
23. Janssen, U., et al., *Improved survival and amelioration of nephrotoxic nephritis in intercellular adhesion molecule-1 knockout mice.* J Am Soc Nephrol, 1998. **9**(10): p. 1805-14.
24. Summers, S.A., et al., *Th1 and Th17 cells induce proliferative glomerulonephritis.* J Am Soc Nephrol, 2009. **20**(12): p. 2518-24.
25. Phoon, R.K., et al., *T-bet deficiency attenuates renal injury in experimental crescentic glomerulonephritis.* J Am Soc Nephrol, 2008. **19**(3): p. 477-85.
26. Steinmetz, O.M., et al., *The Th17-defining transcription factor RORgammat promotes glomerulonephritis.* J Am Soc Nephrol, 2011. **22**(3): p. 472-83.
27. Kitching, A.R., S.R. Holdsworth, and P.G. Tipping, *IFN-gamma mediates crescent formation and cell-mediated immune injury in murine glomerulonephritis.* J Am Soc Nephrol, 1999. **10**(4): p. 752-9.
28. Mukai, H., et al., *Lung Dysfunction and Mortality in Patients with Chronic Kidney Disease.* Kidney Blood Press Res, 2018. **43**(2): p. 522-535.
29. Basu, R.K. and D.S. Wheeler, *Kidney-lung cross-talk and acute kidney injury.* Pediatr Nephrol, 2013. **28**(12): p. 2239-48.
30. Lee, S.A., et al., *Distant Organ Dysfunction in Acute Kidney Injury: A Review.* Am J Kidney Dis, 2018. **72**(6): p. 846-856.
31. Yates, M., et al., *EULAR/ERA-EDTA recommendations for the management of ANCA-associated vasculitis.* Ann Rheum Dis, 2016. **75**(9): p. 1583-94.
32. Ma, T.T., et al., *Late restoration of renal function in patients with severe ANCA-associated glomerulonephritis who were dialysis-dependent at presentation.* Clin Rheumatol, 2018. **37**(8): p. 2143-2150.
33. Petrelli, A., et al., *Generation of Donor-specific T Regulatory Type 1 Cells From Patients on Dialysis for Cell Therapy After Kidney Transplantation.* Transplantation, 2015. **99**(8): p. 1582-9.
34. Mosmann, T.R., et al., *Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins.* J Immunol, 1986. **136**(7): p. 2348-57.
35. Szabo, S.J., et al., *A novel transcription factor, T-bet, directs Th1 lineage commitment.* Cell, 2000. **100**(6): p. 655-69.

36. O'Shea, J.J. and W.E. Paul, *Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells.* Science, 2010. **327**(5969): p. 1098-102.
37. Kitching, A.R., et al., *IL-12p40 and IL-18 in crescentic glomerulonephritis: IL-12p40 is the key Th1-defining cytokine chain, whereas IL-18 promotes local inflammation and leukocyte recruitment.* J Am Soc Nephrol, 2005. **16**(7): p. 2023-33.
38. Neumann, C., et al., *Role of Blimp-1 in programing Th effector cells into IL-10 producers.* J Exp Med, 2014. **211**(9): p. 1807-19.
39. Thieu, V.T., et al., *Signal transducer and activator of transcription 4 is required for the transcription factor T-bet to promote T helper 1 cell-fate determination.* Immunity, 2008. **29**(5): p. 679-90.
40. Afkarian, M., et al., *T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells.* Nat Immunol, 2002. **3**(6): p. 549-57.
41. Nakanishi, K., et al., *Interleukin-18 regulates both Th1 and Th2 responses.* Annu Rev Immunol, 2001. **19**: p. 423-74.
42. Romagnani, S., *Th1/Th2 cells.* Inflamm Bowel Dis, 1999. **5**(4): p. 285-94.
43. Mosmann, T.R. and K.W. Moore, *The role of IL-10 in crossregulation of TH1 and TH2 responses.* Immunol Today, 1991. **12**(3): p. A49-53.
44. Le Gros, G., et al., *Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells.* J Exp Med, 1990. **172**(3): p. 921-9.
45. Kaplan, M.H., et al., *Stat6 is required for mediating responses to IL-4 and for development of Th2 cells.* Immunity, 1996. **4**(3): p. 313-9.
46. Zheng, W. and R.A. Flavell, *The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells.* Cell, 1997. **89**(4): p. 587-96.
47. Ozawa, H., et al., *Immune responses to Nippostrongylus brasiliensis and tuberculin protein in GATA-3-transgenic mice.* Immunol Lett, 2005. **99**(2): p. 228-35.
48. Martinez-Moczygemba, M. and D.P. Huston, *Biology of common beta receptor-signaling cytokines: IL-3, IL-5, and GM-CSF.* J Allergy Clin Immunol, 2003. **112**(4): p. 653-65; quiz 666.
49. Woodruff, P.G., et al., *T-helper type 2-driven inflammation defines major subphenotypes of asthma.* Am J Respir Crit Care Med, 2009. **180**(5): p. 388-95.
50. Malik, S. and A. Awasthi, *Transcriptional Control of Th9 Cells: Role of Foxo1 in Interleukin-9 Induction.* Front Immunol, 2018. **9**: p. 995.
51. Fard, N.A., G. Azizi, and A. Mirshafiey, *The Potential Role of T Helper Cell 22 and IL-22 in Immunopathogenesis of Multiple Sclerosis.* Innov Clin Neurosci, 2016. **13**(7-8): p. 30-6.
52. Aujla, S.J., et al., *IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia.* Nat Med, 2008. **14**(3): p. 275-81.
53. Sonnenberg, G.F., et al., *CD4(+) lymphoid tissue-inducer cells promote innate immunity in the gut.* Immunity, 2011. **34**(1): p. 122-34.
54. Harrington, L.E., et al., *Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages.* Nat Immunol, 2005. **6**(11): p. 1123-32.

55. Ivanov, II, et al., *The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells.* Cell, 2006. **126**(6): p. 1121-33.
56. Krummey, S.M., et al., *Candida-elicited murine Th17 cells express high Ctla-4 compared with Th1 cells and are resistant to costimulation blockade.* J Immunol, 2014. **192**(5): p. 2495-504.
57. Langrish, C.L., et al., *IL-23 drives a pathogenic T cell population that induces autoimmune inflammation.* J Exp Med, 2005. **201**(2): p. 233-40.
58. Park, H., et al., *A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17.* Nat Immunol, 2005. **6**(11): p. 1133-41.
59. Lowes, M.A., et al., *Psoriasis vulgaris lesions contain discrete populations of Th1 and Th17 T cells.* J Invest Dermatol, 2008. **128**(5): p. 1207-11.
60. Esplugues, E., et al., *Control of TH17 cells occurs in the small intestine.* Nature, 2011. **475**(7357): p. 514-8.
61. Huber, S., et al., *Th17 cells express interleukin-10 receptor and are controlled by Foxp3(-) and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner.* Immunity, 2011. **34**(4): p. 554-65.
62. Diefenhardt, P., et al., *IL-10 Receptor Signaling Empowers Regulatory T Cells to Control Th17 Responses and Protect from GN.* J Am Soc Nephrol, 2018. **29**(7): p. 1825-1837.
63. Ivanov, II, et al., *Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine.* Cell Host Microbe, 2008. **4**(4): p. 337-49.
64. Backhed, F., et al., *Host-bacterial mutualism in the human intestine.* Science, 2005. **307**(5717): p. 1915-20.
65. Gomes, T.A., et al., *Diarrheagenic Escherichia coli.* Braz J Microbiol, 2016. **47 Suppl 1**: p. 3-30.
66. Ivanov, II, et al., *Induction of intestinal Th17 cells by segmented filamentous bacteria.* Cell, 2009. **139**(3): p. 485-98.
67. Patel, D.D. and V.K. Kuchroo, *Th17 Cell Pathway in Human Immunity: Lessons from Genetics and Therapeutic Interventions.* Immunity, 2015. **43**(6): p. 1040-51.
68. Omenetti, S., et al., *The Intestine Harbors Functionally Distinct Homeostatic Tissue-Resident and Inflammatory Th17 Cells.* Immunity, 2019. **51**(1): p. 77-89 e6.
69. Nakamoto, N., et al., *Gut pathobionts underlie intestinal barrier dysfunction and liver T helper 17 cell immune response in primary sclerosing cholangitis.* Nat Microbiol, 2019. **4**(3): p. 492-503.
70. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**(7090): p. 235-8.
71. Veldhoen, M., et al., *TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells.* Immunity, 2006. **24**(2): p. 179-89.
72. Manel, N., D. Unutmaz, and D.R. Littman, *The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat.* Nat Immunol, 2008. **9**(6): p. 641-9.

73. McGeachy, M.J., et al., *TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology.* Nat Immunol, 2007. **8**(12): p. 1390-7.
74. Xu, J., et al., *c-Maf regulates IL-10 expression during Th17 polarization.* J Immunol, 2009. **182**(10): p. 6226-36.
75. Sun, M., et al., *RORgammat Represses IL-10 Production in Th17 Cells To Maintain Their Pathogenicity in Inducing Intestinal Inflammation.* J Immunol, 2019. **202**(1): p. 79-92.
76. Lee, J.Y., et al., *Serum Amyloid A Proteins Induce Pathogenic Th17 Cells and Promote Inflammatory Disease.* Cell, 2020. **180**(1): p. 79-91 e16.
77. Chung, Y., et al., *Critical regulation of early Th17 cell differentiation by interleukin-1 signaling.* Immunity, 2009. **30**(4): p. 576-87.
78. Lee, J.S., et al., *Interleukin-23-Independent IL-17 Production Regulates Intestinal Epithelial Permeability.* Immunity, 2015. **43**(4): p. 727-38.
79. Korn, T., et al., *IL-17 and Th17 Cells.* Annu Rev Immunol, 2009. **27**: p. 485-517.
80. Liang, S.C., et al., *Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides.* J Exp Med, 2006. **203**(10): p. 2271-9.
81. Mangan, P.R., et al., *Transforming growth factor-beta induces development of the T(H)17 lineage.* Nature, 2006. **441**(7090): p. 231-4.
82. Lee, Y.K., et al., *Late developmental plasticity in the T helper 17 lineage.* Immunity, 2009. **30**(1): p. 92-107.
83. Hirota, K., et al., *Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model.* J Exp Med, 2007. **204**(12): p. 2803-12.
84. Sun, M., et al., *Microbiota-derived short-chain fatty acids promote Th1 cell IL-10 production to maintain intestinal homeostasis.* Nat Commun, 2018. **9**(1): p. 3555.
85. Brockmann, L., et al., *IL-10 Receptor Signaling Is Essential for TR1 Cell Function In Vivo.* J Immunol, 2017. **198**(3): p. 1130-1141.
86. Fontenot, J.D., et al., *Regulatory T cell lineage specification by the forkhead transcription factor foxp3.* Immunity, 2005. **22**(3): p. 329-41.
87. Chinen, T., et al., *An essential role for the IL-2 receptor in Treg cell function.* Nat Immunol, 2016. **17**(11): p. 1322-1333.
88. Zorn, E., et al., *IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo.* Blood, 2006. **108**(5): p. 1571-9.
89. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3.* J Exp Med, 2003. **198**(12): p. 1875-86.
90. Bennett, C.L., et al., *The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3.* Nat Genet, 2001. **27**(1): p. 20-1.
91. Kluger, M.A., et al., *Stat3 programs Th17-specific regulatory T cells to control GN.* J Am Soc Nephrol, 2014. **25**(6): p. 1291-302.
92. Ostmann, A., et al., *Regulatory T cell-derived IL-10 ameliorates crescentic GN.* J Am Soc Nephrol, 2013. **24**(6): p. 930-42.

93. Berglund, D., et al., *Obtaining regulatory T cells from uraemic patients awaiting kidney transplantation for use in clinical trials.* Clin Exp Immunol, 2013. **173**(2): p. 310-22.
94. Savage, T.M., et al., *Early expansion of donor-specific Tregs in tolerant kidney transplant recipients.* JCI Insight, 2018. **3**(22).
95. Kitching, A.R., et al., *Endogenous interleukin-10 regulates Th1 responses that induce crescentic glomerulonephritis.* Kidney Int, 2000. **57**(2): p. 518-25.
96. Roncarolo, M.G., et al., *Tr1 cells and the counter-regulation of immunity: natural mechanisms and therapeutic applications.* Curr Top Microbiol Immunol, 2014. **380**: p. 39-68.
97. Gagliani, N., et al., *Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells.* Nat Med, 2013. **19**(6): p. 739-46.
98. Apetoh, L., et al., *The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27.* Nat Immunol, 2010. **11**(9): p. 854-61.
99. Maynard, C.L., et al., *Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10.* Nat Immunol, 2007. **8**(9): p. 931-41.
100. Gregori, S., et al., *Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway.* Blood, 2010. **116**(6): p. 935-44.
101. Zhang, P., et al., *Eomesodermin promotes the development of type 1 regulatory T (TR1) cells.* Sci Immunol, 2017. **2**(10).
102. Okamura, T., et al., *CD4+CD25-LAG3+ regulatory T cells controlled by the transcription factor Egr-2.* Proc Natl Acad Sci U S A, 2009. **106**(33): p. 13974-9.
103. Chihara, N., et al., *Induction and transcriptional regulation of the co-inhibitory gene module in T cells.* Nature, 2018. **558**(7710): p. 454-459.
104. Kamanaka, M., et al., *Expression of interleukin-10 in intestinal lymphocytes detected by an interleukin-10 reporter knockin tiger mouse.* Immunity, 2006. **25**(6): p. 941-52.
105. Perruche, S., et al., *CD3-specific antibody-induced immune tolerance involves transforming growth factor-beta from phagocytes digesting apoptotic T cells.* Nat Med, 2008. **14**(5): p. 528-35.
106. Groux, H., et al., *A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis.* Nature, 1997. **389**(6652): p. 737-42.
107. Yao, Y., et al., *Tr1 Cells, but Not Foxp3+ Regulatory T Cells, Suppress NLRP3 Inflammasome Activation via an IL-10-Dependent Mechanism.* J Immunol, 2015. **195**(2): p. 488-97.
108. Desreumaux, P., et al., *Safety and efficacy of antigen-specific regulatory T-cell therapy for patients with refractory Crohn's disease.* Gastroenterology, 2012. **143**(5): p. 1207-1217 e2.
109. Mfarrej, B., et al., *Generation of donor-specific Tr1 cells to be used after kidney transplantation and definition of the timing of their in vivo infusion in the presence of immunosuppression.* J Transl Med, 2017. **15**(1): p. 40.
110. Soranno, D.E., et al., *Delivery of interleukin-10 via injectable hydrogels improves renal outcomes and reduces systemic inflammation following*

ischemic acute kidney injury in mice. Am J Physiol Renal Physiol, 2016. **311**(2): p. F362-72.
111. Scheinin, T., et al., *Validation of the interleukin-10 knockout mouse model of colitis: antitumour necrosis factor-antibodies suppress the progression of colitis.* Clin Exp Immunol, 2003. **133**(1): p. 38-43.
112. Huang, W., et al., *Beyond Type 1 Regulatory T Cells: Co-expression of LAG3 and CD49b in IL-10-Producing T Cell Lineages.* Front Immunol, 2018. **9**: p. 2625.
113. Hastings, W.D., et al., *TIM-3 is expressed on activated human CD4+ T cells and regulates Th1 and Th17 cytokines.* Eur J Immunol, 2009. **39**(9): p. 2492-501.
114. Neumann, K., et al., *The co-inhibitory molecule PD-L1 contributes to regulatory T cell-mediated protection in murine crescentic glomerulonephritis.* Sci Rep, 2019. **9**(1): p. 2038.
115. Wang, Y., et al., *The transcription factors T-bet and Runx are required for the ontogeny of pathogenic interferon-gamma-producing T helper 17 cells.* Immunity, 2014. **40**(3): p. 355-66.
116. Akamatsu, M., et al., *Conversion of antigen-specific effector/memory T cells into Foxp3-expressing Treg cells by inhibition of CDK8/19.* Sci Immunol, 2019. **4**(40).
117. Nistala, K., et al., *Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment.* Proc Natl Acad Sci U S A, 2010. **107**(33): p. 14751-6.
118. Miragaia, R.J., et al., *Single-Cell Transcriptomics of Regulatory T Cells Reveals Trajectories of Tissue Adaptation.* Immunity, 2019. **50**(2): p. 493-504 e7.
119. Hirota, K., et al., *Fate mapping of IL-17-producing T cells in inflammatory responses.* Nat Immunol, 2011. **12**(3): p. 255-63.
120. Komatsu, N., et al., *Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis.* Nat Med, 2014. **20**(1): p. 62-8.
121. Chang, K.K., et al., *IL-27 triggers IL-10 production in Th17 cells via a c-Maf/RORgammat/Blimp-1 signal to promote the progression of endometriosis.* Cell Death Dis, 2017. **8**(3): p. e2666.
122. Penaloza, H.F., et al., *Opposing roles of IL-10 in acute bacterial infection.* Cytokine Growth Factor Rev, 2016. **32**: p. 17-30.
123. Rojas, J.M., et al., *IL-10: A Multifunctional Cytokine in Viral Infections.* J Immunol Res, 2017. **2017**: p. 6104054.
124. Coomes, S.M., et al., *CD4(+) Th2 cells are directly regulated by IL-10 during allergic airway inflammation.* Mucosal Immunol, 2017. **10**(1): p. 150-161.
125. Wang, Z.Y., et al., *Regulation of IL-10 gene expression in Th2 cells by Jun proteins.* J Immunol, 2005. **174**(4): p. 2098-105.
126. Mosmann, T.R., et al., *Isolation of monoclonal antibodies specific for IL-4, IL-5, IL-6, and a new Th2-specific cytokine (IL-10), cytokine synthesis inhibitory factor, by using a solid phase radioimmunoadsorbent assay.* J Immunol, 1990. **145**(9): p. 2938-45.
127. Kuhn, R., et al., *Interleukin-10-deficient mice develop chronic enterocolitis.* Cell, 1993. **75**(2): p. 263-74.

128. Madsen, K.L., et al., *Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice.* Gastroenterology, 1999. **116**(5): p. 1107-14.
129. Rubtsov, Y.P., et al., *Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces.* Immunity, 2008. **28**(4): p. 546-58.
130. Glocker, E.O., et al., *Inflammatory bowel disease and mutations affecting the interleukin-10 receptor.* N Engl J Med, 2009. **361**(21): p. 2033-45.
131. Nunberg, M.Y., et al., *Impaired IL-10 Receptor-mediated Suppression in Monocyte From Patients With Crohn Disease.* J Pediatr Gastroenterol Nutr, 2018. **66**(5): p. 779-784.
132. Weber-Nordt, R.M., et al., *Stat3 recruitment by two distinct ligand-induced, tyrosine-phosphorylated docking sites in the interleukin-10 receptor intracellular domain.* J Biol Chem, 1996. **271**(44): p. 27954-61.
133. O'Farrell, A.M., et al., *IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: evidence for Stat3-dependent and -independent pathways.* EMBO J, 1998. **17**(4): p. 1006-18.
134. Kamanaka, M., et al., *Memory/effector (CD45RB(lo)) CD4 T cells are controlled directly by IL-10 and cause IL-22-dependent intestinal pathology.* J Exp Med, 2011. **208**(5): p. 1027-40.
135. Chaudhry, A., et al., *Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation.* Immunity, 2011. **34**(4): p. 566-78.
136. Fiorentino, D.F., et al., *IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells.* J Immunol, 1991. **146**(10): p. 3444-51.
137. El-Gabalawy, H., L.C. Guenther, and C.N. Bernstein, *Epidemiology of immune-mediated inflammatory diseases: incidence, prevalence, natural history, and comorbidities.* J Rheumatol Suppl, 2010. **85**: p. 2-10.
138. Mehta, R.L., et al., *International Society of Nephrology's 0by25 initiative for acute kidney injury (zero preventable deaths by 2025): a human rights case for nephrology.* Lancet, 2015. **385**(9987): p. 2616-43.
139. Hsu, R.K. and N.R. Powe, *Recent trends in the prevalence of chronic kidney disease: not the same old song.* Curr Opin Nephrol Hypertens, 2017. **26**(3): p. 187-196.
140. Zoccali, C., A. Kramer, and K.J. Jager, *Chronic kidney disease and end-stage renal disease-a review produced to contribute to the report 'the status of health in the European union: towards a healthier Europe'.* NDT Plus, 2010. **3**(3): p. 213-224.
141. Roncarolo, M.G., et al., *Interleukin-10-secreting type 1 regulatory T cells in rodents and humans.* Immunol Rev, 2006. **212**: p. 28-50.
142. Paust, H.J., et al., *CXCR3+ Regulatory T Cells Control TH1 Responses in Crescentic GN.* J Am Soc Nephrol, 2016. **27**(7): p. 1933-42.
143. Yang, C., et al., *The Regulatory T-cell Transcription Factor Foxp3 Protects against Crescentic Glomerulonephritis.* Sci Rep, 2017. **7**(1): p. 1481.
144. Paust, H.J., et al., *Regulatory T cells control the Th1 immune response in murine crescentic glomerulonephritis.* Kidney Int, 2011. **80**(2): p. 154-64.
145. Alikhan, M.A., et al., *Regulatory T cells in renal disease.* Clin Transl Immunology, 2018. **7**(1): p. e1004.

146. Koutrolos, M., et al., *Treg cells mediate recovery from EAE by controlling effector T cell proliferation and motility in the CNS.* Acta Neuropathol Commun, 2014. **2**: p. 163.
147. Symons, A., A.L. Budelsky, and J.E. Towne, *Are Th17 cells in the gut pathogenic or protective?* Mucosal Immunol, 2012. **5**(1): p. 4-6.
148. Chatenoud, L., *CD3-specific antibody-induced active tolerance: from bench to bedside.* Nat Rev Immunol, 2003. **3**(2): p. 123-32.
149. Nicolls, M.R., et al., *Induction of long-term specific tolerance to allografts in rats by therapy with an anti-CD3-like monoclonal antibody.* Transplantation, 1993. **55**(3): p. 459-68.
150. Kluger, M.A., et al., *RORgammat expression in Tregs promotes systemic lupus erythematosus via IL-17 secretion, alteration of Treg phenotype and suppression of Th2 responses.* Clin Exp Immunol, 2017. **188**(1): p. 63-78.
151. Collison, L.W. and D.A. Vignali, *In vitro Treg suppression assays.* Methods Mol Biol, 2011. **707**: p. 21-37.
152. Lyons, A.B., S.J. Blake, and K.V. Doherty, *Flow cytometric analysis of cell division by dilution of CFSE and related dyes.* Curr Protoc Cytom, 2013. **Chapter 9**: p. Unit9 11.
153. Okin, D. and R. Medzhitov, *Evolution of inflammatory diseases.* Curr Biol, 2012. **22**(17): p. R733-40.
154. Gavin, M.A., et al., *Foxp3-dependent programme of regulatory T-cell differentiation.* Nature, 2007. **445**(7129): p. 771-5.
155. Rossi, M. and A. Bot, *The Th17 cell population and the immune homeostasis of the gastrointestinal tract.* Int Rev Immunol, 2013. **32**(5-6): p. 471-4.
156. Korn, T., et al., *IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells.* Proc Natl Acad Sci U S A, 2008. **105**(47): p. 18460-5.
157. Cosmi, L., et al., *Th17 plasticity: pathophysiology and treatment of chronic inflammatory disorders.* Curr Opin Pharmacol, 2014. **17**: p. 12-6.
158. Xu, L., et al., *Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta.* J Immunol, 2007. **178**(11): p. 6725-9.
159. Pot, C., et al., *Induction of regulatory Tr1 cells and inhibition of T(H)17 cells by IL-27.* Semin Immunol, 2011. **23**(6): p. 438-45.
160. Yanaba, K., et al., *A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses.* Immunity, 2008. **28**(5): p. 639-50.
161. Moore, K.W., et al., *Interleukin-10 and the interleukin-10 receptor.* Annu Rev Immunol, 2001. **19**: p. 683-765.
162. Kessler, B., et al., *Interleukin 10 inhibits pro-inflammatory cytokine responses and killing of Burkholderia pseudomallei.* Sci Rep, 2017. **7**: p. 42791.
163. Kluger, M.A., et al., *RORgammat(+)Foxp3(+) Cells are an Independent Bifunctional Regulatory T Cell Lineage and Mediate Crescentic GN.* J Am Soc Nephrol, 2016. **27**(2): p. 454-65.
164. Foster, B., et al., *Detection of intracellular cytokines by flow cytometry.* Curr Protoc Immunol, 2007. **Chapter 6**: p. Unit 6 24.

165. Hirota, K., et al., *Plasticity of Th17 cells in Peyer's patches is responsible for the induction of T cell-dependent IgA responses.* Nat Immunol, 2013. **14**(4): p. 372-9.
166. Penaranda, C., Q. Tang, and J.A. Bluestone, *Anti-CD3 therapy promotes tolerance by selectively depleting pathogenic cells while preserving regulatory T cells.* J Immunol, 2011. **187**(4): p. 2015-22.
167. Shi, G., et al., *Unlike Th1, Th17 cells mediate sustained autoimmune inflammation and are highly resistant to restimulation-induced cell death.* J Immunol, 2009. **183**(11): p. 7547-56.
168. Yu, H., et al., *Intestinal type 1 regulatory T cells migrate to periphery to suppress diabetogenic T cells and prevent diabetes development.* Proc Natl Acad Sci U S A, 2017. **114**(39): p. 10443-10448.
169. Fadok, V.A., et al., *Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF.* J Clin Invest, 1998. **101**(4): p. 890-8.
170. Chung, E.Y., S.J. Kim, and X.J. Ma, *Regulation of cytokine production during phagocytosis of apoptotic cells.* Cell Res, 2006. **16**(2): p. 154-61.
171. Zheng, S.G., et al., *IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells.* J Immunol, 2007. **178**(4): p. 2018-27.
172. Clayburgh, D.R., et al., *Epithelial myosin light chain kinase-dependent barrier dysfunction mediates T cell activation-induced diarrhea in vivo.* J Clin Invest, 2005. **115**(10): p. 2702-15.
173. Karmaus, P.W.F., et al., *Metabolic heterogeneity underlies reciprocal fates of TH17 cell stemness and plasticity.* Nature, 2019. **565**(7737): p. 101-105.
174. Shouval, D.S., et al., *Enhanced TH17 Responses in Patients with IL10 Receptor Deficiency and Infantile-onset IBD.* Inflamm Bowel Dis, 2017. **23**(11): p. 1950-1961.
175. Alfen, J.S., et al., *Intestinal IFN-gamma-producing type 1 regulatory T cells coexpress CCR5 and programmed cell death protein 1 and downregulate IL-10 in the inflamed guts of patients with inflammatory bowel disease.* J Allergy Clin Immunol, 2018. **142**(5): p. 1537-1547 e8.
176. Joller, N., et al., *Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses.* Immunity, 2014. **40**(4): p. 569-81.
177. Anderson, A.C., N. Joller, and V.K. Kuchroo, *Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation.* Immunity, 2016. **44**(5): p. 989-1004.
178. Yang, W.C., et al., *Interleukin-4 Supports the Suppressive Immune Responses Elicited by Regulatory T Cells.* Front Immunol, 2017. **8**: p. 1508.
179. Guenova, E., et al., *IL-4 abrogates T(H)17 cell-mediated inflammation by selective silencing of IL-23 in antigen-presenting cells.* Proc Natl Acad Sci U S A, 2015. **112**(7): p. 2163-8.
180. Cook, H.T., et al., *Interleukin-4 ameliorates crescentic glomerulonephritis in Wistar Kyoto rats.* Kidney Int, 1999. **55**(4): p. 1319-26.

181. Liu, L., et al., *CD4+ T Lymphocytes, especially Th2 cells, contribute to the progress of renal fibrosis.* Am J Nephrol, 2012. **36**(4): p. 386-96.
182. Cao, Q., D.C. Harris, and Y. Wang, *Macrophages in kidney injury, inflammation, and fibrosis.* Physiology (Bethesda), 2015. **30**(3): p. 183-94.
183. Aoki, S., et al., *"Abacterial" and bacterial pyelonephritis. Immunofluorescent localization of bacterial antigen.* N Engl J Med, 1969. **281**(25): p. 1375-82.
184. Zasloff, M., *Antimicrobial peptides, innate immunity, and the normally sterile urinary tract.* J Am Soc Nephrol, 2007. **18**(11): p. 2810-6.
185. Rinninella, E., et al., *What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases.* Microorganisms, 2019. **7**(1).
186. Costes, L.M.M., et al., *IL-10 signaling prevents gluten-dependent intraepithelial CD4(+) cytotoxic T lymphocyte infiltration and epithelial damage in the small intestine.* Mucosal Immunol, 2019. **12**(2): p. 479-490.
187. Jeon, S.G., et al., *Probiotic Bifidobacterium breve induces IL-10-producing Tr1 cells in the colon.* PLoS Pathog, 2012. **8**(5): p. e1002714.
188. Bedke, T., et al., *Title: IL-10-producing T cells and their dual functions.* Semin Immunol, 2019. **44**: p. 101335.
189. Gruarin, P., et al., *Eomesodermin controls a unique differentiation program in human IL-10 and IFN-gamma coproducing regulatory T cells.* Eur J Immunol, 2019. **49**(1): p. 96-111.
190. Vigne, S., et al., *IL-27-Induced Type 1 Regulatory T-Cells Produce Oxysterols that Constrain IL-10 Production.* Front Immunol, 2017. **8**: p. 1184.
191. Tomura, M., et al., *Monitoring cellular movement in vivo with photoconvertible fluorescence protein "Kaede" transgenic mice.* Proc Natl Acad Sci U S A, 2008. **105**(31): p. 10871-6.
192. Pino, M., et al., *Fingolimod retains cytolytic T cells and limits T follicular helper cell infection in lymphoid sites of SIV persistence.* PLoS Pathog, 2019. **15**(10): p. e1008081.
193. Bacchetta, R., et al., *Immunological Outcome in Haploidentical-HSC Transplanted Patients Treated with IL-10-Anergized Donor T Cells.* Front Immunol, 2014. **5**: p. 16.

5.2 List of abbreviations

Ahr	Aryl hydrocarbon receptor
APC	Antigen presenting cell
Blimp-1	PR domain zinc finger protein 1
BSA	Bovine Serum Albumin
C	Cluster
c-maf	c-musculoaponeurotic fibrosarcoma
C°	Degree celsius
CCR	Chemokine receptor
CD49b	Integrin α 2
CNS	Central nervous system
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DNA	Deoxyribonucleic acid
dNTPs	Nucleoside triphosphate
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FIR	FOXP3-IRES-mRFP
Foxp3	Forhead box p3
G	Acceleration due to gravity
g	Gramm
GATA3	Trans-acting T-cell-specific transcription factor GATA-3
GFP	Green fluorescent protein
Gy	Gray
HRP	Horseradish peroxidase
IBD	Inflammatory Bowel Disease
IFN-ɣ	Interferon-gamma
IL	Interleukin
IMIDs	Immune mediated inflammatory diseases

l	Liter
LAG	Lymphocyte-activation gene
LXR	Liver X receptor
M	Molar
m	Milli
nm	Nanometer
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Programmed cell death
PMA	Phorbol-12-myristat-13-acetat
Rα	Receptor alpha
RFP	Red fluorescent protein
RNA	Ribonucleic acid
ROR-ɣt	retinoid acid receptor-related orphan receptor gamma t
SC	Single cell
SEM	Standard error of the mean
SFB	Segmented filamentous bacteria
STAT	Signal transducer and activator of transcription
T cell	Thymocytes cell
T-bet	T-box transcription factor
TCR	T-cell receptor
TGF-β	Transforming growth factor beta
TH	T helper
TIGIT	T-cell immunoglobulin and ITIM domain
TIM3	The transmembrane protein TIM-3
TR1	T regulatory Type 1
Treg	regulatory T cell
YFP	Yellow fluorescent protein
µ	Micro

5.3 List of tables

5.4 List of figures

5.5 Curriculum Vitae

Entfällt aus datenschutzrechtlichen Gründen

Eidesstattliche Erklärung

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

Statement under oath

I hereby declare upon oath that I have written the present dissertation independently and have not used further resources and aids than those stated.

Hamburg, April 2020

Confirmation of linguistic correctness

I hereby declare, that I have read the doctoral thesis from Shiwa Soukou titled “Analysis of TH17-cell plasticity into regulatory fates during crescentic glomerulonephritis in a mouse model” and I confirm its linguistic correctness in English.

Hamburg, April 2020