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Modulating Regulatory T Cells for Treatment of Glomerulonephritis and Acute Kidney Injury

Dissertation

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“Nothing remained but to see what he wanted to see.

Any fool can turn the blind eye, but who knows what the ostrich sees in the sand?”

Samuel Beckett, *Murphy*

TO MY PARENTS

IN ETERNAL LOVE AND GRATITUDE

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2 Introduction

2.1 The Healthy Kidney and Glomerulonephritis

Shape and name of many items of everyday use, such as kidney tables, kidney dishes or kidney beans, remind us of our kidneys and reflect the significance, which these bean-shaped, retroperitoneal organs hold for human and animal life (Radi, 2019). The kidneys play a central part in a variety of essential body functions and non-redundantly contribute to a general homeostatic environment through their excretory and incretionary effects. They are crucial for regulating the volume and electrolyte household as well as upholding osmolarity and acid-base homeostasis. Furthermore, they are central for the excretion of metabolites and toxins. Being equipped with endocrine functions, the kidneys are also relevantly participating in blood pressure control via the renin-angiotensin-system. Furthermore, they activate vitamin D3 for calcium and bone homeostasis and secret erythropoietin which is essential for erythropoiesis (Madrazo-Ibarra and Vaitla, 2020, Wang and Garrett, 2017, Dalal et al., 2020).

Structurally each kidney consists of about 1 million subunits called nephrons (Wang and Garrett, 2017). A nephron comprises the renal corpuscle – made up of the Bowman's capsule and the glomerulus – and a renal tubule which is attached to it (Kazi and Hashmi, 2020). Importantly, inflammation of the glomerulus – i.e. glomerulonephritis (GN) – has a strong effect on the overall kidney function and is one of the most common causes for end-stage renal disease in Europe and North America (Kazi and Hashmi, 2020, Stahl and Hoxha, 2016).

Glomerulonephritis refers to a highly heterogeneous group of immune-mediated diseases. The underlying etiologies are manifold and the course of disease ranges from subclinical cases to the rapid irreversible deterioration of kidney function even in young patients. Such a rapid loss of kidney function is seen in so called 'rapid progressive glomerulonephritis' (RPGN). RPGN constitutes the GN subgroup that overall exhibits the fastest disease progression and the worst prognosis. RPGN is common in ANCA-associated systemic vasculitis, Goodpasture-syndrome or autoimmune diseases like systemic lupus erythematosus (SLE) (Kazi and Hashmi, 2020, Stahl and Hoxha, 2016).

Due to incomplete understanding of the pathophysiology, GN is usually treated with broadly immunosuppressive agents. Thus, current therapeutic regimes are highly unspecific and entail serious side effects. This stresses the need for novel, more specific therapeutic approaches. Strikingly, recent research has demonstrated the key role of Th17 cells and regulatory T cells (Tregs) in in either the pathogenesis of, or the protection from GN, respectively. These cell types are thus promising targets for future therapies (Herrnstadt and Steinmetz, 2020, Krebs and Steinmetz, 2016, Bluestone and Tang, 2018).

2.2 Cisplatin-induced Acute Kidney Injury

Glomerulonephritis, however, is not the only entity that puts our renal health at risk. Many different causes can lead to various forms of kidney injury, of which a relevant proportion is – unfortunately – iatrogenic. Indeed, about 25% of all cases of acute kidney injury (AKI) that occur in the critically ill are attributed to nephrotoxic medication (Bentley et al., 2010). As AKI is associated with high mortality (Bentley et al., 2010), this poses a serious clinical problem.

Cisplatin is a highly effective chemotherapeutic drug. Its clinical use, however, is unfortunately severely limited by its nephrotoxic properties. As it has happened so often in science, the discovery of cisplatin as a potential cytostatic agent was purely triggered by accident. While conducting experiments on the effect of electric fields on E. coli growth in 1965, Rosenberg et al. realized, it was in fact not electricity that led to the inhibition of cell division, but rather the platinum electrodes they had used in their experimental setup (Rosenberg et al., 1965). It would then take about another 13 years until cisplatin had successfully passed clinical studies for testicular and ovarian cancers and reached FDA approval in 1978 (Kelland, 2007). After that cisplatin would soon rise to become one of the most frequently and consistently used chemotherapeutic agents to date. Today – even 40 years later – cisplatin still plays a critical role in therapeutic regimes for a variety of solid tumors. Those include small-, as well as, non-small cell lung cancer (Gatzemeier et al., 2000, Noda et al., 2002), head and neck cancer (Planting et al., 1999), ovarian cancer (Hoskins et al., 2000), testicular cancer (Hartmann et al., 1999) and esophageal cancer (Sasaki et al., 2016). Unfortunately, however, about 20-30% of patients treated with cisplatin develop AKI (Miller et al., 2010, Yao et al., 2007) and are unable to receive adequate cisplatin dosages.

Cisplatin-induced AKI has initially been attributed to effects of direct tubular toxicity. However, infiltration of innate and adaptive immune cells into renal tissue of mice with cisplatin-induced AKI has more recently been observed (Faubel et al., 2004, Lu et al., 2008, Liu et al., 2006), making it likely that immune-mediated effects contribute to renal damage as well. Indeed, T cells infiltrate early into kidneys of cisplatin treated mice (Liu et al., 2006) and T cell-deficiency has been demonstrated to be protective (Akcay et al., 2011, Alikhan et al., 2016, Liu et al., 2006). Furthermore, depletion of regulatory T cells enhanced kidney injury (Lee et al., 2010), whereas adoptive Treg transfer

(Alikhan et al., 2016, Lee et al., 2010) and Treg expansion via an IL-2/IL-33 hybrid cytokine (Stremska et al., 2017) protected from injury. This stresses the importance of T effector- and Treg-mediated effects in cisplatin-induced AKI. However, many aspects remain yet to be elucidated and a deeper and more differentiated understanding of the mechanisms at work is needed. Such insights could lead to novel, T cell-based preventive strategies for clinical cisplatin application and could thus contribute to a better treatment of malignancies.

2.3 Immune Homeostasis and T Cell Heterogenicity

Every second our body is confronted with potential pathogens. To cope with those and to persevere evolutionarily, we have been equipped with ways to defend ourselves. Innate immunity constitutes the first wall of resistance. Innate cells recognize pathogens via evolutionary preserved patterns and keep them contained during the initial stage of infection. Innate immunity, however, is often not sufficient to ultimately eliminate all pathogens. A more specific, more powerful entity is needed – adaptive immunity. Made up of B and T cells, adaptive immune responses can specifically recognize pathogens and highly efficiently orchestrate their clearance through cellular and humoral mechanisms (Yatim and Lakkis, 2015). Diseases with impaired B and T cells, like X-linked severe immunodeficiency (X-SCID) stress the importance of a functioning adaptive immune response. Children born with X-SCID show a general lack of T cells and have nonfunctional B cells, making them extremely susceptible to opportunistic infections (Justiz Vaillant and Qurie, 2019). Without treatment X-SCID patients die within the first 2 years of their life (Allenspach et al., 1993).

On the other hand, overshooting or misdirected immune responses bear the risk to inflict *self*-harm and induce autoimmune diseases (Horwitz et al., 2019). Sophisticated mechanisms are therefore essential for providing an immunological environment, that is able to balance pro- and anti-inflammatory effects.

Immunology research over the past decades has unveiled a complex system of heterogenic and distinctive T cell subpopulations. The two CD4+ T helper cell subclasses first described in 1986 by Timothy Mosmann and Robert Coffman have proven themselves to constitute only the tip of the iceberg (Mosmann et al., 1986). By now the scope of T cell subpopulations has drastically increased and new discoveries are continuously being made.

In addition to the initially discovered Th1 and Th2 cells (Mosmann et al., 1986), we now know of further functionally and phenotypically distinct subclasses, like Th17 cells, T follicular helper cells (Tfh), various regulatory T cell (Treg) populations and many more (Caza and Landas, 2015). The system's complexity is enhanced even further by concepts, suggesting a high degree of T cell plasticity (Caza and Landas, 2015, Bluestone et al., 2009, Huber et al., 2017, Krebs et al., 2016).

A short introduction to some relevant T cell subsets and their clinical significance will be provided in the following paragraphs.

2.3.1 Th1 Cells

Th1 cells are essential for the defense against intracellular pathogens, such as viruses or intracellular bacteria (Caza and Landas, 2015). Th1 immune responses have further been noted to play important pathogenic roles in autoimmunity and inflammatory diseases, including GN (Herrnstadt and Steinmetz, 2020, Summers et al., 2009). Th1 cell lineage commitment is achieved under the influence of IFNγ and IL-12, cytokines produced by natural killer (NK) and dendritic cells (Caza and Landas, 2015). Subsequent phosphorylation of the intracellular transcription factor (TF) STAT1 leads to the activation of the lineage-defining master TF Tbet (Caza and Landas, 2015, Szabo et al., 2000). Th1 cells mediate their effects mainly via the production and release of IFNγ, which is crucial for recruitment and activation of inflammatory M1 type macrophages. Furthermore, Th1 cells are able to facilitate antibody class-switching by B cells towards production of IgG2a and 3 (Caza and Landas, 2015).

2.3.2 Th2 Cells

Upon confrontation with parasites, dendritic cells produce IL-4, which leads to upregulation of the key transcription factor GATA3 in naïve T cells and induces the generation of Th2 cells. Via production of their signature cytokines IL-4, IL-5 and IL-13, Th2 cells orchestrate specific immune responses against parasitic pathogens. Induction of a class-switch by Th2 cells in B cells leads to the production of IgE antibodies. IgE specifically targets parasitic organisms and promotes pathogen clearance via mast cells and eosinophils. Th2 cells are further able to produce the anti-inflammatory cytokine IL-10, hereby inhibiting Th1 responses. Importantly, Th1 as well as Th2 cells each possess mechanisms to reciprocally inhibit one another, stressing the importance of context dependent immune homeostasis. Besides their role in the clearance of parasites, misdirected Th2 cells are strongly associated with allergic responses (Caza and Landas, 2015, Mosmann et al., 1986, Turner et al., 2010).

2.3.3 Follicular Helper T Cells

In order generate robust and prolonged antibody responses that lead to immunity against infectious agents, induction of memory B cells and plasma cells is needed. Selection and final B cell differentiation is promoted in the germinal center of lymphatic B cell follicles by follicular helper T cells (Tfh cells). Tfh cells mediate their effects on B cells in large parts through CD40 ligand (CD40L) and secretion of IL-21. Reciprocal interaction between Tfh and B cells is further characterized by antigen-presentation and expression of ICOS ligand by B cells. Tfh cells are defined by the transcription factor Bcl6, which induces expression of the B cell follicle homing receptor CXCR5 (Ballesteros-Tato et al., 2012, Ji et al., 2020).

2.3.4 Th17 Cells

Th17 cells play a crucial role in the clearance of extracellular bacteria and fungi (Caza and Landas, 2015, Korn et al., 2009). A cytokine mix of TGF-β, IL1β, IL-6 and IL-23 direct naïve CD4+ T cells to differentiate into Th17 cells by induction of STAT3 (Caza and Landas, 2015, Turner et al., 2010). STAT3 in turn induces the expression of the Th17-defining transcription factor RORγt as well as RORα (Yang et al., 2008b). The strong inflammatory properties of Th17 cells, mediated through cytokine production of IL-17A, IL-17F, IL-22 and TNF-α, have been shown to be directly dependent on expression of RORγt (Turner et al., 2010). A similar, but less profound role in the induction of a Th17-specific pro-inflammatory phenotype has been demonstrate for RORα (Ivanov et al., 2006, Jetten, 2009).

Th17 cells have been attributed a major role in a variety of inflammatory and autoimmune diseases. They are considered to play a key role in the development of psoriasis (Di Cesare et al., 2009, Nestle et al., 2009), inflammatory bowel disease (Leppkes et al., 2009), multiple sclerosis (Kaskow and Baecher-Allan, 2018) and rheumatoid arthritis (Hueber et al., 2010). A variety of successful therapeutic approaches targeting mediators of Th17 immunity emphasize their importance in autoimmunity as well as the potential translational opportunities associated (Papp et al., 2012, Sandborn et al., 2012, Hueber et al., 2010).

Importantly, recent research has established strong pathogenic effects of Th17 cells and their mediators in animal models of glomerular nephritis (Krebs et al., 2016, Paust

et al., 2009, Steinmetz et al., 2011, Summers et al., 2014, Turner et al., 2010, Herrnstadt and Steinmetz, 2020, Summers et al., 2009). Associative clinical evidence further stresses the therapeutic potential for the field of nephrology (Turner et al., 2010, Wang et al., 2010, Krebs et al., 2017).

2.3.5 Regulatory T Cells

In order to keep overshooting immune responses in check and to prevent autoimmunity, a way to dampen and modulate inflammation is needed. This feature is provided by regulatory T cells (Tregs). Tregs mediate their strong anti-inflammatory effects via various mechanisms including IL-10 and TGF-β (Lochner et al., 2008) secretion, as well as expression of a number of anti-inflammatory surface molecules. In addition, the release of Amphiregulin (AREG) by activated Tregs has recently been found to contribute to wound healing and tissue regeneration (Zaiss et al., 2019). Furthermore, Tregs can starve T effector cells by competitive IL-2 consumption and can alternative directly kill them by granzyme B and perforin secretion (Shevyrev and Tereshchenko, 2019). Their anti-inflammatory phenotype is crucially dependent on expression of the Treg-defining transcription factor Foxp3 (Fontenot et al., 2003).

The importance of Tregs in immune homeostasis is highlighted, when mutations occur that lead to defective or absent CD4+Foxp3+ Tregs. Patients suffering from X-linked auto-immunity-allergic dysregulation syndrome (XLAAD), a disease were Foxp3 transcription is impaired due to a mutation, present with sever autoimmunity and allergies (Patel, 2001). A similar hyper-lymphoproliferative autoimmune phenotype is seen in *scurfy* and Foxp3 deficient mice (Fontenot et al., 2003). Importantly, exogenous reconstitution with functionally intact regulatory T cells is able to prevent disease development (Fontenot et al., 2003).

Initially considered to be one homogenous cell population responsible for the overall immune homeostasis, recent research has challenged this *one size fits it all* paradigm. Interestingly, Tregs can be divided into specialized subtypes, each specifically adapted to the respective T effector cell type they are supposed to modulate. Specialized Tregs have been reported for Th1 and Th17 responses and were correspondingly named Treg1 and Treg17 cells by us (Kluger et al., 2014, Kluger et al., 2015, Nosko et al., 2017). T cell specific properties appear to be achieved via co-expression of the target

cells' respective master transcription factor – STAT3 in the case of Treg17 cells and T-bet for Treg1 cells (Krebs and Steinmetz, 2016, Nosko et al., 2017, Herrnstadt and Steinmetz, 2020).

With their ability to dampen overshooting immune responses, Tregs play an important role in the prevention from autoimmunity and inflammatory diseases. This principle does indeed apply to inflammatory kidney diseases as well. For instance, functionally impaired or dysbalanced Treg responses in patients have been associate with ANCA-associated vasculitis (Abdulahad et al., 2007, Free et al., 2013), IgA nephropathy (Yang et al., 2015) and SLE (von Spee-Mayer et al., 2016). Furthermore, depletion of Foxp3+ Tregs significantly aggravates disease severity in the model of nephrotoxic nephritis (NTN) (Paust et al., 2011) and the Treg-selective knockout of IL-10 limits the ability of Tregs to protect from kidney injury in the same model (Ostmann et al., 2013). More recently, the importance of subtype specific Tregs – i.e. Treg1 and Treg17 cells – has been recognized and a nuanced picture of their regulatory effects and mechanisms in glomerulonephritis is evolving (Herrnstadt and Steinmetz, 2020).

Due to their central role in the prevention of inflammation, Treg adoptive cell therapy and *in vivo* expansion have been considered promising therapeutic strategies and first translational efforts are already under way (Raffin et al., 2020). Importantly, adoptive Treg transfer has been demonstrated to be highly protective in experimental models of acute glomerulonephritis (Kluger et al., 2016, Wolf et al., 2005), underscoring the translational potential and viability of such strategies for glomerulonephritis.

2.3.6 RORγt+Foxp3+ biTregs

Our lab and others could recently identify a novel and unusual T cell subpopulation characterized by simultaneous activation of RORγt and Foxp3, the transcription factors responsible for the pro-inflammatory Th17 phenotype and anti-inflammatory Tregs, respectively (Lochner et al., 2008, Kluger et al., 2016). Corresponding to their activation of functionally rather opposing transcription factors, RORγt+Foxp3+ double positive T cells have been shown to express a variety of pro- as well as anti-inflammatory genes simultaneously. In particular, they show robust expression of Th17 characteristic IL-17, which is rather unusual for a Treg. Our lab has thus proposed the term *biTregs* as an operational name for this novel cell lineage to pay tribute to their

extraordinary expression profile and the corresponding bi-functional properties they possess.

Importantly, biTregs have been reported to be present in naïve mice (Yang et al., 2016, Ohnmacht et al., 2015) as well as in healthy humans (Ayyoub et al., 2009, Voo et al., 2009). This trans-species conversation indicates physiological significance (Kluger et al., 2016). Furthermore, several studies have linked biTregs to various diseases, again emphasizing their functional and potential therapeutic relevance. Associations have been made for diseases including inflammatory bowel disease (Hovhannisyan et al., 2011, Li and Boussiotis, 2013), juvenile idiopathic arthritis (Pesenacker et al., 2013), psoriasis (Bovenschen et al., 2011), colonic cancer (Blatner et al., 2012, Li and Boussiotis, 2013) and periodontitis (Okui et al., 2012).

In addition to these purely associative studies, recent research has dug deeper and shed some light on the functional role of biTregs in murine models of inflammatory diseases. Adoptively transferred biTregs were shown to be protective in a model of T cell transfer colitis (Yang et al., 2016). Strikingly, the protective effect of transferred biTregs could even exceeded that of conventional RORγt- Tregs (Yang et al., 2016). Protective effects have additionally been demonstrated in experimental autoimmune encephalitis (EAE) (Kim et al., 2017), a murine model of multiple sclerosis. With respect to GN, *Kluger et al.* from our group demonstrated, that adoptively transferred exogenous biTregs could potently ameliorate kidney damage in nephrotoxic nephritis (NTN) (Kluger et al., 2016) – a murine model mimicking acute crescentic GN. The protective effect of exogenous biTregs was reported to match that of adoptively transferred conventional RORγt negative Tregs (cTregs) (Kluger et al., 2016). The same study further established, that the pro-inflammatory effects of biTregs are in large parts dependent on activation of RORγt. NTN induction in $Foxp3^{Cre}xRORC^{fl/fl}$ mice, which exclusively lack RORγt in biTregs, led to significantly less histological and functional kidney damage as compared to control animals (Kluger et al., 2016), demonstrating a relevant degree of pathogenicity mediated by RORγt in biTregs. In line with abrogated RORγt expression, $Foxp3^{Cre}xRORC^{fl/fl}$ mice were completely devoid of IL-17 producing Tregs (Kluger et al., 2016).

In addition, a protective effect for a specific RORγt-deficiency in biTregs was seen in TNBS-induced colitis (Sefik et al., 2015a) as well as in pristane-induced pulmonary vasculitis and lupus nephritis (Kluger et al., 2017). Along with relevantly ameliorated

functional and structural damage in lungs and kidneys, Foxp3^{Cre}xRORC$^{fl/fl}$ mice presented with a significantly reduced influx of pro-inflammatory immune cells into target organs after pristane treatment (Kluger et al., 2017).

BiTregs have further been acknowledged to specifically modulate type 2 immune responses, potentially via expression of CTLA4 and IRF4 (Kluger et al., 2016, Kluger et al., 2017, Ohnmacht et al., 2015) and have therefore been attributed a role in the prevention of allergies (Ohnmacht et al., 2015, Krebs and Steinmetz, 2016).

Collectively, these data suggest a potent, yet janus-faced role for biTregs in immune-meditated diseases. While exhibiting high suppressive capacity and strong protective effects (Kluger et al., 2016, Yang et al., 2016), biTregs are also equipped with pro-inflammatory properties *in vivo* (Kluger et al., 2016, Kluger et al., 2017). Not surprisingly, this is generally in accordance with data gathered from *in vitro* experiments, where suppressive as well as inflammatory effects of biTregs have been noted (Ayyoub et al., 2009, Hovhannisyan et al., 2011, Lochner et al., 2008, Kluger et al., 2016, Pesenacker et al., 2013).

As biTregs share many key characteristics of both, Tregs and Th17 cells, the question naturally arises, whether they constitute a stable, independent cell lineage or whether they are perhaps only an intermediate stage of trans-differentiation. This question has certainly sparked some controversy in the scientific community. Some studies have indeed proposed biTregs to be an unstable cell population, that only arises as a transitory intermediate during Th17/Treg trans-differentiation (Bovenschen et al., 2011, Downs-Canner et al., 2017, Du et al., 2014). However, more recent research has disputed this concept and has made a strong scientific case for biTregs to constitute a phenotypically distinct and stable Treg population (Hagenstein et al., 2019, Kluger et al., 2016, Kluger et al., 2017, Krebs and Steinmetz, 2016, Ohnmacht et al., 2015, Sefik et al., 2015b, Yang et al., 2016).

Much has been learned about biTregs in the past years. However, there are many important aspects that remain yet to be fully elucidated. Future thorough research is especially needed as many newly established or proposed therapeutic strategies target the Th17-axis. Sharing so many key characteristics with Th17 cells, it is more than likely for biTregs to be incidentally affected. Hence, further insights into how

biTregs´ pro- vs. anti-inflammatory effects are mediated and in what context each one arises is of utter importance and could help to develop more specific therapies.

2.3.7 Treg-derived AREG

The highly pleiotropic cytokine AREG is a member of the epidermal growth factor (EGF)-like family and is expressed by a large number of different cell types (Zaiss et al., 2015, Berasain and Avila, 2014). Functionally, AREG has been identified as potent immune modulatory molecule, which mediates both, pro- as well as anti-inflammatory effects (Cook et al., 1997, Yamane et al., 2008, Zaiss et al., 2013, Melderis et al., 2020). Furthermore, an important role in fibrosis but conversely also in tissue regeneration and reparation has been described (Kefaloyianni et al., 2019, Monticelli et al., 2015, Zaiss et al., 2019). Strikingly, as mentioned above, particularly Treg-derived AREG contributes to tissue regeneration and wound healing (Zaiss et al., 2019, Arpaia et al., 2015) and thus constitutes a potential novel therapeutic target.

2.4 Interleukin-2: Pleiotropy, Clinical Utilization and Challenges

One way to target T cells is to interfere with their development, lineage commitment and stabilization of their phenotype. In this context, the interleukin IL-2 has been shown to be of great importance. The following section will thus provide an in-depth discussion of IL-2´s heterogeneous properties and effects. It will give insight into IL-2´s regulatory mechanisms and its multiple effects on T cells. Furthermore, it will outline the role of IL-2 in current and potential future clinical applications.

2.4.1 Pleiotropy and T cells

It has now been more than 40 years since the first description of IL-2 surfaced (Morgan et al., 1976). Over the course of time several seemingly conflicting observations have led away from the initial notion of IL-2 as simply a promoter of T effector cell development and thorough further research has now established a more complex regulatory framework (Ross and Cantrell, 2018, Spolski et al., 2018).

Interestingly, mutations in the IL-2 receptor gamma chain (IL-2Rγ) have been found to lead to X-linked severe combined immunodeficiency (XSCID), resulting in a lack of T cells in patients (Noguchi et al., 1993, Justiz Vaillant and Qurie, 2019). These findings are in line with the idea of IL-2 as a general positive inductor of T cell responses. Strikingly however, IL-$2^{-/-}$ mice as well as mice lacking the IL-2 receptor alpha chain (IL-2Rα, CD25) presented with enhanced T cell activity and an autoimmune phenotype (Ross and Cantrell, 2018, Sadlack et al., 1993). Similarly, several autoimmune diseases in patients have been associated with mutations affecting IL-2 pathways (Ross and Cantrell, 2018). So how can we make sense of these rather contradictory findings?

Differential expression of IL-2 receptor subunits by lymphocytes results in varying responses to IL-2 exposure depending on cell type, activation stage and microenvironmental context. This important phenomenon is termed cytokine pleiotropy and IL-2 serves as a perfect example.

2.4.1.1 Differential Receptor Expression: To Express or Not to Express

The IL-2 receptor possesses three distinct subunits: The IL-2 receptor alpha chain (IL-2Rα, CD25), beta chain (IL-2Rβ) and common gamma chain (IL-2Rγc) (Spolski et al., 2018, Wang et al., 2009). IL-2Rα constitutes a non-signaling unit that - on its own - binds IL-2 with only low affinity. IL-2Rβ and IL-2Rγc form a dimeric receptor with intermediate affinity and functional signaling capacity (Ross and Cantrell, 2018, Spolski et al., 2018). Signaling is initiated through heterodimerization of IL-2Rβ's and IL-2Rγc's cytoplasmatic domains and subsequent activation of Janus family kinase (JAK) 1 and 3 (Nakamura et al., 1994, Spolski et al., 2018). High affinity binding to IL-2 is, however, only achieved through co-expression of all three IL-2R subunits (Ross and Cantrell, 2018, Spolski et al., 2018). Importantly, cells expressing the intermediate affinity IL-2 receptor, i.e. IL2-Rβ and IL-2Rγc, can therefore further increase their sensitivity to IL-2 by additionally expressing IL-2Rα.

Naïve T effector cells do not express IL-2Rα until after T cell receptor (TCR) activation has taken place. With IL2-Rα co-expression affinity to IL-2 increases about 1,000-fold (Chinen et al., 2016), making the activated cell significantly more sensitive to IL-2. Interestingly, IL-2 itself induces expression of IL-2Rα, leading to formation of the high-affinity trimeric IL-2 receptor. Since activated T effector cells secret high amounts of IL-2, T cells further facilitate their activation through an IL-2 driven auto- and paracrine feedback loop (Ross and Cantrell, 2018, Spolski et al., 2018, Kim et al., 2006).

2.4.1.2 Th1, Th2, Th17 and Tfh: Differentiation and Activation

In addition to its role in boosting T effector activation and proliferation, IL-2 plays a key role in the differential development of T effector subsets. IL-2 is known to induce Th1 cell generation by enhancing IFNγ production and expression of IL-12Rβ2. Furthermore, expression of the Th1-defining transcription factor T-bet is induced by STAT5 phosphorylation downstream of IL-2/IL-2R signaling. IL-2 induced STAT5 signaling can also lead to expression of the IL-4 receptor, hereby promoting Th2 priming. In contrast to these effects, that promote type 1 and 2 immunity, IL-2 constitutes a strong inhibitor of Th17 differentiation. Th17 priming is blocked via STAT5 dependent repression of RORγt – the key transcription factor of Th17 cells – as well as repression of IL-6 receptor genes and competition to STAT3 for binding to IL-17a gene loci. The observation that Th17 cells lack IL-2 transcription is in line with this

notion and further emphasizes the inhibitory role of IL-2 in this context. Interestingly however, once priming towards a Th17 phenotype is concluded, IL-2 has been shown to be a driver of Th17 expansion (Kim et al., 2006, Ross and Cantrell, 2018, Spolski et al., 2018).

Follicular helper T cells (Tfh cells) are another cell line negatively regulated by IL-2. Here, IL-2 has been shown to inhibit the expression of Bcl6, the Tfh defining transcription factor. This is most likely mediated through STAT5 dependent induction of Blimp1, which is well known to repress Bcl6 (Ballesteros-Tato et al., 2012, Spolski et al., 2018). Influenza infected mice that were treated with recombinant IL-2 (rIL-2) showed impaired Tfh as well as B cell responses (Ballesteros-Tato et al., 2012), highlighting the functional importance of IL-2's inhibitory role on Tfh cells. Somewhat similar to the effect seen for Th17 cells, the negative impact on Tfh development is most pronounced in early stages of differentiation (Ballesteros-Tato et al., 2012).

2.4.1.3 Regulatory T Cells: Affinity is Key

Unlike naïve T effector cells that only express the trimeric high-affinity IL-2 receptor after TCR activation, regulatory T cells do so constitutively, hence generally making them extremely sensitive to IL-2 (Ross and Cantrell, 2018, Spolski et al., 2018). Importantly, this allows Tregs to be preferentially activated and expanded by IL-2 in comparison to naïve T effector cells (Shevach, 2012, Spolski et al., 2018, Trotta et al., 2018). This feature has been clinically utilized in the treatment of autoimmune diseases (He et al., 2016, Li et al., 2017, Miao et al., 2020, von Spee-Mayer et al., 2016) and will be discussed more thoroughly later on.

IL-2 induced STAT5 signaling plays a crucial role for the expression of Foxp3 and Treg function and differentiation (Chinen et al., 2016, Ross and Cantrell, 2018). Direct competition for IL-2 has been described as an important method for suppressing CD8+ T cells by Tregs, whereas CD4+ Teffs remain relatively unaffected by this mechanism (Chinen et al., 2016). Unlike effector T cells, Tregs do not secrete IL-2 themselves and are therefore rendered dependent on IL-2 production by other sources, as e.g. Teffs (Chinen et al., 2016). This has led to the hypothesis that IL-2 is able to control its own expression through reciprocal interaction between IL-2 production by Teffs and Treg mediated Teff suppression (Spolski et al., 2018).

2.4.1.4 CD8+T Cells, NK and NKT Cells

CD8+ T cells (cytotoxic T lymphocyte, CTL) recognize antigen presented by nucleated cells via the major histocompatibility complex class I (MHC I). If *non-self* antigen – esp. antigen from viruses or tumor cells – is detected, CTLs are able to destroy the presenting cell. IL-2 is a key driver of CTL expansion and differentiation. It equips CD8+ T cells with important effector functions by promoting expression of IFN-γ, TNF-α, perforin and others (Ross and Cantrell, 2018). At the same time, however, IL-2 also plays an important role in the induction of memory CD8+ T cells, as well as in T cell exhaustion (Spolski et al., 2018). Interestingly, CD8+ T cells with low expression of IL-2Rα, i.e. only intermediate affinity to IL-2, tend to become memory cells and those with stronger high-affinity receptor expression preferentially develop into effector cells (Spolski et al., 2018). Induction of effector properties in CD8+ T cells with high IL-2 signaling, is attributed to Blimp-1 dependent suppression of Bcl6, a crucial factor for memory function (Ross and Cantrell, 2018, Kalia et al., 2010). Correspondingly, low-dose IL-2 application has been shown to foster memory cell generation (Manjunath et al., 2001, Ross and Cantrell, 2018). It is important to note, that similar observations regarding memory cell induction have been made for CD4+ T cells (Ross and Cantrell, 2018).

Natural killer (NK) cells constitute another important line of defense against viral and other intracellular infections as well as tumor growth. NK cells recognize a lack of MHC I as *missing-self* and activate their cytotoxic functions. This way they can counteract immune evasion. NK cells are further able to recognize pathogenic proteins and allogenic MHC I (Abel et al., 2018). Since NK cells constitutively express the dimeric intermediate-affinity IL-2R they are sensitive to IL-2, which drives their proliferation and activates their functions. IL-2's effect is further fostered as NK cells start to express the IL-2Rα after activation (Abel et al., 2018, Meazza et al., 2011, Spolski et al., 2018).

Natural killer T (NKT) cells own their name to the fact, that they share many key cell surface molecules with NK cells. Unlike NK cells, however, they also express a T cell receptor (TCR) and are, hence, generally considered to resemble a T cell subpopulation (Pellicci et al., 2020). The TCR repertoire of NKT cells is highly restricted compared to conventional T cells and recognizes specific lipid antigens on Cd1d (La Cava et al., 2006, Pellicci et al., 2020). Upon activation NKT cells can release a variety of regulatory as well as inflammatory mediators, like IL-4, IL-10 or IL-13 and INFγ or

TNFα, respectively (Fujii, 2005). Importantly, NKT cells have been attributed a protective role in a variety of autoimmune diseases, including experimental models of crescentic GN and lupus nephritis (Riedel et al., 2012, Yang et al., 2003, Yang et al., 2008a). Interestingly, NKT cells have been shown to modulate Treg differentiation and function via the release of high amounts of IL-2 (La Cava et al., 2006).

Taken together, IL-2 constitutes an intensely pleiotropic cytokine, that mediates its divergent impacts on T cells, as in particular Th1, Th2, Th17 and Tfh cells as well as iTregs and CTLs through a variety of mechanisms. In addition, indirect effects on B cell development and antibody production have been proposed. The mechanisms through which pleiotropy is achieved include differential receptor affinity, context-dependent IL-2 secretion and distinct down-stream signaling cascades. Differential reactions to IL-2 do not only depend on the respective cell type but are further influenced by differentiation stage and the broader microenvironmental context.

Being a crucial regulator of so many different cell lines known to play pathogenic roles in a variety of diseases, IL-2 is indeed a promising therapeutic target. However, its pleiotropic and heterogenic effects pose serious challenges when it comes to its clinical utility. Comprehensive research and a deep understanding of IL-2's closely intertwined regulatory system is therefore vital to derive precisely tailored and highly specific therapeutic approaches.

2.4.2 Low-Dose IL-2: Current Translational Efforts

The divergent ways through which IL-2 regulates a large number of key players in immunology has been lined out above. The clinical importance of those cells - either by promoting or dampening inflammation – has been discussed as well. It is therefore no surprise that clinical research has aimed to utilize IL-2's effects for the treatment of auto-immune and inflammatory diseases. Especially the observation that regulatory T cells are extremely sensitive to IL-2 and can therefore be preferentially activated over naïve T effector cells (Shevach, 2012, Ross and Cantrell, 2018) has been exploited in clinical studies by applying low-dose IL-2.

First promising results could be seen in patients suffering from chronic graft-vs-host disease (GvHD) (Koreth et al., 2011). Immunological and clinical responses in 23

patients with glucocorticoid refractory chronic GvHD were evaluated after daily subcutaneous application of low-dose IL-2 for 8 weeks. Strikingly, a marked increase of Tregs was observed that remained robust over the time of treatment. Furthermore, in patients that were treated for an extended period a sustained robust increase in Treg frequencies could be noted after up to 52 weeks. Importantly, no alteration was seen for CD8+ T cell, NK cell, NKT cell or B cell responses and amelioration of GvHD as well as reduction of corticosteroids was achieved in a substantial proportion of patients (Koreth et al., 2011). Similar results have been reported for HCV-induced vasculitis (Saadoun et al., 2011), alopecia areata (Castela et al., 2014), type 1 diabetes (Rosenzwajg et al., 2015) and systemic lupus erythematosus (SLE) (Humrich et al., 2015, He et al., 2016, von Spee-Mayer et al., 2016, He et al., 2020).

Therapeutic low-dose IL-2 approaches in SLE have particularly been in the focus of clinical research as pre-clinical studies have suggested that IL-2 disbalance plays a relevant role in SLE pathogenesis (Wofsy et al., 1981, Alcocer-Varela and Alarcón-Segovia, 1982, Humrich et al., 2010, Lieberman and Tsokos, 2010, Mizui et al., 2014). Open-label observational studies in small numbers of patients have produced the first clinical evidence of the efficiency and safety of low-dose IL-2 therapy in SLE (Humrich et al., 2015, He et al., 2016, von Spee-Mayer et al., 2016). These studies consistently reported a pronounced increase of Treg frequencies (Humrich et al., 2015, He et al., 2016, von Spee-Mayer et al., 2016) as well as relevant disease amelioration (Humrich et al., 2015, He et al., 2016) after subcutaneous treatment with low-dose IL-2. Furthermore, a decrease in Th17 and Tfh cells was noted, whereas Th1 and Th2 responses remained unaltered (He et al., 2016). A recent randomized double-blinded placebo-controlled study has further underscored these findings in 60 SLE patients (He et al., 2020).

Even though low-dose IL-2 has shown some impressive effects in clinical trials, its clinical utility is hampered by IL-2's short half-life and the need for daily subcutaneous injection. Potential unspecific effects, due to IL-2's pleiotropic nature, and its context-dependent effects in different stages of activation constitute another obstacle for clinical application. Activated T effector cells, for instance, also start to express the trimeric high-affinity IL-2R (Ross and Cantrell, 2018, Spolski et al., 2018). Furthermore, priming of Th17 cells is known to be inhibited by IL-2. However, once fully differentiated they expand under IL-2 influence (Ross and Cantrell, 2018). Additive calcineurin

inhibitors, acting via the inhibition of IL-2 production by T effector cells, have shown strong effects in SLE therapy (Rovin et al., 2019). This strongly indicates a pathogenic role for IL-2 in the acute inflammatory stage of SLE and suggests an administration at the wrong stage of disease could turn out detrimental.

The development of IL-2 variants and IL-2-antibody complexes has aimed to address these issues. Strikingly, in addition to improved pharmacokinetics, some IL-2 variants and complexes were specifically engineered to selectively expand regulatory T cells (Shanafelt et al., 2000, Boyman et al., 2006, Mitra et al., 2015, Spangler et al., 2015, Peterson et al., 2018, Sockolosky et al., 2018, Spangler et al., 2018, Trotta et al., 2018, Kim et al., 2013, Polhill et al., 2012, Yan et al., 2017). Importantly, selective IL-2 variants have already been demonstrated to be effective in animal models of kidney diseases (Polhill et al., 2012, Kim et al., 2013, Yan et al., 2017), further stressing the translational potential of this approach.

In summary, promising results have been obtained using low-dose IL-2 induced Treg expansion in SLE. However, many questions are yet to be answered and important uncertainties remain. The optimal timing for IL-2 application in SLE, for instance, is currently unknown. Furthermore, nothing is known about the specific impact of low-dose IL-2 on specialized Treg subsets, which have been shown to play an important role in GN and could be the key to more selective therapies (Herrnstadt and Steinmetz, 2020, Kluger et al., 2015, Nosko et al., 2017). Considering their bifunctional properties, special attention should further be focused on the effect IL-2 exhibits on RORγt+Foxp3+ biTregs. Their strong pro-inflammatory features could seriously hamper therapeutic effects. However, if it were possible to selectively exploit biTregs' powerful anti-inflammatory properties, this axis could could be of significant therapeutic benefit (Herrnstadt and Steinmetz, 2020).

3 Aims

3.1 Part One: Treg Expansion via REH Treatment

Previous studies have established an important protective role for regulatory T cells in GN and Treg-expansion is therefore considered a promising therapeutic approach (Bluestone and Tang, 2018, Herrnstadt and Steinmetz, 2020). Interestingly, Tregs can be expanded in vivo by application of low-dose IL-2 (Spolski et al., 2018). Strikingly, Treg expansion by low-dose IL-2 has indeed been shown to be effective in animal and clinical studies (He et al., 2020, He et al., 2016, Humrich et al., 2015, von Spee-Mayer et al., 2016, Rose et al., 2019). However, due to IL-2's pleotropic nature, unspecific immune activation and corresponding side effects are bound to occur, underlining the need for a more selective Treg-expansion strategy and a deeper understanding of IL-2's immunomodulatory effects.

Interestingly, in pioneering experiments, the K. Christopher Garcia lab (Department of Molecular and Cellular Physiology, Stanford University, CA, USA) has developed a mutated human IL-2 protein (a so called mutein, termed ´REH´), which exhibits reduced affinity for the IL-2Rγ subunit. This intricate modification results in selective activation and proliferation of IL-2R^{high} Tregs and spares T effector cells. This dissertation therefore aims to explore the effect of this novel, Treg-selective IL-2 mutein, which was provided by Dr. Garcia as part of a scientific cooperation, in an immunization model and models of inflammatory kidney diseases.

The following questions are addressed using the indicated mouse models:

Preemptive and Therapeutic REH Treatment in a Sheep IgG Immunization Model

- Can Tregs be expanded in vivo after preemptive/therapeutic treatment?
- How does REH influence different Treg subtypes?
- What effect does treatment have on humoral immune responses?
- What effect is seen for immune cells other than Tregs?
- How does the effect of REH compare to that of native IL-2?

Therapeutic REH Treatment in Nephrotoxic Nephritis (NTN)

- Can Tregs be expanded in vivo in NTN?
- Can REH treatment ameliorate disease?

- Which differential effects are seen on Treg subtypes and biTregs?
- What effect is seen for immune cells other than Tregs?
- How does the effect of REH compare to that of native IL-2?

Therapeutic REH Treatment of Lupus Nephritis using the MRL-lpr Mouse Model

- Can Tregs be expanded in vivo in MRL-lpr mice?
- Can REH treatment ameliorate disease?
- Which differential effects are seen on Treg subtypes and biTregs?
- What effect is seen for immune cells other than Tregs?
- How does the effect of REH compare to that of native IL-2?

3.2 Part Two: Cisplatin-induced Acute Kidney Injury

Cisplatin's nephrotoxicity, particularly cisplatin-induced AKI, constitutes a serious and common adverse effect, that can severely limit its clinical applicability in the treatment of malignancies (Miller et al., 2010, Yao et al., 2007). Thus, innovative strategies to prevent cisplatin-induced AKI are urgently needed.

Strikingly, recent studies have established a pathogenic role for T effector cells in cisplatin-induced AKI (Akcay et al., 2011, Alikhan et al., 2016, Liu et al., 2006) and Tregs could be shown to ameliorate damage (Alikhan et al., 2016, Lee et al., 2010, Stremska et al., 2017). This makes T effector cells and Tregs potential targets for novel prevention strategies and warrants further research. We thus aimed to further evaluate the role of Tregs and their effector molecules on Cisplatin induced AKI.

Given, that our group could recently identify RORγt+ Foxp3+ biTregs as potent and crucial novel players of inflammatory renal disease (Kluger et al., 2016, Kluger et al., 2017, Hagenstein et al., 2019), we wanted to specifically analyze their role in AKI. Furthermore, the pleiotropic cytokine Amphiregulin (AREG) was recently identified as key molecule of tissue regeneration and reparation, secreted by activated Tregs (Zaiss et al., 2019). We thus hypothesized that AREG-derived from Tregs might play a protective role in cisplatin induced renal tissue injury.

The second part of this dissertation therefore aims to evaluate the potentially protective effects of biTregs, as well as the Treg effector molecule AREG in a murine model of cisplatin-induced AKI.

The following aspects regarding cisplatin-induced AKI are addressed in this dissertation:

- **Establishment and characterization of cisplatin-induced AKI as a murine model in our lab**
- **Analysis of the dynamics of innate and adaptive immune cell infiltration into kidneys in the murine model of cisplatin-induced AKI**
- **Analysis of renal biTregs in the murine model of cisplatin-induced AKI**
- **Effect of a biTreg selective RORγt-knockout in the murine model of cisplatin-induced AKI**
- **Effect of AREG-deficiency on the murine model of cisplatin-induced AKI**

4 Materials

4.1 Compounds, Chemicals, Nucleotides and Sera

	Source
Aqua (H2O)	B. Braun Melsungen, Melsungen, Hessen, Germany
Brefeldin A	Sigma-Aldrich; St. Louis, MO, USA
Collagenase D	Roche; Basel, Switzerland
Complete Freund's Adjuvant (CFA)	Sigma-Aldrich; St. Louis, MO, USA
Deoxyribonuclease I (DNAse I)	Roche; Basel, Switzerland
Fetal Calf Serum (FCS)	Gibco™; Thermo Fisher Scientific; Waltham, MA, USA
Ionomycin	Calbiochem®, Sigma-Aldrich; St. Louis, MO, USA
LIVE/DEAD Staining	Invitrogen Molecular Probes; Eugene, Oregon, USA
MSA-hIL-2 (8.23 µg/µl)	K. Christopher Garcia; Stanford University; Stanford, CA, USA)
MSA-REH (5.66µg/µl)	K. Christopher Garcia; Stanford University; Stanford, CA, USA)
Nephrotoxic Nephritis Sheep Serum (Batch 11-01)	Seramun Diagnostica; Heidesee, BB, Germany
Normal Mouse Serum	Jackson ImmunoResearch; Ely, Cambridgeshire, UK
Penicillin/Streptomycin	Gibco™; Thermo Fisher Scientific; Waltham, MA, USA
Percoll™ (37%)	Cytiva, formerly GE Healthcare Life Sciences; Marlborough, MA, USA
PMA (Phorbol-12-myristat-13-acetat)	Sigma-Aldrich; St. Louis, MO, USA
ROTI®Histofix (Formaldehyde)	Carl Roth; Karlsruhe, BW, Germany
Sheep Immunoglobulin G (sIgG)	Sigma-Aldrich; St. Louis, MO, USA
Trypan Blue Solution (0.4%)	Gibco™; Thermo Fisher Scientific; Waltham, MA, USA

Table 1. *Compounds, Chemicals, Nucleotides and Sera*

4.2 Buffer and Solutions

	Source/Content
Erylysis Stock Solution 1	Tris(hydroxymethyl)aminomethane at pH 7.6 in H2O
Erylysis Stock Solution 2	144mM ammonium chloride in H2O
Hanks's Balanced Salt Solution (HBSS)	Gibco™; Thermo Fisher Scientific; Waltham, MA, USA
HEPES (1M)	Gibco™; Thermo Fisher Scientific; Waltham, MA, USA
MACS® Buffer	Miltenyi Biotec; Bergisch Gladbach, NRW, Germany
Phosphate-Buffered Saline (PBS)	Gibco™; Thermo Fisher Scientific; Waltham, MA, USA
RPMI 1640 Medium	Gibco™; Thermo Fisher Scientific; Waltham, MA, USA

Table 2. *Buffer and Solutions*

4.3 Antibodies for FACS Surface Staining

Antibody	Source
anti-CCR6	BioLegend; San Diego, CA, USA
anti-CD3	BioLegend; San Diego, CA, USA
anti-CD4	BD Bioscience; Franklin Lakes, NJ, USA
anti-CD45	BD Bioscience; Franklin Lakes, NJ, USA
anti-CD8	BioLegend; San Diego, CA, USA
anti-NK1.1	BioLegend; San Diego, CA, USA
anti-γδTCR	BioLegend; San Diego, CA, USA

Table 3. *Antibodies for FACS Surface Staining*

4.4 Antibodies For FACS Intracellular/Intranuclear Staining

Antibody	Source
anti-Foxp3	Invitrogen™, eBioscience™, Thermo Fischer Scientific; Waltham, MA, USA
anti-GATA3	BioLegend; San Diego, CA, USA
anti-IFNγ	BioLegend; San Diego, CA, USA
anti-IL-10	BioLegend; San Diego, CA, USA
anti-IL-13	BioLegend; San Diego, CA, USA
anti-IL-17A	BioLegend; San Diego, CA, USA
anti-RORγt	Invitrogen™, eBioscience™, Thermo Fischer Scientific; Waltham, MA, USA; BD Bioscience; Franklin Lakes, NJ, USA
anti-Tbet	BioLegend; San Diego, CA, USA

Table 4. *Antibodies for FACS Intracellular/Intranuclear Staining*

4.5 Antibodies for Immunohistochemistry

Antibody	Source
anti-CD3	A0452, Dako; Hamburg, Germany
anti-F4/80	BM8, BMA Biomedicals; Hiddenhausen, NRW, Germany
anti-GR-1	NIMP-R14, Hycult Biotech; Uden, North Brabant, the Netherlands

Table 5. *Antibodies for Immunohistochemistry*

4.6 Commercial Kits

	Source
Antibodies for IgG2c ELISA	Bethyl Laboratories; Montgomery, TX, USA
Antibodies for Total IgG/IgG1 ELISA	Southern Biotech; Birmingham, AL, USA
Antibody-alkaline Phosphatase Kit POLAP	Zytomed; Berlin, Germany
Creatinine Kit	Hengler Analytik; Steinbach, Hesse, Germany

Foxp3 Staining Kit	eBiosciences; St. Diego, CA, USA
Mouse albumin ELISA Kit	Bethyl Laboratories; Montgomery, TX, USA
NucleoSpin Kit	Macherey-Nagel, Düren, NRW, Germany
SYBR™ Green qPCR Kit	Invitrogen™, Thermo Fischer Scientific; Waltham, MA, USA

Table 6. *Commercial Kits*

4.7 Devices and Software

	Source
40µm Cell Strainer	Falcon™, Thermo Fisher Scientific; Waltham, MA, USA
70µm Cell Strainer	Falcon™, Thermo Fisher Scientific; Waltham, MA, USA
BD LSRII Flow Cytometry Cell Analyzer	Becton Dickinson; Heidelberg, BW, Germany
Biometra Thermal Cycler	Biometra, Analytik Jena; Jena, BW, Germany
FlowJo v10	Becton Dickinson; Ashland, Oregon, USA
gentleMACS™ Dissociator	Miltenyi Biotec; Bergisch Gladbach, NRW, Germany
GraphPad Prism v5.01	GraphPad Software; San Diego, CA, USA
Heraeus™ Multifuge™ X3R Centrifuge	Thermo Fisher Scientific; Waltham, MA, USA
K3 EDTA Micro Tube 1.3ml	Sarstedt, Nümbrecht, NRW, Germany
Microsoft Excel v16	Microsoft; Redmond, WA, USA
StepOnePlus™ Real-Time PCR System	Applied Biosystems™, Thermo Fisher Scientific; Waltham, MA, USA
TC20™ Automated Cell Counter	Bio-Rad Laboratories; Hercules, CA, USA

ZEISS Axio Scope.A1	Carl Zeiss Microscopy; Jena, BW, Germany
ZEISS Axiocam HRc	Carl Zeiss Microscopy; Jena, BW, Germany

Table 7. *Devices and Software*

5 Methods

5.1 Compounds

Therapeutic compounds were kindly provided by K. Christopher Garcia from Stanford University. 10 µg of mouse serum albumin (MSA) conjugated to hIL-2 or REH were administered intraperitoneally at indicated time points.

5.2 Models and Animals

5.2.1 Animals

MRL/MpJ-Fas^{lpr}/J mice were kindly provided by Jan-Eric Turner (University Medical Center (UKE), Hamburg, Germany) and were bred in-house.

C57BL/6J mice and RORC$^{fl/fl}$ mice initially derived from Jackson Laboratories (Bar Harbor, Maine, USA). Foxp3$^{YFP\text{-}Cre}$ mice were kindly gifted to us by Alexander Y. Rudensky (Memorial Sloan-Kettering Cancer Centre; New York, NY, USA) and are hereafter referred to as Foxp3^{Cre} mice. Foxp3^{Cre} and RORC$^{fl/fl}$ mice were interbred to generate Foxp3^{Cre} x RORγt$^{fl/fl}$ mice. RORC$^{fl/fl}$, Foxp3^{Cre} and Foxp3^{Cre} x RORγt$^{fl/fl}$ mice were on a C57BL/6J background.

AREG-/- mice initially derived from Matías A. Ávila (University of Navarra; Pamplona, Spain) and were on a C57BL/6J background.

All mice were bred in our facility under specific pathogen-free conditions.

5.2.2 Immunization

Male C57BL/6J mice aged 10-12 weeks were immunized with 500µg sheep immunoglobin G (sIgG) (Sigma-Aldrich; St. Louis, MO, USA) in complete Freund's adjuvant (CFA). In the preemptive treatment model mice were pretreated with either 10µg MSA-REH in PBS (Gibco™; Thermo Fisher Scientific; Waltham, MA, USA) or PBS alone via i.p. injection 7, 4 and 1 day prior to immunization. In one set, an additional group was treated with 10µg MSA-hIL-2. In the therapeutic treatment approach 10µg MSA-REH or PBS were administered at days 5, 8 and 11 or at days 8, 11 and 14 after immunization. Mice were sacrificed at day 7 (preemptive treatment) or

at day 14 or 21 (therapeutic treatment) after immunization and blood as well as spleens were harvested for further analysis.

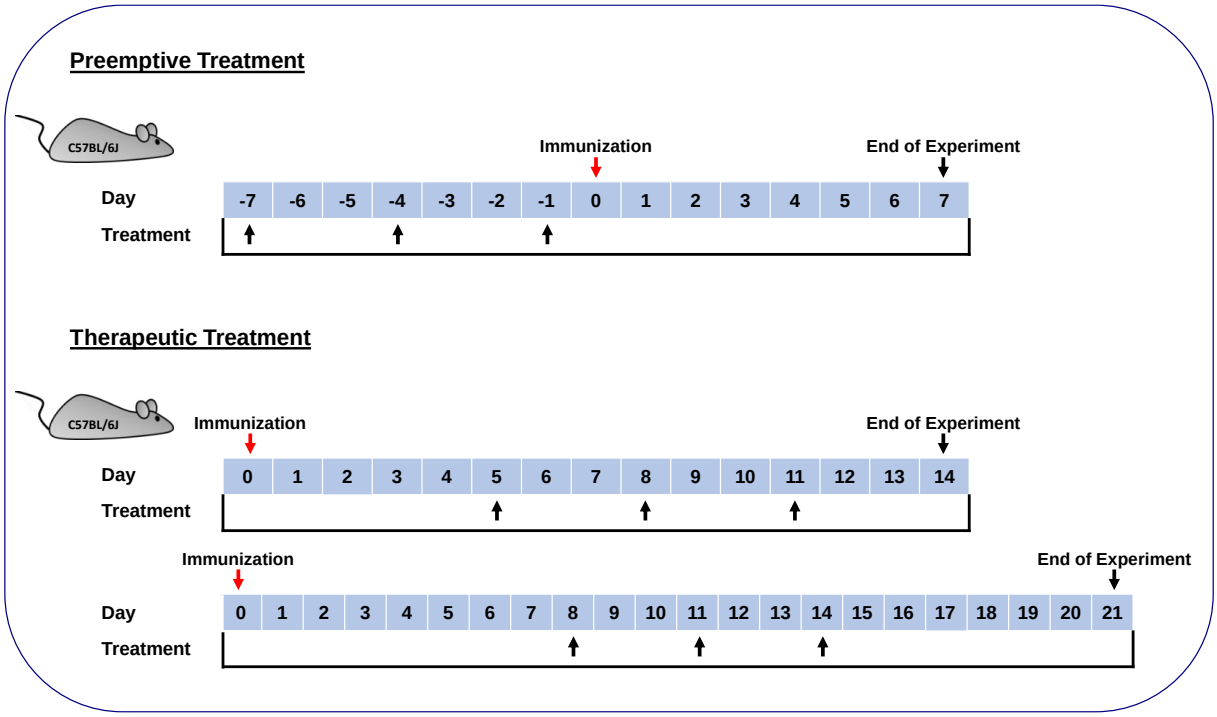

Figure 1. *Timeline Immunization Experiments*

5.2.3 Nephrotoxic Nephritis (NTN)

Nephrotoxic nephritis was induced in male C57BL/6J mice aged 10-12 weeks via i.p. application of nephrotoxic sheep serum (Seramun Diagnostica; Heidesee, BB, Germany). Mice were each subsequently treated with either 10µg MSA-REH or PBS i.p. every three days for a total of 5 doses. Treatment was either started 6 days after induction, resulting in injection at days 6, 9, 12, 15, 18. Urine was collected the day prior to organ removal. Kidneys, spleen and blood were eventually harvested at day 21 after NTN induction.

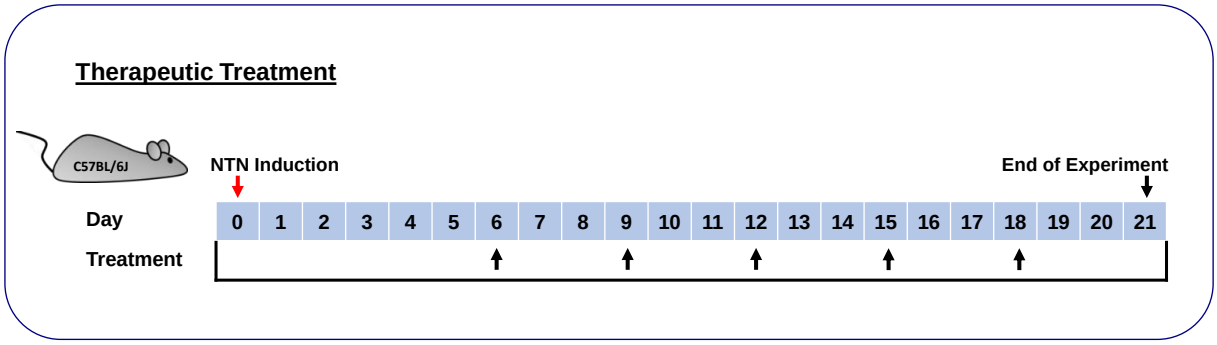

Figure 2. *Timeline NTN Experiments*

5.2.4 MRL-lpr

At the age of 14 weeks, female mice were treated with either 10µg murine serum albumin (MSA) coupled REH (MSA-REH) in PBS, 10µg MSA-hIL-2 in PBS or PBS alone via i.p. injection. Treatment was performed every 3 days for 4 weeks. After 4 weeks of treatment mice were sacrificed and kidneys, spleen, blood and lymph nodes were harvested for further analysis. Clinical appearance of skin and lymph nodes was scored and urine was sampled directly before treatment was started. After onset of treatment clinical scoring and urine collection was performed once every week.

Severity of skin involvement was scored in three areas (face, ears and back) for each mouse. Each area was assigned a score ranging from 0 to 3 corresponding to the grade of skin damage: 0 – complete absence of skin lesions; 1 – mild disease; 2 – moderate disease; 3 – severe disease with heavy ulceration or necrosis. The scores of the three areas were added up, resulting in an overall score with a maximum of 9.

Lymph node involvement was assessed by attributing each mouse a score ranging from 0 to 4 depending on the grade of lymph node enlargement. Submandibular, axillary and inguinal lymph nodes were examined. Scores of 0 to 4 correspond to the following clinical features: 0 - no visible or palpable lymph nodes; 1 – minimal or mild swelling in one region; 2 - mild or moderate swelling in at least two regions; 3 - moderate swelling in two regions or heavy swelling in one region and mild swelling in at least one additional region; 4 - moderate swelling in all regions or heavy swelling in two regions.

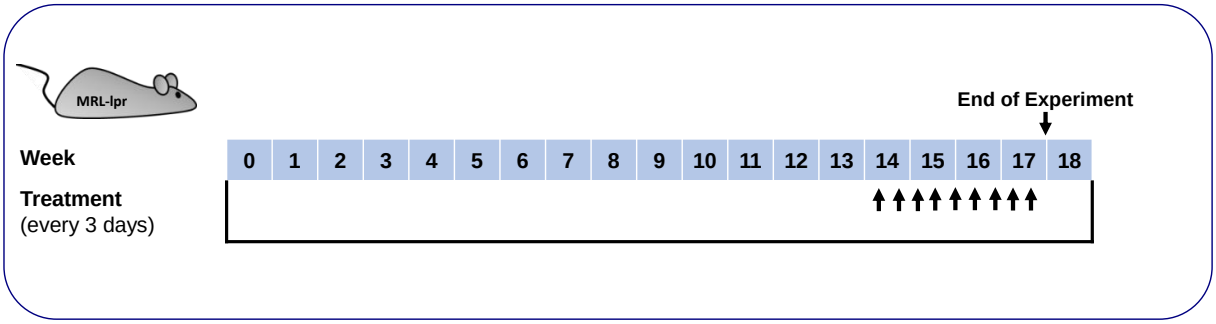

Figure 3. *Timeline MRL-lpr Experiments*

5.2.5 Cisplatin-induced Acute Kidney Injury

Weight-matched C57BL/6J mice aged 9-12 weeks were treated with Cisplatin i.p. to induce acute kidney injury (AKI). For dose finding experiments 20, 25 or 30 mg/kg bodyweight (BW) Cisplatin or PBS (controls) was injected and organ removal was

conducted after 72h. For time course experiments mice received 22 mg/kg BW Cisplatin i.p. or no treatment (naïve controls) and animals were sacrificed after 1h, 3h, 6h, 14h or 38h, 62h, 64h and 70h.

Weight- and age-matched male Foxp3Cre x RORγtfl/fl or AREG-/- mice aged 8-13 weeks and their respective controls were treated with 22 mg/kg BW (4 sets) or 27 mg/kg BW (one set) Cisplatin i.p. and organ removal was conducted between 62 and 70h after treatment as indicated.

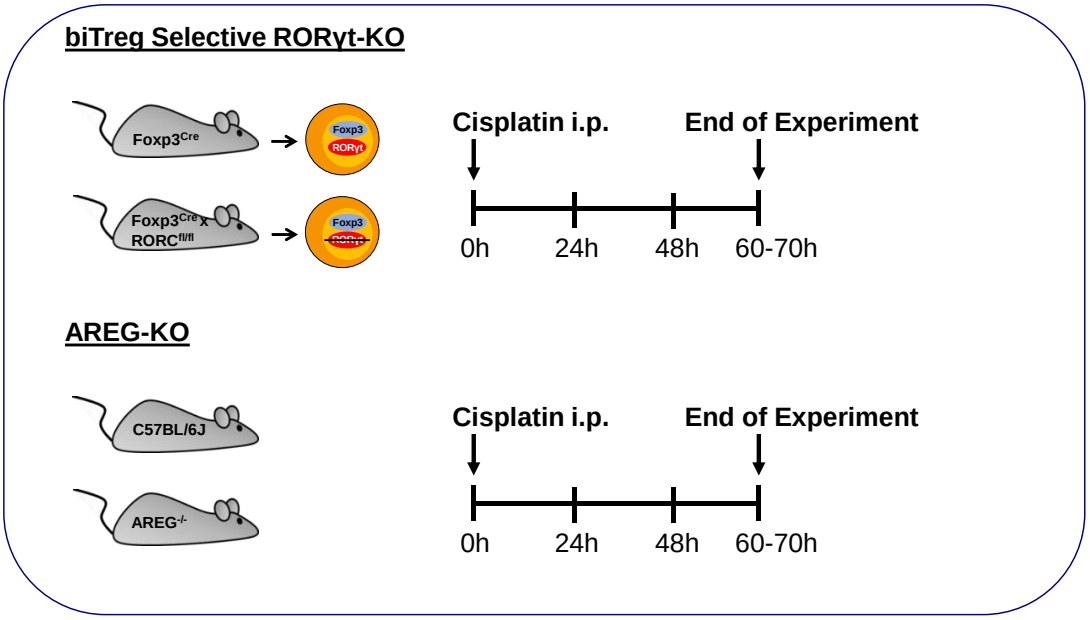

***Figure 4**. Timeline Cisplatin Experiments*

5.2.6 Animal Ethics

Before experiments were started, approval by local authorities was granted (approval codes: N11/19, N63/19, G20/16 and G34/16). All experiments were then conducted in compliance with national and internal animal care and ethics guidelines. Animals were housed in specific-pathogen-free surroundings in our in-house animal facility.

5.3 Single Cell Isolation

5.3.1 Spleen

Spleens were extracted from animals and stored in Hanks balanced salt solution (HBSS) (Gibco™; Thermo Fisher Scientific; Waltham, MA, USA), before being given

over 70µm cell strainer (Thermo Fisher Scientific; Waltham, MA, USA) for purification. Lysis of erythrocytes was conducted using an ammonium chloride based erylysis solution and the samples were subsequently washed with HBSS. Samples were further purified using 40µm cell strainers (Thermo Fisher Scientific; Waltham, MA, USA) and washed again. Live cells were then counted with an automated cell counter (Bio-Rad Laboratories; Hercules, CA, USA), using trypan blue (Gibco™; Thermo Fisher Scientific; Waltham, MA, USA) as a marker of cell death, and ultimately resuspended in PBS for FACS staining or culture. All steps were performed on ice, except for erylysis which was performed at room temperature.

5.3.2 Kidney

Both kidneys were harvested from each mouse and the renal capsule was removed. For some experiments, a small apical piece of one kidney was cut off and stored in liquid nitrogen for RNA-analyses. Furthermore, a bigger transversal slice was stored in 5% formalin (Carl Roth; Karlsruhe, Baden-Württemberg, Germany) for histological staining. Renal parts were then minced and incubated for 45min at 37°C in digestion medium (RPMI 1640 medium, Gibco™; Thermo Fisher Scientific; Waltham, MA, USA) with 10% FCS (Gibco™; Thermo Fisher Scientific; Waltham, MA, USA), 1% HEPES (Gibco™; Thermo Fisher Scientific; Waltham, MA, USA), 1% penicillin/streptomycin (Gibco™; Thermo Fisher Scientific; Waltham, MA, USA), 8µg/ml Collagenase D (Roche; Basel, Switzerland) and 4µg/ml DNase (Roche; Basel, Switzerland). In order to obtain a single-cell suspension, the gentleMACS™ Dissociator (Miltenyi Biotec; Bergisch Gladbach, NRW, Germany) was used. For further enrichment and isolation of leukocytes, cells were centrifuged after dissociation and resuspended in 37% Percoll™-solution (Cytiva, formerly GE Healthcare Life Sciences; Marlborough, MA, USA). A Percoll™-gradient was generated via centrifugation at 500x g for 15min at room temperature. The sedimented cells were then resuspended in PBS for subsequent FACS staining.

5.3.3 Blood

Blood was gathered from the retrobulbar venous plexus and was stored in EDTA tubes (Sarstedt, Nümbrecht, NRW, Germany). Samples were then centrifuged at 3,000rpm

at 4°C for 15min so that serum and cellular components could be seperated. Serum was stored for later analysis. Erylysis was conducted for cellular components as previously described and the single cell suspension was subsequently resuspended in PBS for FACS staining.

5.4 Flow Cytometry

5.4.1 Staining

Right before staining, unspecific binding was reduced through incubation of single cell suspensions with 5% normal mouse serum (Jackson ImmunoResearch; Ely, Cambridgeshire, UK) in MACS buffer (Miltenyi Biotec; Bergisch Gladbach, NRW, Germany) at 4°C for 20min. Samples were then washed and the cell surface was stained via incubation with fluorochrome-labeled antibodies for 20min at 4°C. The antibodies used for surface staining are provided in **Table 3**. Dead cells were labeled with LIVE/DEAD staining (Invitrogen Molecular Probes; Eugene, Oregon). Cells were activated with 50 ng/ml PMA (Sigma-Aldrich; St. Louis, MO, USA) and 1 µg/ml Ionomycin (Calbiochem®, Sigma-Aldrich; St. Louis, MO, USA) in the presence of 10 µg/ml Brefeldin A (Sigma-Aldrich; St. Louis, MO, USA) for 2.5h at 37°C before intracellular and intranuclear staining was performed. Cells were permeabilized with a commercial staining kit (Foxp3 Staining Kit; eBiosciences; St. Diego, CA, USA) so that antibodies could gain access to intracellular and -nuclear sites. After permeabilization cells were stained for 30min at 4°C, washed and ultimately resuspended in PBS for flow cytometric analysis. The antibodies used for intracellular and intranuclear staining are shown in **Table 4**.

5.4.2 FACS Analysis

Samples were measured using a BD LSRII Flow Cytometry Cell Analyzer (Becton Dickinson; Heidelberg, Germany). Gating and analysis were performed with FlowJo v10 (Becton Dickinson; Ashland, Oregon, USA).

5.5 Real-Time Quantitative PCR Analysis

Using a standard Trizol protocol, RNA was isolated from the renal cortex and was subsequently purified via the commercial NucleoSpin kit (Macherey-Nagel, Düren, NRW, Germany). The SYBR™ Green qPCR kit (Invitrogen™, Thermo Fischer Scientific; Waltham, MA, USA) was used to perform real-time PCR. Results were normalized to expression of 18S rRNA and depicted as fold of baseline expression. All primer sequences are provided upon request.

5.6 Histology and Morphologic Studies

5.6.1 PAS Staining of the Kidney

Immediately after organ removal kidney slices were fixated in 5% formalin for 24h. They were then washed and embedded in paraffine for periodic acid-Schiff (PAS) staining or immunohistology, which was conducted according to well established laboratory protocols.

5.6.2 Evaluation of Glomerular and Interstitial Damage in NTN

Kidney injury was scored in 2-µm-thick PAS-stained kidney sections according to previously published methods (Steinmetz et al., 2011). Slides were masked to ensure scoring was conducted in a blinded fashion. To assess glomerular damage, crescent formation and glomerular necrosis were scored in a minimum of 30 glomeruli per animal. Severity of tubulointerstitial damage was rated semi quantitatively with a score ranging from 0-4 (0: no damage; 1: ≤25% damaged; 2: 26-50% damaged; 3: 51-75% damaged; 4: >75% damaged). 20 randomly chosen cortical areas (magnification x200) were scored per animal and tubular dilatation, atrophy, sloughing of tubular epithelial cells or basement membrane thickening were considered to resemble damage. (Steinmetz et al., 2011).

5.6.3 Evaluation of Tubulointerstitial Damage in Cisplatin-induced AKI

For each mouse 20 randomly selected high-power fields (hpf, magnification x400) in the renal cortex were examined in 2-µm-thick PAS-stained kidney sections in a blinded manner. Instances of tubular dilatation, tubular necrosis, cellular debris accumulation, atrophy, sloughing of tubular epithelial cells or basement membrane thickening were

considered to resemble damage. Each HPF was semi quantitatively scored 0-4 according to what area percentage of the tubulointerstitium was damaged (0: no damage; 1: ≤25%; 2: 26-50%; 3: 51-75%; 4: >75%).

5.6.4 Immunohistology

To quantify cell influx into renal interstitium, immunohistology was used. Paraffin-embedded renal slices were incubated with antibodies against CD3 (A0452; Dako; Hamburg, Germany), F4/80 (BM8; BMA Biomedicals; Hiddenhausen, NRW, Germany) or GR-1 (NIMP-R14; Hycult Biotech, Uden, Netherlands). Enzymatic development to visualize primary-antibody binding sites was performed with a secondary antibody alkaline phosphatase kit (POLAP; Zytomed; Berlin, Germany). Positive cells were counted in 20 random cortical tubulointerstitial hpfs (magnification x400) in a blinded fashion.

5.7 Blood and Urine Analysis

Urine samples were collected after mice were housed in metabolic cages with free access to water for 4,5h. A standard albumin ELISA kit (Bethyl Laboratories; Montgomery, TX, USA) was used to measure albuminuria. Blood was collected and processed as described above. For Treg expansion experiments Blood urea nitrogen (BUN) and urinary creatinine were quantified using standard methods in our lab. Quantification of BUN in cisplatin experiments was performed by the Institute of Clinical Chemistry and Laboratory Medicine (University Medical Center (UKE), Hamburg, Germany).

5.8 Analyses of Antibody Production

To analyze humoral immune responses, sheep-globulin-specific IgG, IgG1 (Southern Biotech; Birmingham, AL, USA) and IgG2c (Bethyl; Montgomery, TX, USA) serum titers were measured using commercial ELISA.

5.9 Statistical Analysis

GraphPad Prism v5.01 (GraphPad Software; San Diego, CA, USA) was used for statistical analysis. T tests were performed to compare two groups. To compare more than two groups ANOVA with Tukey post hoc analysis was used. The log-rank test was used for analysis of survival. Statistical significance was defined as a P value ≤ 0.05.

6 Results – Part One: Treg Expansion via REH Treatment

6.1 Immunization Experiments

To investigate immunological *in vivo* effects of the novel IL-2 mutein REH, we performed immunization experiments, where mice were challenged with subcutaneous injections of sheep immunoglobulin (Ig).

6.1.1 Preemptive REH Treatment Expands Tregs *in Vivo* and Reduces T Effector Cell Frequencies in Immunized Mice

In order to evaluate REH's ability to expand Tregs *in vivo*, mice were pretreated with either REH or PBS before being immunized with sheep Ig in CFA. REH treatment resulted in significantly higher frequencies of splenic and blood Foxp3+ Tregs (**Figure 5A/B**) as well as a reduction of splenic and blood T effector cells (**Figure 5C**). Furthermore, expression of the key effector cytokines IFNγ, IL-17A and IL-13 by CD4+ Teffs was reduced (**Figure 5D**), indicating some broad immune-regulatory effects. Similarly, CD8+ T cell frequencies (**Figure 5E**) as well as IFNγ-production by CD8+ T cells (**Figure 5F**) were significantly decreased. Interestingly, no effect was seen for

frequencies of γδ T cells (**Figure 5I**) or NK cells (**Figure 5G**), whereas NKT cells, in contrast, were significantly expanded after REH pretreatment (**Figure 5H**).

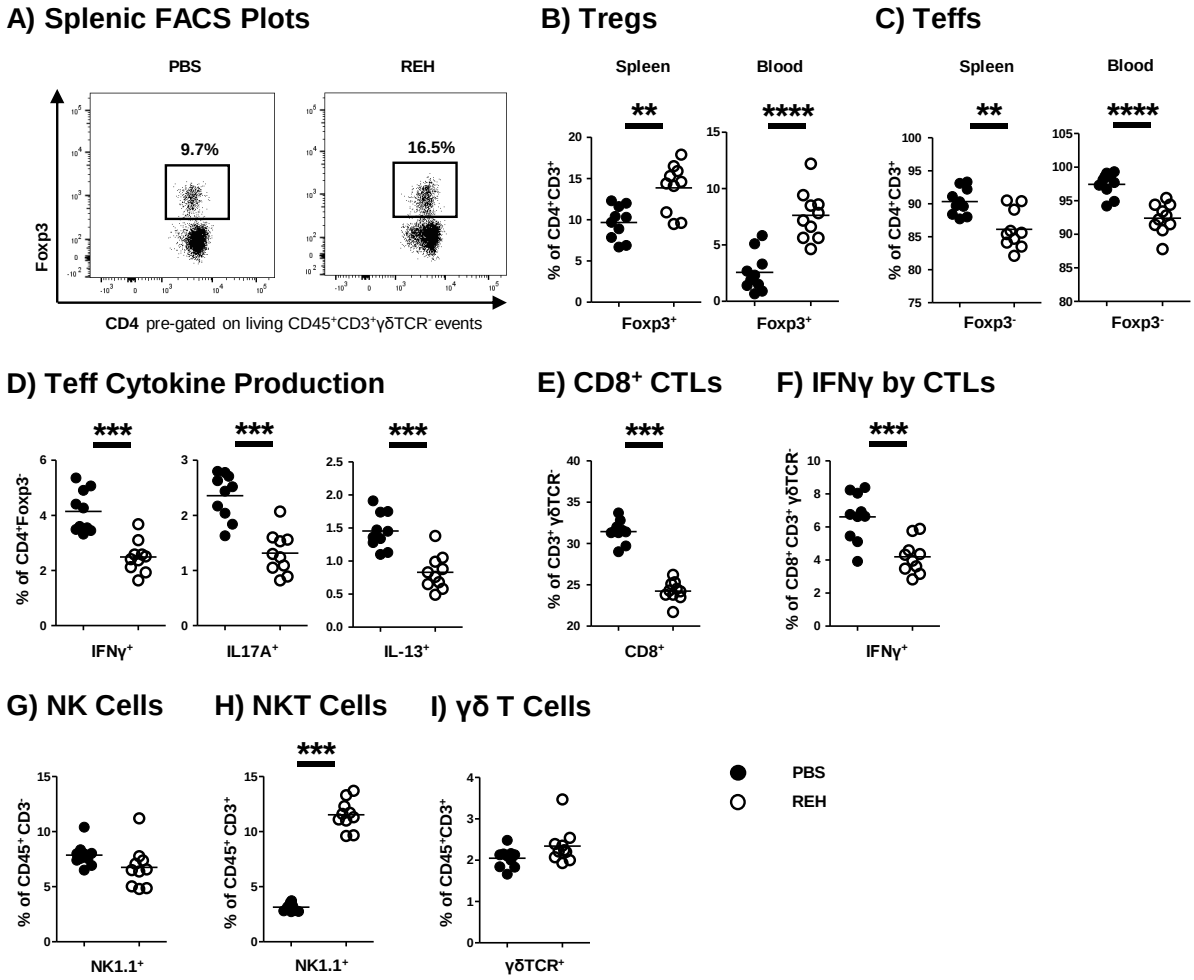

Figure 5. *Effect of Preemptive REH Treatment 7 Days after Immunization*

*Male C57BL/6J mice were pre-treated with REH or PBS 7, 4 and 1 days prior to immunization with sIgG in CFA. (A) Representative FACS plots of splenic Tregs. (B) Quantification of Treg and (C) Teff frequencies in spleen and blood 7 days after immunization. (D) Quantification of Teff cytokine production, (E) CD8+ T cell frequencies and (F) IFNγ production by CTLs as well as (G) frequencies of NK, (H) NKT and (I) γδ T cells in spleens analysed by FACS 7 days after immunization. Circles represent each individual animal, horizontal lines show mean values. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

6.1.2 Treg Expansion via Preemptive REH Treatment Efficiently Reduces Antibody Production

Having shown that REH can indeed expand Tregs *in vivo,* we next sought to further examine its suppressive effects by looking at specific humoral immune responses. ELISA analysis revealed reduced levels of anti-sheep globulin total IgG, IgG1 and IgG2c in mice that were preemptively treated with REH (**Figure 6A**).

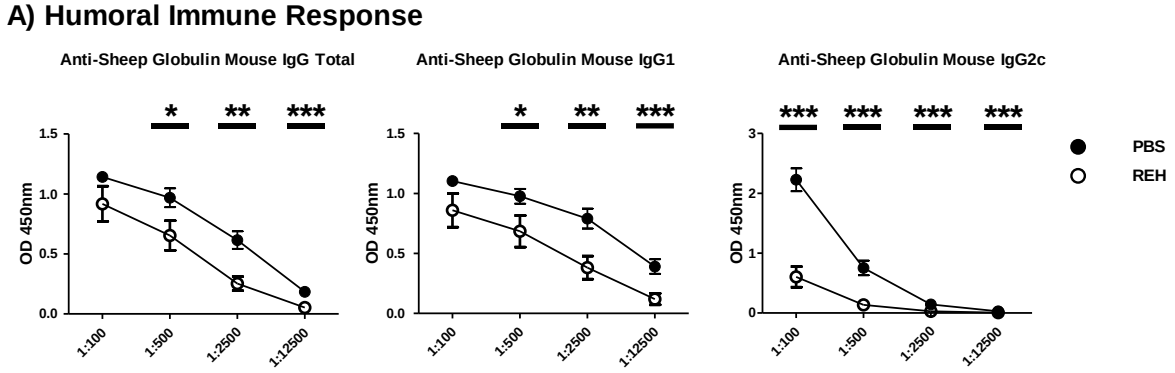

Figure 6. *Effect of Preemptive REH Treatment on Antibody Production*

*(A) Anti sheep globulin specific mouse IgG serum titers at the indicated serum dilutions, as measured by ELISA from male C57BL/6J mice, 7 days post immunization with sIgG in CFA, pre-treated with REH or PBS. Circles indicate means, error bars indicate standard error of the mean (SEM). n=10 for each group. * p<0.05, ** p<0.01, *** p<0.001.*

6.1.3 Therapeutic REH Induces a Massive Expansion of Tregs but Lacks Functional Effects Early After Immunization

Next, we evaluated REH's effect in a therapeutic setting, where mice were treated after immunization had taken place. Strikingly, Tregs were massively expanded in mice treated with REH. Surpassing the effect seen in the preemptive approach, Treg frequencies in spleens and blood had increased more than 3-fold at 14 days after immunization (**Figure 7A/B**). Correspondingly, Teff (**Figure 7C**) as well as CD8+ T cell frequencies (**Figure 7E**) were massively reduced. Unfortunately, however, production of the key effector cytokines IFNγ, IL-17A and IL-13 by CD4+ Teffs (**Figure 7D**) and INFγ expression by CD8+ T cells (**Figure 7F**) were significantly enhanced rather than reduced after therapeutic REH treatment. Furthermore, frequencies of NK (**Figure 7G**), NKT (**Figure 7H**) and γδ T cells (**Figure 7I**) increased and mice treated with REH presented with higher levels of anti-sheep globulin total IgG (**Figure 7J**). This stands

in sharp contrast to our previous findings in preemptively treated mice, where robust suppressive effects by REH could be noted.

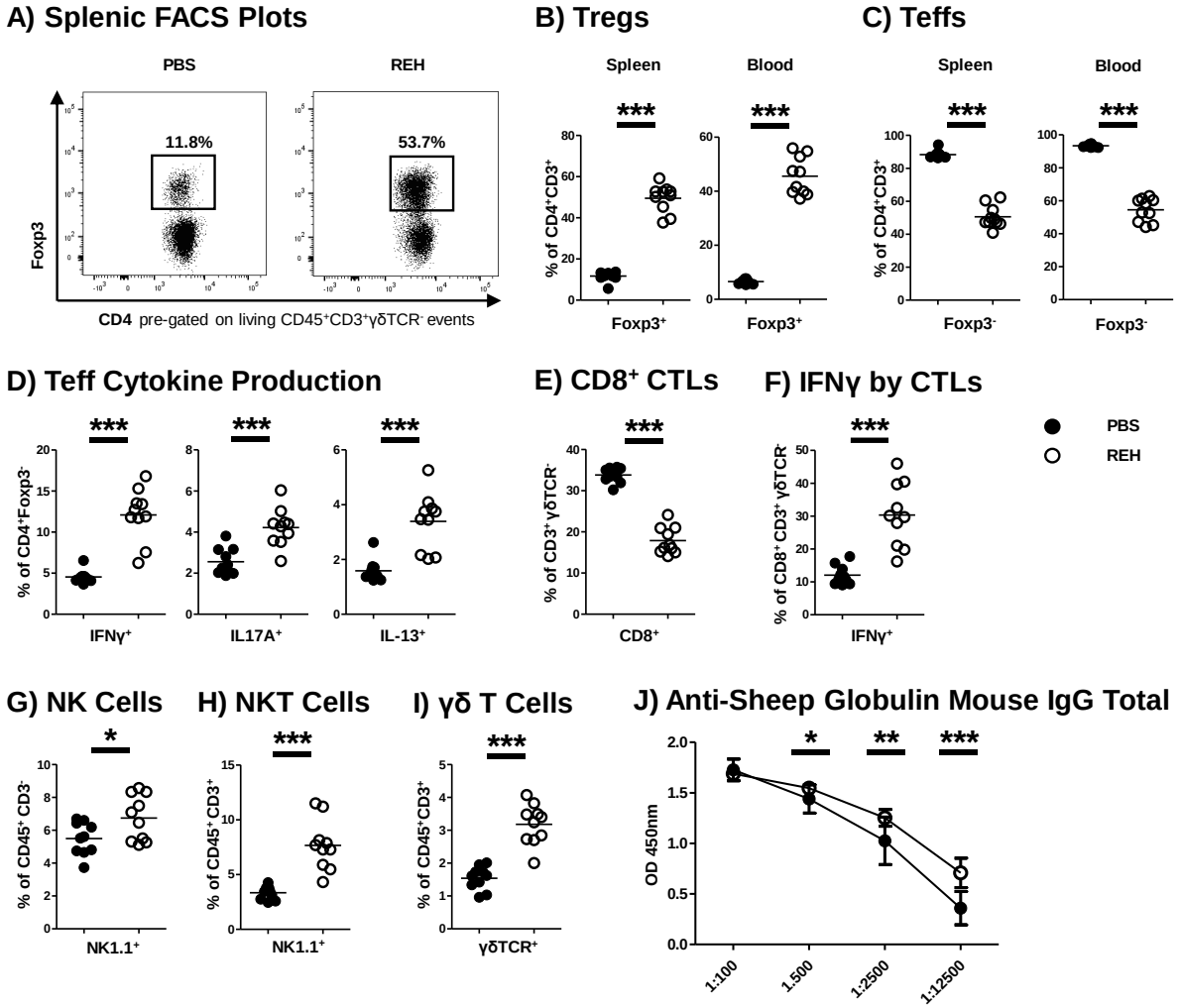

Figure 7. *Effect of Therapeutic REH Treatment 14 Days after Immunization*

*Male C57BL/6J mice were immunized with sIgG in CFA and treated with REH or PBS at days 6, 9 and 12 after immunization. (A) Representative FACS plots of splenic Tregs. (B) Quantification of Treg and (C) Teff frequencies in spleen and blood 14 days after immunization. (D) Quantification of Teff cytokine production, (E) FACS analysis of CD8+ T cell frequencies and (F) IFNγ production by CTLs as well as (G) frequencies of NK, (H) NKT and (I) γδ T cells in spleens 14 days after immunization. Circles represent each individual animal, horizontal lines show mean values. (J) Anti-sheep globulin specific mouse IgG serum titers as measured by ELISA 14 days post immunization at the indicated serum dilutions. Circles indicate means, error bars indicate SEM. n=10 for each group. * p<0.05, ** p<0.01, *** p<0.001.*

6.1.4 The Effects of Therapeutic REH on Immunity Relevantly Change over Time

To better understand the dynamics of Treg expansion and to gain further insight into optimal treatment strategies, we looked at a later time point after immunization and REH treatment. Treg frequencies remained elevated - although on a lower level - even 21 days after Immunization and 7 days after the last REH application (days 8, 11, 14) (**Figure 8A/B**). Similarly, frequencies of Teffs and CD8+ CTLs remained significantly reduced (**Figure 8C/E**). Strikingly, IFNγ expression by Teffs and CD8+ CTLs, which we had previously shown to be elevated at 14 days after immunization (**Figure 7D/F**), was now significantly decreased (**Figure 8D/F**). Furthermore, an increase in IL-17A and IL-13 production by Teffs as demonstrated at day 14 (**Figure 7D**) could not be

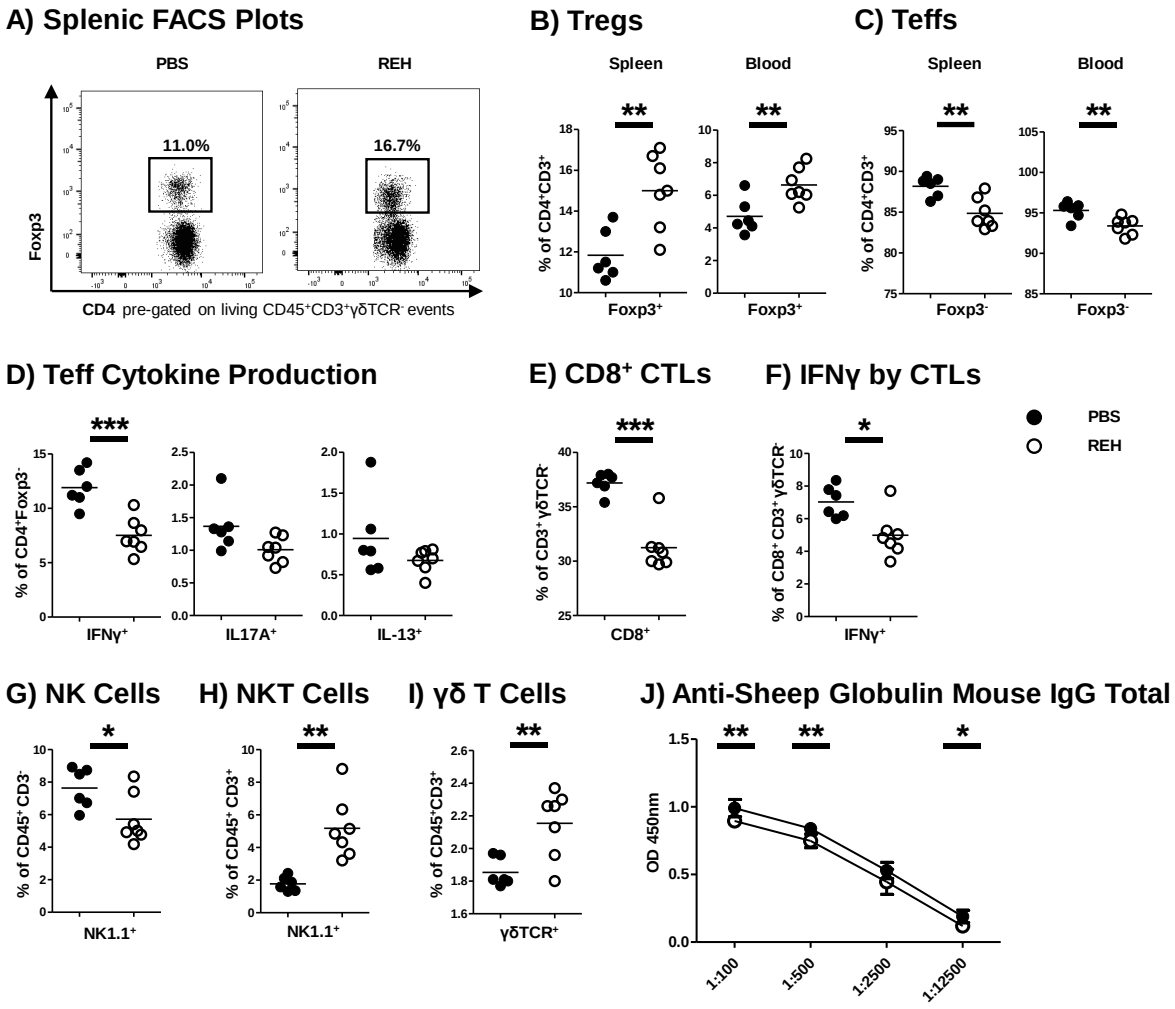

Figure 8. *Effect of Therapeutic REH Treatment 21 Days after Immunization*

*Male C57BL/6J mice were immunized with sIgG in CFA and treated with REH or PBS at 8, 11 and 14 days after immunization. (A) Representative FACS plots of splenic Tregs. (B) Quantification of Tregs and (C) Teff frequencies in spleen and blood 21 days after immunization. (D) Quantification of Teff cytokine production, (E) CD8+ T cell frequencies and (F) IFNγ production by CTLs as well as (G) frequencies of NK, (H) NKT and (I) γδ T cells in spleens by FACS analysis 21 days after immunization. Circles represent individual animals, horizontal lines show mean values. (J) Anti-sheep globulin specific mouse IgG serum titers as measured by ELISA 21 days post immunization at the indicated serum dilutions. Circles indicate means, error bars indicate SEM. PBS n=6, REH n=7. * p<0.05, ** p<0.01, *** p<0.001.*

noted anymore 21 days after immunization (**Figure 8D**). In line with our data from the earlier time point, NKT (**Figure 8H**) and γδ T cell frequencies (**Figure 8I**) were elevated. NK cell frequencies, however, were reduced (**Figure 8F**). Strikingly, opposed to elevated serum levels 14 days after immunization we found a mild reduction of mouse anti-sheep globulin total IgG antibodies at 21 days (**Figure 8J**).

6.1.5 Therapeutic REH Massively Reduces Foxp3+Rorγt+ biTreg Frequencies

As Tregs have recently been found to constitute a highly heterogenous cell population, that is made up of functionally diverse subsets (Herrnstadt and Steinmetz, 2020), we next aimed to characterize REH's differential effect on Treg subsets at 21 days after immunization. No significant change was noted for frequencies of T-bet+ Treg1 cells (**Figure 9A**), whereas CCR6+RORγt- Treg17 frequencies were significantly reduced compared to control animals (**Figure 9B**). Even more importantly, frequencies of Foxp3+Rorγt+ biTregs were strikingly diminished both, among Tregs and among CD4+ T cells (**Figure 9C**). As biTregs have been identified as Tregs with enhanced suppressive capacity with a key role in inflammatory kidney diseases (Kluger et al., 2016, Kluger et al., 2017, Hagenstein et al., 2019) this dramatic decrease of biTregs by REH treatment is particularly noteworthy.

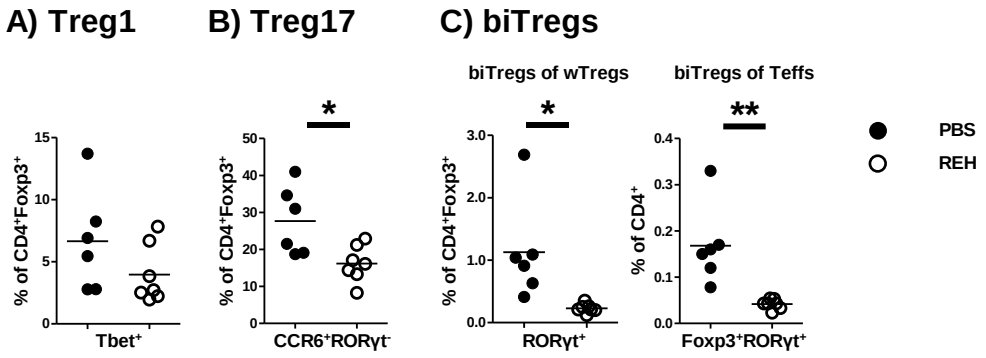

Figure 9. *Effect of Therapeutic REH Treatment on Treg Subsets 21 Days after Immunization*

*Male C57BL/6J mice were immunized with sIgG in CFA and treated with REH or PBS at 8, 11 and 14 days after immunization. Analysis was conducted 21 days after immunization using FACS. (A) Quantification of Treg1 and (B) Treg17 frequencies as percentage of whole Tregs in spleens. (C) Frequencies of biTregs as percentage of whole Tregs or whole CD4+ T cells in spleens. Circles represent each individual animal, horizontal lines show mean values. * p<0.05, ** p<0.01, *** p<0.001.*

6.1.6 Preemptive REH Treatment Appears to be More Potent in Treg Expansions and Immunosuppression than IL-2

As low-dose IL-2 has already shown some promising results in clinical trials (He et al., 2016, Li et al., 2017, Miao et al., 2020, von Spee-Mayer et al., 2016, He et al., 2020), we next sought to directly compare effects of preemptive REH application to IL-2. We thus treated mice with either IL-2, REH or PBS and subsequently immunized them with sIgG in CFA. As expected, splenic Treg frequencies of mice treated with REH were significantly higher than those of PBS controls, whereas no difference was noted after IL-2 treatment (**Figure 10A**). Treg expression of IL-10 was similarly enhanced in both, the REH and IL-2 groups as compared to PBS controls (**Figure 10B**). Interestingly, only REH was able to significantly reduce splenic CD4+ Teff frequencies, whereas IL-2 did not (**Figure 10C**). Furthermore, IFNγ expression by CD4+ Teffs was significantly lower in REH treated animals, as compared to IL-2 treated mice (**Figure 10D**).

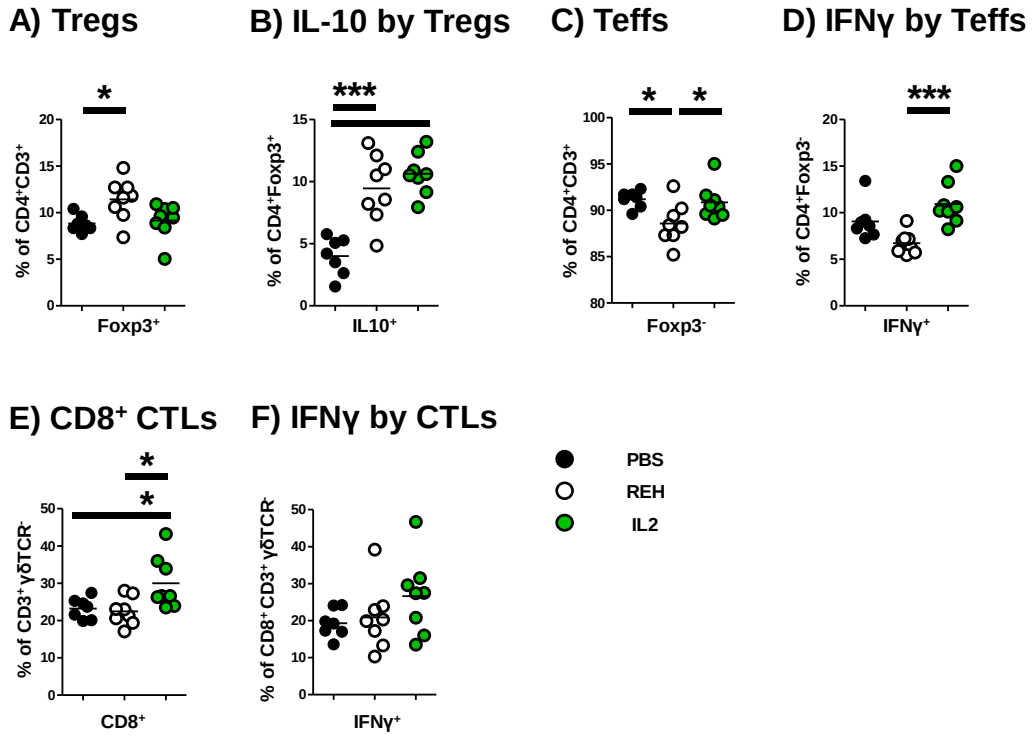

Figure 10. *Effect of Preemptive Treatment with REH and IL-2*

*Mice were pre-treated with either PBS, REH or IL-2 at days 7, 4 and 1 before immunization with sIgG in CFA. Analysis was conducted 7 days after immunization. (A) Quantification of splenic Treg frequencies and (B) Treg IL-10 production by FACS. (C) Quantification of splenic Teff frequencies and (D) INFγ expression by splenic T effector cells by FACS. (E) Frequencies of splenic CD8+ T cells and (F) CD8+ T cell IFNγ expression quantified by FACS. Circles represent each individual animal, horizontal lines show mean values. * p<0.05, ** p<0.01, *** p<0.001.*

6.1.7 IL-2 but not REH Expands CTLs

Activation of CD8+ T cells has been discussed as a potential detriment of low-dose IL-2 therapies, as CTLs express intermediate- and high-affinity IL-2R complexes, similar to the situation on Tregs (Spolski et al., 2018). It is thus noteworthy, that we could show that REH reduces CTL frequencies in a variety of immunization settings (**Figure 5E, Figure 7E** and **Figure 8E**). To investigate whether these effects are unique to REH, we directly compared REH effects on CTLs to those of IL-2 treatment. Interestingly, IL-2 treatment indeed resulted in expansion of splenic CTLs (**Figure 10E**), while REH treatment did not. However, no significant difference regarding IFNγ expression by CTLs could be found after either treatment (**Figure 10F**).

6.2 NTN Experiments

Our above-described experiments established REH's strong ability to expand Tregs in vivo and demonstrated that REH exhibits various, albeit in part opposing, immune regulatory effects on T cells and humoral immunity in preemptive and therapeutic immunization settings. Thus, we next wanted to explore REH's therapeutic potential for kidney diseases, utilizing the nephrotoxic nephritis (NTN) model of acute crescentic GN.

6.2.1 Therapeutic REH Treatment Massively Expands Renal Tregs after NTN Induction

Beginning from day 6 after NTN induction, mice were treated with either REH or PBS every 3 days. In line with our previous results, REH markedly increased frequencies of Tregs (**Figure 11B**) and reduced Teffs in spleens (**Figure 11C**). Strikingly, Tregs were also massively expanded in the nephritic kidneys (**Figure 11A/B/D**) and renal Teff frequencies were reduced (**Figure 11C**). Furthermore, we detected reduced levels of effector cytokines IFNγ and IL-17A in renal Teffs, whereas protective IL-13 levels were increased (**Figure 11E**). Renal frequencies of CD8+ T cells (**Figure 11F**) and their IFNγ expression were also significantly reduced (**Figure 11G**).

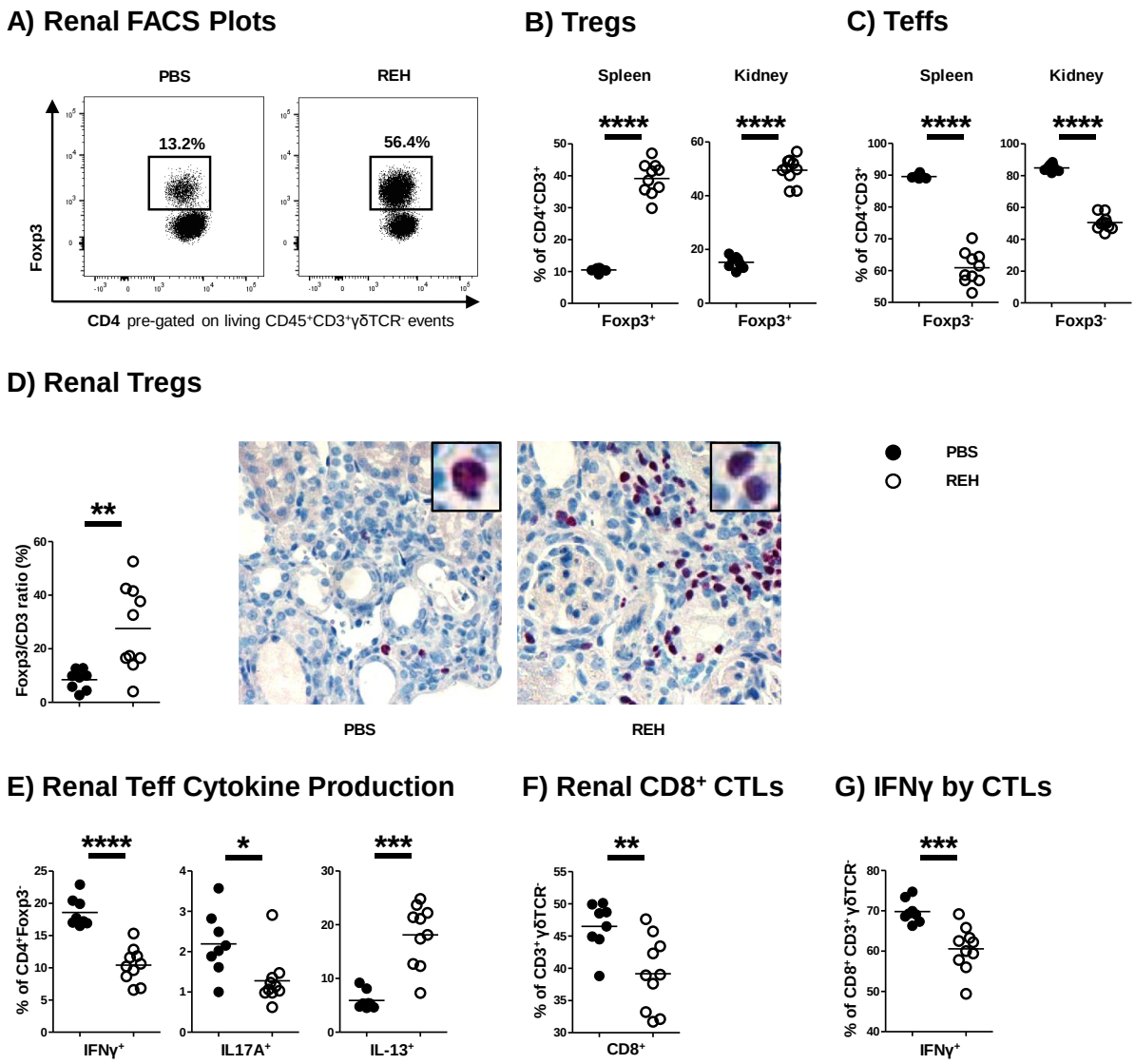

Figure 11. *Effect of Therapeutic REH Treatment after NTN Induction*

*Male C57BL/6J mice were treated with REH or PBS at day 6, 9, 12, 15 and 18 after NTN induction. Analysis was conducted 21 days after NTN induction. (A) Representative FACS plots of renal Tregs. (B) quantification of splenic and renal Treg and (C) Teff frequencies by FACS. (D) Treg to CD3+ T cell ratio as assessed by renal immunohistochemistry and representative kidney sections with immunohistochemical staining for Foxp3. (E) FACS based Quantification of renal Teff frequencies and their IFNγ, IL-17A and IL-13 expression. (F) Quantification of CD8+ T cells and (G) their IFNγ expression in kidneys by FACS. Circles represent individual animals, horizontal lines show mean values. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

6.2.2 Renal biTregs Are Massively Reduced by REH after NTN Induction

Previous studies from our lab have demonstrated, that biTregs play an important immunosuppressive role in the NTN model of acute crescentic GN. The transfer of exogenous biTregs could potently ameliorate kidney damage, indicating net protective effects in this context (Kluger et al., 2016). Importantly, however, our previous immunization studies had shown, that REH treatment led to a dramatic decrease of

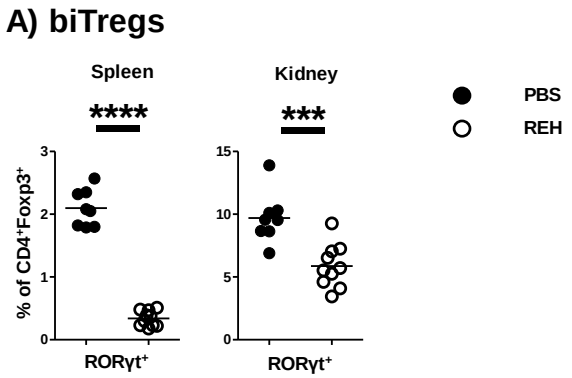

Figure 12. *Effect of Therapeutic REH Treatment on biTregs after NTN Induction*

*Male C57BL/6J mice were treated with REH or PBS at days 6, 9, 12, 15 and 18 after NTN induction. Analysis was conducted 21 days after NTN induction. (A) Quantification of biTreg frequencies in spleen and kidneys by FACS. Circles represent individual animals, horizontal lines show mean values. *** p<0.001.*

biTreg frequencies. Considering the importance of biTregs in NTN we next wanted to explore REH's effect on biTregs in this model of acute kidney inflammation. In line with our previous immunization experiments, splenic as well as renal biTreg frequencies were dramatically diminished by REH treatment (**Figure 12A**).

6.2.3 REH Induced Treg Expansion Shows Mild but Inconsistent Amelioration of NTN

Having demonstrated, that REH is indeed able to expand renal Tregs and exhibits a profound regulatory impact on renal effector cells, we finally wanted to examine if REH treatment can attenuate disease severity. To assess kidney injury, we histologically evaluated interstitial damage as well as glomerular crescent formation in PAS-stained kidney slices. Furthermore, we looked at levels of blood urea nitrogen (BUN) as a functional parameter of kidney injury. Strikingly, histological evaluation of interstitial damage and crescent formation (**Figure 13A**) revealed a small but significant attenuation of kidney damage by REH treatment. Levels of BUN (**Figure 13B**) as well as albuminuria (**Figure 13C**), however, showed no significant difference as compared to PBS controls. Unfortunately, we could not reliably reproduce this protective effect of REH in NTN induced acute crescentic GN in subsequent experiments. These findings are, thus, to be considered preliminary and further research is warranted.

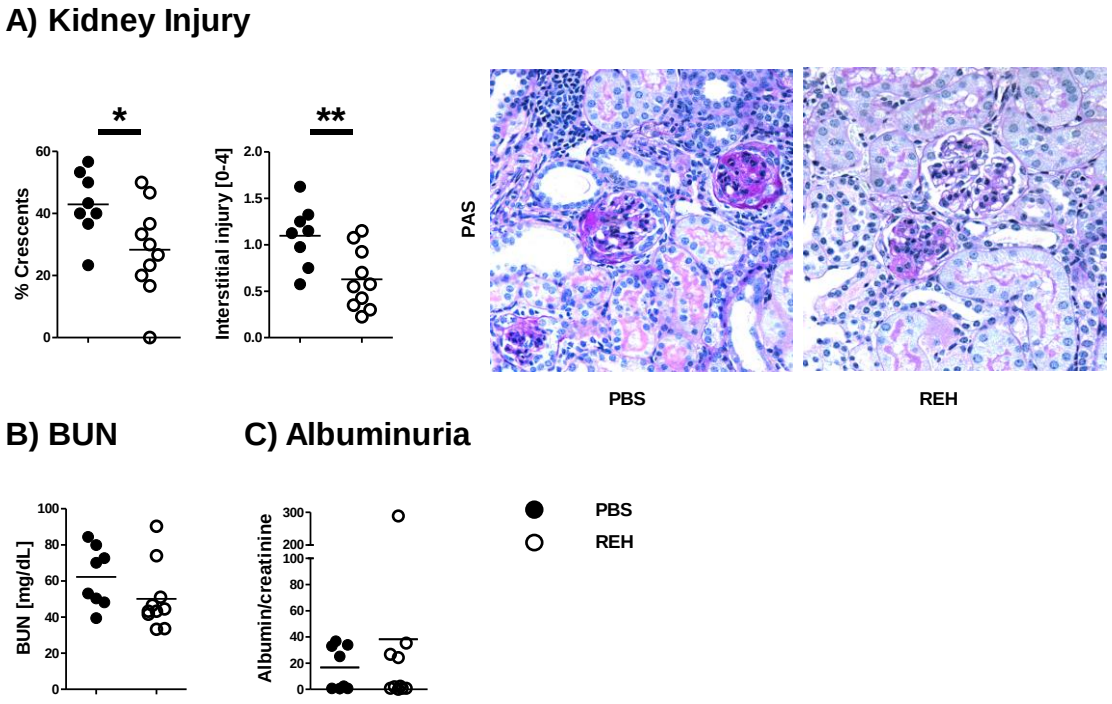

Figure 13. *Clinical Outcome after Treatment with REH after NTN Induction*

*Male C57BL/6J mice were treated with REH or PBS at days 6, 9, 12, 15 and 18 after NTN induction and analysis was conducted at day 21. (A) Quantification of histological renal damage by analysis of crescent formation (left) and interstitial injury (right) and representative photograph of PAS-stained kidney sections. (B) Quantification of blood urea nitrogen levels and (C) albuminuria as an indicator of functional damage. Circles represent individual animals, horizontal lines show mean values. * p<0.05, ** p<0.01, *** p<0.001.*

6.3 MRL-lpr Experiments

As low-dose IL-2 treatment has produced promising results for SLE, we wanted to explore the effect of REH treatment in lupus nephritis. For this purpose, we utilized the MRL-lpr model of lupus nephritis. Notably, previous studies have demonstrated a protective effect for low-dose IL-2 in mouse models of lupus nephritis, including the MRL-lpr model (Mizui et al., 2014, Rose et al., 2019).

6.3.1 REH Shows Only Minimal Effects on Experimental Lupus Nephritis

In line with our previous findings, we noticed a significant increase of Treg frequencies in spleens of MRL-lpr mice treated with REH as compared to PBS controls (**Figure 14A**). Regarding renal Tregs, treatment with both, REH as well as IL-2, resulted in significantly enhanced Treg frequencies (**Figure 14A**). Interestingly, renal Treg expansion after REH treatment significantly exceeded the effect of IL-2 treatment (**Figure 14A**). Furthermore, Teff frequencies in spleens were significantly reduced after REH treatment. Both, REH as well as IL-2 treatment, significantly reduced renal Teffs (**Figure 14B**). The effect in kidneys was, again, significantly more pronounced after REH treatment (**Figure 14B**). Strikingly, while a huge proportion of PBS controls died, all mice treated with REH or IL-2 survived until the end of the experiment (**Figure 14C**), indicating a survival benefit for both treatments. To further assess the clinical outcome of REH treatment, skin involvement and lymph node swelling was scored. In contrast to previous data (Mizui et al., 2014), IL-2 treated mice showed significantly enhanced skin damage (**Figure 14D**). However, no difference was noted for REH treatment in comparison to PBS controls (**Figure 14D**). Interestingly, lymph node swelling was mildly attenuated after REH treatment as compared to PBS controls (**Figure 14D**). BUN levels, as indicators of renal injury revealed no significant difference between all three groups (**Figure 14F**). In line, we did not find differences in histological renal injury (**Figure 14E**). Importantly, since PBS control animals were not simultaneously treated, sacrificed and analyzed, our data are to be treated with caution and further experiments are needed. Furthermore, due to the small number of animals in our MRL-lpr experiments, these results are generally to be taken as preliminary evidence only and further experiments with larger group sizes are necessary.

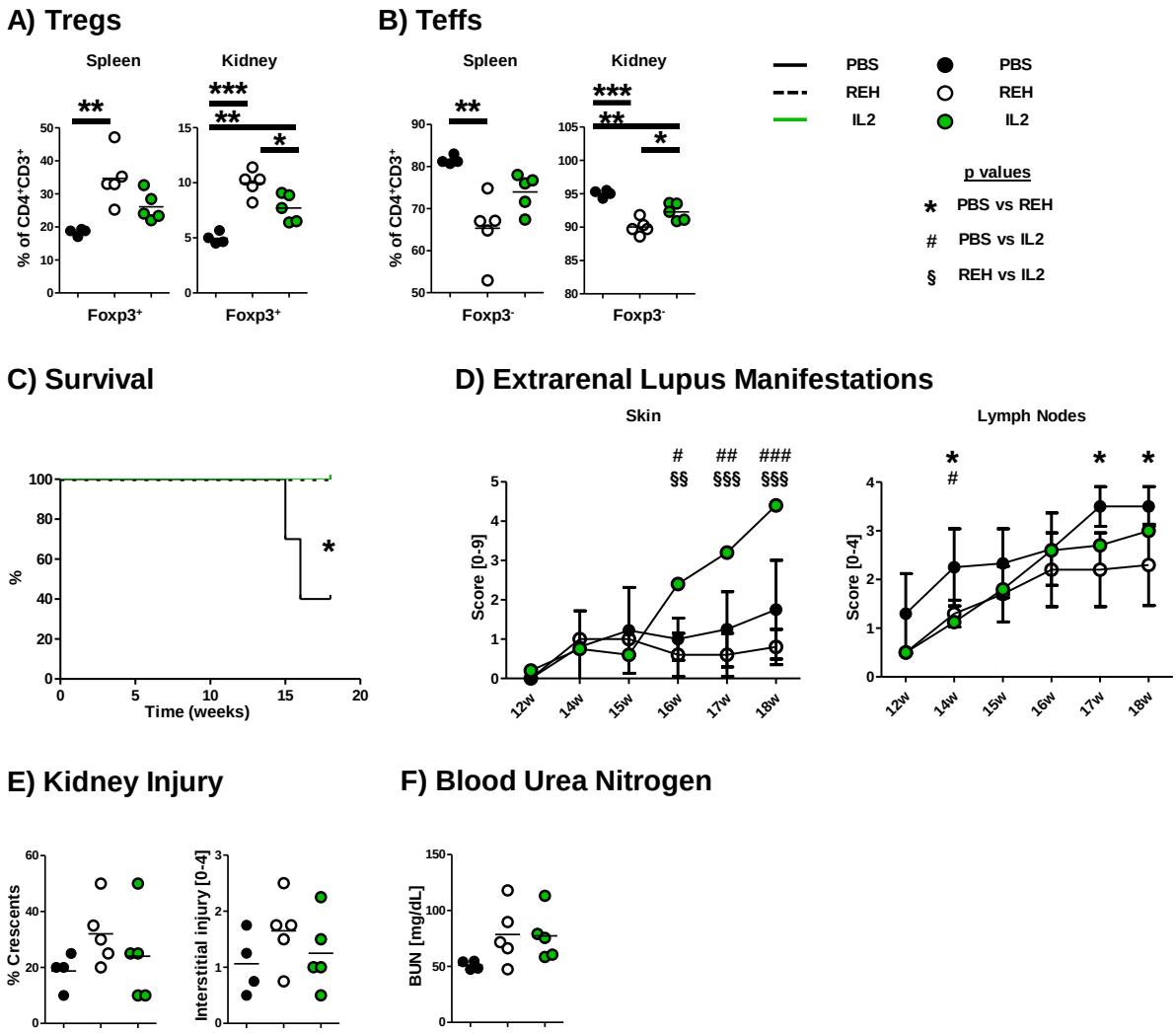

Figure 14. *Effect of REH and IL-2 Treatment in Experimental Lupus Nephritis*

*Starting from the age of 14 weeks MRL/MpJ-Fas^{lpr}/J mice were treated with either REH, IL-2 or PBS every 3 days for 4 weeks. (A) Quantification of renal and splenic Treg and (B) Teff frequencies by FACS. (C) Survival over time as percentage of all animals. PBS n= 10, REH n=5, IL-2 n=5. (D) Clinical score of skin and lymph node involvement over time. (E) Quantification of histological renal damage by analysis of crescent formation (left) and interstitial injury (right) (F) Quantification of blood urea nitrogen levels as an indicator of renal functional damage. (*A, B, D and E*) Circles represent individual animals, horizontal lines show mean values. (D) Circles indicate means, error bars indicate standard deviation. * # § p<0.05, ** ## §§ p<0.01, *** ### §§§ p<0.001.*

7 Results – Part Two: Cisplatin-induced Acute Kidney Injury

In the second part of this dissertation, we aimed to newly establish the model of cisplatin-induced acute kidney injury (AKI). Our aims were to 1) characterize immune cell dynamics in this model, 2) explore the role of biTregs and 3) the role of AREG on the clinical outcome of AKI.

7.1 Establishment of Cisplatin Model

In order to newly establish the murine model of cisplatin-induced AKI, we first performed dose finding experiments in small numbers of animals. Histological examination revealed dose-dependent tubulo-interstitial damage characterized by the loss of tubular epithelial cells, tubular necrosis and tubular cast formation (**Figure 15A**). Judging from the extend of tubulo-interstitial damage, we considered dosages between 20 and 30 mg per kg bodyweight to be suitable for subsequent experiments.

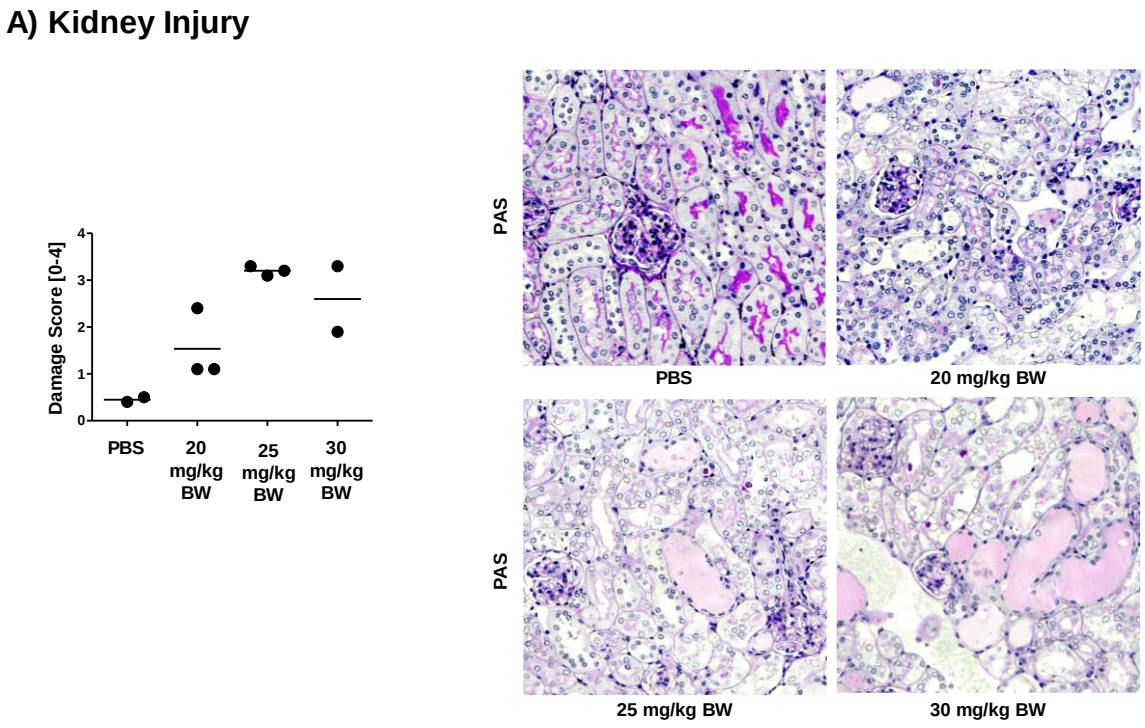

Figure 15. *Murine Model of Cisplatin-induced AKI Dose Finding*

C57BL/6J mice were treated with the indicated doses of cisplatin or PBS i.p. and analysis was conducted after 72h. (A) Quantification of histological renal damage by analysis of tubulo-interstitial injury (left) and representative PAS-stained kidney sections (right). Circles represent individual animals, horizontal lines show mean values. BW: body weight.

7.2 Dynamics of Cisplatin-induced Acute Kidney Injury

To gain further insights into the dynamics of this newly established model, we performed time course experiments in small numbers of mice. As expected, functional and structural damage appears early after cisplatin treatment and increases gradually over time (**Figure 16A**). In line with the literature (Liu et al., 2006), T cell infiltration into kidneys peaked early and subsequently decreased (**Figure 16B**). Strikingly, we noticed a second increase 62 hours after cisplatin treatment (**Figure 16B**). Renal macrophages peaked between 6 and 14 hours and subsequently decreased (**Figure 16B**). Similar to T cell infiltration, a second peak was seen at 62 hours. Neutrophil infiltration only occurred at later stages and reached its peak after 38 hours (**Figure 16B**). Strikingly, renal biTregs were also present at relevant frequencies. A mild peak

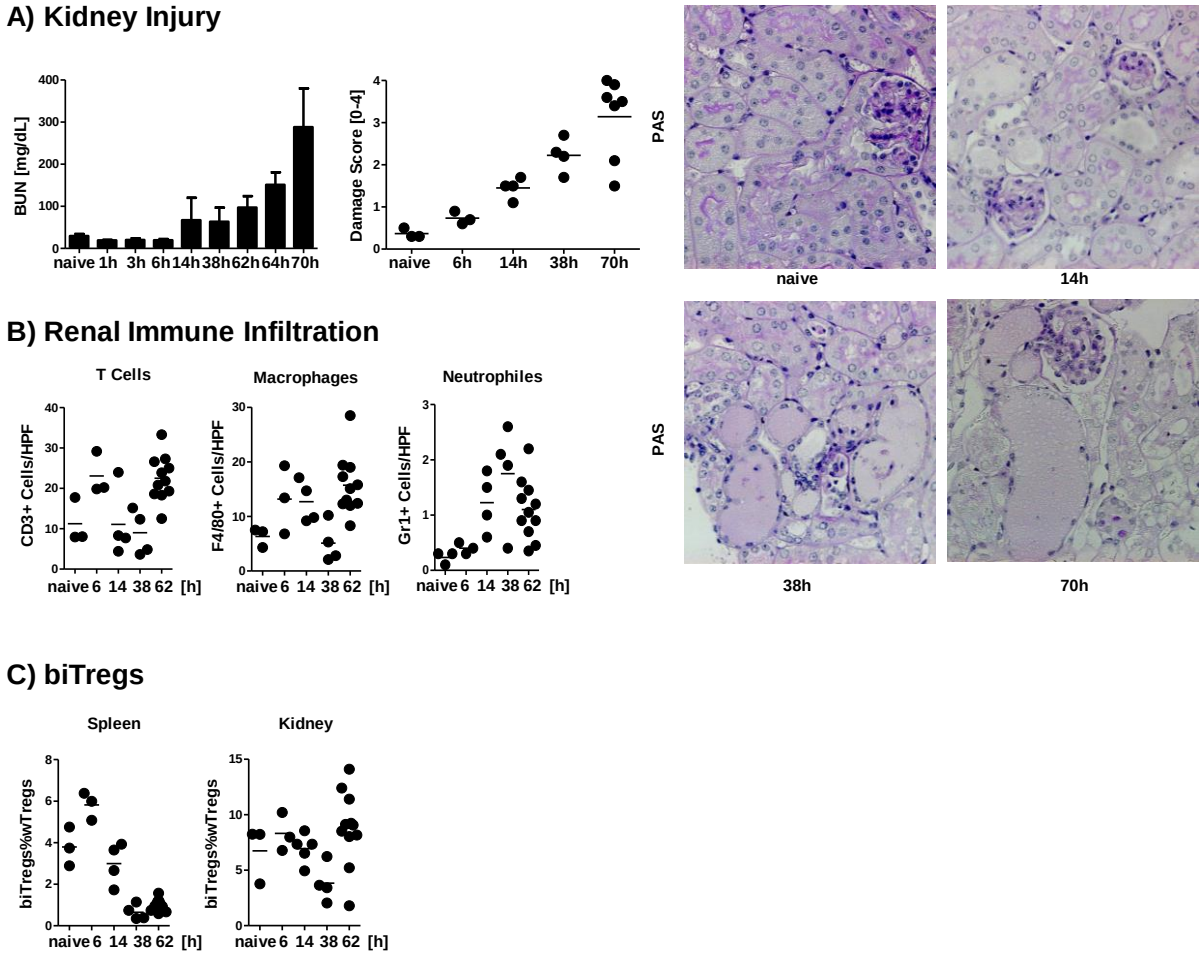

Figure 16. *Dynamics of Cisplatin-induced AKI*

Wildtype mice were treated with 22mg/kg BW Cisplatin i.p.. Naïve controls were not treated. Analysis was conducted at indicated time points after cisplatin treatment. (A) Quantification of blood urea nitrogen levels as an indicator of functional damage (left), Quantification of histological renal damage by analysis of tubulo-interstitial injury (middle), and representative PAS-stained kidney sections (right). (B) Quantification of infiltration of CD3+, F4/80+ and Gr1+ cells into renal interstitium via immunohistochemical staining. (C) FACS based quantification of renal and splenic biTregs at the indicated time points. Circles represent each individual animal, horizontal lines show mean values and error bars indicate standard deviation.

was noted at 6h after cisplatin treatment in spleens and kidneys (**Figure 16C**). While splenic biTreg frequencies remained on a very low level after the initial peak, renal biTregs peaked again after 62 hours (**Figure 16C**).

7.3 A biTreg Selective RORγt Knockout is not Protective in Cisplatin-induced Acute Kidney Injury

Having demonstrated that biTregs are present at low frequencies and peak early after cisplatin treatment, we next sought to evaluate the effect of a biTreg selective RORγt-knockout. Using the Cre/flox system we generated Foxp3^{Cre} x RORγt$^{fl/fl}$ mice, which selectively lack RORγt in biTregs only (**Figure 17A/B**). Unfortunately, no protective effects could be noted for Foxp3^{Cre} x RORγt$^{fl/fl}$ mice regarding tubulo-interstitial damage and BUN level as compared to Foxp3^{Cre} control animals (**Figure 17C**). We can therefore conclude, that a biTreg specific RORγt knockout does not exhibit protective effects in Cisplatin-induced AKI.

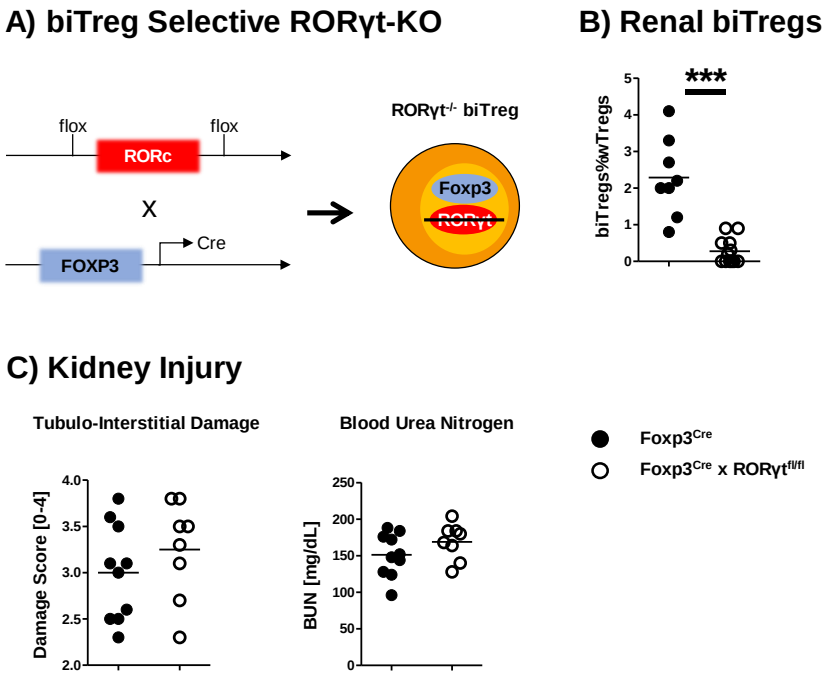

Figure 17. *Foxp3^{Cre} x RORγt$^{fl/fl}$ Mice are not Protected from Cisplatin AKI*

*Foxp3^{Cre} x RORγt$^{fl/fl}$ or Foxp3^{Cre} (controls) mice were treated with 22mg/kg BW Cisplatin i.p.. Analysis was conducted 64 hours after Cisplatin treatment. (A) Generation of mice with RORγt-deficent biTregs via using the Cre/flox system. (B) Quantification of renal biTreg frequencies. (C) Quantification of histological renal damage by analysis of tubulo-interstitial injury (left) and blood urea nitrogen (right). One representative experiment out of 4 independent experiments is shown for renal biTreg frequencies and kidney injury, respectively. Circles represent each individual animal, horizontal lines show mean values. *** p<0.001.*

7.4 AREG Protects from Cisplatin-induced Acute Kidney Injury

Tregs have recently been found to contribute to wound healing and tissue regeneration via the secretion of AREG (Zaiss et al., 2019). We, thus, wanted to explore AREG's role in cisplatin-induced AKI. After cisplatin treatment AREG mRNA expression in kidneys gradually increased and was, indeed, strongly upregulated after 62 hours compared to baseline levels (**Figure 18A**). In order to evaluate whether AREG mediates protective effects in cisplatin-induced AKI, AREG$^{-/-}$ mice and wildtype controls were treated with cisplatin. Strikingly, more deaths occurred in mice lacking AREG compared to wildtype control animals (**Figure 18B**). Furthermore, AREG$^{-/-}$ mice presented with enhanced kidney damage, as characterized by significantly increased tubulo-interstitial damage and elevated BUN levels (**Figure 18C**). This indicates a strong protective effect for AREG in cisplatin-induced AKI.

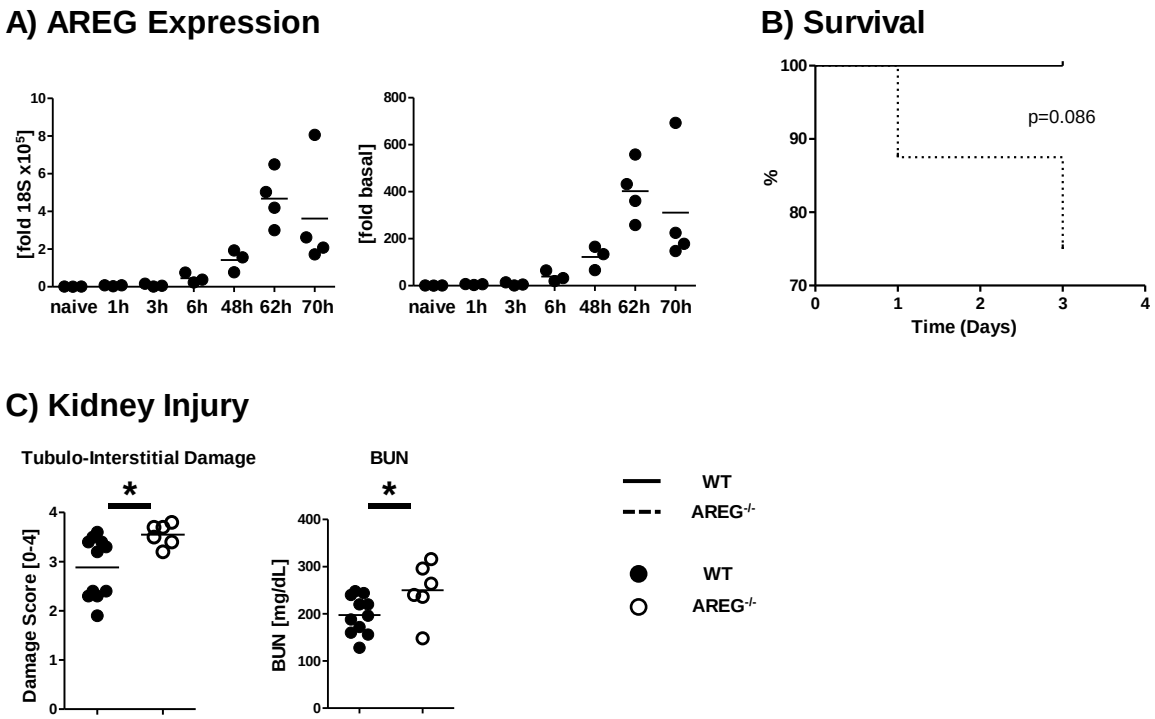

Figure 18. *Effect of an AREG-KO in Cisplatin-induced AKI*

*AREG$^{-/-}$ or wildtype mice were treated with 22mg/kg BW Cisplatin i.p.. Analysis for (A) was conducted at indicated time points after Cisplatin treatment. Analysis for (B and C) was conducted 70h after Cisplatin treatment. (A) Renal expression of AREG mRNA relative to 18s rRNA or baseline levels. (B) Kaplan Meier plot of survival over time as percentage of all animals. WT n=11, AREG$^{-/-}$ n=8. (C) Quantification of renal damage by analysis of tubulo-interstitial injury (left) and blood urea nitrogen levels (right). Circles represent individual animals, horizontal lines show mean values. * p<0.05.*

8 Discussion

New insights into the complexity of our immune system have unveiled the existence of highly diverse and distinct T cell subpopulations. This has paved the way for novel innovative and more specific therapeutic concepts. Particularly regulatory T cells, with their strong immune-suppressive functions, constitute a promising therapeutic option. Indeed, first clinical efforts utilizing Treg adoptive cell therapy or *in vivo* expansion are already on their way (Raffin et al., 2020). As the protective effects of Tregs for inflammatory kidney diseases are well established (Raffin et al., 2020), we here aim to explore the therapeutic utility of in vivo Treg expansion. Furthermore, evidence for a protective role of Tregs in acute kidney injury has recently been described (Alikhan et al., 2016, Lee et al., 2010, Stremska et al., 2017). Thus, we wanted to explore the hitherto unknown role of RORγt+Foxp3+ biTregs and the Treg-derived tissue protective factor AREG in cisplatin-induced AKI.

8.1 Treg Expansion via REH Treatment

IL-2 is a potent mediator of T cell differentiation and activation with highly pleiotropic properties (Ross and Cantrell, 2018). It exerts strong effects on a set of highly diverse lymphocytes and thus plays an important role in a variety of diseases (Ross and Cantrell, 2018, Spolski et al., 2018). This makes the IL-2 axis a promising therapeutic target. Strikingly, Tregs are extremely sensitive to IL-2 and can be preferentially activated and expanded by low-dose IL-2 (Shevach, 2012). First promising clinical results for this strategy have been obtained from studies in autoimmune diseases, including SLE (Humrich et al., 2015, He et al., 2016, von Spee-Mayer et al., 2016, He et al., 2020). However, pleiotropy still hampers IL-2's clinical use. Strikingly, the lab of Dr. K. Christopher Garcia was able to develop a Treg-specific IL-2 mutein, called 'REH'. In comparison to wildtype IL-2, REH shows reduced affinity for the IL2Rγ subunit. This theoretically leads to selective activation and proliferation of Tregs, which have a particularly high IL-2R expression. Thus, we wanted to explore the effect of REH treatment in immunization models and models of inflammatory kidney diseases.

In an immunization model with the model antigen sheep IgG, preemptive REH treatment was indeed able to robustly expand Tregs *in vivo*, while Teff and CTL frequencies were reduced. This underscores REH's Treg-specific activity.

Furthermore, preemptive REH treatment exhibited potent functional effects, as effector cytokine as well as specific antibody production was reduced. We could, thus, establish proof of concept of REH's ability to expand Tregs and have further demonstrated an immune regulatory effect on T effector cells and antibody responses in a preemptive treatment setting *in vivo*. However, in clinical reality it is only on rare occasions that treatment can be started preemptively. It is – unfortunately – rather the regular case that treatment can only be started after disease manifestation has already taken place. This clearly makes therapeutic treatment options clinically more relevant. Therefore, we next evaluated REH's effect in a therapeutic setting, where mice were treated after immunization had taken place.

Strikingly, 3 days after the last dose of therapeutic REH treatment, the extend of Treg expansion even exceeded that of preemptive treatment and Teffs along with CTLs were massively reduced. However, quite to our surprise, we found effector cytokine production and specific antibodies to be increased. In further contrast to preemptive treatment, where only protective NKT cells were expanded, we also noticed an increase of NK and γδ T cells. Since the interval between the end of REH treatment and organ removal was much shorter in our therapeutic treatment experiment than it was in the preemptive treatment setup, this enhancement of effector functions could hint to some non-specific short-term activational properties of REH. As such side effects could potentially pose critical issues for clinical applications, we wanted to gain a better understanding of the effects of therapeutic REH treatment over time. Thus, we next performed experiments, where we looked at a later timepoint after the end of REH treatment. Treg expansion and reduction of Teffs and CTLs persisted on a lower level even 7 days after the last dose of REH. Strikingly, in marked contrast to the earlier time point, INFγ expression by Teffs as well as CTLs, frequencies of NK cells and mouse anti-sheep globulin IgG were now reduced. This indicates favorable long-term outcome after REH treatment.

Interestingly, in the therapeutic treatment setting, Treg expansion had already occurred at 3 days after the end of REH treatment. Suppressive effects on Teff cytokine production, however, became only apparent several days later on. Indeed, REH even seems to exhibit immune stimulatory effects during the early period of treatment, which might be explained by initial unspecific effector cell activation before the functional effects of Treg expansion become relevant. This might lead to early increased

production of pro-inflammatory effector cytokines by Teff, like IFNγ, which is later downregulated by Tregs. These findings further stress the importance to carefully choose the right timing for treatment. Such immune stimulatory effects, even if only temporary, could potentially turn out detrimental, if induced at the wrong time of disease.

Next, in order to further examine the differential effects of REH treatment on Tregs, we looked at expansion of different Treg subpopulations. This is especially important, as recent research has shown distinct roles for different Treg subtypes in GN (Herrnstadt and Steinmetz, 2020). Interestingly, Th17 specific Treg17 cells were reduced by REH treatment. Furthermore, and even more importantly, we observed a dramatic decrease of Foxp3+RORγt+ biTregs. This does not come completely unexpected, since both Treg subtypes develop along a pathway similar to Th17 cells, which are known to be potently suppressed by IL-2.

Since both, Treg17 cells and biTregs possess enhanced suppressive capacity and play an important role in inflammatory kidney diseases (Kluger et al., 2016, Kluger et al., 2017, Hagenstein et al., 2019), their drastic reduction could potentially counteract the protective effect of a REH-induced Treg expansion.

Having established the ability of REH treatment to potently expand Tregs in a variety of immunization settings *in vivo*, we next wanted to directly compare its effect to treatment with wild type IL-2. REH treatment led to increased Treg frequencies and reduced Teffs, whereas IL-2 treatment failed to do so. Furthermore, IL-2 treatment – unlike REH - resulted in enhanced INFγ expression by Teffs and increased frequencies of CTLs. Thus, REH is, indeed, more Treg-specific than IL-2 and shows reduced activity on CTLs.

In order to explore REH's potential for treatment of inflammatory kidney diseases we next went on to study REH's effects on the nephrotoxic nephritis (NTN) model of acute crescentic GN and the MRL-lpr model of lupus nephritis.

Strikingly, REH treatment after NTN induction resulted in the expansion of both, splenic and also renal Tregs. We could thus demonstrate, that REH's ability to expand Tregs is not only limited to lymphatic organs. In line with our previous findings from the immunization studies, frequencies of splenic and renal Teffs, as well as CTLs were reduced. Also, the expression of pro-inflammatory effector cytokines was reduced,

whereas protective IL-13 production was increased. Similar to the findings from our immunization experiments, however, biTregs were strikingly diminished in kidneys and spleens. This observation might pose a relevant restriction which applies to potential treatment with REH.

In terms of histological kidney damage, we found mild amelioration after REH treatment. Unfortunately, however, we could not find any effect regarding BUN levels or proteinuria. Furthermore, amelioration of histological kidney injury could not be stably reproduced in repeat experiments. Therefore, the actual protective effect of REH in NTN appears to be rather low and/or context dependent. Considering the massive expansion of renal Tregs, this lack of protective effect is somewhat surprising. Especially, since strong protective effects of Tregs are well established for NTN (Herrnstadt and Steinmetz, 2020). We hypothesize, that suppression of the development of effector Tregs as Treg17 cells and biTregs by REH is causative for the lack of effect on NTN.

Intrigued to see, whether REH could ameliorate renal injury in a more chronic model of kidney inflammation, we next utilized the MRL-lpr model of lupus nephritis. REH treatment, again, resulted in robust expansion of renal Tregs and, strikingly, survival of REH and IL-2 treated animals exceeded that of controls. However, we found only minimal effects regarding other clinical endpoints and in particular, no effects were seen for kidney injury. Due to the small number of animals used in our experiments, additional experiments are needed to definitely validate the results. However, overall and similar to NTN, the effect of REH treatment in the MRL-lpr model on development of lupus nephritis appears to be minimal at best.

Since REH treatment has resulted in a massive expansion of renal Tregs as well as a robust reduction of renal Teffs and effector cytokines, the lack of protection in both models is highly astonishing. Interestingly, we know from our previous experiments, that REH treatment can lead to increased effector cytokine production early after immunization with sIgG and that immune suppression takes full effect only at later stages. Such early activational properties of REH treatment can profoundly hamper therapeutic effects and could potentially be one of the reasons we did not achieve potent protection from kidney injury. Furthermore, we have shown, that biTregs are dramatically diminished after REH application. As this newly described effector Treg population is known to possess enhanced suppressive capacities in NTN (Kluger et

al., 2016), negative effects of REH on biTreg development, might – at least in part – account for the lack of clinical efficiency. Still, the fact, that such a massive expansion of Tregs, as observed after REH application, does not result in more pronounced protective effects is highly surprising. It appears therefore likely, that while REH treatment is, indeed, able to potently expand Tregs, it might fail to induce proper differentiation of Tregs with full suppressive capacity.

In conclusion, we were able to show a robust expansion of Tregs by preemptive as well as therapeutic application of the novel IL-2 mutein REH in immunization experiments. Preemptive treatment resulted in pronounced functional effects as reflected by decreased frequencies of T effector cells, reduced effector cytokine production and reduced serum levels of mouse anti-sheep globulin IgG. Strikingly, the extent of Treg expansion in mice, treated therapeutically after immunization, even exceeded that of preemptively treated animals. However, increased levels of effector cytokines in treated mice early after immunization constitute evidence of unspecific effector cell activation by REH. In addition, and importantly, biTreg frequencies were massively reduced by REH treatment. Considering the important role of biTregs in inflammatory kidney diseases, this could have serious further implications. When assessing REH as treatment option for GN, we found, that REH treatment also robustly expands Tregs in inflamed kidneys when using the NTN model of acute crescentic GN and the MRL-lpr model of lupus nephritis. Furthermore, renal effector T cell frequencies and their cytokine production were reduced. Unfortunately, however, the protective effects of REH treatment on kidney injury appear to be minimal at best. Considering the massive Treg expansion, this is highly surprising and could potentially be explained by early unspecific activational effects on Teffs, the striking reduction of Treg17 cells and biTregs as well as generally insufficient suppressive function of REH expanded Tregs.

8.2 Cisplatin-induced Acute Kidney Injury

The discovery of cisplatin as potent chemotherapeutic agent has been nothing short of a revolution in oncology. Even today it still constitutes a highly effective therapeutic agent that is used for the treatment of a wide variety of malignancies (Hoskins et al., 2000, Planting et al., 1999, Sasaki et al., 2016, Hartmann et al., 1999). However, its strong anti-neoplastic properties are severely hampered by its nephrotoxicity. Indeed,

in about a third of patients, that are treated with cisplatin, clinically relevant AKI occurs (Miller et al., 2010, Yao et al., 2007). Cisplatin-induced AKI has long been exclusively attributed to direct tubular toxicity. Interestingly, however, recent research has unveiled a pathogenic role for the immune system as well. While T effector cells enhance tissue injury (Akcay et al., 2011, Alikhan et al., 2016, Liu et al., 2006), Tregs were found to be protective (Alikhan et al., 2016, Lee et al., 2010, Stremska et al., 2017). As these findings potentially open up new Treg-based possibilities for preventive strategies, we started off by establishing the experimental mouse model of cisplatin-induced AKI in our lab. Considering the important role RORγt+Foxp3+ biTregs play in inflammatory glomerular diseases (Herrnstadt and Steinmetz, 2020), we aimed to explore their role in AKI as well. In addition, we wanted to assess the role of Treg-derived AREG, as this pleiotropic cytokine was recently described to be crucial for tissue regeneration and wound healing (Zaiss et al., 2019).

We started to establish the experimental model of cisplatin-induced AKI by performing dose finding experiments. Intraperitoneal application of 20 to 30 mg of cisplatin per kg bodyweight reliably resulted in acute kidney injury after about 3 days. Thus, mice were treated accordingly in subsequent experiments. As expected, tubulo-interstitial kidney injury manifested early after treatment and progressed over time. In line with data from the literature, T cell infiltration into renal tissue peaked early at about 6 hours after cisplatin treatment and declined thereafter. Interestingly, we found a second peak of T cell infiltration, that occurred at later stages. A similar dynamic was noted regarding macrophage infiltration. In contrast, neutrophil numbers increased until about 38 hours after treatment and subsequently decreased. Strikingly, we could also show biTregs to be present in kidney tissue in relevant numbers after cisplatin induced injury. Similar to T effector cells, biTreg frequencies peaked early and subsequently declined, only to finally rise to new heights in the late stages of disease. Systemically, biTregs peaked similarly early but remained low after that.

To further explore biTregs's potential functional role, we wanted to find out, which effect a Treg selective RORγt knockout exhibited on the outcome after cisplatin induced AKI. As RORγt is known to equip biTregs with strong pro-inflammatory properties (Kluger et al., 2016, Kluger et al., 2017), we hypothesized, that selective RORγt-deficiency in biTregs, which completely abrogates their expression of pathogenic IL-17, should result in amelioration of AKI. Unfortunately, however, we failed to observe any

protective effects. Thus, silencing of RORyt in biTregs, which previously showed potent anti-inflammatory effects on glomerular kidney diseases (Kluger et al., 2016, Kluger et al., 2017), appears to play no role in cisplatin-induced AKI.

It is, however, important to consider that this does not necessarily prove that biTregs in general do not play a role in AKI. Selective RORγt-deficiency in biTregs, as used in our studies, does not equal complete absence of biTregs. Thus, future research, using models to mimic complete biTreg-deficiency, is needed to fully characterize their functional role in cisplatin-induced AKI.

Finally, we aimed to explore the role of Treg-derived AREG in cisplatin-induced AKI, since AREG is known to contribute to wound healing and tissue regeneration. Interestingly, we found a strong upregulation of AREG mRNA expression in renal tissue after cisplatin induced injury. This indicates that AREG might indeed exhibit some functional effects in the course of disease. To evaluate potential protective effects, we evaluated AKI in AREG$^{-/-}$ mice. Strikingly, AREG-deficiency resulted in reduced survival and aggravated functional and structural kidney damage. We can thus conclude, that AREG exhibits protective effects in cisplatin-induced AKI. As Tregs are known to protect from cisplatin-induced kidney injury (Alikhan et al., 2016, Lee et al., 2010) and Treg-derived AREG has recently been shown to contribute to wound healing and tissue repair (Zaiss et al., 2019), we hypothesize, that Treg-derived AREG is protective in cisplatin-induced AKI. However, as AREG is expressed by a wide variety of cells, further research is needed to explicitly prove that the protective effects we could demonstrate here, are indeed attributed to AREG from Tregs and not from other cellular sources. Based on my findings from this dissertation, our group has started to address this aspect in more detail and a first manuscript incorporating some of my data is currently in preparation. Finally, our studies open up perspectives for future translational research, as our findings strongly indicate that the AREG/EGFR axis constitutes a promising therapeutic target for the prevention and attenuation of cisplatin- and/or immunologically- mediated AKI. Indeed, prevention of AKI by such novel strategies could eventually improve the outcome of cisplatin based oncologic therapies.

9 Summary

The kidney constitutes a highly complex and vital organ for the organism‘s wellbeing. Unfortunately, however, its function can be put at risk by inflammatory diseases, like Glomerulonephritis (GN), or toxic side effects of drugs such as cisplatin-induced acute kidney injury (AKI). Strong protective effects of regulatory T cells (Tregs) for GN are well established and Tregs have recently also been shown to protect from AKI. Tregs are a diverse population and can be subdivided into a number of specialized subtypes, including the newly described, highly active RORγt+Foxp3+ biTregs. In addition to their immunosuppressive functions, Tregs have recently been shown to be also involved in tissue regeneration through the secretion of amphiregulin (AREG). Since Tregs constitutively express the trimeric, high-affinity IL-2 receptor, they can be preferentially activated and expanded via treatment with low-dose IL-2. The lab of K. Christopher Garcia could recently design the IL-2 mutein “REH”, which selectively expands Tregs and has only minimal effects on T effector cells (Teffs). Therefore, one aim of this dissertation was to evaluate the therapeutic effect of REH treatment in immunization experiments as well as in models of inflammatory kidney diseases. We could demonstrate, that REH treatment led to a massive expansion of Tregs after immunization, as well as in the NTN model of acute crescentic GN and the MRL/lpr model of lupus nephritis. Unfortunately, however, REH treatment did not result in protection from kidney injury. This discrepancy between the massive expansion of Tregs and lack of renoprotection might potentially be explained by early unspecific activational effects on Teffs. Furthermore, we observed generally insufficient suppressive function of REH expanded Tregs with much reduced percentages of biTregs and other effector Treg populations. Secondly, we aimed to investigate the effects of biTregs as well as of Treg-derived AREG on cisplatin-induced AKI. In this respect, we newly established the model of cisplatin-induced AKI in our lab. Unfortunately, we found that Treg-specific deficiency of RORγt did not result in any effects on the outcome of AKI. In contrast, however, we were able to demonstrate a strong protective effect of AREG. Further studies are currently being carried out in our lab to investigate, whether Treg-derived AREG might serve as target for preventive strategies against cisplatin-induced AKI.

10 Zusammenfassung

Die Niere ist ein hochkomplexes und lebensnotwendiges Organ. Ihre Funktion kann jedoch durch immunologische Erkrankungen wie die Glomerulonephritis (GN) oder toxische Medikamentennebenwirkungen wie das Cisplatin-induzierte akute Nierenversagen (ANV) stark beeinträchtigt werden. Ein ausgeprägter protektiver Einfluss von regulatorischen T Zellen (Tregs) ist für die GN gut belegt. Neuerdings wird zudem vermutet, dass Tregs auch vor dem Gewebeschaden im Rahmen eines ANV schützen. Tregs sind eine diverse Zellpopulation, die in spezialisierte Subtypen, wie die neu beschriebenen, hoch aktiven RORγt+Foxp3+ biTregs, unterteilt werden können. Zusätzlich zu ihren immunsuppressiven Fähigkeiten konnte kürzlich auch ein Einfluss auf die Gewebeheilung durch die Sekretion von Amphiregulin (AREG) nachgewiesen werden. Da Tregs konstitutiv den trimeren, hoch-affinen IL-2 Rezeptor exprimieren, können sie durch niedrig dosiertes IL-2 präferentiell aktiviert und expandiert werden. Die Arbeitsgruppe um K. Christopher Garcia konnte kürzlich die IL-2 Mutante „REH“ synthetisieren, welche Tregs hoch-selektiv expandiert und nur einen minimalen Einfluss auf T Effektor Zellen (Teffs) ausübt. Das Ziel dieser Dissertation war es daher, den therapeutischen Effekt einer Behandlung mit REH in Immunisierungsexperimenten und Modellen inflammatorischer Nierenerkrankungen zu ergründen. Wir konnten zeigen, dass die Behandlung mit REH sowohl nach Immunisierung als auch im NTN Modell der akuten GN und dem MRL/lpr Modell der Lupus Nephritis zu einer massiven Expansion von Tregs führte. Unglücklicherweise ließ sich insgesamt jedoch kein protektiver Effekt auf den Nierenschaden finden. Die Diskrepanz zwischen massiver Treg-Expansion und fehlender Protektion erklärten wir mutmaßlich durch unspezifische aktivierende Eigenschaften von REH auf Teff, sowie fehlende suppressive Fähigkeiten der expandierten Tregs mit einem deutlich reduzierten Anteil an biTregs und weiteren Effektor-Treg Populationen. Im zweiten Teil dieser Dissertation untersuchten wir den Einfluss von biTregs sowie die Sekretion von AREG auf das Cisplatin-induzierte ANV. Hierfür konnten wir das Modell des Cisplatin-induzierten ANV erfolgreich etablieren. Unglücklicherweise stellten wir fest, dass eine Treg spezifische RORγt-Defizienz keinen Einfluss auf das Ausmaß des ANVs ausübte. Weitere Experimente zeigten jedoch einen deutlichen protektiven Effekt von AREG auf den ANV induzierten Gewebeschaden. In aktuellen Folgestudien versuchen wir derzeit zu ergründen, inwiefern sich von Tregs sezerniertes AREG als neues therapeutisches Ziel für die Prävention des Cisplatin-induzierten ANV eignet.

11 Index of Abbreviations

AKI	*Acute kidney injury*
ANCA	*Anti-neutrophil cytoplasmic antibody*
ANOVA	*Analysis of variance*
ANV	*Akutes Nierenversagen*
AREG	*Amphiregulin*
Bcl6	*B-cell lymphoma 6 protein*
biTregs	*Bifunctional regulatory T cells*
Blimp1	*B lymphocyte-induced maturation protein-1*
BUN	*Blood urea nitrogen*
BW	Bodyweight
CD	*Cluster of differentiation*
CFA	*Complete Freund's adjuvant*
CTL	*Cytotoxic T lymphocyte*
CTLA4	*Cytotoxic T-lymphocyte-associated protein 4*
cTregs	*Conventional regulatory T cells*
DNase	*Deoxyribonuclease*
E. coli	*Escherichia coli*
EAE	*Experimental autoimmune encephalitis*
EDTA	*Ethylenediaminetetraacetic acid*
FACS	*Fluorescence-activated cell sorting*
FCS	*Fetal calf serum*
Foxp3	*Forkhead box P3*
GN	*Glomerulonephritis*
GvHD	*Graft-vs-host disease*
HBSS	*Hanks balanced salt solution*
HCV	*Hepatitis C virus*
HEPES	*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*
hpf	*High-power field*
i.p.	*Intraperitoneally*
IFNγ	*Interferon gamma*
IgE	*Immunoglobulin E*
IgG	*Immunoglobulin G*
IL-	*Interleukin-*

IL-2R	*IL-2 receptor*
IL-2Rα	*IL-2 receptor alpha chain*
IL-2Rβ	*IL-2 receptor beta chain*
IL-2Rγ	*IL-2 receptor gamma chain*
IL-2Rγc	*IL-2 receptor common gamma chain*
IRF4	*Interferon regulatory factor 4*
iTregs	*Induced regulatory T cells*
JAK	*Janus family kinase*
MACS	*Magnetic-activated cell sorting*
MHC	M*ajor histocompatibility complex*
MRL-lpr	*Murphy Roths Large-lymphoproliferation*
MSA	*Mouse serum albumin*
NK	*Natural killer*
NKT	*Natural killer T*
NTN	*Nephrotoxic nephritis*
PAS	*Periodic acid-Schiff*
PBS	*Phosphate-buffered saline*
PCR	*Polymerase chain reaction*
PMA	*12-O-Tetradecanoylphorbol-13-acetat*
qPCR	*Quantitative polymerase chain reaction*
rIL-2	*Recombinant IL-2*
RNA	*Ribonucleic acid*
RPGN	*Rapid progressive glomerulonephritis*
rRNA	Ribosomal RNA
SLE	Systemic lupus erythematosus
STAT	*Signal transducer and activator of transcription*
Tbet	*T-box expressed in T cells*
TCR	*T cell receptor*
TF	*Transcription factor*
Tfh	*T follicular helper cells*
TGF-β	*Transforming growth factor beta*
Th	*T helper*
TNBS	*Trinitrobenzenesulfonic acid*
TNF-α	*Tumor necrosis factor alpha*

Tregs	*Regulatory T cells*
XLAAD	*X-linked auto-immunity-allergic dysregulation syndrome*
X-SCID	*X-linked severe immunodeficiency*

12 Table of Figures

12.1 Tables

12.2 Figures

13 Bibliography

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15 Curriculum Vitae

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