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The function of RORγt^{+}Foxp3^{+} biTregs in glomerulonephritis

Dissertation

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dedicated to my parents with deep gratitude

1 Table of contents

2 Published data

2.1 Part one

Partial results of this dissertation were published with the title "RORγt expression in Tregs promotes systemic lupus erythematosus via IL-17 secretion, alteration of Treg phenotype and suppression of Th2 responses" as co-author in the journal „Clinical and Experimental Immunology ", 2017, volume 188, pages 63-78.

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RORγt expression in T_{regs} promotes systemic lupus erythematosus via IL-17 secretion, alteration of T_{reg} phenotype and suppression of Th2 responses

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Summary

Systemic lupus erythematosus (SLE) is a common autoimmune disorder with a complex and poorly understood immunopathogenesis. However, a pathogenic role for the T helper type 17 (Th17) axis was demonstrated by many studies, while regulatory T cells (T_{regs}) were shown to mediate protection. Recently, we and others characterized a novel and independent T cell population expressing both the T_{reg} characteristic transcription factor forkhead box protein 3 (FoxP3) and the Th17-defining retinoic acid receptor-related orphan nuclear receptor γt (RORγt). Studies in a model of acute glomerulonephritis unveiled potent regulatory, but also proinflammatory, functions of RORγt$^+$FoxP3$^+$ T_{regs}. This bi-functional nature prompted us to suggest the name 'biT_{regs}'. Importantly, the pathogenic biT_{reg} effects were dependent upon expression of RORγt. We thus aimed to evaluate the contribution of RORγt$^+$FoxP3$^+$ biT_{regs} to pristane-induced SLE and explored the therapeutic potential of interference with RORγt activation. Our analyses revealed expansion of IL-17 producing biT_{regs} in a distinctive time–course and organ-specific pattern, coincident with the development of autoimmunity and tissue injury. Importantly, specific ablation of RORγt activation in endogenous biT_{regs} resulted in significant amelioration of pristane-induced pulmonary vasculitis and lupus nephritis. As potential mechanisms underlying the observed protection, we found that secretion of IL-17 by biT_{regs} was abrogated completely in FoxP3^{Cre} × RORC$^{fl/fl}$ mice. Furthermore, T_{regs} showed a more activated phenotype after cell-specific inactivation of RORγt signalling. Finally, and remarkably, biT_{regs} were found to potently suppress anti-inflammatory Th2 immunity in a RORγt-dependent manner. Our study thus identifies biT_{regs} as novel players in SLE and advocates RORγt-directed interventions as promising therapeutic strategies.

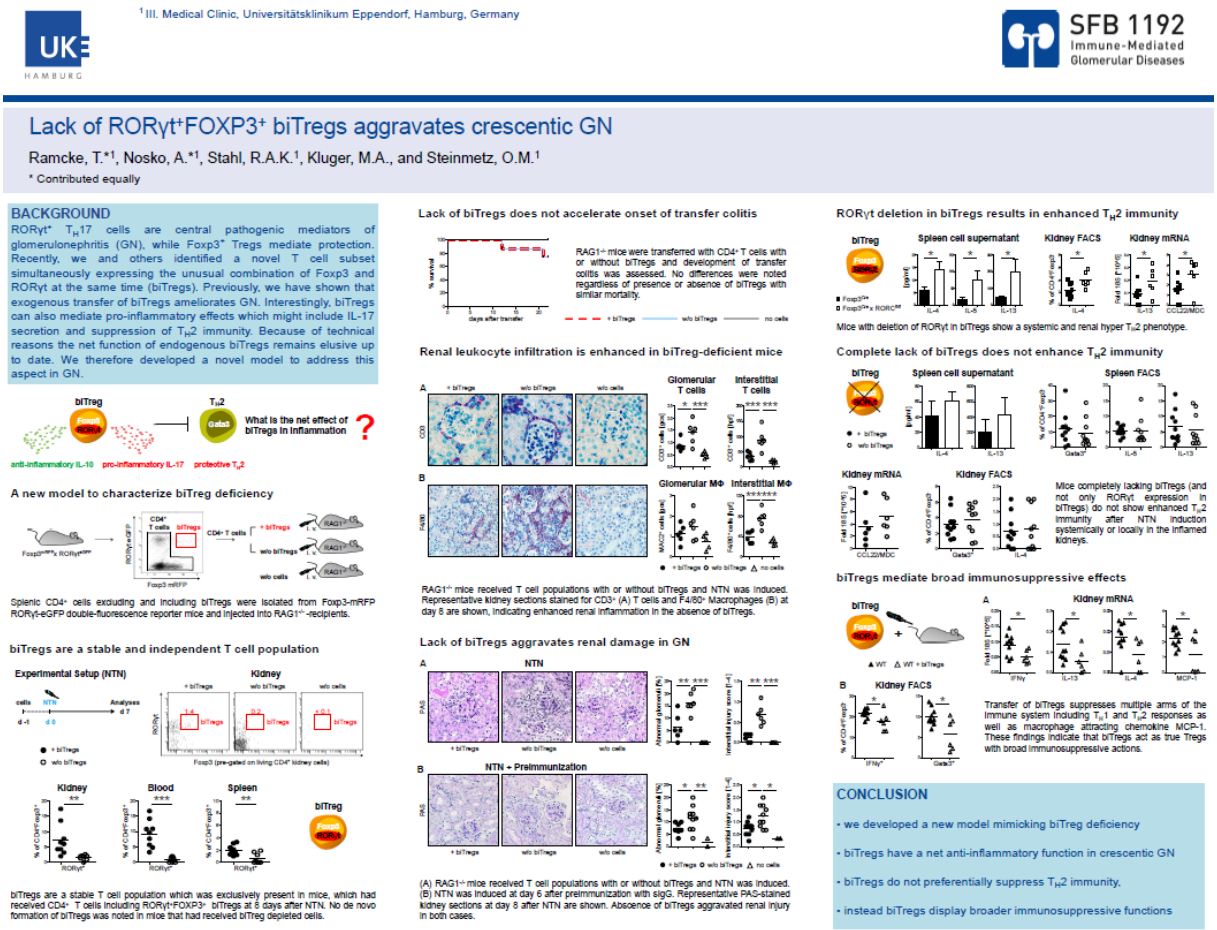
Keywords: immunology, lupus nephritis, lymphocytes, systemic lupus, transcription factors

Introduction

Systemic lupus erythematosus (SLE) is a complex and relatively common autoimmune disorder, which can affect multiple organs including skin, joints, lungs, kidneys and the central nervous system [1,2]. As SLE causes high morbidity and mortality in a rather young collective of patients, the search for new therapeutic strategies is a priority for scientists worldwide [3,4]. Despite intensive research, the events which lead to development of SLE and eventually cause the associated organ pathologies still remain widely elusive [5,6]. However, a central role for CD4$^+$ T helper cells in disease pathogenesis has been demonstrated by multiple studies in mice and humans, including our own [7–11]. Recently, we and others particularly highlighted the importance of the T helper type 17/interleukin-17 (Th17/IL-17) axis for systemic autoimmunity and lupus nephritis [10,12,13]. Importantly, mice deficient in IL-17A or F were shown to be protected from disease in

2.2 Part two

Partial results of this dissertation were presented with the title "Lack of RORγt⁺Foxp3⁺ biTregs aggravates crescentic GN" as poster presentation at the DGfN congress on September 15, 2017 in Mannheim and at the ASN congress on November 02, 2017 in New Orleans. The presented data were honoured by the ´poster award´ of the DGfN.



3 Introduction

3.1 Architecture and function of the kidney

Similar to organs such as lung, heart or the central nervous system, the kidneys are essential for human living. They are a pair of organs situated in the retroperitoneum (Schünke, 2012), which provide several vital functions for the human organism. The renal function ensures a strictly regulated homeostatic environment, comprising the salt and volume household, the blood pressure and the acid-base balance. Moreover, the kidney is crucial for elimination of toxic, water soluble-substances and it is involved in bone metabolism and erythropoiesis (Hoenig and Zeidel, 2014, Blaine et al., 2015, Zeisberg and Kalluri, 2015).

At microscopic level, the kidney is composed of many functional subunits called nephrons. The nephron is for histologic and functional reasons subdivided in renal corpuscle, proximal and distal tubules. The comprehension of this microscopic architecture is helpful to understand, how the kidney fulfils its role in maintaining the homeostasis in our body. The glomerulus, as part of the renal corpuscle, is the localisation, where the primary urine is filtered from the blood. The ultrafiltrate resembles plasma composition, however compared to plasma, the primary urine does not contain larger proteins or cells, which are held back by the glomerular filtration barrier. The proximal and distal tubules modify the primary urine by utilizing active and passive transport mechanisms to recover or secrete substances and water, depending on current needs of the organism. This need-orientated function serves to maintain a homeostatic internal environment (Hoenig and Zeidel, 2014).

Taken together, the kidneys represent a complex organ system, whose extrinsic and intrinsic functions are crucial for the entire human organism.

3.2 Glomerulonephritis (GN)

Some human diseases result in inflammation of the renal glomerulus; this state is called glomerulonephritis (GN). Since the ultrafiltrate production represents the elementary step for the renal function, it is obvious that inflamed glomeruli threaten and

restrict the proper function of the kidney. In accordance with these considerations, glomerulonephritis has a high clinical relevance in the field of nephrology, since it is a frequent cause of acute and/or chronic renal failure (Stahl and Hoxha, 2016).

Glomerulonephritis represents a heterogenous group of diseases combining different underlying etiopathologic entities. However, all forms of glomerulonephritis have in common, that the endogenous immune system is dysregulated, which finally results in inflammation and destruction of the glomeruli (Couser, 2016).

A particularly severe form of human glomerulonephritis is clinically classified as RPGN (rapidly progressive glomerulonephritis), which rapidly results in renal failure. At microscopic level, RPGN is characterized by glomerular necrosis and crescent formation. This form of GN is found in the course of different disease entities including ANCA vasculitis, systemic lupus erythematosus (SLE), IgA vasculitis or Goodpasture syndrome (Stahl and Hoxha, 2016). The therapeutic options for these diseases are limited, non-specific and highly toxic, so that novel strategies are urgently needed. One promising target cell population are regulatory T cells (Tregs), which have been shown to potently protect from renal tissue injury in multiple experimental models of GN (Wolf et al., 2005, Ooi et al., 2011, Paust et al., 2011, Kluger et al., 2014, Alikhan et al., 2018). Even more importantly, recent pioneering studies have also shown great promise of Treg directed therapies for human autoimmune diseases (Konig et al., 2016, Bluestone and Tang, 2018, Duggleby et al., 2018).

3.3 RORγt$^+$Foxp3$^+$ T cells (biTregs) - novel players of the immune system

In autoimmune diseases like GN, the immune system, which normally attacks foreign threats in order to defend the body, is dysregulated and attacks endogenous structures. As a consequence, vital organs, like the kidneys, are getting destroyed and are not capable anymore to sustain their biological function. This inflammatory, self-destructive process is mediated by both, the innate and adaptive arm of the immune system (Kurts et al., 2013).

Many studies of the past have revealed, that RORγt$^+$ Th17 cells and T-Bet$^+$ Th1 cells play a crucial role in initiating and promoting the self-destructive autoimmune process leading to glomerular dysfunction (Steinmetz et al., 2011, Disteldorf et al., 2015, Hunemorder et al., 2015, Paust et al., 2015, Schmidt et al., 2015). On the contrary, it

has also been shown that Foxp3$^+$ regulatory T cells (Tregs) are able to supress inflammation and thereby attenuate the course of glomerulonephritis (Hagenstein et al., 2019, Nosko et al., 2017, Kluger et al., 2016, Kluger et al., 2014, Paust et al., 2011, Ooi et al., 2011, Wolf et al., 2005).

Recently, a novel and unique T cell population has been identified by our lab group and others, showing the unusual co-activation of the Th17 cell defining transcription factor RORγt together with the Treg defining transcription factor Foxp3 (Lochner et al., 2008, Kluger et al., 2016). This unconventional transcriptional equipment was shown to mediate both anti- but also pro-inflammatory functions in a context dependent manner. We thus proposed the operational name bifunctional Tregs (biTregs) (Kluger et al., 2016). In order to get an impression, what is known about this fascinating T cell subtype so far, relevant scientific findings on RORγt$^+$Foxp3$^+$ T cells, especially in the context of autoimmunity, are summarized below.

3.3.1 biTregs occur in health and several murine and human diseases

After discovery and first characterisation in healthy mice (Lochner et al., 2008) and humans (Ayyoub et al., 2009, Voo et al., 2009), further reports could demonstrate that biTregs can be found in the course of various disease states with different pathogenesis. In detail, RORγt$^+$Foxp3$^+$ cells were detected in human periodontitis (Okui et al., 2012) and in the course of human chronic inflammatory bowel diseases (Sefik et al., 2015, Hovhannisyan et al., 2011). In addition, biTregs can be found in the course of tumour diseases; e. g. in the environment of human (Blatner et al., 2012) or murine colorectal cancer (Downs-Canner et al., 2017) and in the course of ovarian cancer in mice (Downs-Canner et al., 2017). Moreover, biTregs expand during the course of several autoimmune diseases including human psoriasis (Bovenschen et al., 2011), human childhood arthritis (Pesenacker et al., 2013) and murine autoimmune-triggered diabetes (Tartar et al., 2010). Especially of note, biTregs were also found to massively expand in the course of acute crescentic glomerulonephritis in mice (Kluger et al., 2016). In line with their presence in different murine and human disease entities, it is conceivable that biTregs play a key disease-modifying role in above mentioned pathologies.

3.3.2 Expression profile of biTregs

Considering that biTregs activate the Th17 cell defining transcription factor RORγt together with the Treg defining transcription factor Foxp3, it is an interesting issue, whether biTregs show the ability to produce characteristic gene products of both T cell subsets. Indeed, in accordance with their transcriptional profile, biTregs activate Treg- and Th17-cell characteristic genes at the same time (Kluger et al., 2016, Ohnmacht et al., 2015, Lochner et al., 2008).

In fact, several reports revealed that biTregs show high expression levels of Th17 cell-associated genes like **IL-17** (Kluger et al., 2017, Kim et al., 2017, Kluger et al., 2016, Hovhannisyan et al., 2011, Bovenschen et al., 2011, Voo et al., 2009, Osorio et al., 2008), **IL-21** (Hovhannisyan et al., 2011), **IL-22** (Kim et al., 2017, Hovhannisyan et al., 2011), **IL1R1** (Kim et al., 2017, Yang et al., 2016), **IL-23-receptor** (Kim et al., 2017, Sefik et al., 2015, Pesenacker et al., 2013, Osorio et al., 2008), **CCR6** (Kim et al., 2017, Kluger et al., 2016, Pesenacker et al., 2013, Hovhannisyan et al., 2011, Voo et al., 2009, Ayyoub et al., 2009, Lochner et al., 2008) and **Ikzf3** ***(Aiolos)*** (Downs-Canner et al., 2017, Yang et al., 2016).

On the contrary, in line with its Foxp3 activation, biTregs also show high expression levels of Treg-phenotypical genes including **IL-10** (Yang et al., 2016, Kluger et al., 2016), the **subunit EBI-3 of IL-35** (Kluger et al., 2016), **CD39** (Downs-Canner et al., 2017, Ohnmacht et al., 2015), **CD73** (Ohnmacht et al., 2015), **CD101** (Hovhannisyan et al., 2011), **CD103** (Yang et al., 2016, Kluger et al., 2016, Hovhannisyan et al., 2011), **CTLA-4** (Kim et al., 2017, Yang et al., 2016, Kluger et al., 2016, Ohnmacht et al., 2015), **ICOS** (Kim et al., 2017, Downs-Canner et al., 2017, Yang et al., 2016, Kluger et al., 2016, Ohnmacht et al., 2015, Li et al., 2012, Lochner et al., 2008) and **PD-1** (Downs-Canner et al., 2017, Kluger et al., 2016).

In addition, biTregs are characterised by enhanced levels of **Runx1, Runx2, Runx3** (Li et al., 2012) and **c-Maf** (Xu et al., 2018, Yang et al., 2016), proteins considered to equip biTregs with characteristic functions, which will be delineated in detail later. Collectively, these data demonstrate impressively that RORγt^{+}Foxp3^{+} biTregs co-express Th17 cell- and cTreg-characteristic genes and proteins (Lochner et al., 2008). However, it should be noted that biTregs also activate genes such as **Runx3**, which are switched off in both, Th17 cells and cTregs (Li et al., 2012), indicating that biTregs indeed represent a stable and independent T cell lineage.

3.3.3 RORγt^{+}Foxp3^{+} T cells – an independent T cell population or an intermediate between cTreg and Th17 cell transdifferentiation?

Next, due to the fact that biTregs activate two different master transcription factors, it is a fascinating question, whether RORγt^{+}Foxp3^{+} cells represent a stable T cell population with distinct effector functions, or just an unstable intermediate during a transdifferentiation processes between cTregs and Th17 cells. In this regard, a strong debate in the field of biTreg research was ignited. Some reports propose that biTregs are able to convert into RORγt single positive Th17 cells, in particular during autoimmune processes (Komatsu et al., 2014, Bailey-Bucktrout et al., 2013, Bovenschen et al., 2011, Zhou et al., 2009). Another report, which concentrated on investigating immune cell dynamics during tumour diseases, revealed that biTregs are able to cease RORγt expression and consequently serve as precursor cell for Foxp3 single positive cTregs (Downs-Canner et al., 2017).

On the other hand, there are also several findings rather in favour of the hypothesis, that biTregs represent an individual and stable T cell population with distinct effector functions. In detail, molecular analyses show, that RORγt^{+}Foxp3^{+} double positive cells exhibit epigenetic characteristics of a stable regulatory T cell population including a highly demethylated Treg specific demethylation region (TSDR) at the Foxp3 locus (Yang et al., 2016, Pesenacker et al., 2013) as well as highly demethylated Treg-characteristic genes, such as Ctla-4, Gitr, Eos, and Helios (Yang et al., 2016).

Additionally, fate reporter studies demonstrated that biTregs do not convert into RORγt or Foxp3 single positive cells (Yang et al., 2016, Kluger et al., 2016), suggesting that biTregs do not transdifferentiate into their transcriptional relatives Th17 cells nor into cTregs *in vivo*.

Taken together, there is still some debate, whether biTregs represent an intermediate between Th17 cell and cTreg transdifferentiation or an independent, stable T cell population. However, the vast majority of more recent studies indicate, that biTregs represent an independent T cell lineage (Yang et al., 2016, Kluger et al., 2016).

3.3.4 biTregs mediate immunoregulative effects *in vitro* and *in vivo*

It is well established, that activation of the transcription factor Foxp3 is essential for regulatory T cells to acquire immunoregulatory effector mechanisms (Feuerer et al.,

2009). Considering that biTregs also activate Foxp3, it was crucial to characterize, if this novel T cell is capable to mediate immunosuppression, too.

As a matter of fact, several *in vitro* experiments revealed unanimously that human (Pesenacker et al., 2013, Hovhannisyan et al., 2011, Voo et al., 2009, Ayyoub et al., 2009) and murine (Li et al., 2012, Lochner et al., 2008) biTregs, sometimes defined as IL-17 producing Tregs, have the ability to supress proliferation of stimulated $CD4^+$ T cells. Consequently, these results advise that biTregs acquire *in vitro* immunoregulatory capacity. Additionally, several assumptions have been made to what extent other intrinsic molecules, in addition to Foxp3, contribute to biTreg's regulatory capacity. Some studies propose that biTreg's co-defining transcription factor RORγt impairs the suppressive capacity of biTregs (Blatner et al., 2012). In contrast, other reports rather suggest that activation of RORγt may even enhance the suppressive function of biTregs (Yang et al., 2016, Li et al., 2012, Lochner et al., 2008). Beside the unknown effects of RORγt activation on biTreg suppressive capacity, another highly-ranked study indicates, that activation of the transcription factor c-Maf, could also enhance suppressive functions (Xu et al., 2018).

Importantly, in addition to being immunosuppressive *in vitro*, several studies revealed that $ROR\gamma t^+Foxp3^+$are also able to mediate potent immunoregulative effects *in vivo*, including the NTN model of GN, colitis and insulitis (Lochner et al., 2008, Sefik et al., 2015, Kluger et al., 2016, Kim et al., 2017, Xu et al., 2018).

3.3.5 RORγt-deletion in biTregs results in a hyper type 2 immune phenotype *in vivo*

After having ascertained that biTregs are able to suppress T cells *in vitro* (Lochner et al., 2008) and *in vivo* (Lochner et al., 2008, Kluger et al., 2016), it was of course important to elicit, whether distinct immune players are controlled by biTregs. Interestingly, a highly-ranked study by Ohmacht et al. suggest that biTregs may represent a specific and potent suppressor of Th2 cell immunity, since RORγt-deletion in (bi)Tregs *($Foxp3^{Cre}$ x $RORC^{fl/fl}$ mice)* resulted in a pronounced Th2 cell phenotype in the gut and consequently caused aggravation of Th2 cell-driven intestinal pathologies (Ohnmacht et al., 2015). Ohmacht et al. proposed, that biTregs counteract type 2 immunity by modulating expression of the co-stimulatory proteins CD80 and CD86 on antigen-presenting cells in a CTLA-4-dependant manner (Ohnmacht et al., 2015). Furthermore, in

this context, it has been speculated that activation of the transcription factor IRF4 qualifies biTregs to mediate Th2 cell-specific regulatory effects (Ohnmacht et al., 2015). Interestingly and conversely to data by Ohnmacht et al., another highly ranking report by Sefik et al., which also studied mice bearing RORγt-deficient (bi)Tregs, could not confirm any skewing of Th2 responses (Sefik et al., 2015). biTreg effects on Th2 responses thus remained a matter of debate.

Moreover, in this context, some studies proposed, that biTregs primarily utilize their suppressive function to immunoregulate Th17 and Th1 cell dominant immune responses, in particular in the context of autoimmune diseases (Sefik et al., 2015). In line with the concept that some Treg subclasses are tailor-made to supress distinct immune responses, it was hypothesized, that biTregs are specialized for downregulating Th17 cells (Lochner et al., 2008, Kim et al., 2017, Xu et al., 2018). However, no study has so far provided any evidence for this hypothesis.

In summary, the scientific community is not in agreement, which particular T cell subtype is preferentially suppressed by biTregs. Alternatively, it would not be groundless to consider, that biTregs may mediate broad-immunoregulation, supressing T effector cells independently of the subtype.

3.3.6 biTregs mediate Th17 cell-like, pro-inflammatory effects

Besides Treg-characteristic Foxp3 activation, biTregs simultaneously upregulate the Th17 cell defining transcription factor RORγt. Of course, this extraordinary transcriptional profile raised the central question, if biTregs have the capability to mediate pro-inflammatory effects, apart from immunoregulation. Indeed, it has been shown that biTregs are able to secrete IL-17, the hallmark pro-inflammatory cytokine of Th17 immune responses (Osorio et al., 2008, Voo et al., 2009, Bovenschen et al., 2011, Hovhannisyan et al., 2011, Kluger et al., 2016, Kim et al., 2017). To better characterize potential pro-inflammatory effects mediated by RORγt activation in biTregs, *in vivo* studies were performed by us, which used mice bearing RORγt-deficient (bi)Tregs (*Foxp3^{Cre} x RORC$^{fl/fl}$ mice)*. Interestingly, these studies revealed that the transcription factor RORγt indeed equips biTregs with pro-inflammatory effects. Specific RORγt deletion in biTregs ameliorated the course of acute crescentic NTN glomerulonephritis (Kluger et al., 2016). Remarkably, this experiment also demonstrated that IL-17 production by biTregs was completely dependent on RORγt activation. Taken together,

available literature suggests, that biTregs display a bifunctional role in autoimmune processes. On the one hand, several reports showed, that biTregs, in line with their Foxp3 activation, are able to mediate protective immunregulation (Lochner et al., 2008, Blatner et al., 2012, Kluger et al., 2016, Yang et al., 2016, Xu et al., 2018). On the other hand, in association with RORγt activation, biTregs are also able to mediate Th17 cell-characteristic, pro-inflammatory effects (Kluger et al., 2016, Blatner et al., 2012, Voo et al., 2009). The net effect of biTregs in inflammation, as well as the conditions under which pro- and/or anti-inflammatory biTreg functions dominate, currently remain unclear.

4 Investigated object

As explained above, past studies have uncovered, that biTregs have immunoregulatory properties (Lochner et al., 2008, Kluger et al., 2016). Interestingly, however, RORγt deletion in Tregs prevented, rather than aggravated glomerular injury in the NTN model of acute GN. This finding indicated, that RORγt activation equipped biTregs with additional pro-inflammatory features *(Figure 1),* as e.g. production of IL-17 (Kluger et al., 2016). The net function of biTregs in inflammation thus remains unclear to date.

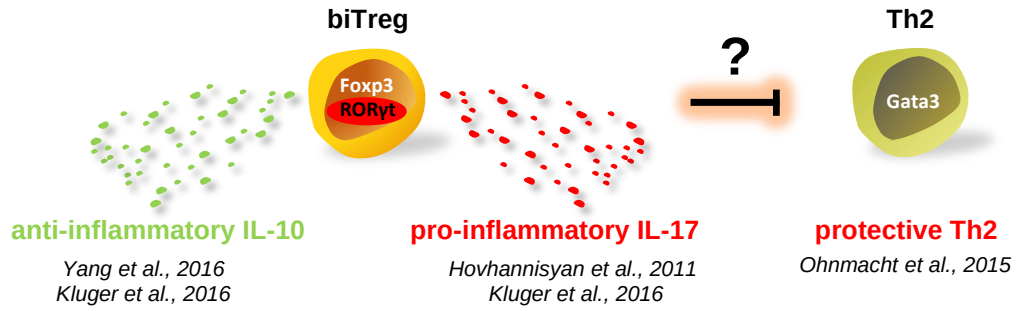


Figure 1. biTregs display pro- and anti-inflammatory effects

Previous studies have identified a novel and unique Treg subpopulation, which is characterized by expression of the Treg characteristic transcription factor Foxp3 together with the Th17 characteristic RORγt. In line with this unusual combination of transcription factors, biTregs were shown to produce anti-inflammatory mediators as IL-10, IL-35 and TGF-ß but also pro-inflammatory IL-17. Furthermore, recent studies have speculated, that RORγt^{+}Foxp3^{+} Tregs suppress protective Th2 responses. Given these dual functions, we have proposed the operational name bifunctional Tregs (biTregs).

Importantly, it also becomes clear from these data, that RORγt-deletion in biTregs is probably not the same as complete absence of biTregs. The effects of complete biTreg absence have not been studied so far. Furthermore, the role of biTregs in chronically developing forms of nephritis as e.g. pristane-induced lupus nephritis *(Figure 2)* also remains unknown.

This dissertation therefore aimed to address these open questions and analysed *(Figure 2)*

1) function of biTregs in lupus nephritis

2) effects of biTregs on immune responses with a focus on Th2 and Th17 immunity

3) the net effect of complete absence of biTregs in the course of acute glomerulonephritis.

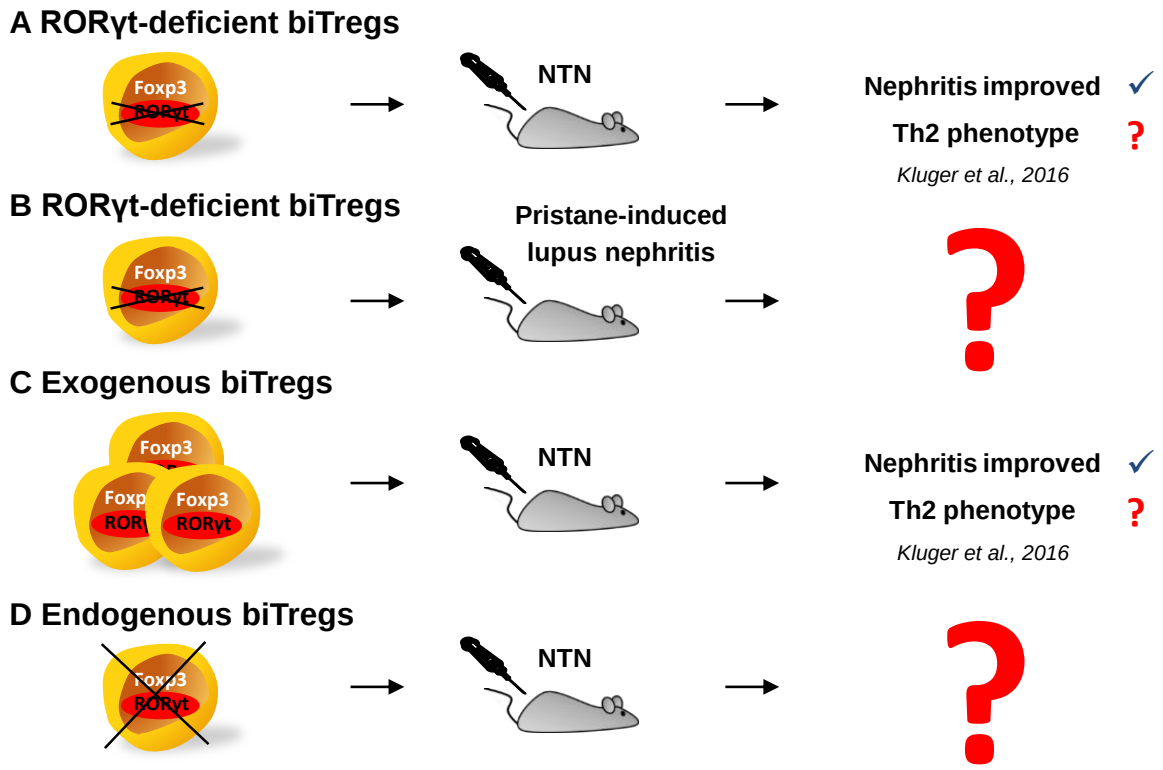


Figure 2. Characterisation of biTreg functions in glomerulonephritis

(A) A recent study has shown, that RORγt equips biTregs with pro-inflammatory properties in the course of acute glomerulonephritis, since RORγt-deleted biTregs ameliorated the course of NTN. (B) Whether RORγt deletion in biTregs would also ameliorate the course of chronically developing lupus nephritis remained unknown. (C) Infusion of exogenous biTregs protects the kidney from NTN glomerulonephritis. Whether exogenous biTregs affect type 2 immunity has not yet been studied (D) The role of endogenous biTregs in acute and chronic glomerulonephritis is completely unknown to date.

Unfortunately, for technical reasons, a selective biTreg knock-out model is currently not available. Therefore, we initially needed to establish a novel model, mimicking the absence of biTregs.

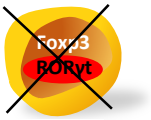
4.1 Overview of the goals of this dissertation

1. **Characterize the role of RORγt activation in biTregs in pristane-induced lupus nephritis**

pristane lupus

- How does RORγt activation in biTregs influence the course of pristane-induced lupus nephritis?
- Does RORγt deletion in Tregs change the regulatory capacity and/or IL-17 productive capacity of Tregs?
- Does RORγt-deletion in biTregs result in altered type 2 immune responses in pristane-induced lupus?

2. **Establishing a murine model which allows to characterize the function of endogenous biTregs**



NTN

- Do biTregs represent a stable and independent T cell lineage or do they transdifferentiate from or into Th17 cells or cTregs?
- Are endogenous biTregs protective or disease-promoting as net effect in NTN glomerulonephritis?
- Do endogenous biTregs influence Th1, Th2, Th17 cell or other Treg responses?

5 Material

5.1 Chemicals, nucleotides and sera

Item	Manufacturer
Brefeldin A	**Sigma-Aldrich**; *St. Louis, Missouri, USA*
Collagenase D	**Roche**; *Basel, Switzerland*
Complete Freund's Adjuvant	**Sigma-Aldrich**; *St. Louis, Missouri, USA*
DNase I *(Deoxyribonuclease I)*	**Roche**; *Basel, Switzerland*
Ethanol *(ethanol washing row for histology)*	**Th. Geyer**; *Hamburg, Germany*
Fetal calf serum *(10 %)*	**Gibco™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
Formaldehyde *(37 %)*	**Carl Roth**; *Karlsruhe, Germany*
HEPES *(1 M) 2-(4-(2-hydroxy-ethyl)-1-piperazineethanesul-fonic acid)*	**Gibco™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
Live/dead staining *(detection with violet laser at 405 ηm)*	**Gibco™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
Nephrotoxic nephritis (NTN) serum *(charge SS 10-11)*	**Eurogentec**; *Seraing, Liège, Belgium*
Normal mouse serum	**Jackson Immuno Research**; *Ely, UK*
Paraplast plus® *(tissue embed-ding medium)*	**McCormick Scientific**; *St. Louis, Missouri, USA*
PCR primers *(sequences upon request available)*	**Thermo Fisher Scientific**; *Waltham, Massa-chusetts, USA*
PCR water *(H_2O)*	**B. Braun Melsungen**; *Melsungen, Germany*
Penicillin-Streptomycin	**Gibco™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
Percoll® solution *(37 %)*	**GE Healthcare**; *Chicago, Illinois, USA*
PMA *(Phorbol-12-myristate-13-acetate)*	**Merck**; *Darmstadt, Germany*
Pristane oil *(2,6,10,14-Tetra-methylpentadecane)*	**Sigma-Aldrich**; *St. Louis, Missouri, USA*
Sheep immunoglobulin G	**Sigma-Aldrich**; *St. Louis, Missouri, USA*
ß-Mercaptoethanol	**Gibco™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
SYBR™ Green qPCR Mix	**Invitrogen™, Thermo Fisher Scientific**; *Wal-tham, Massachusetts, USA*
Trypan Blue *(cell counting)*	**Gibco™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*

Table 1. Overview of applied chemicals, nucleotides and sera

5.2 Buffers and solutions

Buffer/Solution	Ingredients	Manufacturer
Erylysis buffer component 1	*Tris(hydroxymethyl)-amino-methane in distilled H_20 at PH 7,6 (4,12 g Tris solved in 170 ml H_20, 4 ml 25 % HCl added and finally filled with 200 ml distilled water)*	**Self-made**
Erylysis buffer component 2	*144 mM NH_4Cl in distilled water*	**Self-made**
HBSS *(Hank's balanced salt solution)*	*0,14 M NaCl; 0,005 M KCl; 0,001 M $CaCl_2$; 0,0004 M $MgSO_4$-7H_2O; 0,0003 M Na_2HPO_4-2H_2O; 0,0005 M $MgCl_2$-6H_2O; 0,0004 M KH_2PO_4; 0,004 M $NaHCO_3$; 0,006 M Glucose in distilled water*	**Gibco™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
MACS® buffer	*0,5 % bovine serum albumin, 2 mM EDTA in PBS*	**Miltenyi Biotec**; *Bergisch Gladbach, Germany*
PBS *(Phosphate-buffered saline)*	*0,137 M NaCl; 0,0027 M KCl; 0,01 M Na_2HPO_4; 0,0018 M KH_2PO_4 in distilled water*	**Gibco™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
RPMI-1640 medium	*Including amino acids, vitamins and glucose (detailed information on the manufacture's internet page)*	**Gibco™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*

Table 2. Overview of applied buffers and solutions

5.3 Antibodies for Flow Cytometry - Surface staining

FACS antibody	Manufacturer
anti-CD25 antibody	**BD Bioscience**; *Franklin Lakes, New Jersey, USA*
anti-CD3 antibody	**BD Bioscience**; *Franklin Lakes, New Jersey, USA*
anti-CD4 antibody	**BD Bioscience**; *Franklin Lakes, New Jersey, USA*
anti-CD44 antibody	**BD Bioscience**; *Franklin Lakes, New Jersey, USA*
anti-CD45 antibody	**BD Bioscience**; *Franklin Lakes, New Jersey, USA*
anti-CD62L antibody	**BD Bioscience**; *Franklin Lakes, New Jersey, USA*
anti-CD69 antibody	**BD Bioscience**; *Franklin Lakes, New Jersey, USA*
anti-CD8 antibody	**BD Bioscience**; *Franklin Lakes, New Jersey, USA*
anti-CTLA4 antibody	**BD Bioscience**; *Franklin Lakes, New Jersey, USA*
anti-IL-4 antibody	**eBioscience™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
anti-IL-5 antibody	**eBioscience™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*

Table 3. Overview of applied surface antibodies used for Flow Cytometry

5.4 Antibodies for Flow Cytometry - Intracellular staining

FACS antibody	Manufacturer
anti-Foxp3 antibody	**eBioscience™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
anti-Gata-3 antibody	**BioLegend**; *San Diego, California, USA*
anti-IFNγ antibody	**eBioscience™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
anti-IL-13 antibody	**eBioscience™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
anti-IL-17 antibody	**eBioscience™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
anti-Ki67 antibody	**eBioscience™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
anti-RORγt antibody	**BD Bioscience,** *Franklin Lakes, New Jersey, USA*
anti-T-Bet antibody	**eBioscience™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*

Table 4. Overview of applied intracellular antibodies utilized for Flow Cytometry

5.5 Antibodies applied for immunohistologic analyses

Antibody	Manufacturer
anti-CD3 antibody	**Dako**; *Hamburg, Germany*
anti-Foxp3 antibody	**eBioscience™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
anti-GR-1 antibody	**Hycult Biotech**; *Uden, The Netherlands*
anti-Ki67 antibody	**Cell Signaling Technology**; *Danvers, Massachusetts, USA*
anti-Mac-2 antibody	**Cedarlane-Laboratories**; *Burlington, Ontario, Canada*
anti-F4/80 antibody	**BMA Biomedicals**; *Hiddenhausen; Germany*

Table 5. Overview of applied antibodies for immunohistologic analyses

5.6 Devices, instruments and software

Device/Instrument/Software	Manufacturer
Axio Vison software	**Carl Zeiss Microscopy**; *Jena, Germany*
AxioCam HRc	**Carl Zeiss Microscopy**; *Jena, Germany*
Axioscope Light Microscopy	**Carl Zeiss Microscopy**; *Jena, Germany*
BD^{TM} ARIAIII Cytometer	**BD Bioscience**; *Heidelberg, Germany*
BD^{TM} LSRII Flow Cytometry system	**BD Bioscience**; *Heidelberg, Germany*
FlowJo software	**Tree Star**; *Ashland, Oregon, USA*
GraphPad Prism 5 software	**GraphPad Software**; *San Diego, California, USA*

HERAcell® CO_2 Incubator	**Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
Meshes *(50 µm and 70 µm)*	**Falcon™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
Microsoft® Office Word	**Microsoft**; *Redmond, Washington, USA*
Multifuge X3R *(Centrifuge)*	**Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
StepOnePlus Real-Time PCR System	**Applied Biosystems™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
TC20™ Automated Cell Counter	**Bio-Rad**; *Hercules, California, USA*
Thermocyler	**Biometra**; *Göttingen, Germany*

Table 6. Overview of applied devices, instruments and software

5.7 Commercial kits

Kit	Manufacturer
Albumin ELISA kit	**Bethyl Laboratories**; *Montgomery, Texas, USA*
Antibody-alkaline phosphatase kit	**Zytomed Systems**; *Berlin, Germany*
Cytometric Bead Array (CBA) kit	**BD Bioscience**; *Franklin Lakes, New Jersey, USA*
$CD4^+$ T cell isolation kit	**Miltenyi Biotec**; *Bergisch Gladbach, Germany*
Foxp3 staining kit	**eBioscience™, Thermo Fisher Scientific**; *Waltham, Massachusetts, USA*
gentleMACS™ Dissociator	**Miltenyi Biotec**; *Bergisch Gladbach, Germany*
IL-13 ELISA kit	**Invitrogen™, Thermo Fisher Scientific***; Waltham, Massachusetts, USA*
IL-4 ELISA kit	**BioLegend**; *San Diego, California, USA*
IL-5 ELISA kit	**BioLegend**; *San Diego, California, USA*
Immunoglobulin E ELISA kit	**BioLegend**; *San Diego, California, USA*
LEGENDplex™ kit	**BioLegend**; *San Diego, California, USA*
Micro tube 1.3 ml K3E *(EDTA tube)*	**Sarstedt**; *Nümbrecht, Germany*
NucleoSpin© technology	**Machery-Nagel**; *Düren, Germany*

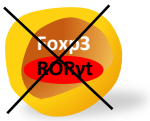
Table 7. Overview of applied commercial kits

6 Methods

6.1 Deletion of RORγt in biTregs and induction of pristane-induced SLE and NTN glomerulonephritis

RORC$^{fl/fl}$ mice were obtained from Jackson Laboratory (Jackson Laboratory; *Bar Harbor, Maine, USA*). Foxp3$^{YFP\text{-}Cre}$ mice were a generous gift from Alexander Y. Rudensky (Memorial Sloan-Kettering Cancer Centre; *New York, USA*). FoxP3$^{Cre\ x}$ RORC$^{fl/fl}$ mice were generated by intercrossing. In these mice, a Cre recombinase is activated under control of the Foxp3 locus, which results in selective excision of RORC in Tregs. To induce systemic lupus erythematosus (SLE), Foxp3$^{Cre\ x}$ RORC$^{fl/fl}$ mice or Foxp3$^{Cre\ x}$ RORC$^{wt/wt}$ control mice were treated intraperitoneally with 500 µl pristane oil *(2,6,10,14-Tetramethylpentadecane)* (Sigma-Aldrich; *St. Louis, Missouri, USA*). Organ removal and analyses were performed at the indicated time points. In order to study NTN, Foxp3$^{Cre\ x}$ RORC$^{fl/fl}$ mice and *Foxp3$^{Cre\ x}$ RORC$^{wt/wt}$* control mice were challenged intraperitoneally with nephrotoxic sheep serum (Assmann et al., 1985). For accelerated NTN (aNTN), mice were s.c. pre-immunized with 0,5 mg normal sheep globulin in Freund´s complete adjuvant. Subsequently, NTN was induced by intraperitoneal injection of nephrotoxic serum. Organ removal and analyses were performed at the indicated time points.

6.2 Establishment of a model mimicking biTreg deficiency



In order to mimick absence of biTregs, we transferred biTreg-depleted or bi-Treg competent CD4^{+} T cells into RAG1$^{-/-}$ recipient mice as explained below. BAC transgenic Ror(γt)-GFP^{TG} x FIR (Foxp3-IRES-mRFP) mice (Foxp3-RORγt double reporter), kindly provided by Gerard Eberl (Institut Pasteur; Paris, France) and Mathias Lochner (Department of Infection and Immunology; Hanover, Germany), were used to allow separation of biTregs from non-biTreg CD4^{+} T cells.

6.2.1 Donor cell isolation

To isolate cells, spleens from Foxp3-RORγt double reporter mice were harvested and stored in HBSS medium (Gibco™, Thermo Fisher Scientific*; Waltham, Massachusetts;*

USA). To get access to individual cells, spleens were passed through 70 µm meshes. Crushed remnants in the meshes were washed with HBSS. In the next step, spleen cell suspensions were centrifuged, decanted and resuspended. Subsequently, the Miltenyi Biotec $CD4^+$ isolation kit (Miltenyi Biotec*; Bergisch Gladbach, Germany*) was used to separate $CD4^+$ T cells according to the manufacturers´ manual. Subsequently, $CD4^+$ T cell enriched suspensions were centrifuged, resuspended in 3 ml MACS® buffer (Miltenyi Biotec; *Bergisch Gladbach, Germany*) and incubated for 30 min with fluorescent anti-CD4-antibodies (BD Bioscience*; Franklin Lakes, New Jersey, USA*) and anti-CD3 antibodies (BD Bioscience; *Franklin Lakes, New Jersey, USA*). After 30 min incubation time, centrifugation and resuspension in phosphate-buffered saline (PBS) (Gibco™, Thermo Fisher Scientific*; Waltham, Massachusetts; USA*), cells were handed to the institutional FACS core unit (University Medical Center Hamburg-Eppendorf (UKE); *Hamburg, Germany*) to sort biTreg-deficient $CD4^+$ T cells and biTregs. Cells were sorted on a BD ARIAIII Cytometer (BD Bioscience; *Heidelberg, Germany*).

6.2.2 Transfer of donor cells and induction of NTN GN

One group of $RAG1^{-/-}$ mice was i.v. injected with $CD4^+$ T cells including biTregs, whereas the other group of $RAG1^{-/-}$ mice received biTreg-depleted $CD4^+$ T cells. Then, transferred mice were challenged with NTN serum. In some experiments, accelerated NTN (aNTN) was induced. Different modifications, in terms of injected cell composition, pre-immunisation *(aNTN model)*, dose of NTN serum, days between transfer, NTN induction and organ removal have been performed. The individual modalities are illustrated in *Table 8*.

	Composition of transferred cells	sIgG	NTN *(charge SS 10-11)*	Organ Removal
Set 1	1,6 x 10^6 biTreg-deficient $CD4^+$ cells + 400.000 biTregs vs. 2 x 10^6 biTreg-deficient $CD4^+$ cells	500 µg, 8 days after transfer	0,8 ml 14 days after transfer	16 days after transfer
Set 2	2 x 10^6 $CD4^+$ biTreg-deficient $CD4^+$ cells + 500.000 biTregs vs. 2 x 10^6 biTreg-deficient $CD4^+$ cells	500 µg, 6 days after transfer	0,6 ml 12 after transfer	20 days after transfer
Set 3	1,8 x 10^6 biTreg-deficient $CD4^+$ cells + 200.000 biTregs vs. 2 x 10^6 biTreg-deficient $CD4^+$ cells	-	0,7 ml one day after transfer	8 days after transfer
Set 4	2 x 10^6 biTreg-deficient $CD4^+$ cells + 200.000 biTregs vs. 2 x 10^6 biTreg-deficient $CD4^+$ cells	-	-	22 days after transfer
Set 5	1,5 x 10^6 biTreg-deficient $CD4^+$ cells + 200.000 biTregs vs. 1,5 x 10^6 biTreg-deficient $CD4^+$ cells	-	0,65 ml NTN 7 days after transfer	15 days after transfer

Table 8. Transfer model to study endogenous biTregs: Overview of set specific modalities

6.3 Transfer of exogenous biTregs to treat NTN

200.000 biTregs FACsorted from Ror(γt)-GFP^{TG} x FIR (Foxp3-IRES-mRFP) double reporter mice, were i.v. injected into the tail vein of C57BL/6 wildtype mice. The control group was treated with phosphate-buffered saline (PBS). To induce NTN, nephrotoxic sheep serum was injected intraperitoneally one day later. Organs were removed and analysed at day 7.

6.4 Animal ethics

Animal experiments were performed in accordance with institutional and national animal and ethical guidelines. Approvals for experiments were confirmed by local comities *(approval codes G37/11, G45/12, 73/14 and 07/15)*. All mice were on C57BL/6 background and kept in pathogen-free surroundings.

6.5 Cell isolation from various organs

6.5.1 Spleen

Spleens were harvested and stored in HBSS medium. In the next step, organs were passed through 70 µm sieves. Afterwards, erythrocytes were depleted by using 2 ml erylysis working solution *(combination of erylysis stock solution 1 and 2; in relation 1:9)* containing ammonium chloride. To get rid of remaining cell detritus, spleen cell suspensions were passed through 40 µm meshes. Finally, spleen cells were counted, utilizing an automatic cell counter, and resuspended in PBS to perform flow cytometry analyses or cell culture studies.

6.5.2 Kidney

Firstly, after organ removal, the capsula fibrosa renalis was manually removed and a transversal renal slice was kept in formalin. After formalin fixation overnight, this part of the kidney was washed with ethanol and consequently embedded with paraffine to perform periodic acid Schiff (PAS) staining, allowing to evaluate the microscopic morphologic shape of the kidney. The processing of samples for histologic analyses was performed using standard laboratory protocols. For FACS analysis, the remaining part

of the kidneys was minced and incubated for 45 min at 37 °C in digestion medium (*RPMI-1640 medium* (Gibco™, Thermo Fisher Scientific*; Waltham, Massachusetts; USA*) *including 10 % fetal calf serum* (Gibco™, Thermo Fisher Scientific*; Waltham, Massachusetts; USA*)*, 1 % HEPES* (Gibco™, Thermo Fisher Scientific*; Waltham, Massachusetts; USA*)*, 1 % Penicillin-Streptomycin* (Gibco™, Thermo Fisher Scientific*; Waltham, Massachusetts; USA*)*, 8 µg/ml Collagenase D (*Roche*; Basel, Switzerland) and 0,4 µg/ml DNase I (*Roche*; Basel, Switzerland))* to get rid of extracellular matrix components. Subsequently, digested kidneys were dissociated into single cell suspensions by using a commercial tissue dissociation system (Miltenyi Biotec; Bergisch *Gladbach, Germany*). Afterwards, to enrich renal cells and to separate cell detritus, cells were resuspended in 37 % Percoll®-solution (GE Healthcare; *Chicago, Illinois, USA*). The Percoll®-cell solution was centrifugated for 20 min at room temperature at 500 × g to concentrate alive renal cells at the bottom of the tube. Finally, after decantation, purified renal cells were resuspended in PBS to perform flow cytometric analyses.

6.5.3 Lung

After lung removal, a pulmonary lobe was separated and filled with 500 µl formalin to prepare histologic and immunohistologic analyses. After fixation overnight, this part of the lung was washed with ethanol and consequently embedded with paraffine to perform periodic acid Schiff (PAS) staining allowing to evaluate pulmonary morphology. The remaining part of the lung was utilized to perform single cell analyses using flow cytometry. To get a single cell suspension for flow cytometric analysis, the remaining lung was minced after organ removal and incubated with digestion medium (*RPMI-1640 medium* (Gibco™, Thermo Fisher Scientific*; Waltham, Massachusetts; USA*) *including 10 % fetal calf serum* (Gibco™, Thermo Fisher Scientific*; Waltham, Massachusetts; USA*)*, 1 % HEPES* (Gibco™, Thermo Fisher Scientific*; Waltham, Massachusetts; USA*)*, 1 % Penicillin-Streptomycin* (Gibco™, Thermo Fisher Scientific*; Waltham, Massachusetts; USA*)*, 8 µg/ml Collagenase D (*Roche*; Basel, Switzerland) and 0,4 µg/ml DNase I (*Roche*; Basel, Switzerland)*) for 45 min at 37 °C to reduce matrix components. Afterwards, lung samples were dissociated into single cell suspensions, using a commercial tissue dissociation kit (Miltenyi Biotec; *Bergisch Gladbach, Germany*). To remove left-over cell detritus, cell suspensions were passed through 70

µm sieves. Afterwards, pulmonary cells were further purified by applying Percoll®-density gradient (GE Healthcare; *Chicago, Illinois, USA*). The Percoll®-cell solution was centrifugated at 500 × g for 20 min at room temperature to enrich alive lung cells at the bottom of the tube. After decantation, purified lung cells were resuspended in PBS, allowing to perform flow cytometric analyses.

6.5.4 Blood cells

Retrobulbar blood samples were collected in conventional EDTA tubes (Sarstedt; *Nümbrecht, Germany*) immediately before sacrifice. In the next step, blood samples were centrifugated to separate cellular blood components from serum. To perform flow cytometric analyses, blood cells were depleted of erythrocytes by using ammonium chloride *(method described above, 6.5.1.)*, washed and resolved in PBS. Serum was used for studying blood urea nitrogen concentration and immunoglobulin titers.

6.5.5 Peritoneum

To analyse peritoneal cell composition, 5 ml of phosphate-buffered saline (PBS) were injected into the peritoneal space and removed after 10 seconds. Afterwards, the single cell solution was analysed by flow cytometry.

6.6 **Flow cytometry**

At first, single cell suspensions from blood and different organs were activated with PMA (50 ng/ml; Sigma-Aldrich), Ionomycin (1 µg/ml; Calbiochem-Merck) and Brefeldin A (10 µg/ml; Sigma-Aldrich) for 3 h to enrich expressed intracellular molekules for subsequent intracellular staining procedure. After washing, cells were incubated with 0,25 µg/µl normal mouse serum (Jackson Immuno Research; *Ely, UK*) for 20 min at 4 °C to reduce unspecific antibody-antigen binding. Then, after washing, probes were incubated for 20 min at room temperature with fluorescent antibodies provided by BD Bioscience (BD Bioscience*, Franklin Lakes; New Jersey; USA*) to mark the cell surface molecules CD3, CD4, CD8, CD25, CD44, CD45, CD62L, CD69 and CTLA4. After washing with PBS, dead cells were marked with live/dead staining provided by Thermo Fisher Scientific following manufacture’s advice (Thermo Fisher Scientific*; Waltham, Massachusetts; USA*).

To perform intracellular and intranuclear staining, cells were permeabilized utilizing the commercial Foxp3 staining kit provided by Eboscience (eBioscience™, Thermo Fisher Scientific; *Waltham, Massachusetts, USA*). Cells were incubated for 30 min at 4 °C with fluorescent-labelled antibodies against IL-4, IL-5, IL-13, IL-17, IFNγ, Foxp3, Ki67, T-Bet (eBioscience™, Thermo Fisher Scientific; *Waltham, Massachusetts, USA*), Gata-3 (BioLegend; *San Diego, California, USA*) and RORγt (BD Bioscience; *Franklin Lakes, New Jersey, USA*). After washing and resuspension in PBS, samples were analysed on a BD LSRII Flow Cytometry system (BD Bioscience; *Heidelberg, Germany*) and consequently assessed using FlowJo software (Tree Star; *Ashland, Oregon, USA*).

6.7 **Morphologic studies**

6.7.1 Evaluation of renal morphology

Renal damage was quantified analysing PAS-stained renal slices in a blinded manner as described previously (Steinmetz et al., 2011). Glomerular damage was determined, evaluating 50 glomeruli per kidney slice. Abnormal glomeruli included glomerular hypercellularity, crescent formation, fibrinoid necrosis, segmental proliferation, hyalinosis and capillary wall thickening. Interstitial morphology was studied by analysing 15 cortical, randomly chosen high power fields (HPF) at ×200 magnification. Interstitial damage was quantified, evaluating tubular morphology *(epithelial cell oedema, tubular dilatation, tubular cell atrophy and tubular cell-sloughing)*, interstitial cell infiltration and expansion of renal tubular basement membrane. Correlating with the amount of injury, each cortical area was scored with whole-numbers between 0 and 4. Kidney sections showing no interstitial damage were scored with „0“. The declared score „1“ implies that the interstitial damage affected less than 25 % of the observed area, „2“ affected between 25 % and less than 50 %, „3“ affected between 50 % and less than 75 % and „4“ affected 75 % or more of the observed high power field (Steinmetz et al., 2011).

6.7.2 Evaluation of pulmonary morphology

Pulmonary damage was determined by quantifying the extent of granuloma formation, pulmonary haemorrhage or alveolar-wall thickening using PAS-stained lung slices. A semiquantitative score was applied (0= 0 % affected, 1= less than 25 % affected, 2=

25 % - less than 50 % affected, 3= 50% - less than 75 % affected, 4= 75 - 100 % affected). The damage score was averaged for 15 high power fields at ×200 magnification. The number of pulmonary granulomas was counted in 10 low power fields at ×100 magnification. The size of the granuloma was measured by using Axio Vison software (Carl Zeiss; *Jena, Germany*).

6.7.3 Immunohistochemical investigations

Renal or lung slices were embedded in paraffine and afterwards coated with antibodies against CD3 (clone A0452; Dako; *Hamburg, Germany*), F4/80 (clone BM8; BMA Biomedicals; *Hiddenhausen, Germany*), GR-1 (clone NIMP-R14; Hycult Biotech; *Uden, The Netherland*s), Foxp3 (clone FJK-16s; eBioscience™, Thermo Fisher Scientific; *Waltham, Massachusetts, USA*), Mac-2 (clone M3/38; Cedarlane-Laboratories; *Burlington, Ontario, Canada*) or Ki67 (clone D3B5; Cell Signaling Technology; *Danvers, Massachusetts, USA*). To visualize bound antibodies, a secondary antibody-alkaline phosphatase kit was used (Zytomed Systems; *Berlin, Germany*). Cell infiltration was analysed by counting 30 randomly chosen cortical fields at ×200 magnification. Sections were analysed by using an Axioscope Light Microscopy (Carl Zeiss Microscopy; *Jena, Germany*). Histological photographies were captured with an AxioCam HRc (Carl Zeiss Microscopy; *Jena, Germany*) at indicated magnifications.

6.8 Assessment of renal function

To obtain urine, mice were kept in metabolic cages for 5 hours with free access to water. Albuminuria was quantified, using a standard albumin ELISA kit (Bethyl Laboratories; *Texas, USA*). Blood urea nitrogen and urinary creatinine measurments were performed by the institutional clincal chemistry (University Medical Center Hamburg-Eppendorf (UKE); *Hamburg, Germany*).

6.9 Analysis of cytokines from spleen cell cultures

4 x 10^6 splenocytes per ml were co-incubated with 10 µg/ml sheep Immunoglobulin G (IgG)-antibodies (Sigma-Aldrich; *Taufkirchen, Germany*) for 72 hours under standard cell culture conditions *(RPMI* (Gibco™, Thermo Fisher Scientific*; Waltham,*

Massachusetts; USA) *including 10 % fetal calf serum* (Gibco™, Thermo Fisher Scientific*; Waltham, Massachusetts; USA*)*, 1 % HEPES* (Gibco™, Thermo Fisher Scientific*; Waltham, Massachusetts; USA*)*, 1 % Penicillin-Streptomycin* (Gibco™, Thermo Fisher Scientific*; Waltham, Massachusetts; USA*) *and 0,1 % ß-Mercaptoethanol* (Gibco™, Thermo Fisher Scientific*; Waltham, Massachusetts; USA*). Subsequently, spleen cell supernatants were harvested and analysed by applying commercial ELISA kits to quantify IL-4 (BioLegend*; San Diego, California; USA*), IL-5 (BioLegend; *San Diego, California, USA*) and IL-13 (Invitrogen™, Thermo Fisher Scientific; *Waltham, Massachusetts, USA*) burden. Alternativly, cytokine concentrations of spleen cell supernatants were quantified by using Cytometric Bead Array (CBA)-technique (BD Bioscience; *Franklin Lakes, New Jersey, USA*).

6.10 **Analysis of humoral immune responses**

Immunoglobin E (IgE) concentration in the serum was analysed by using a commercial ELISA kit (BioLegend; *San Diego, California, USA*). Analyses were performed at indicated dilutions.

6.11 **Semiquantitative real time mRNA analysis**

RNA was analysed using quantitative real-time polymerase chain reaction (qRT-PCR). Renal RNA was isolated from cortex by applying the commercial NucleoSpin© kit (MACHEREY-NAGEL; *Düren, Germany*). Afterwards, renal RNA was transcribed into complementary DNA on a Biometra thermocycler (Biometra; *Göttingen, Germany*) using a standard protocol. Real time polymerase chain reaction was performed for 40 cycles in presence of 1,5 µl complementary DNA (cDNA), 6,25 µl of SYBR™ Green qPCR Mix (Invitrogen™, Thermo Fisher Scientific; *Waltham, Massachusetts, USA*), 2,25 µl PCR water (B. Braun Melsungen; *Melsungen, Germany*) and 1,25 µl (0,9 µmol/l) of each murine primer pair (Thermo Fisher Scientific; *Waltham, Massachusetts, USA*). One cycle included the following procedure: 1. Initial denaturation for 10 min at 95 °C; 2. Denaturation procedure for 15 seconds at 95 °C; 3. Primer annealing and elongation for 1 min at 60 C°. Samples were analysed in duplicate and normalized to parallel running levels of 18S ribosomal RNA. The qRT-PCR procedure was performed

on a StepOnePlus Real-Time PCR System (Applied Biosystems™, Thermo Fisher Scientific; *Waltham, Massachusetts, USA*).

6.12 Statistical Analysis

GraphPad Prism 5 software (GraphPad Software; *San Diego, California, USA*) was used to calculate statistical parameters. A statistically significant difference was declared as p-value ≤ 0.05. If two groups were compared, the two-tailed t test was used. More than two groups were compared by ANOVA with Tukey post hoc testing.

7 Results

7.1 The role of RORγt activation in biTregs during pristane-induced lupus

In order to study the role of RORγt activation in Tregs during the course of lupus nephritis, FoxP3$^{Cre\ x}$ RORC$^{fl/fl}$ mice and respective FoxP3^{Cre} controls were i.p. treated with pristane oil (Reeves et al., 2009).

7.1.1 Characterization of pathologic changes during pristane-induced lupus

To better understand the time course of pathologic changes in pristane-induced lupus, occurrence of organ manifestations was studied. At the early disease state, mice develop an acute sterile peritonitis and non-immune complex pulmonary capillaritis. After three weeks, lympho-granuloma formation was detected in murine lungs, indicating that the adaptive cellular immune response, was taking place. In week 8, first measurable levels of autoantibodies appeared in the serum, showing that a humoral autoimmune component had been established. 5 months after disease induction, mice started to develop an immune-complex nephritis as a consequence of renal auto-antibody deposition and nephritogenic autoimmunity. An overview of organ-specific disease manifestations is demonstrated in *Figure 3*.

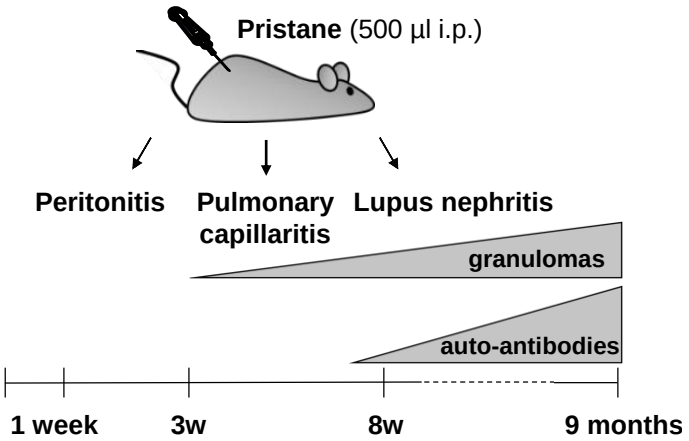


Figure 3. Overview of organ-specific disease manifestations in the course of the pristane-induced lupus

The temporal course of pristane induced inflammation and organ specific pathologies is shown. Intraperitoneal injection leads to development of acute innate mediated peritonitis and non-immune complex pulmonary capillaritis. Subsequently, peritoneal and pulmonary granulomas develop and auto-antibody formation is initiated. During the following months, immune complex lupus nephritis develops.

Figure published in Clinical and Experimental Immunology, 2017, volume 188, 63-78.

7.1.2 RORγt expression in biTregs aggravates the course of pulmonary and renal damage in the pristane-induced lupus model

Next, we aimed to evaluate, whether RORγt deletion in biTregs influences the course of pristane-induced disease manifestations. Importantly, *Figure 4* demonstrates that pulmonary vasculitis and granuloma formation were significantly reduced in Foxp3^{Cre} x RORC$^{fl/fl}$ mice. In line with ameliorated injury, immunohistological analyses revealed that pulmonary infiltration of neutrophils and T cells was reduced in mice containing RORγt-deficient biTregs.

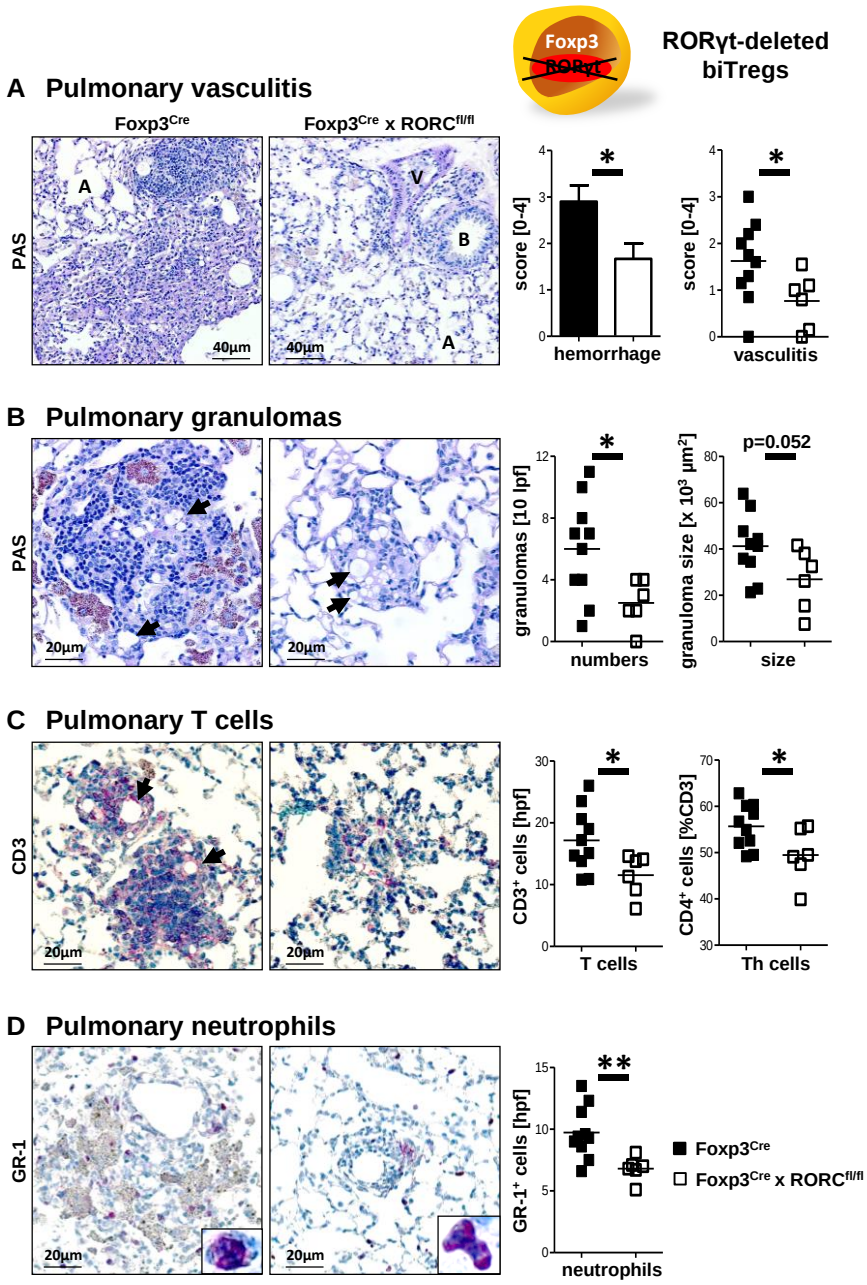


Figure 4. Deletion of RORγt in biTregs ameliorated pulmonary disease manifestation in the pristane-induced lupus model

(A) Representative photograph of periodic acid-Schiff (PAS)-stained lungs from indicated mouse strains at 8 weeks after pristane injection (left). Quantification of macroscopic hemorrhage as well as vasculitis score is shown (right) (original magnification ×200). (B) Representative photographs of pulmonary granulomas and quantification of granuloma numbers and size (original magnification ×400). (C) Immunohistochemical staining for pan T cell marker CD3 (left). Quantification of pulmonary CD3$^+$ T cell numbers by immunohistochemistry and CD4$^+$ T helper cell percentages by FACS as indicated (original magnification ×200). (D) Immunohistochemical staining and quantification of infiltrating granulocyte-differentiation antigen-1 (GR-1$^+$) neutrophils (original magnification ×200). A: alveolus, B: bronchus, V: vessel, arrows indicate pristane droplets. Squares represent individual animals; horizontal lines indicate means. Bars show mean ± SEM. * p<0.05, ** p<0.01

Figure published in Clinical and Experimental Immunology, 2017, volume 188, 63-78.

Taken together, these results consistently show, that activation of the transcription factor RORγt in biTregs aggravates pulmonary capillaritis in the pristane-induced lupus model.

Subsequently, we aimed to study, whether RORγt-deletion in biTregs could change the course of immune complex glomerulonephritis in pristane-induced lupus. Indeed, GN was significantly ameliorated in mice containing RORγt-deficient biTregs, as evidenced by less glomerular injury. In addition, Foxp3^{Cre} x RORC$^{fl/fl}$ mice revealed reduced glomerular proliferating cells. Amelioration of morphologic renal damage was accompanied by better preservation of renal function in mice containing RORγt-deleted biTregs, since blood urea nitrogen levels and albuminuria were significantly lower *(Figure 5)*.

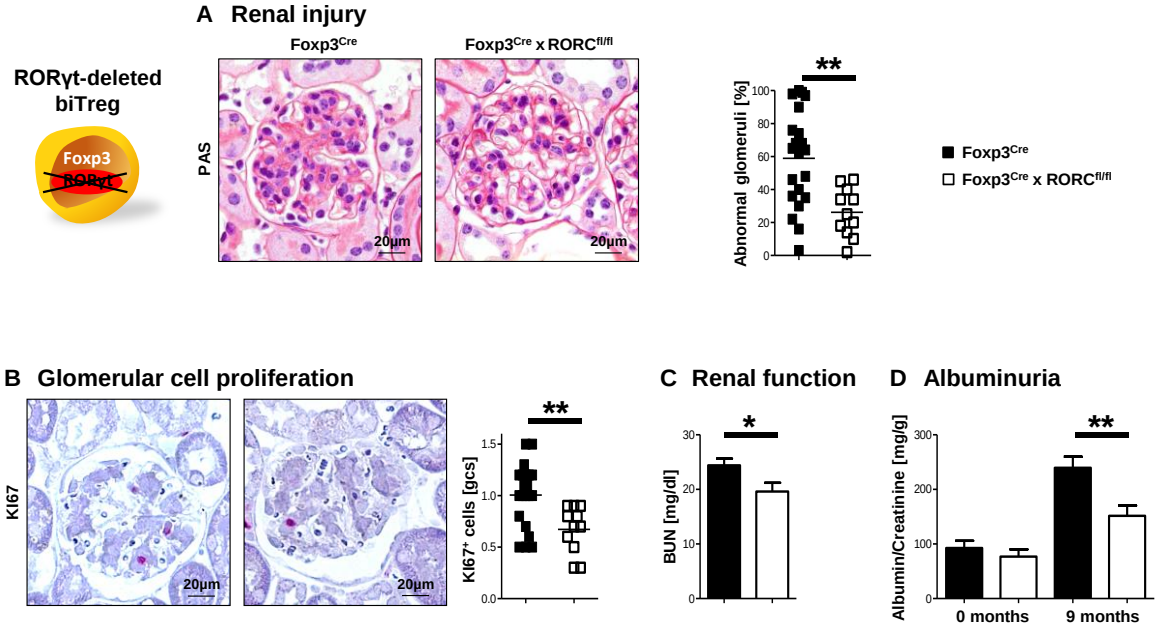


Figure 5. RORγt expression in biTregs aggravates immune complex glomerulonephritis

(A) Representative photographs of periodic acid-Schiff (PAS)-stained kidney sections at 9 months after pristane injection, as well as quantification of glomerular injury (original magnification ×400). (B) Representative photographs of immunohistochemical staining of KI67 and quantification of glomerular KI67^{+} cells (original magnification ×400). (C) Quantification of serum BUN levels. (D) Quantification of urinary Albumin/Creatinine ratios at the indicated time points before and after injection of pristane. Bars show mean ± SEM. Squares represent individual animals; horizontal lines indicate means. * p<0.05, ** p<0.01.

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Additionally, in line with morphologic and functional amelioration, Foxp3^{Cre} x RORC$^{fl/fl}$ mice revealed a significantly lower infiltration of adaptive and innate immune cells into the kidney, compared to wildtype mice. This once more showed, that the pro-inflammatory, kidney-destructive immune process was ameliorated by deletion of RORγt in

biTregs *(Figure 6)*. Taken together, in accordance with our previous report studying the NTN model (Kluger et al., 2016), deletion of RORγt in biTregs resulted in broad anti-inflammatory effects during the course of pristane-induced lupus.

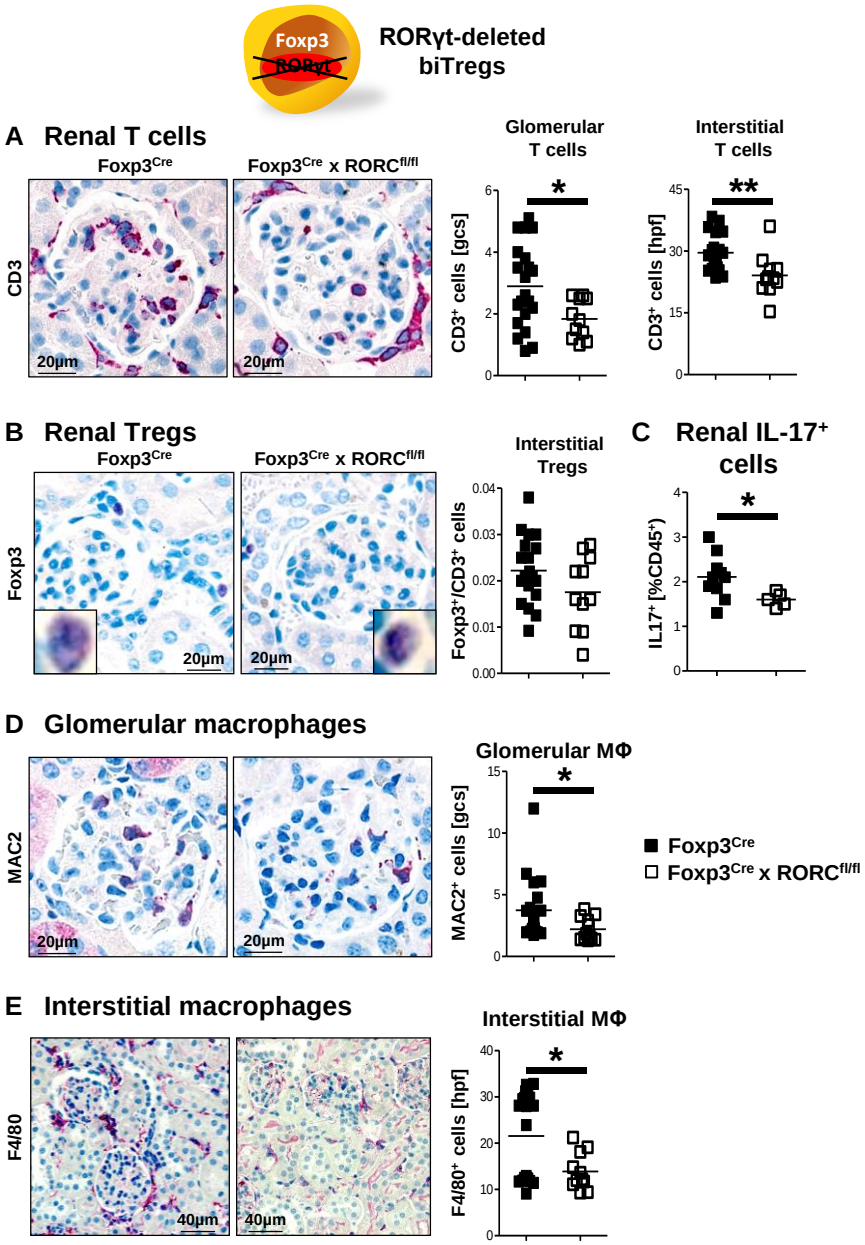

Figure 6. RORγt deletion in biTregs resulted in reduced immune cell infiltration into the kidneys

Representative photographs and quantification of glomerular and interstitial renal (A) $CD3^+$ T cell and (B) forkhead box protein ($FoxP3^+$) regulatory T cell (T_{reg}) infiltration at 9 months after pristane injection (original magnification ×400). (C) Fluorescence activated cell sorter (FACS) analysis of total renal infiltrating interleukin (IL)−17^+ leucocytes (one of two sets shown). (D) Representative photographs and quantification of glomerular MAC-2^+ monocyte/macrophage infiltration (original magnification ×400). (E) Representative photographs and quantification of renal interstitial $F4/80^+$ monocyte/macrophage infiltration (original magnification 200x). Squares represent individual animals; horizontal lines indicate means. * $p<0.05$, ** $p<0.01$.

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7.1.3 RORγt-deleted Tregs lose the ability to produce IL-17 and show enhanced immunoregulatory capacity in the course of SLE

Next, we aimed to identify the mechanisms leading to the observed amelioration of organ manifestation in pristane induced SLE, if Tregs lack RORγt activation.

It is well known that RORγt initiates production of several pro-inflammatory cytokines, among others, the Th17 hallmark cytokine IL-17. Therefore, we focused on investigating, whether the absence of RORγt influences the IL-17 secretion profile of regulatory T cells. Importantly, as shown in *Figure 7*, all regulatory T cell in Foxp3^{Cre} x RORC$^{fl/fl}$ mice did not activate RORγt, indicating that the applied genetic mouse model was fully functional. In the next step, analyses of wild type mice revealed that a significant part of Foxp3^{+} Tregs produced IL-17. In contrast, and notably, RORγt-deleted Tregs were no longer able to secrete pro-inflammatory IL-17 in all organs studied. Hence, these results show that RORγt is crucial for Tregs to be able to produce Th17's hallmark cytokine IL-17.

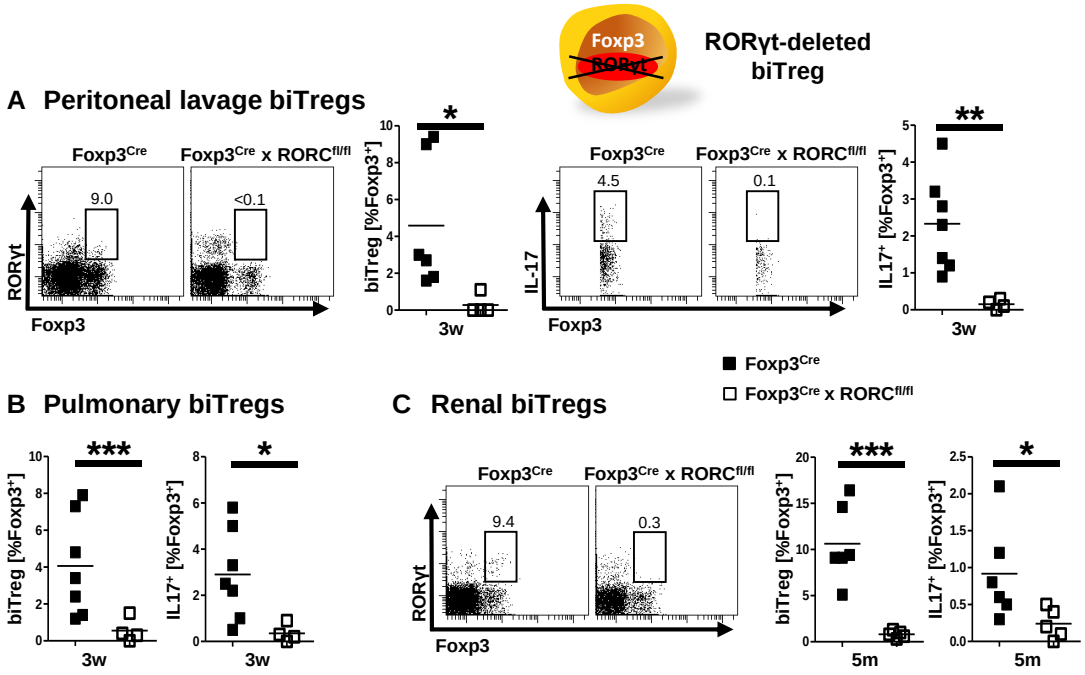

Figure 7. RORγt-deficient Tregs are not able to produce IL-17 in the course of pristane-induced lupus

(A) A representative FACS plot and quantification of peritoneal lavage biTregs (left) as well as IL-17 production by biTregs (right) at 3 weeks after pristane injection into Foxp3^{Cre} wildtype and Foxp3^{Cre} x RORC$^{fl/fl}$ mice (right). (B) Quantification of pulmonary biTregs (left) and IL-17 production (right) at 3 weeks after pristane injection into the indicated mouse strains. (C) A representative FACS plot and quantification of renal biTregs (left) and their IL-17 production (right) at 5 months after pristane injection. Numbers in FACS plots indicate percentages of Foxp3^{+} cells. Squares represent individual animals; horizontal lines indicate means. * p<0.05, ** p<0.01, *** p<0.001.

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In addition, our analyses concentrated on characterizing in detail to what extent the regulatory capacity of the whole Treg compartment changes, if RORγt activation is specifically interrupted in Foxp3$^+$ Tregs *(Figure 8)*. Our analyses indicated, that RORγt-deleted Tregs are characterized by higher levels of Foxp3. Furthermore, Tregs, which are not able to activate RORγt, reveal higher amounts of Treg-characteristic surface markers, a pronounced activation status and an enhanced proliferation tendency. Taking these observations into account, the results indicate, that RORγt deleted Tregs show an enhanced regulatory phenotype in the course of SLE.

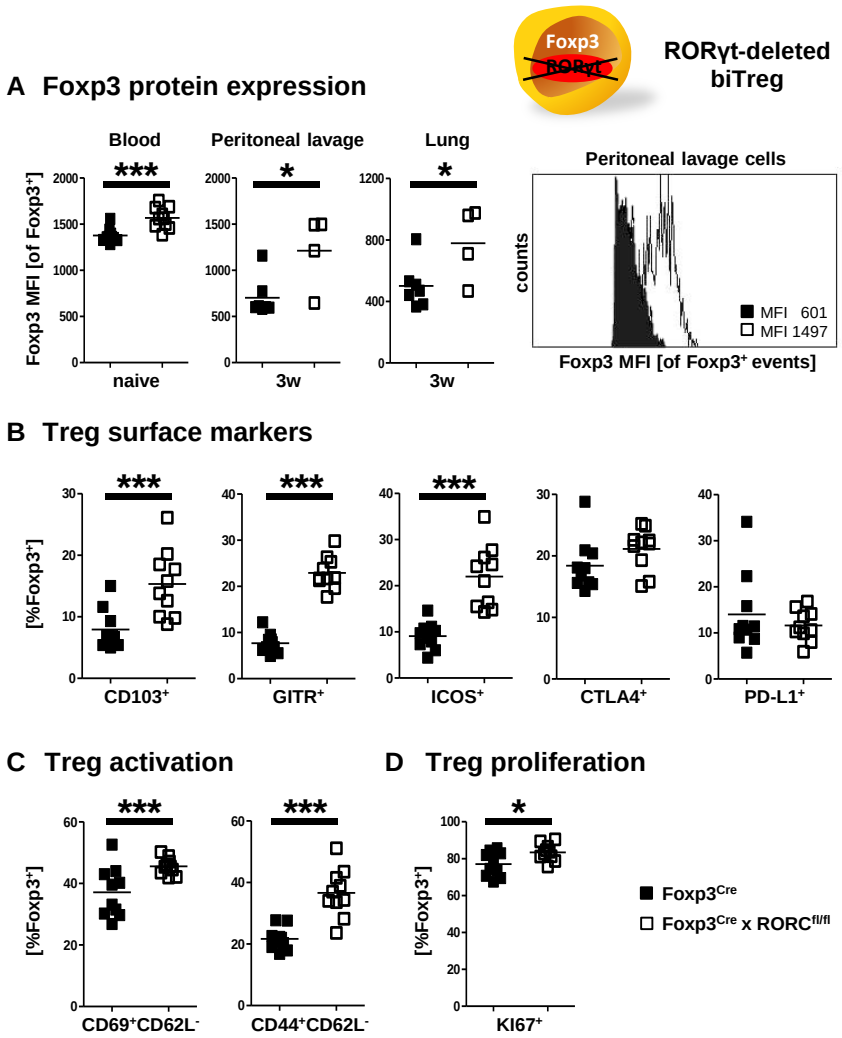

Figure 8. Foxp3+ Tregs are more activated and show higher levels of immunoregulatory surface markers, if their RORγt activation is interupted

(A) Quantification of Foxp3 MFI in Tregs from blood of naïve mice as well as peritoneal lavage and pulmonary Tregs at 3 weeks after pristane injection from indicated mouse strains (left) and a representative FACS plot of Foxp3 MFI on peritoneal lavage Tregs (right). (B) Analysis of the indicated surface markers on Tregs from blood of naïve mice. (C) Quantification of activation markers on Tregs from blood of naïve mice of the indicated genotypes. (D) Assessment of Treg proliferative activity by FACS analysis of KI67 expression on naïve blood Tregs. Squares represent individual animals; horizontal lines indicate means. * p<0.05, *** p<0.001.

In conclusion, our results indicate that amelioration of lupus in mice bearing RORγt-deleted Tregs may be due to two facts. Firstly, deletion of RORγt in Tregs ceases production of pro-inflammatory IL-17. Secondly, RORγt-lacking Tregs possess enhanced regulatory *in vivo* capacity.

7.1.4 biTregs supress type 2 immunity in a RORγt-dependent manner in the course of pristane-induced lupus

Next, inspired by a previous report, showing that Foxp3^{Cre} x RORC$^{fl/fl}$ mice revealed a hyper type 2 immune phenotype in the gut (Ohnmacht et al., 2015), it was aimed to characterize whether Foxp3^{Cre} x RORC$^{fl/fl}$ mice also revealed an exacerbated Th2 phenotype in the course of the pristane-induced lupus model. First of all, Th2 responses were studied in naive Foxp3^{Cre} x RORC$^{fl/fl}$ mice. Indeed, genetic abrogation of biTregs´ RORC expression resulted in a pronounced hyper type 2 immune phenotype in the blood *(Figure 9)*. To specify, whether this observation was preserved in the

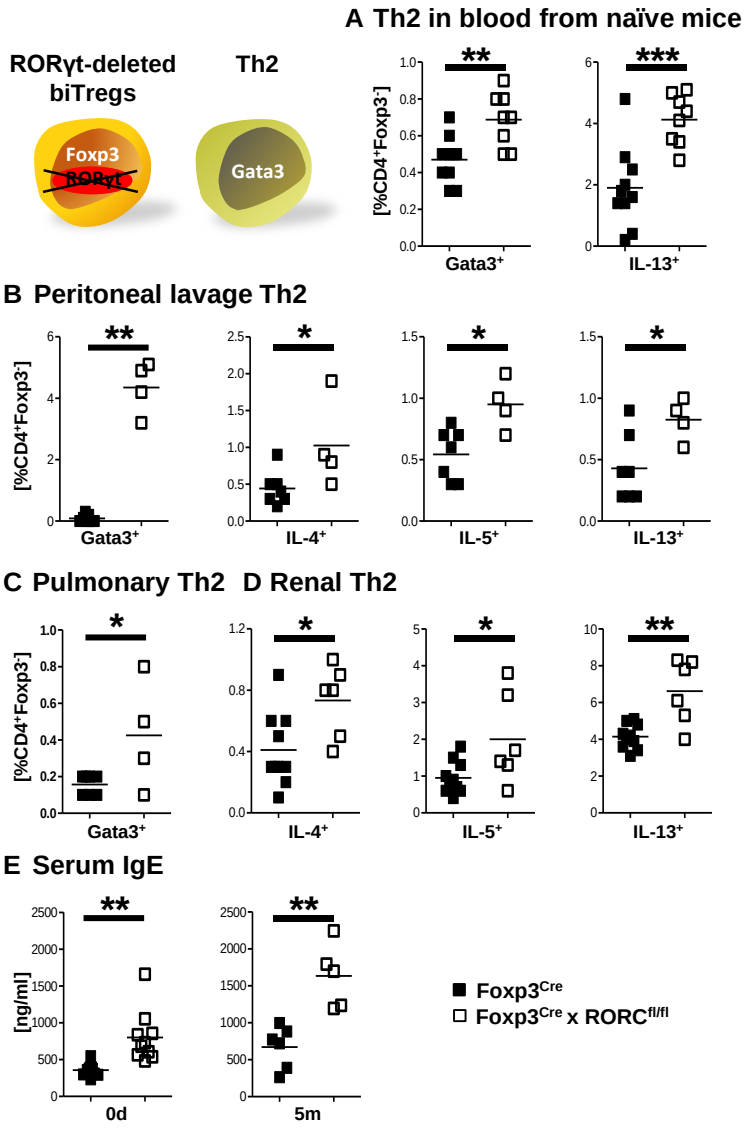

Figure 9. Type 2 immunity was exacerbated in Foxp3^{Cre} x RORC$^{fl/fl}$ mice in the course of pristane-induced lupus

(A) Quantification of T helper (Th) cells expressing the Th2-characteristic transcription factor Gata3 or the Th2 prototype cytokine IL-13 in peripheral blood from naïve mice by flow cytometry. (B) Flow cytometric analysis of peritoneal T helper cells expressing Gata3 or the indicated Th2 prototype cytokines from peritoneal lavage cells 3 weeks after pristane injection. (C) Gata3 expression by T helper cells in lungs at 3 weeks after pristane injection. (D) Quantification of renal T helper cells expressing the indicated Th2 cytokines at 2 months after pristane injection. (E) Quantification of immunoglobulin (Ig)E levels by enzyme-linked immunosorbent assay (ELISA) from serum of naïve mice and at 5 months after pristane injection. Squares represent individual animals; horizontal lines indicate means. * p<0.05, ** p<0.01, *** p<0.001.

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course of pristane-induced lupus, type 2 immunity was intensively investigated after pristane application. Again, cells expressing Th2-hallmark molecules showed higher frequencies in animals containing RORγt-deficient biTregs. This observation was consistently confirmed in analyses from tissues derived from lung, peritoneal space and kidneys. Interestingly, the enhanced type 2 phenotype was not restricted to the cellular component, since titres of immunoglobulin E (IgE), which represents the humoral part of type 2 immunity, were also elevated in Foxp3^{Cre} x RORC$^{fl/fl}$ mice.

In conclusion, these results show that RORγt-deletion in biTregs led to a systemically exacerbated type 2 immune phenotype under healthy conditions and in the course of pristane-induced lupus.

7.1.5 biTregs supress type 2 immunity in a RORγt-dependent manner also in the NTN glomerulonephritis model

Having seen that Foxp3^{Cre} x RORC$^{fl/fl}$ mice revealed a hyper Th2 phenotype in pristane induced nephritis, the question was raised, whether type 2 immunity in Foxp3^{Cre} x RORC$^{fl/fl}$ mice is also upregulated in the NTN model. Indeed, Foxp3^{Cre} x RORC$^{fl/fl}$ mice, challenged with NTN serum, revealed a pronounced hyper Th2 phenotype in kidney tissue, as well as renal and splenic leukocytes. Hallmark Th2 cytokines and chemokines were elevated in mice bearing RORγt-deficient biTregs as illustrated in *Figure 10*.

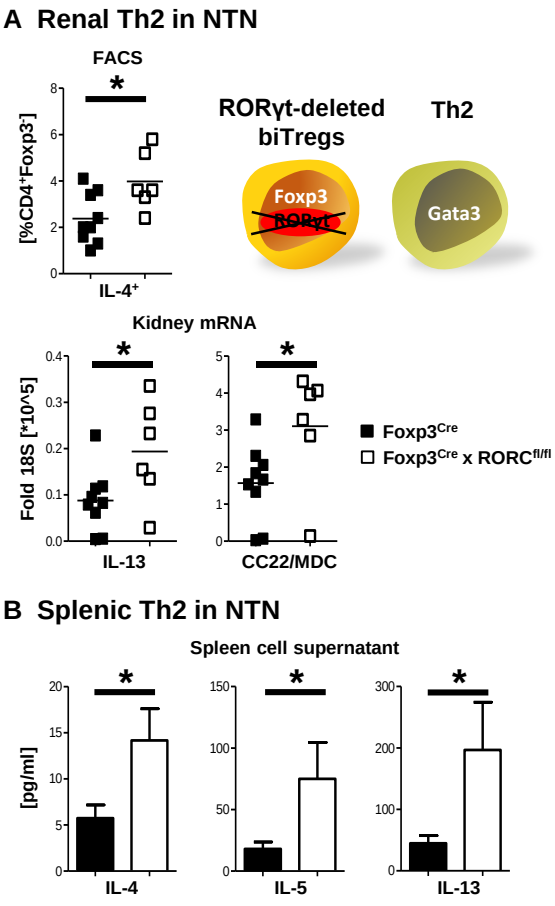


Figure 10. Type 2 immunity was exacerbated in Foxp3^{Cre} x RORC$^{fl/fl}$ mice in the course of NTN

Foxp3^{Cre} x RORC$^{fl/fl}$ mice and wildtype mice were analysed 7 days after NTN induction. (A) Quantification of renal T helper cells expressing the Th2 prototype cytokine IL-4 and renal mRNA analyses of Th2 characteristic molecules IL-13 and the chemokine CCL22 by qRT-PCR. (B) Analyses of Th2 cell hallmark cytokines in spleen cell supernatants by ELISA. Squares represent individual animals; horizontal lines indicate means. Bars show mean ± SEM. * p<0.05.

7.2 A novel model to evaluate the function of endogenous biTregs

Our previous studies analysed the function of RORyt in biTregs. Next, we wanted to assess the effects of complete absence of biTregs. Since no specific biTreg knockout mouse is currently available, we aimed to establish a cell transfer model. For this purpose, we used lymphocyte deficient RAG1$^{-/-}$ mice, which received biTreg-deficient or competent CD4^{+} T cells, harvested by FACsorting from spleens of naïve RORyt Foxp3 double fluorescence reporter mice *(Figure 11)*.

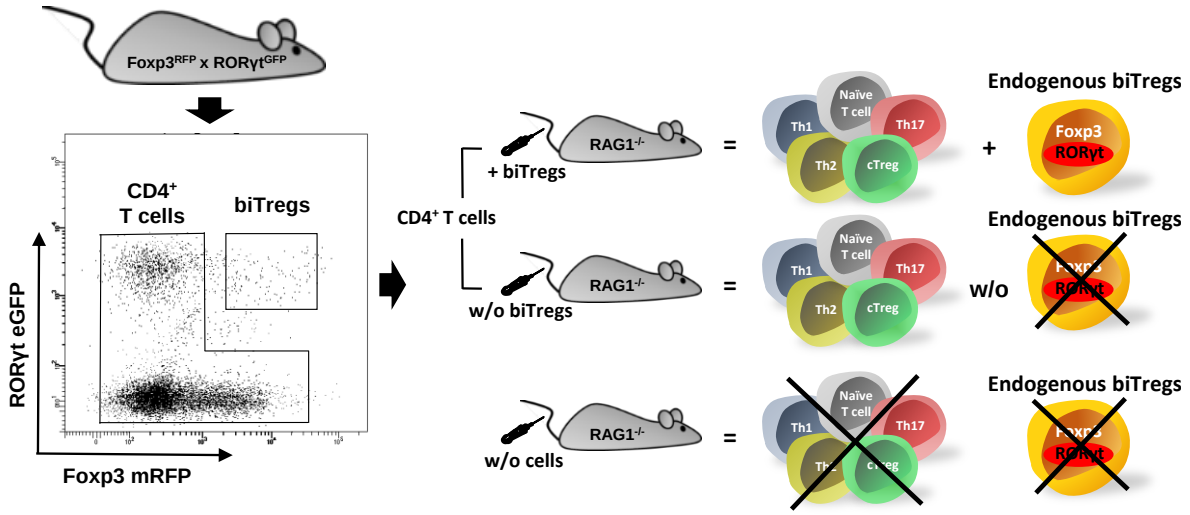


Figure 11. Experimental setup to study absence of endogenous biTregs

A novel transfer model was established, allowing to mimick absence of endogenous biTregs in mice. In detail, Foxp3-RORγt double-reporter donor mice were utilized to purify biTregs and biTreg-depleted CD4^{+} T cells. In the next step, one group of RAG1$^{-/-}$ mice received biTreg-competent CD4^{+} T cells, the other group of RAG1$^{-/-}$ mice was injected with biTreg-deficient CD4^{+} T cells. A RAG1$^{-/-}$ control group was just treated with PBS and did not receive any cells. Detailed descriptions of varying transfer modalities are outlined in chapter 8.2.3.

7.2.1 biTregs are stable and do not transdifferentiate from Th17 cells nor from conventional Foxp3 single positive Tregs

First of all, it was necessary to confirm that our transfer model is indeed suitable to characterize absence of biTregs. For this purpose, it was mandatory that only mice, which had received biTreg-competent CD4^{+} T cells, should harbour biTregs at the day of organ removal. In contrast, biTregs should not be detectable in mice that had received biTreg-deficient CD4^{+} T cells. To answer this central question, organs of RAG1$^{-/-}$ host mice were analysed at different time points after cell transfer and induction of glomerulonephritis *(Figure 12).* Studies were performed in the non-accelerated and

also the accelerated NTN model. FACS analyses of spleen, blood and kidneys at different time points of both models consistently showed that biTregs were only present in mice, which had received biTregs, whereas animals which had received biTreg-depleted CD4$^+$ T cells completely lacked these cells.

In conclusion, our analyses indicate two important aspects. Firstly, biTregs represent a stable T cell population up until day 22 after transfer. Secondly, biTregs did not develop from their transcriptional relatives, Th17 cells and RORγt negative conventional regulatory T cells (cTregs). Thirdly and most importantly, a novel murine transfer model was established, enabling us to characterize the role of endogenous biTregs.

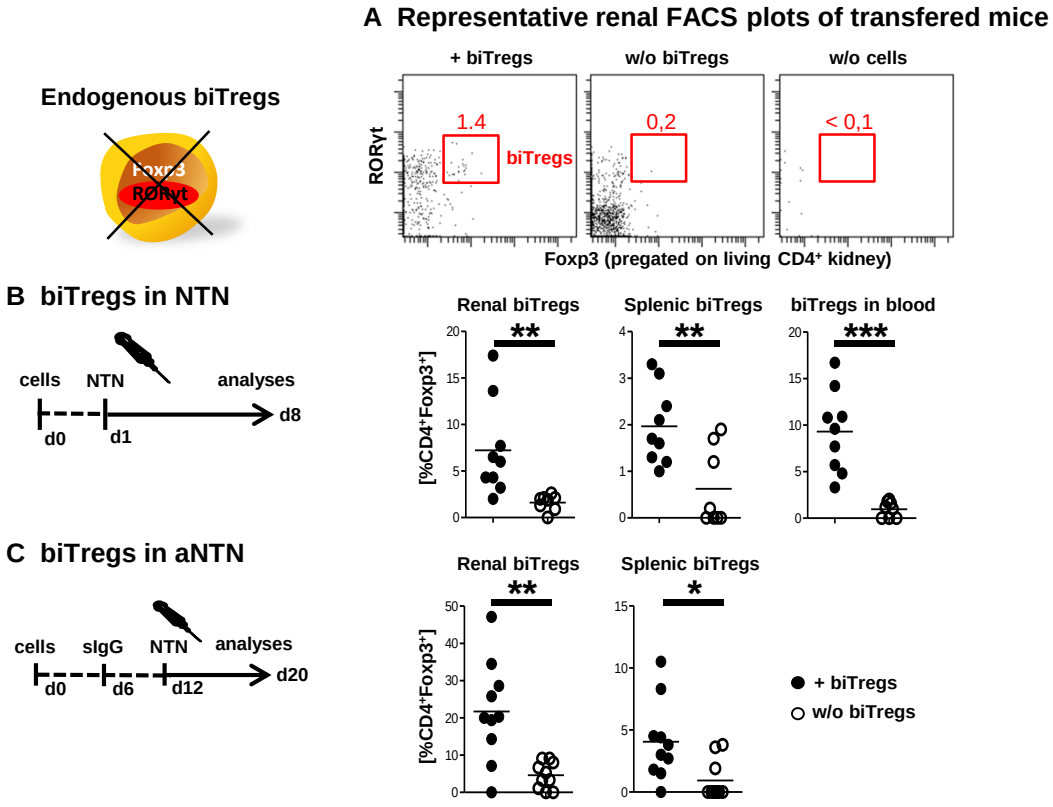


Figure 12. biTregs are a stable and independent T cell lineage

(A) Representative FACS plots of renal biTregs from mice after transfer of biTreg-competent CD4$^+$ T cells, biTreg-depleted CD4$^+$ T cells or no cells at day 8 of aNTN. (B) 1.8 x 10^6 biTreg-depleted CD4$^+$ T cells plus 2oo.ooo biTregs or 2 x 10^6 CD4$^+$ T cells excluding biTregs were transferred into RAG1$^{-/-}$ mice. Nephrotoxic nephritis (NTN) was induced one day after cell transfer. Organs were removed and analysed 7 days after glomerulonephritis induction. On the right, frequencies of biTregs are quantified as percentages of CD4$^+$Foxp3$^+$ cells. (C) 2 x 10^6 biTreg-depleted CD4$^+$ T cells plus 5oo.ooo biTregs or without biTregs were transferred into RAG1$^{-/-}$ mice. Mice were pre-immunized with sheep IgG 6 days after cell transfer and challenged with NTN serum 6 days after preimmunization (accelerated NTN (aNTN)). Organs were removed and analysed 8 days after aNTN induction. On the right frequencies of biTregs are quantified as percentages of CD4$^+$Foxp3$^+$ cells. Horizontal lines show mean values, circles represent individual mice. * p<0.05, ** p<0.01, *** p<0.001.

7.3 The role of endogenous biTregs in disease

Having established our novel model, we consequently focused on characterizing the net effect of endogenous biTregs in disease and also studied how and to what extend they influence other types of immune responses.

7.3.1 Endogenous biTregs do not influence the survival rate in transfer colitis

It is known, that RAG1$^{-/-}$ mice, which have been transferred with CD4^{+} T cells, develop colitis (Ostanin et al., 2009). We thus wanted to know, whether absence of biTregs possibly accelerates this process. Indeed, many mice started to suffer from diarrhoea beginning at day 10 after cell transfer. Interestingly, however, the onset of symptoms nor the survival rate differed between biTreg-competent and biTreg-deficient mice *(Figure 13)*. At day 22, all remaining mice needed to be sacrificed, since severe diarrhoea and weight loss were no longer ethically acceptable.

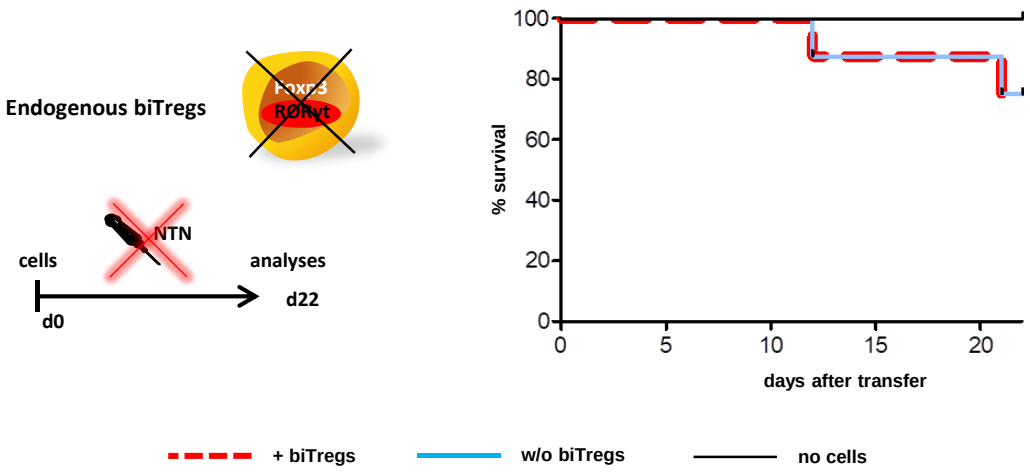


Figure 13. Absence of biTregs did not influence the survival in transfer colitis

RAG1$^{-/-}$ mice were transferred with 2 x 10^{6} biTreg-depleted CD4^{+} T cells plus 2oo.ooo biTregs or without biTregs. At around day 10 mice developed symptoms of transfer colitis. The experiment had to be terminated prematurely at day 22, since mice were in an unacceptably bad shape. On the right, the survival rates are illustrated by Kaplan-Meyer Plot.

In conclusion the results revealed, that endogenous biTregs did not have a significant impact on the course and outcome of transfer colitis.

7.3.2 Endogenous biTregs are protective as net effect in glomerulonephritis

Next, it was an important goal to characterize the net effect of endogenous biTregs in the course of GN. Therefore, NTN was analysed in mice receiving $CD4^+$ T cells including or selectively lacking biTregs. Histologic analyses revealed, that absence of endogenous biTregs resulted in significant aggravation of morphologic renal damage with more crescentic glomeruli and interstitial damage. This finding was reproduced using the model of accelerated NTN *(aNTN)*, a more severe version of NTN *(Figure 14).*

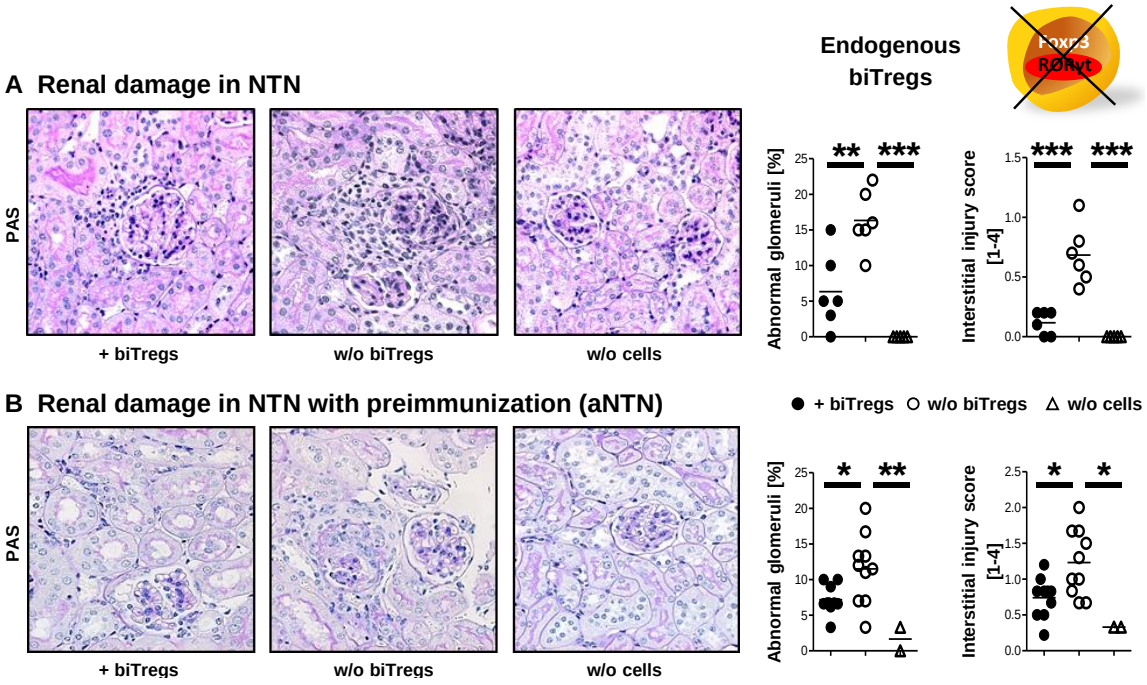


Figure 14. Absence of biTregs aggravated histologic renal damage in NTN

On the left, representative PAS-stained kidney sections are shown, illustrating histological renal damage of $RAG1^{-/-}$ mice after transfer of biTreg-competent or depleted $CD4^+$ T cells or no cells at day 8 of (A) NTN and (B) aNTN (original magnification ×400). On the right, corresponding semiquantitative analyses of glomerular and interstitial damage are shown. Circles represent individual animals; horizontal lines indicate means. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

In concert with tissue protection by biTregs, renal immune cell infiltration was lower in kidneys of biTreg-competent mice. Quantitative analyses revealed that biTreg-depleted mice displayed a much higher infiltration of $CD3^+$ T cells in the glomerulus and in the interstitial space *(Figure 15)*. Moreover, biTreg-depleted mice were characterized by a much enhanced renal infiltration of innate immune cells, indicating that renal inflammation was aggravated in absence of biTregs.

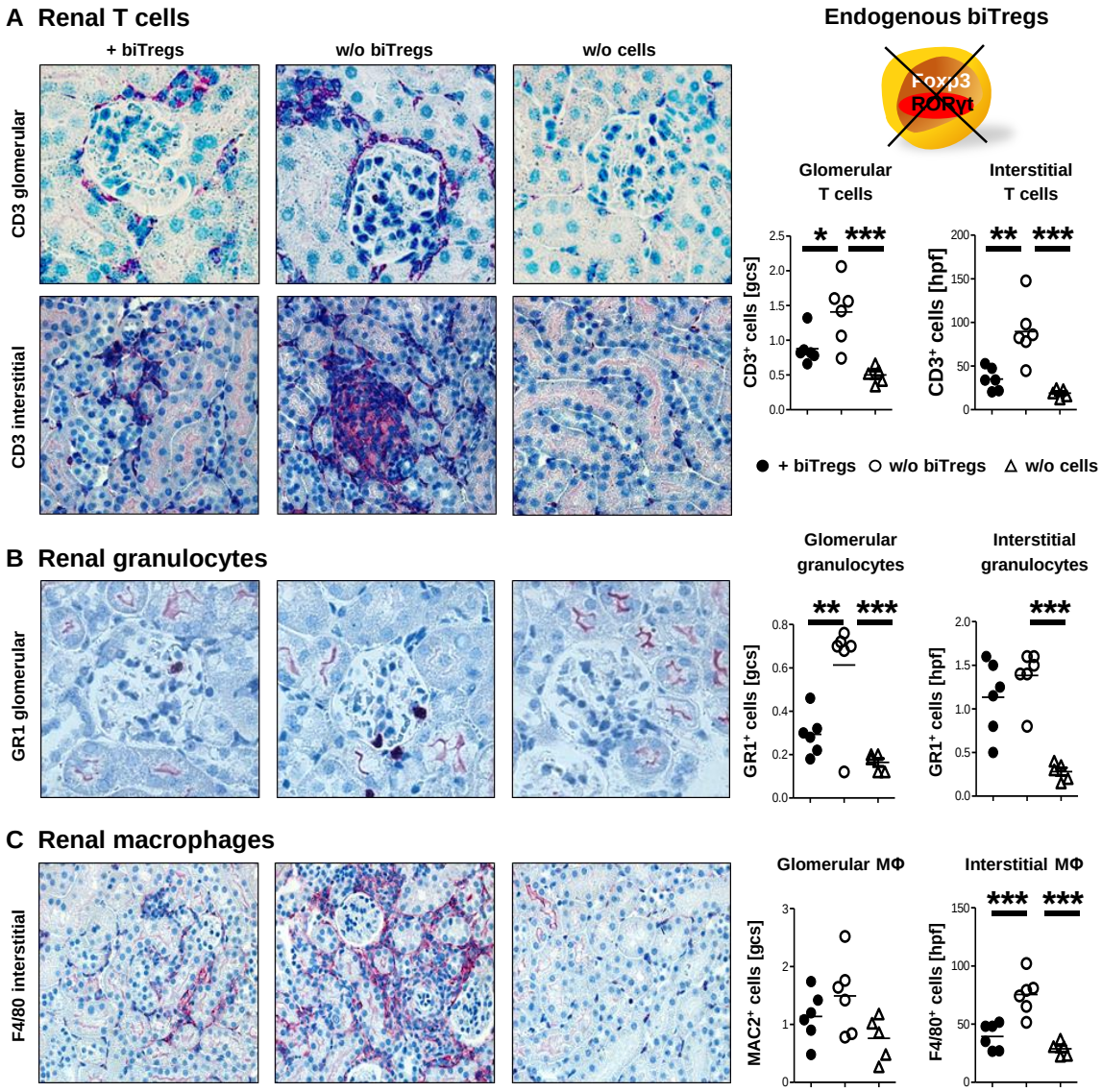


Figure 15. Lack of biTregs resulted in enhanced renal infiltration of adaptive and innate immune cells

RAG1$^{-/-}$ mice were transferred with biTreg-competent CD4$^+$ T cells, biTreg-depleted CD4$^+$ T cells or no cells. Representative photographs of immunohistochemical analyses of kidneys at day 8 of NTN are shown on the left. On the right, the corresponding analyses of cell numbers are shown. (A) CD3$^+$ T cell infiltration in glomeruli and interstitium. (B) On the left, illustration of representative photographs showing the glomerular GR-1$^+$ granulocyte infiltration. On the right quantitative analyses of glomerular and interstitial GR-1$^+$ granulocyte infiltration. (C) On the left, representative photographes of F4/80$^+$ monocyte/macrophage infiltration in the renal interstium. On the right, quantitative analyses of glomerular MAC-2$^+$ monocyte/macrophage infiltration and interstitial F4/80$^+$ monocyte/macrophage infiltration. Photographs, showing glomerular infiltration were captured at ×400 magnification, interstitial cell infiltration was captured at ×200 magnification. Horizontal lines show mean values, symbols represent individual animals. * p<0.05, ** p<0.01, *** p<0.001.

In conclusion, our data showed that biTregs display a net anti-inflammatory effect and dampen renal infiltration of pro-inflammatory immune cells with consequent protection from renal tissue injury.

7.3.3 Endogenous biTregs do not change frequencies of cTregs, Th17 or Th1 cells

Next, potential effects of biTregs on other participating immune cell types were studied. First, it was assessed whether biTregs change frequencies of conventional Foxp3^{+}RORγt^{-} Tregs (cTregs). Interestingly, although NTN was ameliorated in the presence of endogenous biTregs, frequencies of cTregs remained unchanged *(Figure 16)*. Also, as illustrated by *Figure 17*, we found that the Th17 cell response in spleen, kidney and blood did not differ between both groups. In addition, analyses of type 1 immune responses, representing the second major pro-inflammatory T cell response in NTN, also did not show differences in the absence of biTregs. In conclusion, these results implicate, that endogenous biTregs do not mediate immnosuppression via preferential upregulation of RORγt^{-} conventional Treg responses nor downregulation of frequencies of pro-inflammatory Th17 and Th1 cells. It thus still remains unanswered, how biTregs exert their strong anti-inflammatory and tissue protective effect.

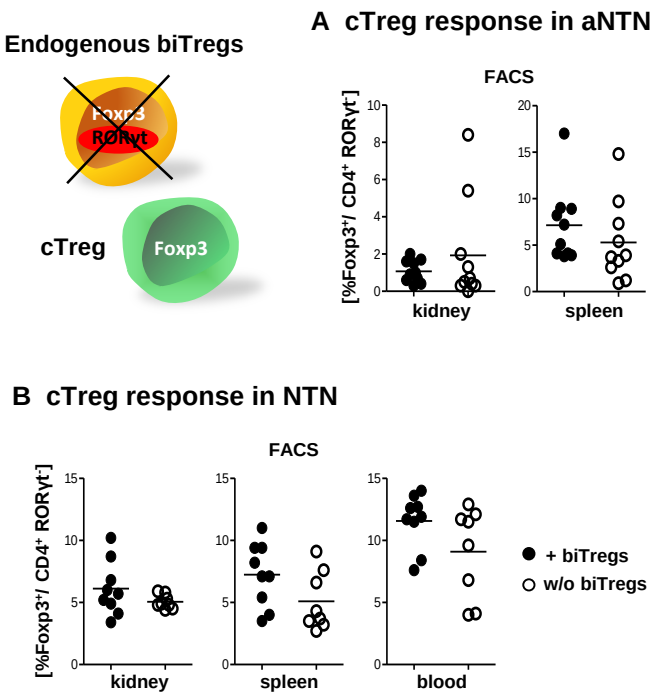


Figure 16. Endogenous biTregs do not influence numbers of conventional Tregs

FACS quantification of Foxp3^{+}RORγt^{-} conventional (c)Tregs from RAG1$^{-/-}$ mice after transfer of biTreg-competent or depleted CD4^{+} T cells. (A) Frequencies of cTregs in kidney and spleen at day 8 after aNTN induction. (B) Renal, splenic and blood cTreg quantification 7 days after NTN induction. Horizontal lines show mean values, circles represent individual mice.

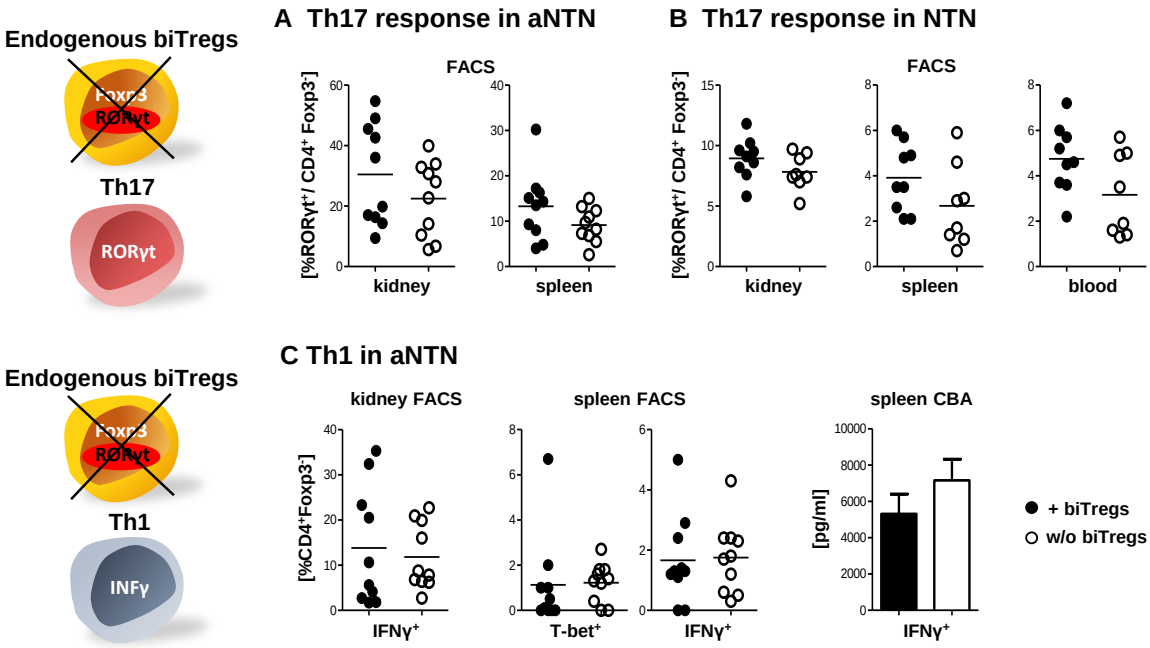


Figure 17. Endogenous biTregs neither influence Th17 nor Th1 response

Analyses of Th17 and Th1 responses from RAG1$^{-/-}$ mice after transfer of biTreg-competent or biTreg-depleted CD4^{+} T cells. (A) Th17 cell quantification in kidney and spleen at day 8 after aNTN induction. (B) Renal, splenic and blood Th17 cell quantification 7 days after NTN induction. (C) Analyses of type 1 immunity at day of organ removal at day 8 of aNTN. On the left, renal and splenic FACS analyses are illustrated quantifying frequencies of Th1 cells (T-bet) and frequencies of cells expressing the Th1 cell hallmark cytokine IFNγ. Concentration of IFNγ in spleen cell supernatants analysed by cytometric bead assay (CBA) is illustrated on the right. Horizontal lines show mean values, circles represent individual mice. Bars show mean ± SEM.

7.3.4 Endogenous biTregs do not supress type 2 immunity

In further experiments, it was thus aimed to evaluate, whether endogenous biTregs influence type 2 immunity. This was especially interesting, in light of the results from Foxp3^{Cre} x RORC$^{fl/fl}$ mice, which indicated, that biTregs mediate Th2-suppressive effects in the gut (Ohnmacht et al., 2015), in pristane-induced lupus and NTN, as presented above. Based on these observations, we hypothesized, that endogenous biTregs may be key modulators of type 2 immunity. Our analyses, displayed in *Figure 18,* however, revealed that biTreg-deficient recipient mice did not show an altered type 2 immune phenotype during the course of NTN. Spleen cell supernatants and FACS analyses of spleen and kidney leukocytes did not show a significant difference regarding Th2 responses. This unchanged type 2 phenotype was also observed in the aNTN model (*Figure 19).*

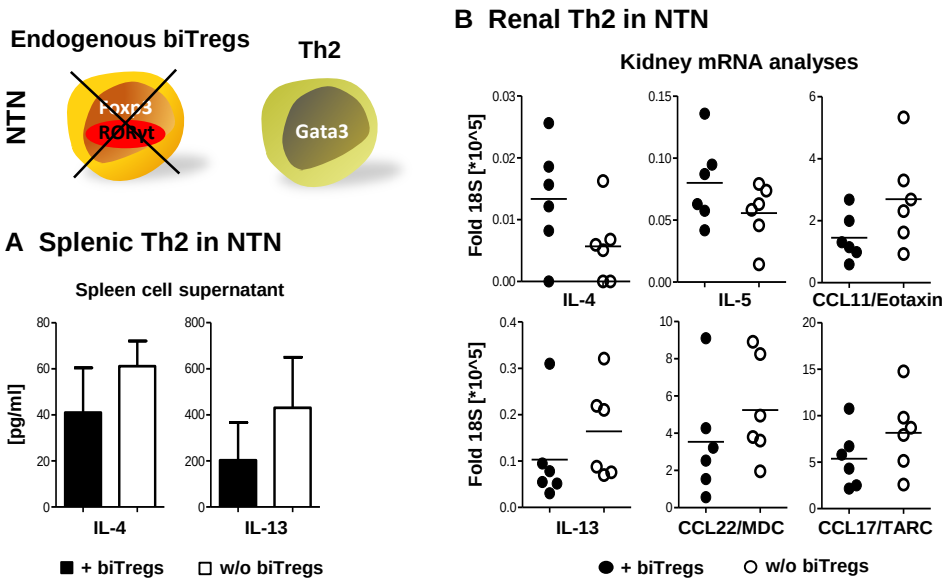


Figure 18. Th2 responses remain unchanged in the absence of endogenous biTregs during NTN

Analyses of Th2 responses from RAG1$^{-/-}$ mice after transfer of biTreg-competent or biTreg-depleted CD4^{+} T cells in the course of NTN. (A) Analyses of Th2 hallmark cytokines in spleen cell culture supernatants at day 8 of NTN quantified by ELISA. (B) Renal qRT-PCR mRNA analyses of Th2 characteristic cytokines and chemokines at day 8 of NTN. Horizontal lines show mean values, circles represent individual mice. Bars show mean ± SEM.

In sum, we somewhat unexpectedly found, that complete absence of RORγt^{+}Foxp^{+} cells did not affect Th2 immunity. This finding once more highlights, that deletion of RORyt in Tregs does not equal absence of biTregs. Furthermore, the mechanisms of immune regulation by biTregs still remained elusive.

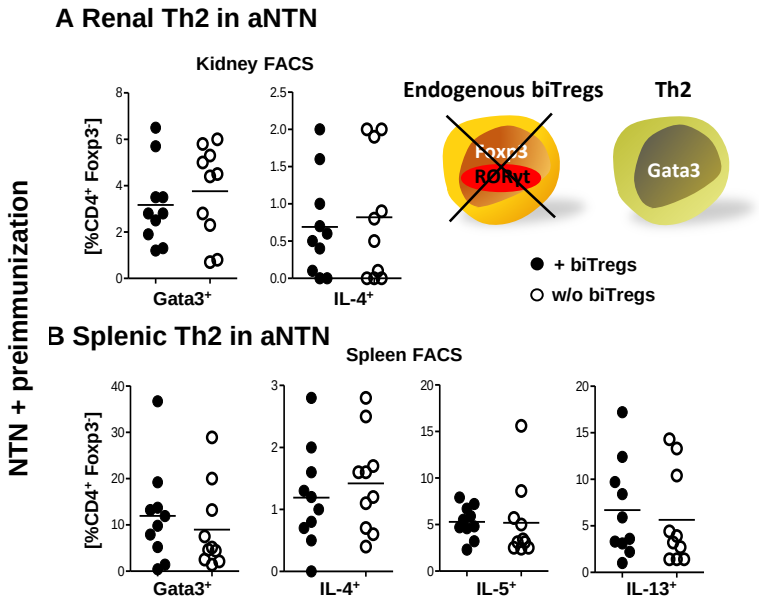


Figure 19. Th2 responses remain unchanged in the absence of endogenous biTregs during aNTN

Analyses of Th2 responses from RAG1$^{-/-}$ mice after transfer of biTreg-competent or biTreg-depleted CD4^{+} T cells in the course of aNTN. FACS analyses of (A) renal and (B) splenic leukocytes, quantifying Th2 cells (Gata3^{+}) and expression of hallmark Th2 cell cytokines IL-4, IL-5 and IL-13. Horizontal lines show mean values, circles represent individual mice.

7.4 Exogenous biTregs mediate broad immunoregulatory effects in NTN

Having seen that RORγt-deletion in biTregs and complete absence of biTregs had differing effects on immune responses, especially regarding type 2 immunity, we aimed to study, how and to what extend therapeutic injection of exogenous biTregs influences immunity. A previous study by us has shown, that injection of exogenous biTregs was protective as net effect in the course of NTN (Kluger et al., 2016), but whether and to what extend the distinct immune responses were altered remained unknown. We therefore performed new transfer experiments and injected FACsorted biTregs into mice, prior to induction of NTN. Subsequent detailed analyses, illustrated in *Figure 20,* revealed that the pro-inflammatory Th17 cell immune response remained unaffected.

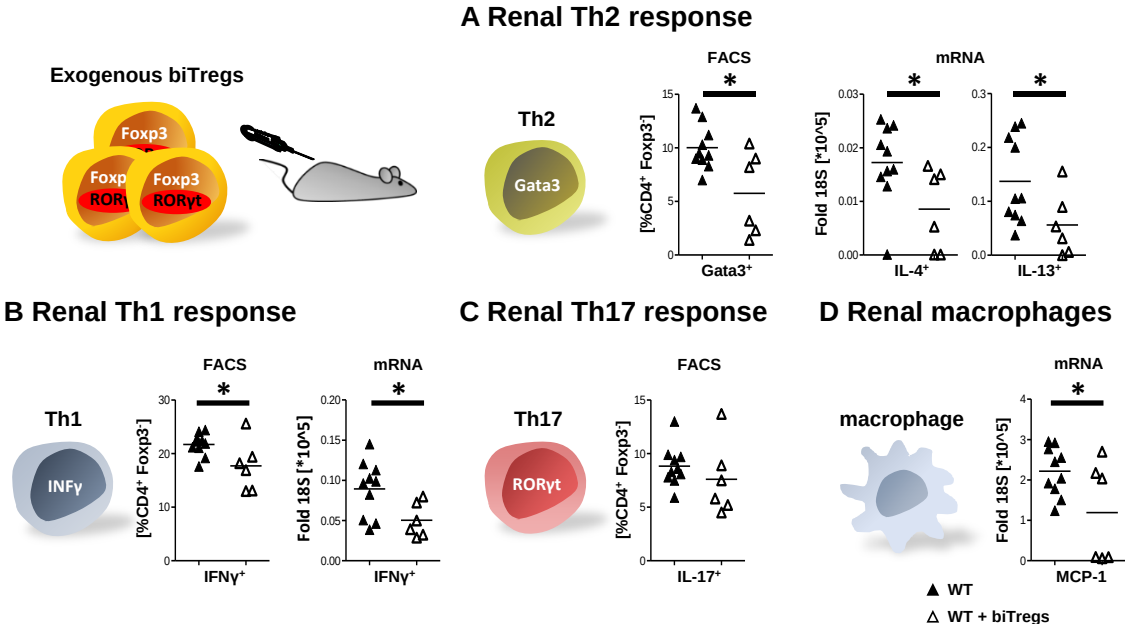


Figure 20. Exogenous biTregs show broad immunosuppressive functions during NTN

200.000 biTregs or PBS were injected into wildtype mice. NTN was induced one day later and outcome was studied at day 7. (A) On the left, FACS quantification of renal Th2 cells (Gata3$^+$). On the right, renal mRNA quantification of Th2 cell protype cytokines, analysed by qRT-PCR is shown. (B) On the left, FACS analyses of renal T helper cells expressing the Th1 hallmark cytokine IFNγ are shown. On the right renal mRNA quantification of IFNγ is shown. (C) FACS quantification of renal T helper cells expressing the Th17 cell characteristic cytokine IL-17. (D) Renal mRNA analysis of M1 type Monocyte attracting chemokine MCP-1/CCL2. Horizontal lines show mean values, symbols represent individual mice. * p<0.05.

However, and interestingly, type 1 immunity was downregulated in kidneys of mice, which received exogenous biTregs. This was evidenced by reduced percentages of T effector cells producing the hallmark Th1 cytokine IFNγ as well as reduced renal IFNγ mRNA expression. In conclusion, these data indicate that exogenous biTregs may

ameliorate nephritis, at least partially, by suppressing pro-inflammatory Th1 responses.

Additionally, it was studied, whether exogenous biTregs influence type 2 immunity, since differing effects between RORγt-deficiency in biTregs and complete absence of endogenous biTregs had been documented as discussed above. In this respect, FACS and mRNA analyses uniformly revealed, that exogenous biTregs indeed potently suppressed type 2 immunity. Last but not least, to get an idea, if exogenous biTregs might also affect the innate immune system, mRNA analyses of MCP-1, a chemokine which is known to chemoattract pro-inflamatory M1 type macrophages, were performed. Interestingly, MCP-1 was significantly reduced in mice receiving exogenous biTregs. This indicates, that exogenous biTregs may also supress M1 type macrophage responses.

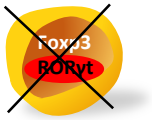
Summarised, these analyses indicate, that the previously observed amelioration of NTN (Kluger et al., 2016) may be due to broad suppression of diverse types of immune responses by exogenously injected biTregs, including innate and adaptive immune effectors. No preference for a certain type of immune response was documented. It is, however, of special note, that while Th1 and Th2 seem to be regulated by biTregs, in particular Th17 responses are not.

7.5 Compilation of the results

1. The role of RORγt activation in biTregs in pristane-induced lupus and NTN

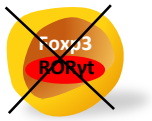
- RORγt deletion in biTregs ameliorates the course of pristane-induced pulmonary capillaritis and lupus nephritis
- RORγt deletion in biTregs enhances Treg suppressive capacity and ceases Treg capacity to produce IL-17
- Type 2 immunity is enhanced by RORγt deletion in biTregs during pristane-induced lupus and NTN

2. A novel transfer model to study endogenous biTregs was established



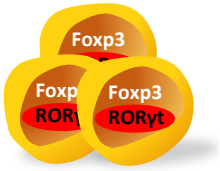
- Our new transfer model for the first time allows to mimic complete biTreg deficiency

3. Characterisation of endogenous biTregs



- biTregs are an independent Treg population and do not develop from Th17 cells or cTregs
- biTregs represent a stable effector Treg population
- lack of endogenous biTregs does not accelerate mortality of transfer colitis
- The net effect of endogenous biTregs in NTN glomerulonephritis is protective
- Lack of endogenous biTregs does not alter Th1, Th2, Th17 or general Treg responses

4. Characterisation of exogenous biTregs



- Transfer of exogenous biTregs does not affect Th17 responses
- Exogenous biTregs have broad non-specific immunosuppressive effects on Th1 and Th2 responses as well as macrophages

8 Discussion

Recently, a novel T cell population has been identified, showing the simultaneous activation of the Th17 cell master transcription factor RORγt and the Treg master transcription factor Foxp3 (Lochner et al., 2008, Kluger et al., 2016). In line with this unusual combination of transcription factors, several studies, including in acute crescentic glomerulonephritis, have shown, that RORγt^{+}Foxp3^{+} cells are able to mediate both, pro- and anti-inflammatory effects depending on the context (Lochner et al., 2008, Voo et al., 2009, Bovenschen et al., 2011, Kluger et al., 2016). To emphasize this bifunctional capacity, we have proposed the name bifunctional Tregs (biTregs) for RORγt^{+}Foxp3^{+} cells (Kluger et al., 2016).

The experiments, presented in this dissertation, aimed to gain a deeper insight into biTreg's immunobiological function, in particular in the context of acute crescentic GN and chronically developing lupus nephritis.

8.1 The role of RORγt activation in biTregs during chronically developing Lupus nephritis

A previous study by our lab group has shown, that deletion of the Th17 cell master transcription factor RORγt selectively in Tregs results in amelioration of acute crescentic glomerulonephritis in mice. This observation demonstrated that RORγt expression equips biTregs with pro-inflammatory, disease-promoting effects during acute GN (Kluger et al., 2016). However, it remained elusive, whether RORγt activation in Tregs also results in pro-inflammatory effects in the course of chronically developing lupus nephritis. To approach this important question, pristane induced Lupus was studied in mice with RORγt-deficient Tregs *(Foxp3^{Cre} x RORC$^{fl/fl}$ mice)*.

Indeed, analyses revealed that pulmonary capillaritis and lupus nephritis, were both ameliorated in mice bearing RORγt-deleted Tregs. Thus, we found that RORγt activation indeed equips biTregs with additional, pro-inflammatory effects in the course of pristane-induced SLE. As next step, it was of course important, to think about the mechanisms leading to amelioration of lupus nephritis in mice containing RORγt-deficient Tregs. Considering that the transcription factor RORγt represents the molecular key to induce the pro-inflammatory Th17 cell fate in naïve T cells (Ivanov et al., 2006), it is conceivable that RORγt activation in biTregs may also initiate production of pro-

inflammatory, Th17-cell associated genes leading to exacerbation of glomerulonephritis. Indeed, we found that a considerable proportion of wild-type biTregs secreted pro-inflammatory IL-17, which was completely abrogated in Foxp3^{Cre} x RORC$^{fl/fl}$ mice. A second mechanism leading to amelioration of nephritis in Foxp3^{Cre} x RORC$^{fl/fl}$ mice, could be due to the fact that RORγt inactivation improves the fitness and suppressive capacity of Tregs (Blatner et al., 2012). Supporting this concept, it has previously been shown, that RORγt inhibits Foxp3 by interaction on the molecular level (Li et al., 2012). In line, our data demonstrated enhancement of Foxp3 expression in Tregs as well as Treg activation and proliferation in Foxp3^{Cre} x RORC$^{fl/fl}$ mice.

Furthermore, a recent study by Ohnmacht et al. has shown, that RORγt-deletion in biTregs (Foxp3^{Cre} x RORC$^{fl/fl}$ mice) causes a pronounced hyper Th2 immune phenotype in the gut (Ohnmacht et al., 2015). Since Th2 responses mediate anti-inflammatory effects in proliferative forms of GN (Masutani et al., 2001), this could be a third mechanism, by which RORγt activation in biTregs mediates GN. We thus decided, to study this aspect in more detail. Indeed, our analyses in naïve mice revealed, that genetic deletion of RORγt in biTregs resulted in exacerbation of Th2 responses in all investigated organs, including the kidney. Noteworthy, this hyper Th2 phenotype was preserved during pristane-induced SLE. Interestingly, our analyses unveiled, that besides enhanced Th2 cell frequencies and elevated levels of Th2 cytokines, also Th2 dependent Immunoglobulin E titers were elevated in mice containing RORγt-deleted Tregs. Moreover, to complete the characterisation, we also studied type 2 immunity of mice bearing RORγt-deleted biTregs during the course of acute NTN. Again, in accordance with our data from pristane induced SLE, Foxp3^{Cre} x RORC$^{fl/fl}$ mice demonstrated a pronounced hyper Th2 phenotype.

Considering these results, it is of course imaginable to conclude in a more general way that endogenous biTregs may supress the type 2 immune axis physiologically. However, the mentioned studies only evaluated RORγt-deficiency in biTregs but not complete absence of biTregs. In other words, RORγt was inactivated in biTregs, but the RORγt-deleted biTreg-“remnants” were still present.

For this reason, we aimed to answer the central question, to what extent endogenous biTregs influence type 2 immunity. To clarify this aspect, it was mandatory to establish a model, which allows to study absence of endogenous biTregs. Moreover, such a model would be ideal to answer the currently unanswered key question, whether

RORγt^{+} bifunctional Tregs (biTregs) are net-protective or pro-inflammatory in autoimmune diseases, including glomerulonephritis.

8.2 The role of endogenous biTregs in the course of acute crescentic GN

Since biTreg-specific molecular targets are not yet known, it was technically impossible to study absence of endogenous biTregs by simply utilizing a biTreg-selective knockout mouse. To bypass this methodical obstacle, we established a novel transfer model. This transfer approach was based on T and B cell-depleted RAG1$^{-/-}$ mice, which were transferred with either biTreg-competent, or selectively biTreg-deficient CD4^{+} T cells. Having developed this concept, it needed to be ensured, if our transfer model is indeed suitable to study the absence of biTregs. This was a precarious issue, since the origin of biTregs is at debate in the scientific field. If transferred biTreg depleted-CD4^{+} T cells contain cells which are able to convert into biTregs, then the absence of biTregs would not be guaranteed in our model. On the one hand, some studies have suggested that RORγt^{-}Foxp3^{+} conventional (c)Tregs could serve as precursor cell for biTreg's development (Osorio et al., 2008, Voo et al., 2009, Hovhannisyan et al., 2011, Kim et al., 2017). On the other hand, there is also a report proposing, that biTregs derive from Th17 cells by upregulating Foxp3 (Downs-Canner et al., 2017). In contrast to both observations, congenic transfer studies performed by our lab group demonstrated, that neither transferred Th17 cells, nor cTregs served as precursor cells for biTreg's development (Kluger et al., 2016). We thus analysed host mice receiving CD4^{+} T cells excluding biTregs and indeed found, that these RAG1$^{-/-}$ recipients did not de novo develop RORγt^{+}Foxp3^{+} biTregs in any of the analysed organs at the day of organ removal. RAG1$^{-/-}$ mice, which had received biTreg-competent CD4^{+} T cell, however, displayed a clearly definable biTreg-population. These results confirmed, that 1) transferred biTregs are stable, 2) biTregs are absent in recipients of biTreg deficient CD4^{+} T cells and 3) biTregs do not arise from transferred Th17 cells nor cTregs.

Importantly, these results also raised many new questions facing the origin of biTregs. While excluding a Th17 cell or cTreg origin, our data also implicate, that biTregs do not develop from naïve splenic T cells, since these were also present in the transferred cell population. In summary, it thus still remains completely elusive, from which precursor cells biTregs derive. Nevertheless, a peripheral T cell origin should not be com-

pletely excluded. Possibly, biTregs are only generated at certain live periods. This hypothesis could explain why naïve T cells did not give rise to biTregs in our transfer model, because our experiment used only adult donor and recipient mice. Alternatively, biTreg development might need more time and/or certain metabolites, which were not present at the right dose or composition in our transfer setting. Further studies are therefore essential to characterize the exact precursor cells of biTregs.

Having confirmed, that absence of biTregs was guaranteed in our newly-established transfer model, we started to study the function of endogenous biTregs in the course of acute crescentic NTN glomerulonephritis. This was an issue that remained unclear, since multiple studies of the past revealed, that biTregs are capable to mediate both, pro-inflammatory (Ayyoub et al., 2009, Kluger et al., 2016, Yang et al., 2016, Kluger et al., 2017) and immunoregulative effects (Lochner et al., 2008, Ayyoub et al., 2009, Voo et al., 2009, Hovhannisyan et al., 2011, Li et al., 2012, Pesenacker et al., 2013, Yang et al., 2016) depending on the model and context. Using our approach, multiple independent experiments with varying conditions demonstrated consistently that acute NTN nephritis was aggravated in biTreg-depleted mice. Thus, our investigations showed for the first time, that endogenous RORγt^{+}Foxp3^{+} biTregs are protective as a net effect during GN. During the course of this dissertation, two further reports were published, approaching the net effect of RORγt^{+}Foxp3^{+} cells in autoimmune diseases using a methodologically similar transfer concept. Kim et al. studied the role of biTregs in autoimmune encephalitis. They transferred disease-promoting IL-17^{+} T cells *(Th17fm cells)* together with either ICOS^{high}CCR6^{+}Foxp3^{+} cells, which they used as surrogate markers for biTregs, or ICOS^{-}CCR6^{-}Foxp3^{+} cells (non-biTregs) into RAG1$^{-/-}$ mice and induced experimental autoimmune encephalitis (EAE). Results of this study indicated, that co-transfer with biTregs was more effective in ameliorating the course of encephalitis than co-transfer with non-biTregs (Kim et al., 2017). However, the setup of this study has some shortcomings, since as delineated above, biTregs were not identified in the unequivocal manner by detecting RORγt. Instead, surrogate parameters, like ICOS and CCR6 expression in Foxp3^{+} Tregs were utilized to define biTregs. Consequently, the transferred cell population was likely to contain a significant fraction of non-biTregs. Furthermore, transferred Th17 cells were only defined by positivity for IL-17 regardless of their Foxp3 status. Since large percentages of biTregs also pro-

duce IL-17, it is actually inevitable, that a relevant number of biTregs was also transferred with the population labelled ´Th17´cells. As a consequence, complete absence of endogenous biTregs was unlikely to be established in this study.

A second recent study, performed by Yang et al., aimed to study the net-role of biTregs in the course of transfer colitis, a model induced by transfer of naive $CD4^+$ T effector cells into RAG-deficient mice (Ostanin et al., 2009). To study the effect of biTregs during this disease, transfer of colitis-triggering T effector cells was either combined with $ROR\gamma t^-Foxp3^+$ cTregs or with biTregs. Their analyses displayed, that co-transfer with biTregs was even more protective than co-transfer with cTregs. This indicated, that biTregs have a stronger net-immunoregulatory capacity than cTregs in this setting (Yang et al., 2016). This approach is similar to the transfer setup designed by us, but differs in some important aspects. Yang et al. focused on comparison of disease-modifying effects mediated by cTregs directly with those of biTregs. For this reason, either biTregs or cTregs were exclusively transferred together with T effector cells. This means, that mice transferred with biTregs lacked cTregs and mice with cTregs lacked biTregs. In contrast, our transfer approach studied the absence and presence of endogenous biTregs in fully cTreg-competent mice. Furthermore, the study by Yang et al used non-physiologic Teff to Treg ratios of 1:4 which dramatically over-represented Tregs. Our studies in contrast used a 10:1 ratio, resembling the normal ratio of a wild type mouse. Despite the methodical differences, however, both mentioned studies are in concert with our findings and found, that endogenous biTregs have a net protective effect in different autoimmune diseases (Kim et al., 2017, Yang et al., 2016).

Given, that analyses by Yang et al. had revealed, that transfer of biTregs protected more potently from transfer colitis than cTregs (Yang et al., 2016), we wanted to assess, whether selective absence of biTregs would accelerate onset of transfer colitis. For this reason, we transferred cTreg-competent $CD4^+$ T cells either with or without biTregs into $RAG1^{-/-}$ recipients and assessed development of colitis. Unexpectedly, onset of colitis symptoms was similarly quick in both groups of mice and survival was unchanged by presence or absence of biTregs. Thus, our study does not indicate an additional disease-modifying role of endogenous biTregs in the presence of cTregs in the course of transfer colitis. However, it must be noted, that our studies just focused on survival, and no detailed immunologic or histologic analyses of the gut have been performed. Furthermore, as mentioned above, our mice received a 1:10 Treg to Teff

ratio, while the standard ratio for prevention of transfer colitis is the non-physiologic 4:1.

In the next step, we used our new transfer model, to answer the question if endogenous biTregs have a relevant influence on type 2 immunity. This hypothesis was based on experiments by us and others (Ohnmacht et al., 2015), which had shown, that mice bearing RORγt-deficient Tregs displayed an enhanced type 2 immune phenotype.

However, broad analyses of immune responses from our transfer model revealed, that absence of endogenous biTregs did not change renal or systemic Th2 responses, including multiple cytokines, chemokines and transcription factors. This result demonstrates impressively, that selective deletion of RORγt in biTregs is not the same as complete absence of biTregs. The reason for these different findings, however, remained unclear. Possibly, constitutive absence of RORyt in Tregs from birth on, might cause counter regulatory effects, augmenting or reducing the activation of other transcription factors. Possibly, use of the recently developed RORγt-blocking agents (Huh et al., 2013, Kojetin and Burris, 2014, Skepner et al., 2014), might help to more exactly characterize the role of RORyt activation in Tregs in the nearer future.

After ruling out major effects on Th2 responses, we wanted to better understand the interaction of biTregs with other types of immune responses. Various authors suggested, that biTregs are tailor-made to supress Th17 cells in different models of inflammation (Lochner et al., 2008, Kim et al., 2017, Xu et al., 2018). None of these studies, however, has provided any robust evidence for this hypothesis, since selective alteration of Th17 responses was either not assessed or not reported.

For this reason, we for the first time studied Th17 immunity in the absence of endogenous biTregs. Interestingly, our results did not show any effects of biTregs on systemic or organ specific Th17 responses.

In addition, our results from multiple sets of experiments revealed, that absence of endogenous biTregs also did not affect frequencies of Th1 cells or cTregs.

In summary, our data for the first time provide evidence, that endogenous biTregs are net protective and act as anti-inflammatory Tregs. Interestingly, biTregs do not seem to be specialized for regulation of a distinct type of immune response. In particular, they do not alter Th1, Th2 nor Th17 responses. The mechanisms explaining their protective effects in acute GN thus currently remain elusive and need to be further clarified in follow-up studies.

8.3 The effects of exogenous biTregs in the course of acute crescentic GN

Having found, that RORγt-deficiency in biTregs and complete absence of biTregs had differing effects on immune responses, especially with respect to type 2 immunity, we decided to study how injection of exogenous biTregs might influence immune responses in the course of NTN. A recent finding by our lab group has shown that transfer of exogenous biTregs ameliorated the course of NTN (Kluger et al., 2016). It remained elusive, however, which effects exogenous biTregs had on immune responses.
In line with our results from studies mimicking lack of endogenous biTregs, transfer of exogenous biTregs had no influence on Th17 immunity. However, and interestingly, transfer of exogenous biTregs suppressed Th2 responses. The reason, why RORγt deletion in biTregs as well as injection of exogenous biTregs, but not endogenous biTregs, affect type 2 immunity remained completely elusive and will need to be the focus of future studies. However, effects on Th2 responses were not exclusive, as exogenous biTregs also supressed Th1 and macrophages responses, which are both key glomerulonephritis-promoting factors.
In summary, exogenous biTregs exhibit broad-immunosuppressive effects on the adaptive and innate immune system, remarkably with the exception of Th17 responses. It still remains unclear, however, why mice lacking endogenous biTregs did not show these effects.

8.4 Clinical implications

Experiments presented in this dissertation aimed to better characterise the role of RORγt^{+}Foxp3^{+} biTregs in the course of acute and chronically developing glomerulonephritis. This characterisation is an essential step to establish, Treg directed novel therapies to treat GN patients. We here provide data, that RORγt activation in biTregs, similar to RORγt activation in T effector cells (Steinmetz et al., 2011), plays a disease-aggravating role in acute GN and lupus nephritis. Therefore, currently developed RORγt-blocking agents (Huh et al., 2013, Kojetin and Burris, 2014, Skepner et al., 2014) represent a promising approach to treat human GN and lupus nephritis. In addition, using our newly established transfer model we found, that endogenous biTregs

are net-protective in murine glomerulonephritis. These data further highlight the potential role of (bi)Treg directed therapies for GN and immune mediated diseases in general as was recently again summarized (Bluestone and Tang, 2018). It is of special note, that there have been several approaches showing that reinfusion of autologlus ex-vivo expanded Tregs represents a promising target in treating immune mediated diseases (Marek-Trzonkowska et al., 2012, Bluestone and Tang, 2018, Miyara et al., 2014). In this context, autologous biTreg therapy for acute GN could be considered. Another promising Treg centered therapeutic approach is based on low-dose IL-2 treatment. Diverse reports in mice and humans have revealed, that therapy with IL-2 restores disturbed Treg homeostasis and results in amelioration of disease severity (Humrich et al., 2010, Saadoun et al., 2011, Humrich et al., 2015, He et al., 2016). It would thus be worthwhile to investigate whether and to what extent IL-2 treatment could be refined to effectively expand biTregs.

9 Summary

Glomerulonephritis (GN) represents a group of immune mediated diseases, which frequently causes end stage renal failure, despite broad-immunosuppressive treatment (Stahl and Hoxha, 2016). Previous studies have identified RORγt^{+} Th17 cells as pro-inflammatory mediators of GN, whereas Foxp3^{+} Tregs protect the kidney from immune mediated injury (Steinmetz et al., 2011, Kluger et al., 2014). Recently, a novel T cell population has been discovered, defined by the unusual co-activation of RORγt and Foxp3. Since this unconventional T cell population was shown to mediate both, pro- and anti-inflammatory effects in GN, we proposed to call these cells bifunctional (bi)Tregs (Kluger et al., 2016). The underlying dissertation aimed to better characterize the role of biTregs during acute and chronically developing GN. First, we could demonstrate, that RORγt deletion in biTregs ameliorates chronically developing lupus nephritis. Mechanistically, we found that RORγt expression in biTregs supresses Treg activation as well as protective renal and systemic type 2 immunity and results in secretion of pro-inflammatory IL-17. Furthermore, we established a novel transfer model, which for the first time allows to study effects of the absence of biTregs. This approach revealed that biTregs are a stable and independent T cell population which does not develop from Th17 cells nor from cTregs. In addition, using our newly established model, we studied the net effect of endogenous biTregs in disease. Although absence of endogenous biTregs did not influence the survival in transfer colitis, our studies revealed, that endogenous biTregs are net protective in acute GN. Interestingly, absence of endogenous biTregs did not result in amelioration of Th1, Th17 or general Treg responses. Surprisingly, Th2 immunity was also unchanged in the absence of endogenous biTregs, although RORγt-deletion in biTregs caused a hyper Th2 phenotype as described above. The reason for these diverging observations remained elusive and require further studies. Last but not least, transfer of exogenous biTregs resulted in broad immunosuppression including Th1, Th2 and macrophage response. Remarkably, Th17 responses were not affected. Taken together, we have here shown, that **1)** deletion of RORγt in biTregs results in hyper Th2 responses and protects from lupus nephritis, **2)** the net effect of biTregs in GN is protective and **3)** biTregs act broadly immunosuppressive but do not regulate Th17 responses.

10 Zusammenfassung

Glomerulonephritden (GN) stellen eine Gruppe immunvermittelter Erkrankungen dar, die häufig, trotz aggressiver Immunsuppression, ein terminales Nierenversagen verursachen (Stahl and Hoxha, 2016). Es wurde gezeigt, dass RORγt^{+} Th17-Zellen das Entzündungsgeschehen fördern, während Foxp3^{+} Tregs protektiv wirken (Steinmetz et al., 2011, Kluger et al., 2014). Kürzlich wurde eine neuartige T-Zellpopulation entdeckt, die durch die ungewöhnliche Co-Aktivierung von RORγt und Foxp3 definiert ist. Da diese unkonventionelle T-Zelle sowohl pro- als auch anti-entzündliche Effekte im Verlauf einer GN vermitteln kann, schlugen wir vor, sie als bifunktionelle (bi)Tregs zu bezeichnen (Kluger et al., 2016). Die vorliegende Promotion zielte darauf ab, die Rolle von biTregs bei akuten und chronischen GNs besser zu verstehen. Wir konnten zeigen, dass die Deletion von RORγt in biTregs den Verlauf der chronischen Lupusnephritis verbessert. Als Mechanismus fanden wir, dass die RORγt-Expression in biTregs die Treg Aktivierung und die protektive Typ 2 Immunität unterdrückt sowie zur Sekretion von pathogenem IL-17 führt. Des Weiteren haben wir ein neuartiges Transfermodell etabliert, mit dem erstmals die resultierenden Effekte einer Abwesenheit von biTregs untersucht werden können. Wir konnten hiermit zeigen, dass biTregs eine stabile und unabhängige Zellpopulation darstellen, die weder aus Th17-Zellen noch aus cTregs entsteht. Ferner konnten wir den Nettoeffekt endogener biTregs bei inflammatorischen Erkrankungen untersuchen. Zwar hatte die Abwesenheit endogener biTregs keinen Einfluss auf das Überleben im Transferkolitis-Modell, allerdings zeigten sich endogene biTregs im Modell der akuten GN stark protektiv. Interessanterweise hatte das Fehlen endogener biTregs keinen Einfluss auf die Balance der Th1, Th17 oder allgemeinen Treg Antwort. Überraschenderweise war auch die Th2 Immunität in Abwesenheit endogener biTregs unverändert, obwohl die RORγt-Deletion in biTregs, wie oben beschrieben, einen Hyper Th2-Phänotyp auslöste. Der Grund für diese divergenten Beobachtungen blieb unklar und erfordert weitere Studien. Interessanterweise führte der Transfer exogener biTregs zur breiten Unterdrückung der pro-inflammatorischen Immunität. Nennenswerterweise blieb jedoch einzig die Th17 Antwort unbeeinflusst. Zusammengefasst zeigt diese Dissertation, dass **1)** die Deletion von RORyt in biTregs zu einer Hyper Th2 Antwort führt und vor Lupusnephritis schützt, **2)** körpereigene biTregs im Rahmen der akuten GN protektiv sind und **3)** biTregs breitgefächerte immunsuppressive Effekte aufweisen, aber nicht die Th17 Antwort regulieren.

11 Register of illustrations

11.1 Tables

11.2 Figures

12 List of abbreviations

%	Per cent
=	Equal
≈	Approximately equal
°C	Degree Celsius
µ	Micro (x 10^{-6})
ANOVA	Analysis of variance
aNTN	Accelerated nephrotoxic nephritis
BAC	Bacterial Artificial Chromosome
biTregs	Bifunctional regulatory T cells
BUN	Blood urea nitrogen
$CaCl_2$	Calcium chloride
CBA	Cytometric bead assay
TARC (CCL17)	Thymus and activation-regulated chemokine
CCL…	Chemokine (C-C motif) ligand…
MDC (CCL22)	Macrophage derived chemokine
CCR…	CC chemokine receptor…
CD…	Cluster of differentiation…
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
Cre	Cre recombinase
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
cTreg	Conventional regulatory T cell ($Foxp3^+RORγt^-$)
D	Day
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
EAE	Experimental autoimmune encephalitis
EBI-3	Epstein-Barr virus induced gene 3
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FIR	Foxp3-IRES-mRFP
Fl	Floxed (flanked by loxP)
Foxp3	Forkhead-Box-Protein P3
g	Gram
GBM	Glomerular basement membrane
GCS	Glomerular cross-section
GFP^{TG}	Green fluorescent protein tagged
GITR	Glucocorticoid-induced TNFR-related protein
GN	Glomerulonephritis
GR-1	Granulocyte-differentiation antigen-1
H_20	Water
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPF	High power field at 200 X magnification
ICOS	Inducible T cell co-stimulator
IFNγ	Interferon-γ
IgE	Immunoglobulin E
IgG	Immunoglobulin G
Ikzf3 (Aiolos)	Ikaros family zinc finger protein 3
IL-…	Interleukin…
IL1R1 (CD121a)	Interleukin 1 receptor, type I
IRF4	Interferon regulatory factor 4
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
LPF	Low power field at 100 X magnification
M	Molar mass
m	Mili (x 10^{-3})
m	Month
MCP-1 (CCL-2)	Monocyte Chemotactic Protein 1

$MgSO_4$	Magnesium sulfate
min	Minute
l	Litre
Mϕ	Macrophage
mRFP	monomeric red fluorescent protein
mRNA	Messenger ribonucleic acid
n	Nano (x $10^{-9)}$
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NH_3	Ammonia
NH_4^+	Ammonium
NH_4Cl	Ammonium chloride
Nrp1	Neuropilin 1
NTN	Nephrotoxic nephritis
PAS	Periodic acid Schiff
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PMA	Phorbol-12-myristate-13-acetate
Pristane oil	2,6,10,14-Tetramethylpentadecane
p-value	Probability value
qPCR	Quantitative polymerase chain reaction
RAG	Recombination-activating gene
RORC	RAR-related orphan receptor C
RORγt	RAR-related orphan receptor gamma
RPGN	Rapidly progressive glomerulonephritis
rtPCR	Real-time polymerase chain reaction
Runx	Runt-related transcription factor
SEM	Standard error of the mean
sIgG	Sheep immunoglobulin E
SLE	Systemic lupus erythematosus
STAT3	Signal transducer and activator of transcription 3
TGF-ß	transforming growth factor beta
Th1 cells	Type 1 helper T cell
Th17 cells	Type 17 helper T cell
Th2 cells	Type 2 helper T cell
Th17fm cells *(Kim et al., 2017)*	$CD4^+YFP^{hi}$ cells from IL-$17F^{Cre}$x$Rosa26^{YFP}$ mice
Treg	Regulatory T cell
Treg 17 cell	Regulatory T cell specialised on supressing Th17 cells
TSDR	Treg specific demethylation region
w	Week
w/o	Without
wt	Wild type
X	Crossed
× g	Units of times gravity
YFP	Yellow fluorescent protein

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Curriculum vitae

“Lebenslauf wurde aus datenschutzrechtlichen Gründen entfent”

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