UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

Zentrum für Innere Medizin
Institut für Experimentelle Immunologie und Hepatologie

Frau Prof. Dr. rer. nat. Gisa Tiegs

The effect of Rolipram in a murine model of immune-mediated hepatitis

Dissertation

zur Erlangung des Grades eines Doktors der Medizin
an der Medizinischen Fakultät der Universität Hamburg

vorgelegt von

Fabian A. Flottmann
aus Herford

Hamburg 2011

Angenommen von der
Medizinischen Fakultät der Universität Hamburg am 28.2.2013

Veröffentlicht mit Genehmigung der
Medizinischen Fakultät der Universität Hamburg

Prüfungsausschuss, die Vorsitzende:
Prof. Dr. G. Tiegs

Prüfungsausschuss, zweiter Gutachter:
Prof. Dr. F. Koch-Nolte

Prüfungsausschuss, dritter Gutachter:
Prof. Dr. H.-W. Mittrücker

Contents

List of Figures

1 Introduction

1.1 Regulatory T cells are the main mediators of immune tolerance

The immune system defends the body against a variety of pathogens, while reactions to the body's endogenous structures are avoided. The immune system in mammals is divided into innate immunity and adaptive immunity. Innate immune cells carry evolutionary conserved receptors that non-specifically recognize molecular patterns typical for pathogens, whereas adaptive immune cells carry receptors with highly variable regions enabling them to specifically recognize potential antigens. Innate and adaptive immunity are strongly cross-linked. Due to their highly variable receptors, adaptive immune cells are principally able to react to constituents of the own body. Thus, a mechanism called "self-tolerance" is required to avoid such autoimmune reactions. Failure of self-tolerance may lead to autoimmune disease, which affects about 5% of the population (Whitacre, 2001).

At present, the etiology of most autoimmune diseases is unknown. However, T cells are known to be key mediators of inflammation in a variety of autoimmune disorders (Sakaguchi et al., 2007). Interestingly, there is a particular subset of cluster of differentiation 4^+ ($CD4^+$) T cells called regulatory T cells (T_{regs}) which are specialised in maintaining self-tolerance by suppressing immune responses. Sakaguchi et al. (1995) identified the α-chain of the interleukin-2 (IL-2) receptor (also known as CD25) as a surface marker for T_{regs}. Later, forkhead box protein 3 (Foxp3) was identified as the major transcription factor of T_{regs} in the murine system (Hori et al., 2003). The importance of Foxp3 was first highlighted by the fact that Foxp3-deficient mice show a severe autoimmune disorder (Brunkow et al., 2001).

T_{regs} can be divided into two groups: natural T_{regs} (nT_{regs}) and adaptive or induced T_{regs} (iT_{regs}) (Figure 1.1). Natural T_{regs} develop in the thymus by high-avidity interactions and permanently express Foxp3. They prevent autoimmunity and raise the activation threshold for immune responses. Induced T_{regs} originate from naive $CD4^+$ T cells and represent a heterogenous pool of cell subsets ($Foxp3^+$ and $Foxp3^-$). They are essential in mucosal immune tolerance and control chronic allergic inflammation, and can be induced *in vitro* by TGF-β (de Lafaille & Lafaille, 2009).

As most findings of T_{reg} physiology were made in the murine system, it is questionable to what extent these findings are transferable to humans. The expression of Foxp3 characterizes T_{regs} in the murine, but not in the human system. Accordingly, specific expression patterns of other surface markers are used to identify highly immunosuppressive T_{regs} in humans, for instance the low expression of CD127 (Liu et al., 2006). However, the general importance of FOXP3 and T_{regs} in the human system has already been demonstrated. Patients suffering from a mutation in the FOXP3 gene locus suffer from a fatal autoimmune disorder due to impaired T_{reg} function: the immune dysregulation, polyendocrinopathy, enteropathy and X-linked inheritance (IPEX) syndrome (Bennett et al., 2001).

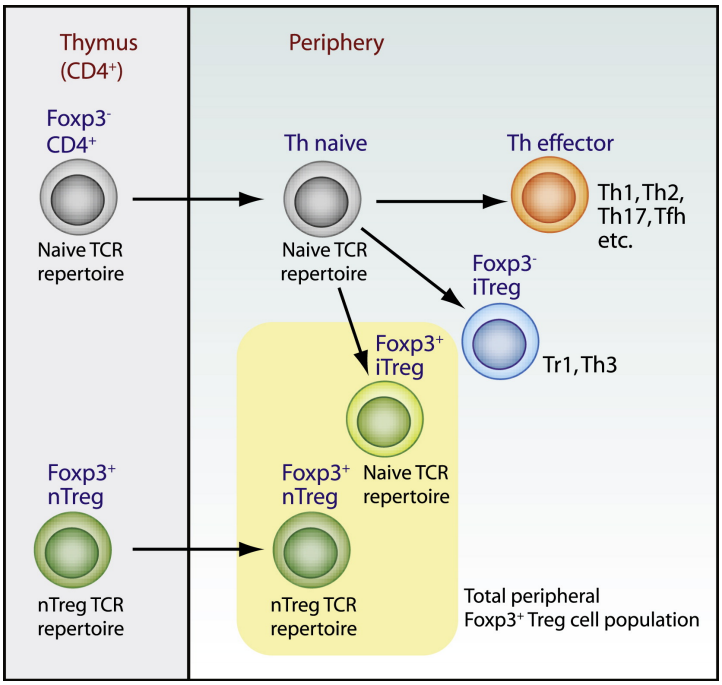

Figure 1.1: Thymic (nT_{reg}) and peripheral (iT_{reg}) generation of Foxp3$^+$ T_{regs}
Two groups of T_{regs} can be distinguished: natural T_{regs} (nT_{regs}) and adaptive T_{regs}(iT_{regs}). Natural T_{regs} differentiate in the thymus, whereas iT_{reg} cells differentiate in secondary lymphoid organs and tissues. From de Lafaille & Lafaille (2009)

1.2 The role of T_{regs} in the therapy of autoimmune disorders: opportunities and obstacles

Recent studies have provided evidence for an association between defects in T_{reg} function and/or number and a variety of human autoimmune diseases (Buckner, 2010). Thus, a therapeutic option to restore self-tolerance in patients suffering from autoimmune disorders might be the improvement of T_{reg}-mediated suppression.

The two main approaches to augment T_{reg} activity are *in vitro* expansion of T_{regs} followed by adoptive transfer and *in vivo* pharmacological therapies.

In case of cellular immunotherapies, T_{reg} cells are isolated from a patient, enriched, expanded *in vitro*, and reinfused (Riley et al., 2009). Although an efficient *in vitro* expansion of isolated T_{regs} can be achieved (Hoffmann et al., 2004), there are several challenges associated with cellular immunotherapies. The protocols used for expansion of patient-derived T_{regs} are complicated and have to be adopted specifically for every patient. Furthermore, the purity and antigen specificity of expanded T_{regs} are crucial: A contamination with effector T cells and expansion of autoreactive T cells could lead to autoimmunity, whereas the expansion of T_{regs} with inappropiate antigen specificity could lead to anergy towards pathogens or tumor cells. The stability of *in vitro* expanded T_{regs} is unknown. They might lose their FOXP3 expression *in vivo* and convert into pro-inflammatory effector T cells. Finally, *in vitro* expansion always contains a risk of malignant transformation or contamination with pathogens (Taams et al., 2006).

In vivo pharmacological therapies aim at augmenting T_{reg} numbers and/or potency locally in the organism in order to improve the suppressive function of T_{regs}. As there is no *in vitro* expansion involved, some of the disadvantages of adoptive cell therapy can be avoided by design. However, results with pharmacological agents in improving T_{reg} function vary a lot from bench to bedside. TGN1412 is a superagonistic CD28 antibody that showed very promising results on *in vivo* expansion of T_{regs} in different animal models (Schmidt et al. (2003), Beyersdorf et al. (2005)). Nevertheless, TGN1412 caused a septic shock in six healthy adults when it was tested in a phase I clinical trial (Suntharalingam et al., 2006). Eastwood et al. (2010) explained this accident by the finding that TGN1412 activates pro-inflammatory cytokine secretion from $CD4^+$ memory T cells in humans. This demonstrates the variability between animals and humans towards the therapy with immunomodulatory drugs. Another problem for *in*

vivo expansion is the assessment of T_{reg} function. While the determination of T_{reg} numbers in patients is clinical routine, the assessment of T_{reg} potency requires *in vitro* suppression assays that are not easily reproducable (Buckner, 2010).

Despite these obstacles, several pharmacological agents have proved their potential to improve T_{reg} function *in vivo*. These pharmacological agents either expand the T_{reg} population or convert effector T cells into iT_{regs}. Both pathways result in an increase of the ratio of T_{regs} to effector T cells. Drugs that improve T_{reg} function in humans include different substances such as Rapamycin, corticosteroids, statins, antithymocyte globulin, and Alemtuzumab (Fort & Narayanan, 2010). Vasoactive Intestinal Peptide (VIP) serves as an example for a cyclic adenosine monophosphate (cAMP) rising drug that is able to generate T_{regs} *in vivo* in a murine animal model (Chorny et al., 2006). The effect of selected immunosuppressive drugs on T_{reg} potency and frequency is shown in Figure 1.2.

The conversion of $CD4^+CD25^-$ T cells into iT_{regs} is a physiological part of immune regulation. It depends on the way in which T cells encounter antigen, for example as soluble or peptide antigen, in a low dose, or in the presence of immunosuppressive drugs (Taams et al., 2006). Interestingly, Prakken et al. (2004) demonstrated that patients with recent-onset rheumatoid arthritis (RA) developed a regulatory-type T cell phenotype when treated orally for 6 months with low doses of a peptide that induces proinflammatory T cell responses in naive RA patients. The hypothesis that the *in vivo* conversion of T cells into iT_{regs} can be supported by pharmacological agents is supported by the findings of Belghith et al. (2003) who demonstrated that anti-CD3 monoclonal antibody (mAb) therapy in non-obese diabetic (NOD) mice might work via the induction of T_{regs}. Interestingly, patients with type I diabetes treated with humanized anti-CD3 mAb maintained residual beta cell function and required lower insulin doses 18 months after beginning of the treatment compared to the control group (Keymeulen et al., 2005).

1.3 The murine model of Concanavalin A (ConA) induced immune hepatitis

The model of ConA-induced hepatitis was used in this study to investigate the effect of Rolipram on T_{regs}. Concanavalin A is a plant lectin from the Jack Bean first isolated in

1919. It is a strong T cell mitogen which activates T cells *in vitro* in an antigen unspecific manner (Palacios, 1982). In vivo, a single intravenous injection of Concanavalin A leads to acute hepatitis in mice (Tiegs et al., 1992) as a consequence of immune activation: Firstly, natural killer (NK) T cells are locally activated in the liver (Kaneko et al. (2000), Takeda et al. (2000)). They produce large amounts of interferonγ (IFNγ) (Küsters et al., 1996), which is crucial for development of disease (Tagawa et al., 1997). $CD4^+$ lymphocytes are attracted and Kupffer cells are activated to secrete tumor necrosis factor-α (TNF-α) which results in apoptosis of hepatocytes and the subsequent release of alanine transaminase (ALT) and aspartate transaminase (AST) from their cytoplasm (Schümann et al. (2000), Gantner et al. (1995)). IL-12 inducing IFNγ production (Nicoletti et al., 2000) and IL-18 play an important role for T-cell mediated hepatitis in the ConA model (Faggioni et al., 2000). T cells are crucial for the development of liver injury, as demonstrated by the fact that severe combined immunodeficiency (SCID) mice suffering from impaired T and B cell development as well as athymic nude mice lacking only T cells are insusceptible towards injection of ConA (Tiegs et al., 1992). Furthermore, $CD4^+$ T cells are specifically important, as depletion of $CD4^+$ T cells prevents ConA-mediated liver injury, whereas depletion of $CD8^+$ T cells does not. Furthermore, a Th1 response is crucial for disease development, as shown by the fact that both STAT1-deficient and T-bet-deficient mice are insusceptible towards ConA-induced hepatitis (Siebler et al., 2003).

Erhardt et al. (2007) could demonstrate that pretreatment with a sublethal dose of ConA induces tolerance towards a second ConA stimulation in C57BL/6 mice. Tolerance develops within 8 days and lasts at least up to 42 days. It is characterized by suppression of the Th1 response typically found after ConA injection and an increased production of IL-10. IL-10 plays a crucial role, as tolerance induction is fully reversible in IL-$10^{-/-}$ mice and after treatment with an anti-IL10 receptor antibody, agreeing with previous findings on the importance of IL-10 for ConA hepatitis (Marco et al. (1999), Louis et al. (1997)). T_{regs} and Kupffer cells are the sources of IL-10 production as shown by depletion studies. Furthermore, T_{regs} from ConA-tolerant mice are more effective in suppressing T-cell responses *in vitro* than T_{regs} from control mice. Moreover, cellular immune therapy with $CD4^+CD25^+$ cells prevented ConA-induced liver injury, with higher protection by T_{regs} from ConA-tolerant mice (Figure 1.3, Erhardt et al. (2007)). Ye et al. (2009) and Wei et al. (2008) demonstrated a protective role of Tr1-type T_{regs} in ConA tolerance.

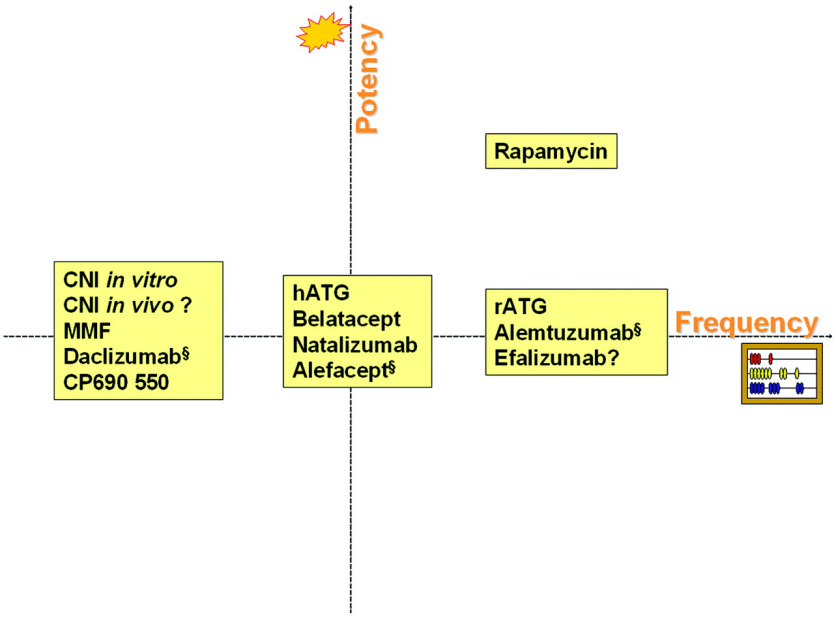

Figure 1.2: Influence of immunosuppressive drugs on T_{reg} potency (y-axis) and frequency (x-axis)
The effect of immunosuppressive drugs on T_{reg} frequency is represented on the horizontal axis and the effect on T_{reg} potency on the vertical axis, each axis delineating a negative versus positive effect.
§: no data available for potency. From Serres et al. (2009)
CNI, calcineurin inhibitors, e.g. Tacrolimus or cyclosporin A, inhibit T-lymphocyte activation by interfering with calcineurin, a key enzyme in the T-cell signal transduction cascade (Clipstone & Crabtree, 1992). **MMF**, mycophenolate mofetil, is bioactivated to mycophenolic acid which inhibits the de novo synthesis of guanosine nucleotides especially in T- and B-lymphocytes, thus limiting DNA synthesis and cell proliferation (Allison & Eugui, 2000). **Daclizumab**, a humanized antibody directed against the IL-2 receptor α-chain (CD25), inhibits IL-2 mediated proliferation of T-lymphocytes (Vincenti et al., 1998). **CP690,550** is a highly selective inhibitor of Janus kinase 3, an enzyme pivotal for signalling by multiple cytokines via the common γ-chain (such as IL-2, -4, -7, -9, -15, -21) (Changelian et al., 2003). **ATG**, anti-thymocyte globulin, a polyclonal antibody generated by immunizing animals with human T-cell lines, acts immunosuppressive mainly by rapidly depleting T cells (Mohty, 2007). **hATG**, human anti-thymocyte globulin. **rATG**, rabbit anti-thymocyte globulin (rATG). **Belatacept**, an immunoglobulin fusion protein construct of CTLA4, binds B7 molecules (CD80 and CD86) and thus inhibits their interection with co-stimulatory T cell receptors (Larsen et al., 2005). **Natalizumab** is a monoclonal antibody which blocks alpha4beta1 integrin and hinders T cells from invading inflamed tissues (Steinman, 2005). **Alefacept**, an immunomodulatory fusion protein composed of LFA-3 and IgG1, induces CD2/CD16-dependent apoptosis of CD2+ cells (i.e. T cells) (Silva et al., 2002). **Alemtuzumab**, also known as Campath-1H, is a monoclonal antibody directed against CD52 which depletes T cells (Flynn & Byrd, 2000). **Efalizumab** is a humanized monoclonal antibody that binds LFA-1 and inhibits the activation of T cells (Lebwohl et al., 2003). **Rapamycin** binds to mTOR and blocks IL-2 dependent signal transduction and cell cycle progression especially in T cells (Sehgal, 1998).

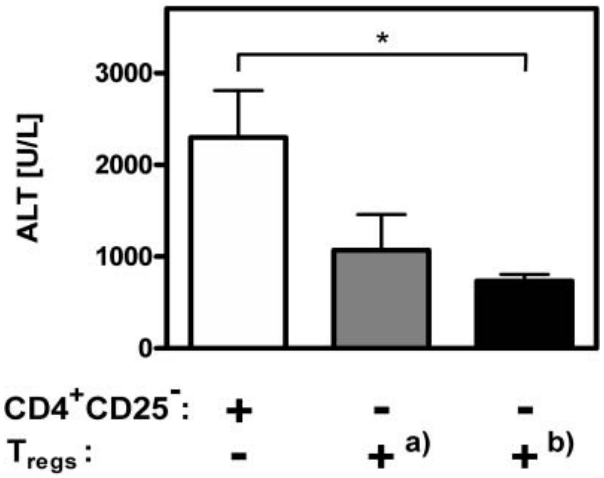

Figure 1.3: Significant suppression of liver injury by adoptively transferred T_{regs} from ConA-pretreated mice
Mice were injected with 1x10^6 FACS-sorted T_{regs} from either saline (a) or ConA pretreated (b) mice, or with 1x10^6 $CD4^+CD25^-$ cells as control 24 hours before ConA treatment. After 8 hours, plasma ALT activities were measured. From Erhardt et al. (2007)

1.4 Rolipram, an anti-inflammatory phosphodiesterase-4-inhibitor

Rolipram is a selective inhibitor of the phosphodiesterase-4 (PDE4) and was firstly described in 1979 (Karppanen et al., 1979). PDE4 is an isoenzyme from the group of cyclic nucleotide phosphodiesterases. It catalyzes the decomposition of cAMP to AMP by degrading the phosphodiester bond of cAMP. PDE4 in its different subforms is located in a variety of organs such as the brain, liver, lung, trachea, placenta, heart and different types of immune cells. By inhibiting PDE4, Rolipram rises intracellular cAMP levels. cAMP is a second messenger to a multitude of cellular signal cascades (Müller et al., 1996).

The anti-inflammatory effects of Rolipram have been demonstrated *in vitro*, where it strongly suppresses inflammatory responses in T cells (Jimenez et al., 2001), as well as *in vivo* in several animal models, such as in experimental autoimmune encephalomyelitis (EAE) in mice (Sommer et al., 1995), colitis in mice (Hartmann et al., 2000) and asthma in monkeys (Turner et al., 1994).

Rolipram has been given in patients as an anti-depressive drug with some clinical success. However, there were severe side effects such as nausea and vomiting which severely reduced patient compliance (Hebenstreit et al., 1989), a problem that also occurred in a recent study on Rolipram for the treatment of multiple sclerosis (Bielekova et al., 2009). A successor of Rolipram, the selective PDE4 inhibitor Roflumilast, has a wider therapeutic range and was recently admitted for the therapy of chronic obstructive pulmonary disease (COPD) in humans (Giembycz & Field, 2010).

The influence of Rolipram on T_{regs} has been studied by Bopp et al. (2009). They could demonstrate that Rolipram improves T_{reg} -mediated suppression of effector T cell responses in a model of allergic airway inflammation. Application of Rolipram improves T_{reg} functionality both *in vitro* and *in vivo*, which is accompanied by increased intracellular cAMP levels.

Interestingly, Rolipram protects from liver damage in the ConA hepatitis model. Upon a single injection of ConA, AST and ALT plasma transaminase activities are decreased under treatment with Rolipram in a dose-dependent manner (Figure 1.4.A). Furthermore, plasma levels of pro-inflammatory cytokines IL-2, IL-4 and TNF-α are lowered.

IL-10 as an anti-inflammatory cytokine is significantly increased (Figure 1.4.B) (Gantner et al., 1997).

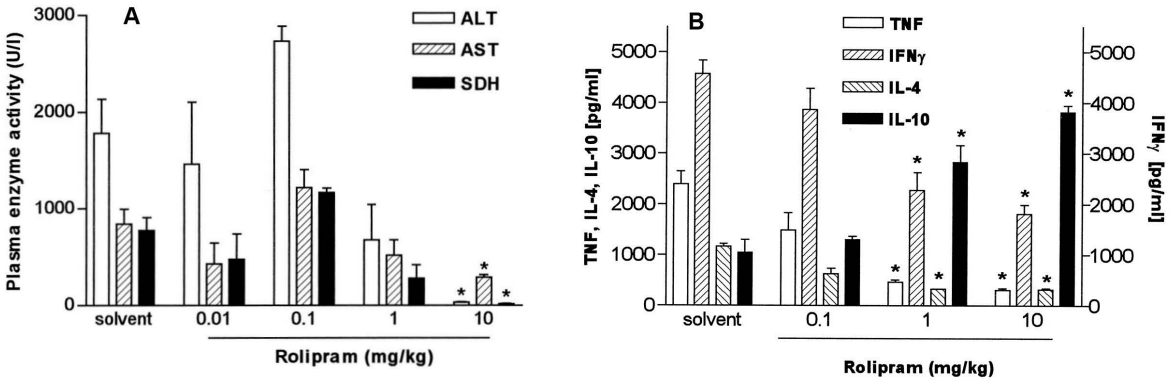

Figure 1.4: Dose-dependent protection from ConA hepatitis (A) and modification of cytokine pattern (B) by Rolipram
Protection from T cell-dependent murine liver failure by rolipram. Liver damage was induced by injection of ConA (25 mg/kg i.v.). Rolipram was administered i.p. 30 min before challenge at the doses indicated. The corresponding volume of solvent was injected into control animals. Plasma samples for the determination of TNF, IL-4 and IL-10 were taken 2 hours after ConA injection. Eight hours after ConA challenge, plasma enzyme activities of ALT, AST and succinate dehydrogenase (SDH) as well as IFNγ concentration were determined. From Gantner et al. (1997)

1.5 Aims of this study

A single sublethal injection of ConA to C57BL/6 mice leads to immune-mediated hepatitis (Tiegs et al., 1992) and induces tolerance towards ConA induced liver damage within 8 days. Tolerance is known to be mediated by T_{regs} (Erhardt et al., 2007). Moreover, Rolipram administered prior to a single injection of ConA protects from hepatitis (Gantner et al., 1997). Recently it has been shown that Rolipram improves T_{reg}-mediated suppression in a model of allergic airway disease (Bopp et al., 2009). Hence, this study investigated the effect of Rolipram on T_{regs} in the ConA hepatitis model, especially a possible *in vivo* conversion of effector T cells into T_{regs}.

The main questions adressed in this study were whether Rolipram further attenuates hepatitis by improving T_{reg}-mediated suppression upon ConA restimulation, and to what extend the protective effect of Rolipram depends on T_{regs}. Rolipram increases intracellular cAMP levels (Bopp et al., 2007). VIP, another cAMP-increasing drug, is able to induce T_{regs} *in vivo* (Chorny et al., 2006). Therefore, a focus of this study was to clarify if Rolipram increases the number of T_{regs}, which could indicate a conversion of effector T cells into iT_{regs}. Furthermore, the effect of Rolipram on the suppressive capacity of T_{regs} was analyzed.

In human $CD4^+$ T cells isolated from multiple sclerosis patients orally treated with Rolipram, *in vitro* proliferation is inhibited (Bielekova et al., 2009). In this study, it was investigated if Rolipram shows an anti-proliferative effect on $CD4^+$ T cells derived from healthy human donors.

2 Materials and Methods

2.1 Mice

Male C57BL/6 wild-type (wt), male IL10$^{-/-}$ (Kühn et al., 1993) or female DEREG (Lahl et al., 2007) mice (6-12 weeks old) weighing 20 to 30 g were used for this study. C57BL/6 wt and IL-10$^{-/-}$ mice were obtained from the internal animal facilities of the University Medical Center Hamburg Eppendorf. DEREG mice were kindly provided by Tim Sparwasser, Hanover. Animals received humane care according to the criteria of the "Guide for the Care and Use of Laboratory Animals" prepared by the US Academy of Sciences and published by the National Institutes of Health, and according to legal requirements in Germany. Animals were maintained under controlled conditions (22° C, 55% humidity, 12–hour day/night rhythm) and fed standard laboratory chow.

2.2 Animal treatment

2.2.1 ConA administration

In the present study, the murine model of ConA-induced liver injury was used. T cell-dependent liver damage was induced by ConA (Sigma-Aldrich, Taufkirchen, Germany) which was administered intravenously in pyrogen-free, cold saline. Mice received a single ConA injection of 8-14 mg/kg in a total volume of 100 µl/10 g mouse. Control mice were injected with saline. In order to induce tolerance, animals were restimulated with ConA on day 8 or 14.

2.2.2 Rolipram administration

Rolipram (Sigma-Aldrich, Taufkirchen, Germany) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) in a concentration of 33.3 mg/ml (stocking solution). Finally,

the working solution of Rolipram was diluted in a total volume of 100 µl/10 g mouse and was administered intraperitoneally in a dose of 5 mg/kg 30 minutes or 1 hour before ConA administration.

2.2.3 Depletion of Regulatory T cells

To deplete T_{regs} in DEREG mice, 1 µg of diphtheria toxin (DT, Calbiochem, Merck, Darmstadt, Germany) was administered intraperitoneally in a total volume of 100 µl PBS one day before ConA injection.

2.3 Sampling of material

Eight hours after ConA injection, mice were anesthetized lethally by i.v. injection of 75 µl/10 g mouse of the following Ketamin-Xylazin mixture: 10 ml contained 0.8 ml of Sedaxylan (Xylazin 200 mg/ml), 1.2 ml Ketamin (115.3 mg/ml), 6 ml NaCl, and 2 ml Heparin (5000 IU/ml). After opening the abdomen and peritoneum, the diaphragma was cut through and cardiac blood was taken for determination of plasma cytokine and transaminase levels. One sample from the cranial part of the left lobe of the liver was removed and frozen in liquid nitrogen for RNA isolation and subsequent qRT-PCR. In case of FACS analysis, freshly isolated livers were removed and stored in Hanks balanced salt solution (HBSS: 5.4 mM KCl; 0.3 mM Na_2HPO_4 x 7 H_2O; 0.4 mM KH_2PO4; 4.2 mM $NaHCO_3$; 1.3 mM $CaCl_2$; 0.5 mM $MgCl_2$ x 6 H_2O; 0.6 mM $MgSO_4$ x 7 H_2O; 137 mM NaCl; 5.6 mM D-glucose; pH 7.4; all chemicals were purchased from Carl Roth GmbH, Karlsruhe, Germany) for isolation of hepatic mononuclear cells. Moreover, freshly excised spleens and portal lymph nodes were also stored in HBSS for subsequent isolation of splenocytes or lympho-nodal cells and subsequent FACS analysis.

2.4 Isolation of cells

2.4.1 Isolation of $CD4^+$ effector T cells and $CD4^+CD25^+$ T_{regs}

Hepatic leukocytes were isolated via density gradient as described previously (Liu et al., 2000). Livers were passed through 100 µm nylon meshes (Becton Dickinson GmbH, Heidelberg) in HBSS and centrifuged at 500 x g for 5 min. The cell pellet was resuspended

in isotonic 36% Percoll/HBSS (Percoll: GE Healthcare) solution containing 100 U/l heparin and centrifuged at 800 x g for 20 min. To lyse red blood cells, the cell pellet was resuspended in NH_4Cl-solution (139 mM NH_4Cl and 19 mM Tris [Carl Roth GmbH]), incubated for 10 min at room temperature and centrifuged at 500 x g for 5 min. After a final washing step with HBSS, the cell pellet was resuspended in cold FACS buffer for flow cytometry analysis. Single cell suspensions from spleens and lymph nodes were prepared by passing the organs through 100 µm nylon meshes in HBSS. After centrifugation at 500 x g for 5 min, red blood cells were lysed, the pellet was washed twice in HBSS, and resuspended in FACS buffer, HBSS, or RPMI medium (Invitrogen, Gibco Cell Culture Products), according to the following procedure. Single cell suspensions from blood were prepared by extracting 80 µl of fresh heart blood, subsequently lysing red blood cells and final washing with HBSS. Cells were then resuspended in FACS buffer for flow cytometry analysis.

2.4.2 Isolation of $CD4^+CD25^-$ responder cells and $CD4^+CD25^+$ T_{regs}

For isolation of splenic $CD4^+CD25^-$ responder cells and $CD4^+CD25^+$ T_{regs}, a combined sorting procedure using magnetic-bead separation (MACS, $CD4^+$ T-Cell-Isolation Kit, mouse; Miltenyi Biotec) and FACS sorting was carried out. Therefore, single cell suspensions from several spleens were pooled and cells were washed with HBSS. $CD4^+$ T cells were enriched by removing all other cell types: $CD8^+$ T cells, macrophages, B cells, NK cells and erythrocytes were marked with a biotinylated antibody cocktail against CD8A, CD11b, CD45R, DX5 and Ter-199. Anti-biotin microbeads were added to allow magnetic depletion of these cells. For the subsequent FACS sorting, cells were stained with fluorescent anti-CD4-APC/Cy7 and anti-CD25-PE antibodies. The sorting procedure was performed in PBS on a FACSAria (BD Biosciences, Heidelberg, Germany). Due to the higher risk of contamination during the FACS sorting procedure cells were captured in 2 ml of medium containing 2% penicillin and streptomycin.

2.4.3 Isolation of human peripheral blood mononuclear cells (PBMC)

Human PBMC from venous blood of a healthy donor were isolated by Ficoll gradient centrifugation (30 min, 20° C, 2000 rpm). Cells were counted and frozen in freezing medium for later use, by adding ½ of end volume of freezing medium 1 (RPMI 90%; fetal bovine serum 10%) and ½ of end volume of freezing medium 2 (RPMI 40%; fetal bovine serum 40%; DMSO 20%).

2.5 In vitro experiments

2.5.1 Co-cultivation of $CD4^+CD25^-$ responder cells and $CD4^+CD25^+$ T_{regs}

T_{regs} from ConA-pretreated or control animals with or without Rolipram treatment and splenic responder T cells were co-cultured in a 96-well round-bottom plate (Thermo Fisher Scientific, Roskilde, Denmark), at a ratio of 1:4 ($0.25x10^5$ Tregs and $1x10^5$ Tresp) and 1:8 ($0.125x10^5$ Tregs and $1x10^5$ Tresp). Single cultures of responder T cells ($1x10^5$ cells or $1.5x10^5$ cells) or T_{regs} ($0.5x10^5$ cells) were performed as controls. Cells were stimulated via activation of the CD3 co-receptor complex by plate bound anti-CD3 monoclonal antibody (5 µg/ml, BD) and stimulation of the CD28 co-receptor complex via soluble anti-CD28 antibody (1 µg/ml, BD Pharmingen, Heidelberg, Germany). Cells were cultured in RPMI medium (Invitrogen, Darmstadt, Germany) containing 2% penicillin and streptomycin, and 10% FCS. Cytokine concentrations of IL-2, IL-10 and IFN-γ were measured using ELISA.

2.5.2 Cultivation and proliferation of human PBMC

$1x10^7$ human PBMC were thawed and washed two times with PBS to remove any serum. For eFlour labeling, cells were resuspended in 1 ml PBS containing 2 µM eFlour (eBioscience, San Diego, CA, USA), vortexed, and incubated at 37° C in the dark for 10 minutes. Labeling was stopped by adding 4-5 volumes of cold RPMI media (Invitrogen, Darmstadt, Germany) including 10% FCS, and incubating cells on ice for 5 minutes. Cells were washed 3 times with complete media and cultured on a 96-well round-bottom

plate (Thermo Fisher Scientific, Roskilde, Denmark). Cells were stimulated via activation of the CD3 co-receptor complex by soluble anti-CD3 antibody (OKT3, 500 ng/ml, BioXCell, West Lebanon). Unstimulated cells were used as controls. Rolipram was added in a concentration of 3.3 mM (0.91 mg/ml). Respective control cultures with PBS and DMSO (solvent control) were performed. Cells were incubated at 37° C in the dark. At day 4, 5, or 6, cells were analysed using flow cytometry.

2.6 Analysis of plasma transaminases

Liver damage was quantified by automated measurement of plasma activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) 8 or 24 hours after ConA administration as described by Bergmeyer (Bergmeyer 1978) using reagents from Roche diagnostics and a COBAS Mira or COBAS c111 System (both from Roche, Mannheim, Germany).

2.7 Plasma cytokine determination by enzyme-linked immunosorbent assay (ELISA)

Plasma levels of TNF-α, IFN-γ, IL-2, IL-6, IL-10, and IL-17 were determined via ELISA using Greiner 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany). Antibodies for detection of IL-2, IL-6, and IL-10 and TMB-Substrate Reagent Set were purchased from BD Pharmingen (Heidelberg, Germany). Abs for detection of TNF-α, IFN-γ, and IL-17 were purchased from R&D Systems (Wiesbaden, Germany). Microtiter plates were coated with diluted capture Abs and incubated over night at 4° C. Between each step, plates were washed four times with washing buffer (0,05% Tween20 in PBS). To avoid unspecific binding on the surface, blocking solution (1% BSA [Serva, Heidelberg], 0,05% NaN_3 (Carl Roth GmbH] in PBS) was added at room temperature for two hours. Afterwards, standard and samples were applied for two hours and antigens were captured by the plate-bound primary Ab. Finally, detection antibody which bound the capture Antibody-antigen-complex of interest was added for two hours. The biotinylated detection Ab was marked with streptavidin-peroxidase (R&D Systems GmbH, Minneapolis) and TMB substrate (BD Pharmingen). After stopping the reaction with

1 M H_2SO_4, the resulting colour change was measured using a TECAN automatic photometer (TECAN Group, Männedorf, Switzerland).

2.8 Flow cytometry

Typically 1x10^6 lymphocytes isolated from liver, spleen or liver lymph nodes were stained. Cells were washed in FACS buffer prior to blocking with Mouse Seroblock Fc (AbD Serotec, Düsseldorf, Germany) in a volume of 50 µl FACS Buffer per sample. Antibody was added in another 50 µl of FACS buffer for fluorescence staining of surface molecules. The following mouse Abs were used: anti-mouse CD4-FITC, anti-mouse CD25-PE-Cy7, anti-mouse Ki67, anti-mouse Foxp3-PE (all from BD Pharmingen). Finally, cells were washed and taken up in 500 µl FACS buffer for analysis. For intracellular Foxp3 staining the Foxp3 staining buffer set and the anti Foxp3-PE-Cy5.5 antibody were used (eBioscience, San Diego, CA, USA). Human PBMC were stained with anti-human CD4-Pacific Blue (BD). Cells were analyzed with a FACS CantoII (BD Biosciences, Heidelberg, Germany) and the BD FACSDiva 6.0 software.

2.9 Quantitative real-time PCR (qRT-PCR)

RNA was isolated from liver samples by using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). The kit allows RNA extraction from frozen tissue samples as well as removal of contaminating DNA using the included DNase enzyme. Concentration of isolated RNA was quantified by photometric measurement according to Gallagher & Desjardins (2008). Isolated RNA was reverse transcribed using the ABgene VersoTM RT-PCR Kit (Thermo Fisher Scientific, Hamburg, Germany) on a MyCycler (BioRad, Munich, Germany). Quantification of transcribed RNA was performed on a CFX 96 Real-Time System and C1000 Thermal Cycler (BioRad) using Absolute QPCR SYBR Green Mix in a total volume of 10 µl. The overall fluorescence of SYBR Green was measured as it increased proportionally to the double-stranded DNA concentration. Melting curves of PCR products were analyzed to confirm amplification specifity. Relative mRNA levels were calculated by means of $\triangle\triangle$CP (CP after normalization to reference β-actin levels). Quantification is reported as the x-fold differences relative to the CP of a respective control group of mice. Primer pairs are listed in table 2.1. The

NCBI Primer-Blast database was queried to confirm that the 5' and 3' primers of each primer pair bind to different exons.

Table 2.1: list of primer pairs (T = annealing temperature, time = elongation time)

name	**sequence**	**T [°C]**	**time [s]**
β-actin 5'	TGG AAT CCT GTG GCA TCC ATG AAA	56	16
β-actin 3'	TAA AAC GCA GCT CAG TAA CAG TCC G	56	16
IL-2 5'	AAC CTG AAA CTC CCC AGG AT	60	12
IL-2 3'	TCA TCA TCG AAT TGG CAC TC	60	12
IL-6 5'	GCC TAT TGA AAA TTT CCT CTG	53	12
IL-6 3'	GTT TGC CGA GTA GAT CTC	53	12
IL-10 5'	ATG CCT GGC TCA GCA C	58	16
IL-10 3'	GTC CTG CAT TAA GGA GTC G	58	16
IL-17 5'	TCC AGA AGG CCC TCA GAC TA	60	17
IL-17 3'	AGC ATC TTC TCG ACC CTG AA	60	17
IFN-γ 5'	GAA CGC TAC ACA CTG CAT	56	16
IFN-γ 3'	GAG CTC ATT GAA TGC TTG G	56	16
TNF-α 5'	CAC CAA ACG AGG GAT GAG AAG TTC CCA A	57	12
TNF-α 3'	CTC ATC CCT GTG GTT TGC TAC GAC GTG	57	12

2.10 Haematoxylin/eosin staining of liver sections

For histological analysis of tissue structures livers were fixed overnight in 4% formalin (Carl Roth GmbH) and subsequently embedded in paraffin. Sections were stained with Haematoxylin/Eosin using a standard procedure and analyzed by light microscopy.

2.11 Statistical analysis

Groups were compared with the two-tailed Student's t test included in Graphpad Prism 5. All data in this study are expressed as mean ± SEM. A p value of 0.05 was considered significant with the following ranges: * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

3 Results

3.1 Effect of Rolipram on liver damage and cytokine secretion

3.1.1 Protection from ConA induced liver damage

To assess the protective effect of Rolipram against liver damage in ConA-tolerant mice, animals were pretreated with ConA or saline, on day 0. On day 8, animals were treated with Rolipram or solvent 30 minutes prior to ConA (re-)stimulation.

Plasma transaminase activities of both ALT and AST determined 8 hours after ConA injection are diagnostic markers for liver damage and correlate with histological findings (Tiegs et al., 1992). Accordingly, mice treated with a single ConA injection displayed high plasma transaminase activities (Figure 3.1: white bars). Mice pretreated with ConA on day 0 showed lower plasma transaminase activities (Figure 3.1: light grey bars). These findings suggest that ConA-pretreated mice developed tolerance as demonstrated recently (Erhardt et al., 2007).

Upon treatment with Rolipram, mice were significantly protected against liver damage (Figure 3.1: dark grey bars). However, in the ConA-pretreated, Rolipram-treated group, plasma transaminase activity was not further reduced, compared to the ConA-tolerant control (Figure 3.1: black bars).

3.1.2 Increased Interleukin-10 (IL-10) production upon ConA restimulation

The tolerant state following ConA hepatitis is mediated by an anti-inflammatory cytokine milieu (Erhardt et al., 2007). Rolipram decreases pro-inflammatory cytokine levels and increases anti-inflammatory IL-10 level upon a single ConA injection (Gant-

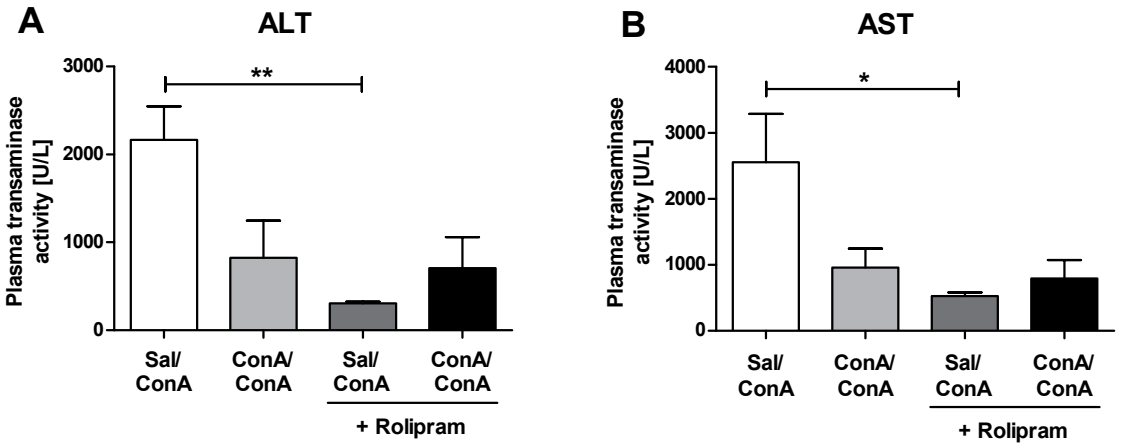

Figure 3.1: Treatment with Rolipram protected mice against liver damage, but did not improve tolerance induction
Mice were pretreated with 10 mg/kg ConA or saline on day 0, injected with 5 mg/kg Rolipram on day 8 and restimulated with 14 mg/kg ConA 30 minutes later. After 8 hours, plasma levels of ALT (A) and AST (B) were determined. Data from one single experiment reported as mean ± SEM (n≥4, * = $p \leq 0.05$, ** = $p \leq 0.01$).

ner et al., 1997). To investigate the cytokine milieu upon Rolipram treatment in the model of ConA tolerance, cytokine levels were measured 2 hours as well as 8 hours after ConA re-stimulation in plasma and liver tissue. Plasma levels of pro-inflammatory cytokines IL-2, IL-6, IFN-γ and TNF-α as well as anti-inflammatory IL-10 were determined by ELISA. Furthermore, intrahepatic mRNA expression of cytokines was quantified by qRT-PCR.

Upon a single ConA injection, the cytokine milieu corresponded to elevated plasma transaminase activities, reflecting the T-cell mediated ConA induced liver damage already described before (Tiegs et al., 1992). The pro-inflammatory cytokines IL-2, IL-6, IFN-γ and TNF-α were upregulated (Figure 3.3, white bars) compared to healthy animals (Sass et al. (2002), data not shown). Upon ConA restimulation, the cytokine milieu showed contradictory results. Although plasma transaminase activities were reduced suggesting tolerance induction, there was an increase of the pro-inflammatory cytokines IL-6 and TNF-α, compared with saline-pretreated animals (Figure 3.3.B and E, light grey bars). However, the release of the anti-inflammatory cytokine IL-10 was increased (Figure 3.3.C).

Upon a single ConA injection and Rolipram treatment, the pro-inflammatory cytokines IL-6 and TNF-α were reduced compared with non-Rolipram treated animals (Figure 3.3.B and E, white and dark grey bars). However, IL-2 and IFN-γ remained unchanged (Figure 3.3.A and D). IL-10 was slightly increased (Figure 3.3.C). Upon ConA restimulation and Rolipram treatment, all pro-inflammatory cytokines were decreased except for IFN-γ (Figure 3.3, light grey and black bars). Interestingly, the plasma level of IL-10 was increased 8 hours after ConA restimulation. This increase was also detectable in liver tissue (Figure 3.3.C).

The increase of IL-10 production reached statistical significance ($p \leq 0.01$) 2 hours after ConA restimulation (Figure 3.2, black bar).

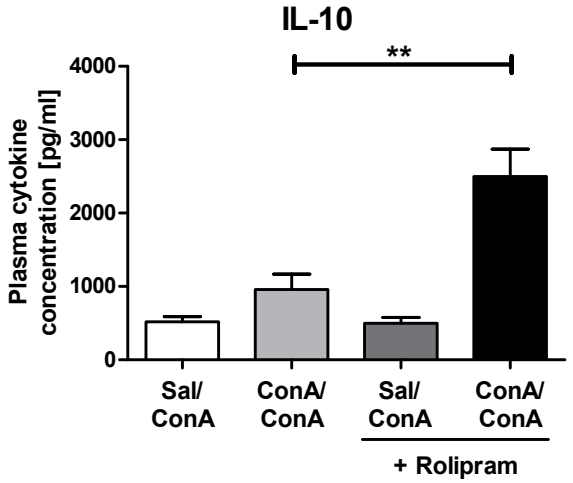

Figure 3.2: IL-10 was strongly increased in Rolipram-treated mice 2 hours after ConA restimulation
Mice were pretreated with 10 mg/kg ConA or saline on day 0, injected with 5 mg/kg Rolipram on day 8 and restimulated with 14 mg/kg ConA 30 minutes later. After 2 hours, the plasma concentration of IL-10 was determined. Data from one single experiment reported as mean ± SEM ($n \geq 4$, ** = $p \leq 0.01$).

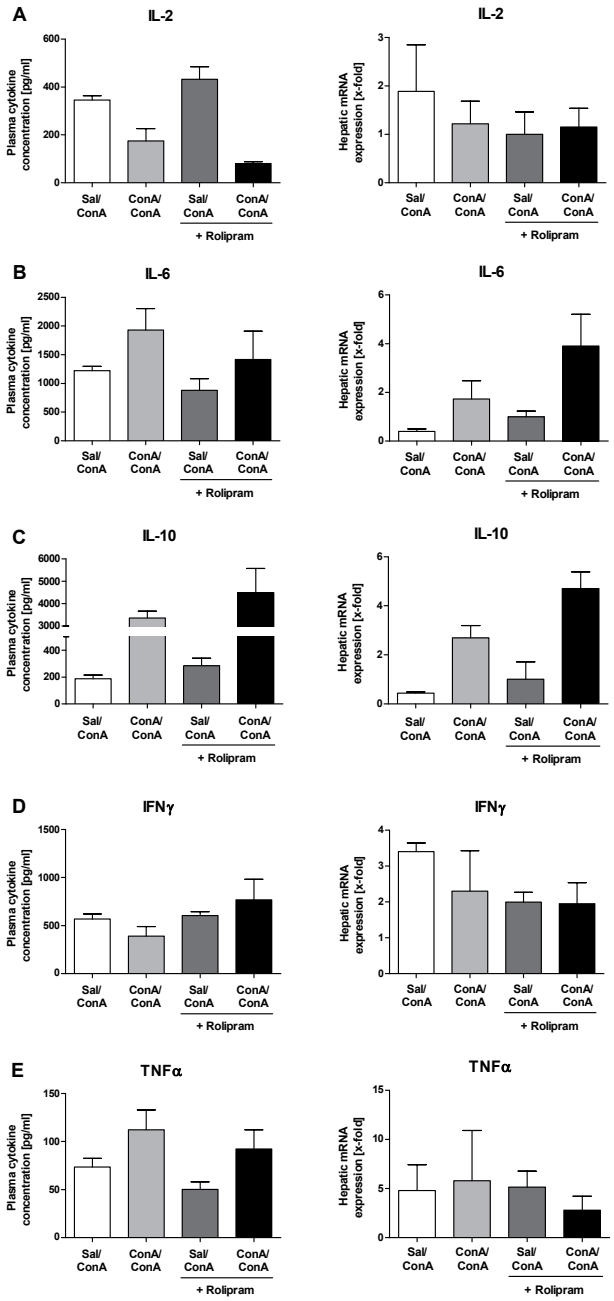

Figure 3.3: Rolipram increased IL-10 levels upon ConA restimulation
Mice were pretreated with 10 mg/kg ConA or saline on day 0, injected with 5 mg/kg Rolipram on day 8 and restimulated with 14 mg/kg ConA 30 minutes later. After 8 hours, plasma concentrations of the cytokines IL-2 (A), IL-6 (B), IL-10 (C), IFN-γ (D), and TNF-α (E) were determined. Liver samples were excised for mRNA extraction and subsequent qRT-PCR analysis. Data from one single experiment reported as mean $\pm$ SEM (n$\geq$4).

3.2 Alteration of T_{reg} frequencies and Forkhead-Box-Protein P3 (Foxp3) expression upon Rolipram treatment

3.2.1 Increased intrahepatic T_{reg} frequencies

T_{regs} are main producers of IL-10 (Uhlig et al., 2006). We observed an increase of plasma IL-10 levels in Rolipram-treated, ConA-tolerant mice. Furthermore, there is evidence that PDE4-inhibition promotes T_{reg} expansion (Paintlia et al., 2008). Thus, the observed increase of plasma IL-10 levels upon Rolipram treatment might be due to an increase in T_{reg} frequencies. To assess T_{reg} frequencies, we isolated leukocytes from spleen, liver, and portal lymph nodes and performed FACS analysis. Cells were stained with anti-CD4, anti-CD25 and anti-Foxp3 antibodies.

T_{reg} frequencies were measured as percentage of $CD4^+CD25^+Foxp3^+$ cells among all $CD4^+$ cells, because CD25 and Foxp3 are the most specific markers for T_{regs} in the murine system (Sakaguchi, 2005).

ConA restimulation increased T_{reg} frequencies in the spleen and portal lymph nodes (Figure 3.4.B and C, light grey bars). Rolipram increased T_{reg} frequencies in the spleen upon a single ConA injection (Figure 3.4.B, dark grey bar). Interestingly, the frequency of T_{reg} was significantly increased in the liver of Rolipram-treated, ConA-tolerant mice (Figure 3.4.A, black bar).

3.2.2 Increased T_{reg}-specific Foxp3 expression in the liver

The mean of fluorescence intensity (MFI) of Foxp3 reflects the Foxp3 expression on single cell level. As Foxp3 is the main transcription factor of T_{regs} in the murine system (Sakaguchi, 2005), an upregulation of Foxp3 might induce higher suppressive capacity of T_{regs}.

ConA restimulation did not alter the MFI of Foxp3 (Figure 3.5.A and B, light grey bars). Upon a single ConA injection, treatment with Rolipram increased the MFI of Foxp3 in the liver and more pronounced in the spleen (Figure 3.5.A and B, dark grey bars). However, Rolipram significantly increased the MFI of Foxp3 in the liver of ConA-tolerant mice (Figure 3.5.A, black bar).

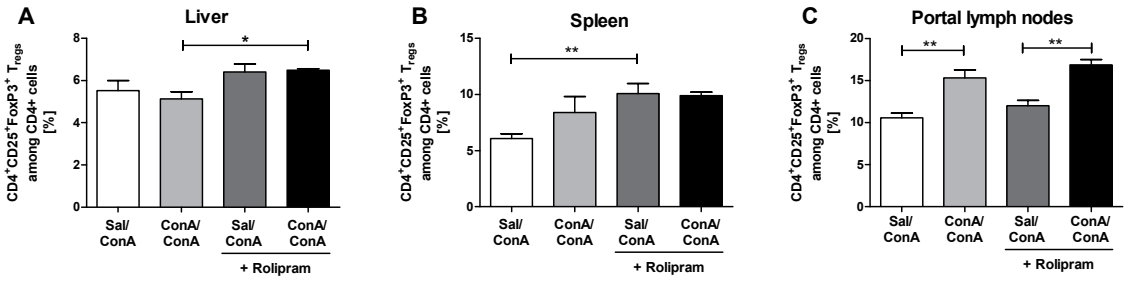

Figure 3.4: Rolipram increased T_{reg} frequencies in liver of ConA-tolerant mice and spleen upon a single ConA injection

Mice were pretreated with 11 mg/kg ConA or saline on day 0, injected with 5 mg/kg Rolipram on day 8 and restimulated with 14 mg/kg ConA 30 minutes later. After 2 hours, leukocytes from liver (A), spleen (B), and portal lymph nodes (C) were analyzed by flow cytometry. Data from one single experiment reported as mean ± SEM (n=4, * = $p \leq 0.05$, ** = $p \leq 0.01$).

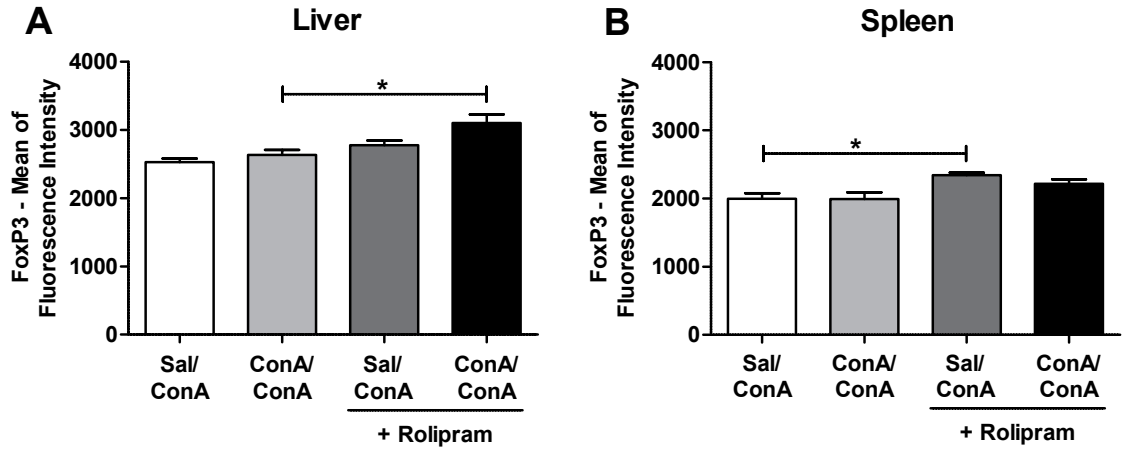

Figure 3.5: Rolipram increased the MFI of Foxp3 in liver of ConA-tolerant mice and spleen upon a single ConA injection

Mice were pretreated with 11 mg/kg ConA or saline on day 0, injected with 5 mg/kg Rolipram on day 8 and restimulated with 14 mg/kg ConA 30 minutes later. After 2 hours, leukocytes were isolated from liver (A) and spleen (B) and analyzed for Foxp3 expression. Data from one single experiment reported as mean ± SEM (n=4, * = $p \leq 0.05$, ** = $p \leq 0.01$).

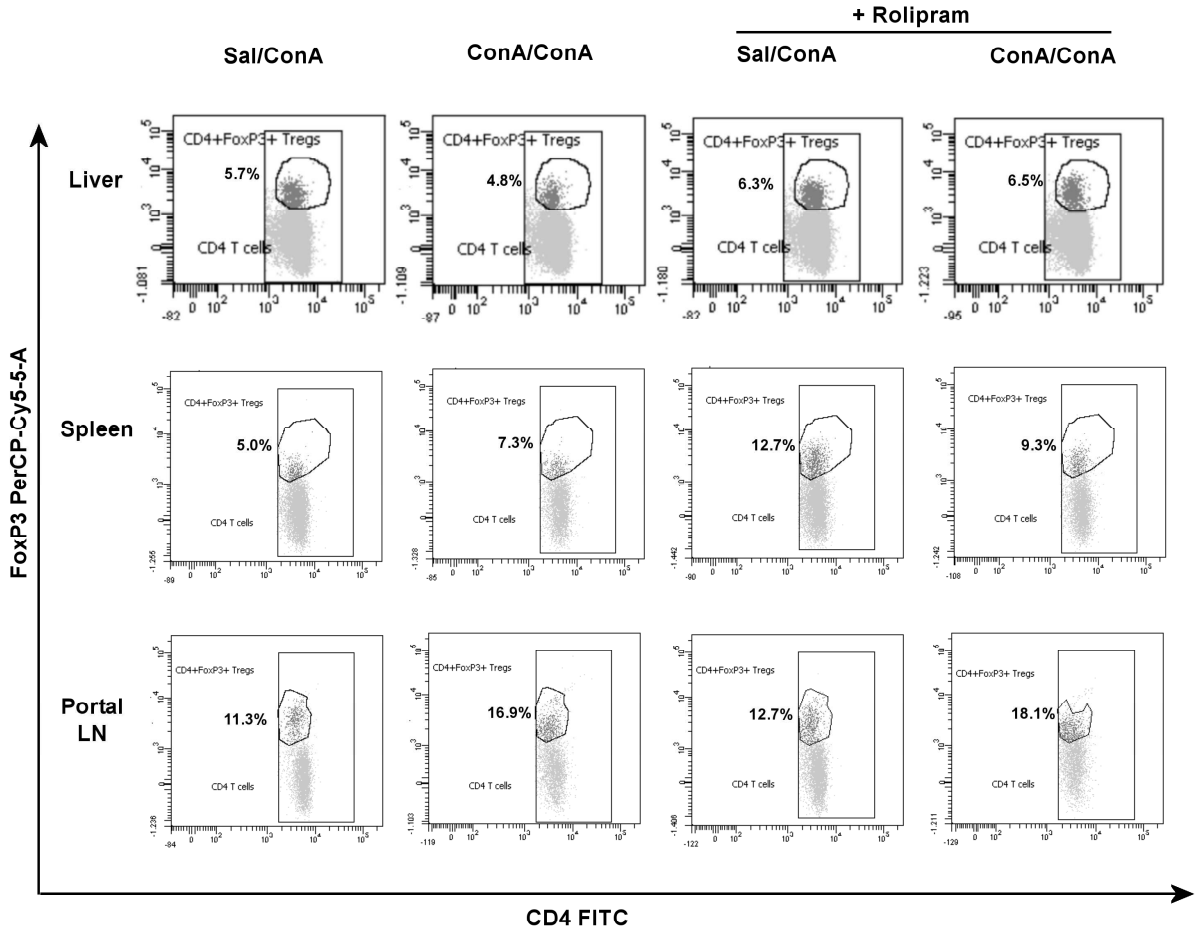

Figure 3.6: Representative dot plots for flow cytometry data presented in figures 3.4 and 3.5

Mice were pretreated with 11 mg/kg ConA or saline on day 0, injected with 5 mg/kg Rolipram on day 8 and restimulated with 14 mg/kg ConA 30 minutes later. After 2 hours, leukocytes from liver, spleen, and portal lymph nodes were analyzed by flow cytometry. Percentages represent the proportion of $CD4^+FoxP3^+$ T_{regs} among all $CD4^+$ cells. One representative dot plot from each experimental group is shown. Data from one single experiment.

3.3 Suppressive capacity of T_{regs} *in vitro*

3.3.1 T_{regs} from Rolipram-treated animals strongly suppressed IL-2 production by $CD4^+$ responder cells

To clarify if the increased expression of Foxp3 per T_{reg} cell results in a higher suppressive capacity of T_{regs}, an *in vitro* suppression assay was performed: $CD4^+CD25^+$ T_{regs} and $CD4^+CD25^-$ responder cells (Tresp) were co-cultivated and stimulated for three days. The concentration of IL-2 in supernatant was measured to quantify the proliferative capacity of Tresp.

Single-cultured Tresp produced large amounts of IL-2 upon stimulation (Figure 3.7, shaded bar). T_{regs} alone did not produce any IL-2 upon stimulation (Figure 3.7). The release of IL-2 from Tresp was slightly suppressed by T_{regs} from ConA-pretreated (Tregs ConA), but not from untreated animals (Tregs Sal, Figure 3.7, light grey vs white bars). T_{regs} from Rolipram-treated non-tolerant animals (Tregs Sal/Roli) significantly reduced IL-2 production of Tresp in a concentration of 1:8 (Figure 3.7, dark grey bars). This effect was even more pronounced in co-cultures with T_{regs} from Rolipram-treated ConA-tolerant mice (Tregs ConA/Roli, Figure 3.7, black bars).

In all four co-culture groups, lower numbers of Tregs suppressed IL-2 production to a stronger degree than higher numbers of Tregs (Figure 3.7, co-culture groups, respective right bar ($0.125*10^5$ Tregs) vs. left bar ($0.25*10^5$ Tregs)).

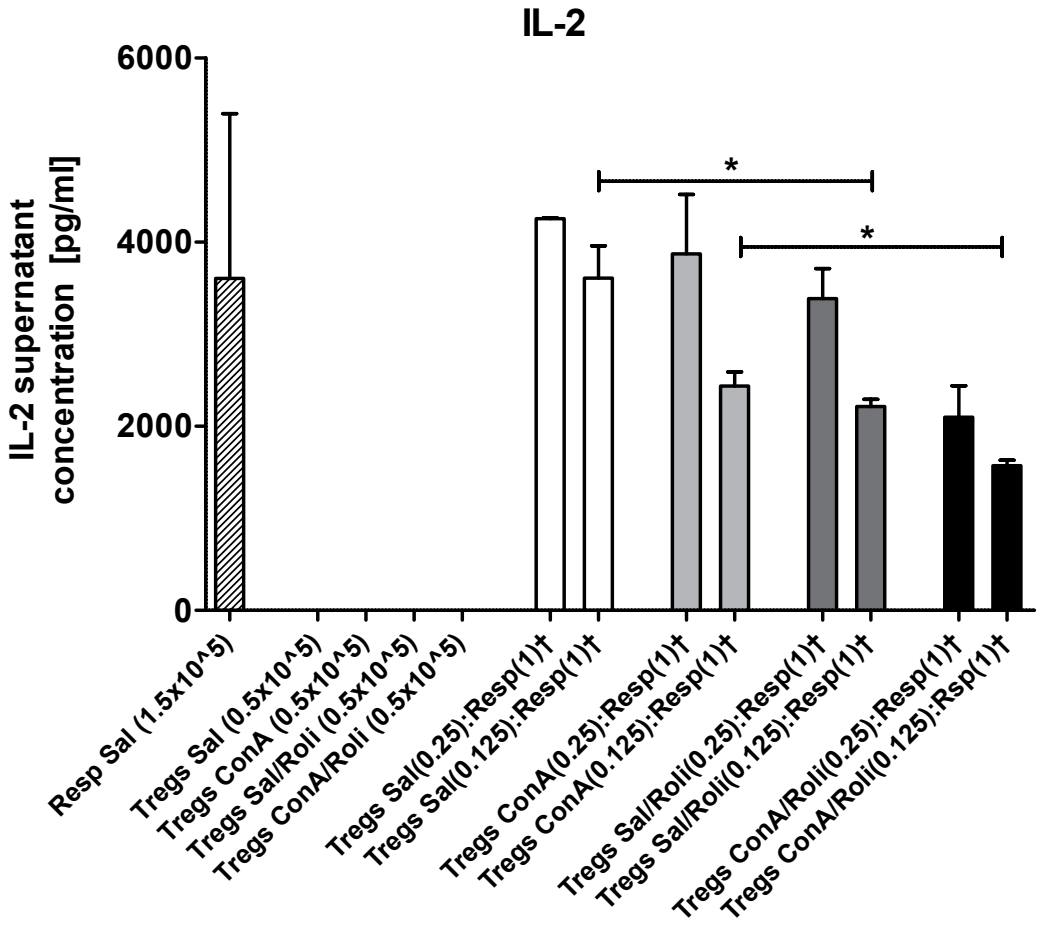

Figure 3.7: T_{regs} from Rolipram-treated animals lowered IL-2 production in co-cultures

Mice were pretreated with ConA or saline on day 0 and treated with Rolipram or solvent on day 8. Leukocytes were isolated from the spleen and sorted for $CD4^+CD25^-$ responder cells (Tresp) and $CD4^+CD25^+$ T_{regs}. Tresp from untreated animals and T_{regs} from non-tolerant (Tregs Sal), ConA-tolerant (Tregs ConA), Rolipram-treated non-tolerant (Tregs Sal/Roli) and Rolipram-treated ConA-tolerant (Tregs ConA/Roli) animals were cultured alone or co-cultured at a ratio of 1:4 and 1:8. After 3 days, cytokine concentration in supernatant was measured by ELISA. Every bar represents a cell culture triplet. Data from one single experiment reported as mean ± SEM (n=3, * = $p \leq 0.05$). †: Numbers in brackets are to be multiplied by $1x10^5$ and represent the absolute cell count per well.

3.3.2 T_{regs} from Rolipram-treated animals produced more IL-10

IL-10 is an important anti-inflammatory cytokine that is produced, amongst others, by T_{regs} and has different functions, including the control of T cell activation (Grütz, 2005). The concentration of IL-10 was determined in supernatants of co-cultures of T_{regs} and Tresp to further quantify the suppressive capacity of T_{regs}.

Tresp alone produced low levels of IL-10 (Figure 3.8, shaded bar). Single-cultured T_{regs} from ConA-tolerant animals (Tregs ConA) produced detectable amounts of IL-10 (Figure 3.8, dotted bar). This production was significantly increased in T_{regs} isolated from Rolipram-treated ConA-tolerant animals (Tregs ConA/Roli, Figure 3.8, grey dotted bar). Accordingly, co-cultures of Tresp and T_{regs} from ConA-tolerant, Rolipram-treated mice produced high amounts of IL-10 (Figure 3.8, black bars). Notably, IL-10 production was also increased in co-cultures of T_{regs} from Rolipram-treated non-tolerant animals (Figure 3.8, dark grey bar).

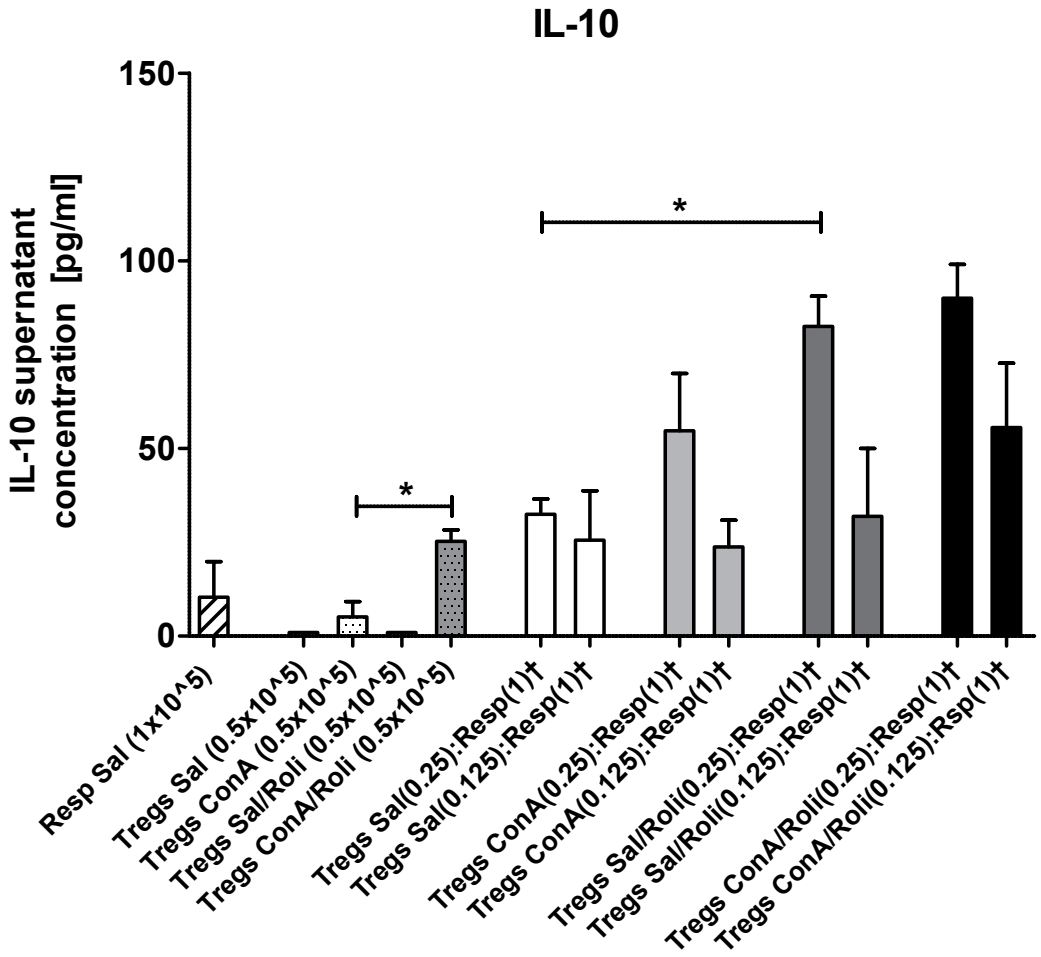

Figure 3.8: T_{regs} from Rolipram-treated animals produced IL-10 in co-cultures Same experimental setting as in Figure 3.7. Every bar represents a cell culture triplet. Data from one single experiment reported as mean ± SEM (n=3, * = $p \leq 0.05$). †: Numbers in brackets are to be multiplied by $1x10^5$ and represent the absolute cell count per well.

3.4 Effect of Rolipram in T_{reg}-depleted mice

3.4.1 T_{regs} were depleted by injection of diphtheria toxin (DT)

DEREG mice are bacterial artificial chromosome-transgenic mice expressing a diphtheria toxin (DT) receptor-enhanced green flourescent protein (GFP) fusion protein under the control of the Foxp3 gene locus (Lahl et al., 2007). They allow selective and efficient depletion of Foxp3$^+$ T_{regs} by injection of DT.

DT (1 µg/mouse) was injected into DEREG mice on day 0. Rolipram or control solvent and ConA were administered on day 1. To demonstrate the efficacy of T_{reg} depletion, splenic cells were isolated and stained for CD4. Subsequently, cells were analyzed using flow cytometry.

DT-treated mice showed reduced numbers of CD4$^+$GFP$^+$Foxp3$^+$ T_{regs} (Figure 3.9). Thus, T_{regs} were successfully depleted by injection of DT in contrast to control mice (0.9% vs 6.5%).

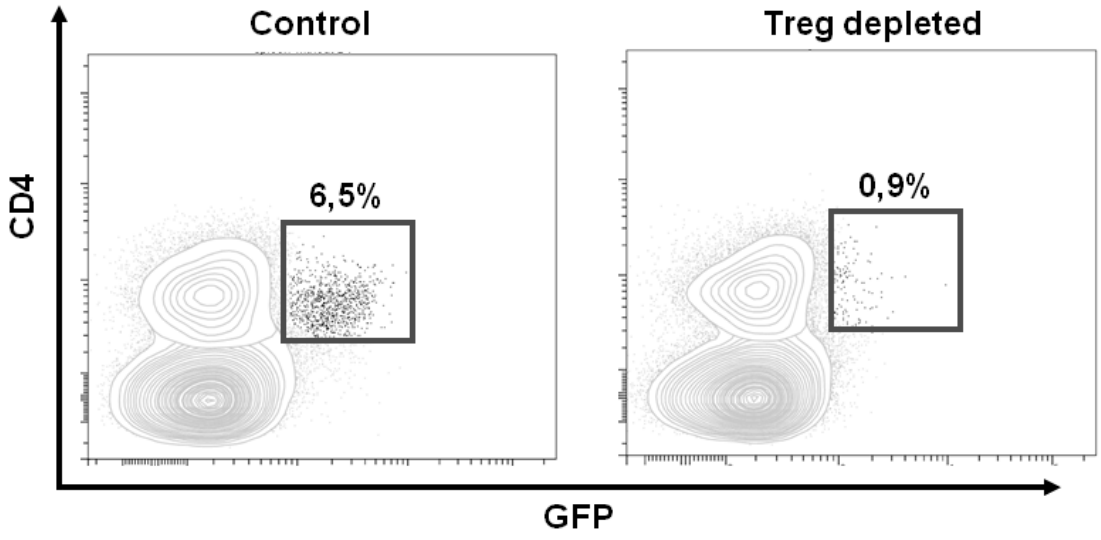

Figure 3.9: DT successfully depleted T_{regs} in DEREG mice
Splenocytes were isolated from an untreated DEREG mouse as control (left panel) and a DT-treated DEREG mouse 24 hours after DT injection (right panel). Splenic cells were stained for CD4 and gated for lymphocytes. T_{regs} are in the respective upper right black squares. T_{reg} frequency is presented as percentage of all CD4$^+$ T cells. Representative contour plots from one single experiment are depicted.

3.4.2 Increased liver damage in T_{reg}-depleted mice

Depletion of T_{regs} aggravated liver damage following a single injection of ConA (Figure 3.10, light grey bar). In the presence of T_{regs}, treatment with Rolipram did not ameliorate liver damage significantly, but it slightly decreased mean ALT levels (Figure 3.10, dark grey bar). In the absence of T_{regs}, Rolipram did not ameliorate liver injury (Figure 3.10, black bar). Interestingly, T_{reg}-depleted mice had higher levels of IL-10 eight hours after ConA injection (data not shown).

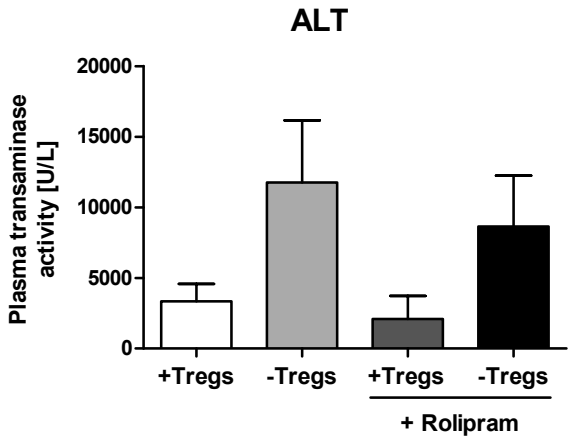

Figure 3.10: Increased liver damage and abrogated protection by Rolipram in T_{reg}-depleted DEREG mice
T_{regs} were depleted in DEREG mice (-T_{regs}) by injection of 1 µg DT on day 0. Controls did not receive DT (+T_{regs}). Mice were treated with 5 mg/kg Rolipram on day 1. All animals were challenged with a a single injection of 8 mg/kg ConA 30 minutes later. Plasma transaminase activity was determined 8 hours after ConA administration. Data from one single experiment reported as mean ± SEM (n≥4).

3.5 Tolerance induction upon Rolipram treatment prior to the first ConA challenge

A single ConA injection induces a fulminant hepatitis and induces subsequent tolerance within one week (Erhardt et al., 2007). To investigate whether a single injection of ConA under protection of Rolipram induces tolerance, we injected mice with Rolipram and pretreated them with a single dose of ConA. Fourteen days later, mice were (re-)stimulated with ConA.

A single injection of ConA led to an increased plasma transaminase activity of ALT (Figure 3.11.A, light grey bar). However, Rolipram treatment protected from liver damage, since ALT levels were significantly declined (Figure 3.11.A, black bar). Furthermore, the concentration of IL-10 in plasma was significantly increased in Rolipram-treated animals (Figure 3.11.B). Interestingly, plasma levels of IFN-γ were elevated in Rolipram-treated animals (mean of 1558 pg/ml in Rolipram-treated animals vs. 570 pg/ml in non-treated animals, data not shown).

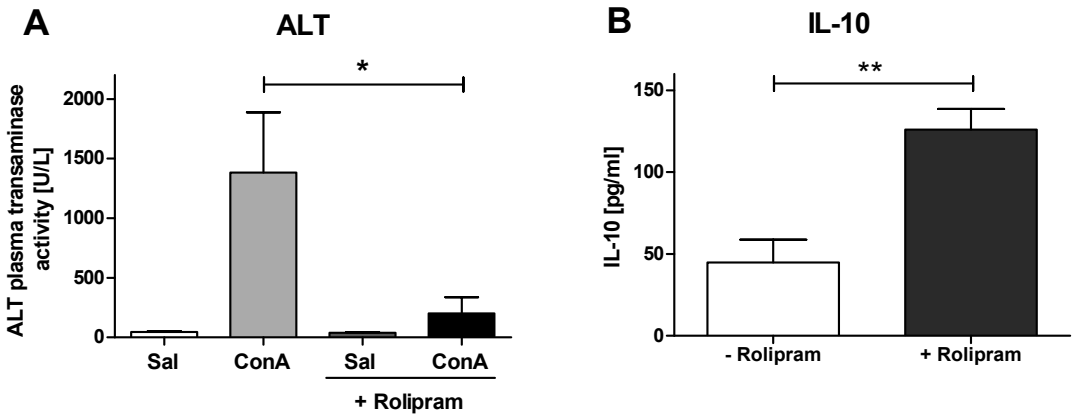

Figure 3.11: Rolipram protected from liver damage upon first ConA injection, and it increased IL-10 levels
Mice were injected with 5 mg/kg Rolipram on day 0 and treated with 10 mg/kg ConA 30 minutes later. Plasma ALT (A) and IL-10 (B) levels were determined 8 hours after ConA injection. Data from one single experiment reported as mean ± SEM (n=6, * = $p \leq 0.05$, ** = $p \leq 0.01$).

Fourteen days after the first ConA injection, mice were (re-)stimulated with ConA. Mice that had not been pretreated with ConA were susceptible towards ConA injection

and developed hepatitis, as reflected by their high ALT and AST levels (Figure 3.12, white bars). In the ConA-tolerant groups, ALT and AST levels were significantly reduced (Figure 3.12, light grey and black bars). Thus, mice that had not suffered from hepatitis on day 0 due to Rolipram treatment were also able to develop tolerance, although the Th1 immune response had been suppressed in these mice. Notably, Rolipram was completely eliminated from plasma 14 days after injection, assuming a plasma half life of 3 hours (Krause & Kühne, 1988). Accordingly, saline-pretreated mice developed hepatitis 14 days after Rolipram injection (Figure 3.12, dark grey bar).

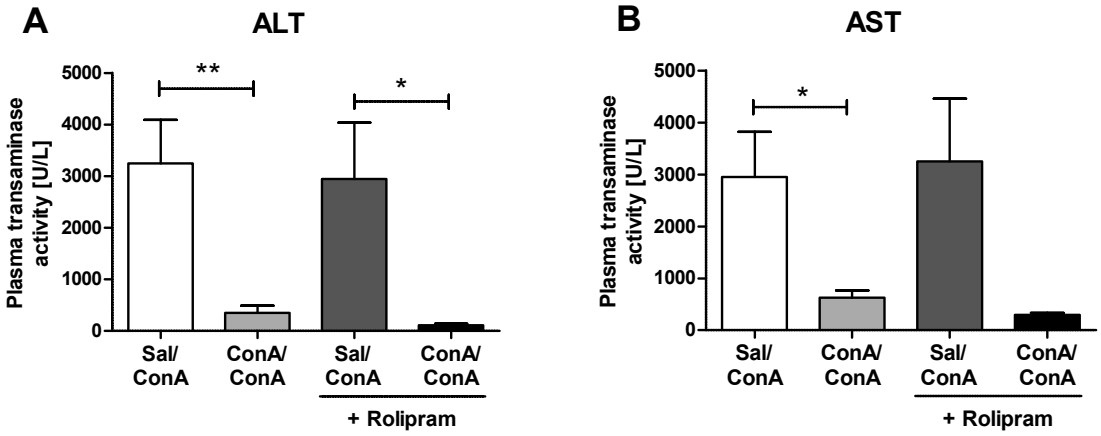

Figure 3.12: Mice protected from hepatitis by Rolipram on day 0 were tolerant 14 days later

Mice were injected with 5 mg/kg Rolipram on day 0 and treated with 10 mg/kg ConA or saline 30 minutes later. After 14 days, all animals were (re-)stimulated with 15 mg/kg ConA. Plasma transaminase activity of ALT (A) and AST (B) was measured 8 hours after ConA injection on day 14. Data from one single experiment reported as mean ± SEM (n=6, * = $p \leq 0.05$, ** = $p \leq 0.01$).

Development of ConA tolerance was verified by measuring the cytokine milieu both 2 hours and 8 hours after the second ConA injection. Both ConA pretreated groups displayed strong reductions of IL-2, IL-6, IL-17, and TNF-α compared with non-tolerant groups (Figure 3.13.A, B, D, E, and G, light grey vs. black bars). However, IL-10 was slightly increased in Rolipram-treated, ConA-tolerant mice (Figure 3.13.C, black bar), whereas IL-10 was significantly upregulated in the ConA-tolerant control group (Figure 3.13.C, light grey bar). Furthermore, the levels of IFN-γ were slightly increased in all

tolerant mice, except for the ConA-tolerant Rolipram-treated group 2 hours after ConA restimulation (Figure 3.13.F, black bar).

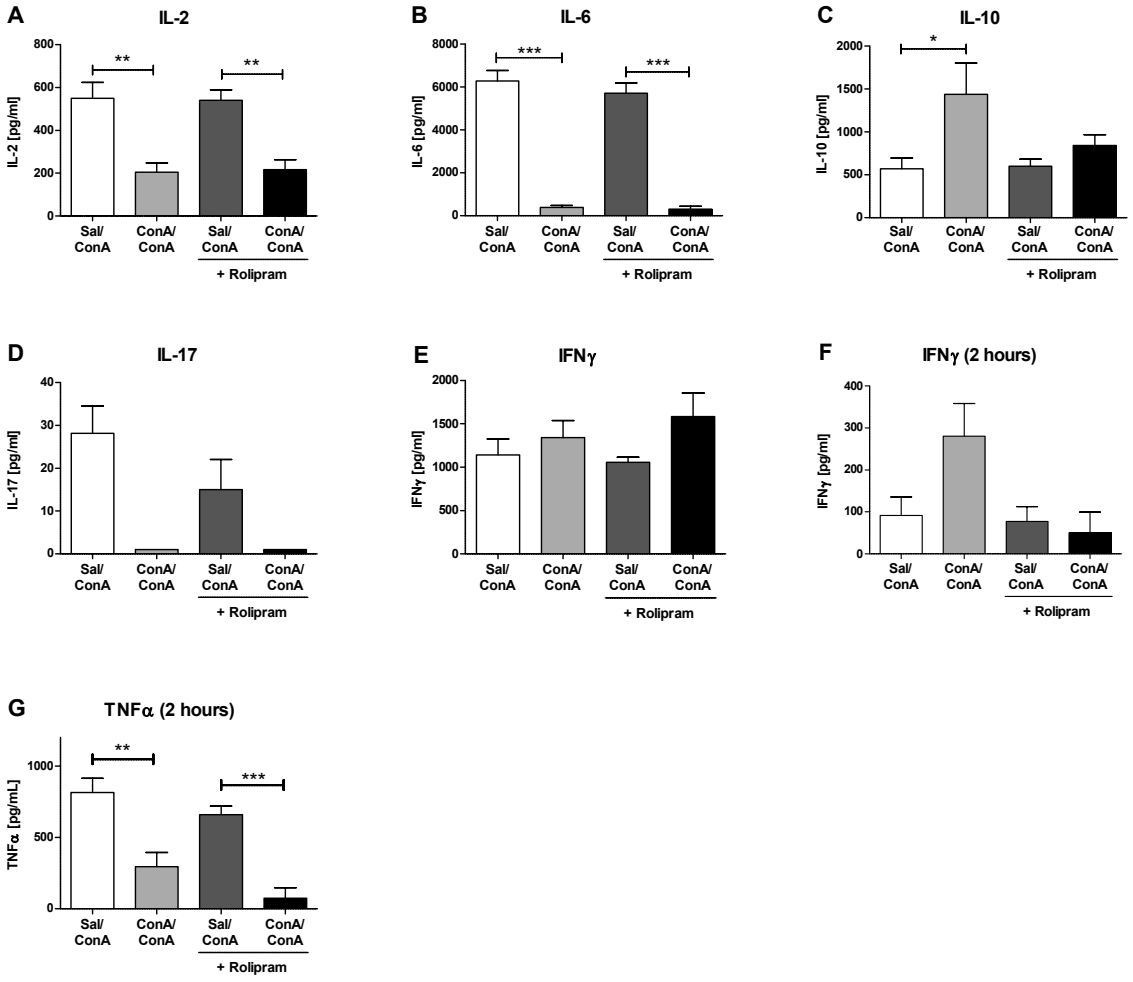

Figure 3.13: Cytokine milieu reflects tolerant state in both ConA-pretreated groups

Mice were injected with 5 mg/kg Rolipram on day 0 and treated with 10 mg/kg ConA or saline 30 minutes later. After 14 days, all animals were (re-)stimulated with 15 mg/kg ConA. Plasma cytokine levels of IL-2 (A), IL-6 (B), IL-10 (C), IL-17 (D), IFN-γ (E and F), and TNF-α (G) were determined 2 hours or 8 hours after ConA restimulation, respectively. Data from one single experiment reported as mean $\pm$ SEM (n=6, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$).

3.6 Rolipram inhibited the proliferation of human CD4$^+$ T cells *in vitro*

In order to investigate the effect of Rolipram on the proliferative capacity of human immune cells, an *in vitro* proliferation assay was conducted. PBMC were isolated from the blood of a healthy donor. Cells were stained with eFlour, and stimulated with anti-CD3 antibody for 4, 5 or 6 days in the presence or absence of Rolipram. On the respective last day, cells were stained for CD4 and analysed by FACS.

In Figure 3.14.A, the gating strategy of CD4$^+$ T cells is demonstrated. CD4$^+$ T cells that had not been stimulated with anti-CD3 antibody did not proliferate, as demonstrated by their consistently high intensity of eFlour (see Figure 3.14.B, unstimulated group). CD4$^+$ T cells that had been stimulated with anti-CD3 antibody did proliferate (Figure 3.14.B, solvent control group). Proliferation was lower in the presence of Rolipram on day 4, 5, and 6 compared with the control group: 91.0% to 82.2% on day 4, 88,3% to 76.8% on day 5 and 88.1% to 75.7% on day 6 (Figure 3.14.B, solvent control vs. Rolipram group).

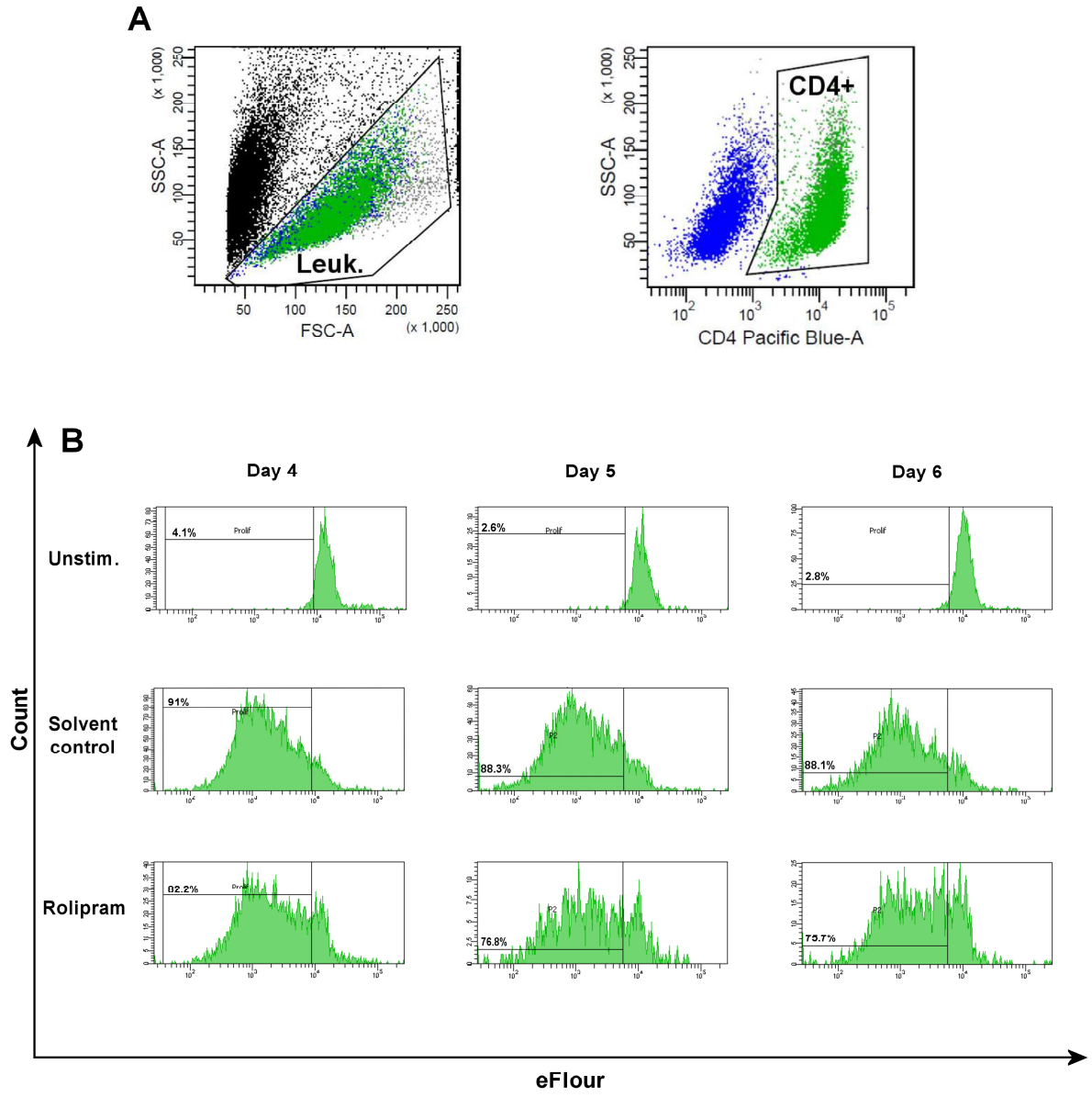

Figure 3.14: Rolipram inhibited the proliferation of human CD4$^+$ T cells *in vitro*

PBMC were stained for CD4 and gated for leukocytes and CD4$^+$ T cells. Proliferation is calculated as percentage of CD4$^+$ T cells with lowered eFlour intensity in comparison to non-proliferating cells in the unstimulated control. Leuk.: Leukocytes. Dot plots from day 4 and histograms of single wells from day 4, 5 and 6 are depicted. Data from one single experiment.

4 Discussion

4.1 The role of T_{regs} in ConA tolerance

T_{regs} play an important role in maintaining self-tolerance and preventing autoimmune diseases (Miyara & Sakaguchi, 2011). In this study, the model of ConA-induced hepatitis was used, resembling human autoimmune hepatitis. Upon a single ConA injection, T_{regs} seem to play an important role in protecting from liver damage, as depletion of T_{regs} before a single ConA injection aggravated ConA hepatitis shown in this study.

Upon ConA restimulation, the role of T_{regs} with respect to tolerance induction has to be elucidated. Erhardt et al. (2007) provided evidence for mediation of tolerance by T_{regs}: T_{regs} from ConA-tolerant animals exhibited an improved *in vitro* suppressive capacity and increased IL-10 production. Moreover, they significantly protected from ConA hepatitis after adoptive transfer into WT animals.

The following observations suggest that T_{regs} might not be the exclusive cell population mediating ConA tolerance: Firstly, given the fact that Rolipram improves T_{reg}-mediated suppression *in vivo* in an allergic airway model (Bopp et al., 2009) as well as in the *in vitro* suppression assay conducted for this study, one would expect a synergistic protective effect in Rolipram-treated, ConA-tolerant animals. However, this expectation was not supported by the results in this study. The experiment should be repeated in order to validate data. Secondly, the depletion of T_{regs} on day 8 before ConA restimulation did aggravate hepatitis, but depletion on day 14 did not (data not shown), demonstrating the involvement of other cell populations in long-lasting tolerance. Thirdly, treatment with Rolipram prior to ConA pretreatment did result in tolerance after 14 days. This is remarkable, considering the fact that treatment with Rolipram inhibited the Th1 immune response and protected from hepatitis. However, the Th1 immune response is necessary for the generation of T-bet$^+$CXCR3$^+$IL-10$^+$Foxp3$^+$ T_{regs} that mediate tolerance upon restimulation (Erhardt et al., 2011). The hypothesis that these cells are

absent in Rolipram-pretreated mice is further supported by the fact that these mice show lower plasma levels of IL-10 as well as of the Th1 cytokine IFN-γ when restimulated with ConA on day 14. Despite the absence of this crucial type of T_{regs}, Rolipram-treated, ConA-tolerant animals demonstrated strong protection from hepatitis with low plasma transaminase activities and typical cytokine patterns.

The results suggest that ConA tolerance is mediated by different cell types depending on the time of ConA restimulation. A possible hypothesis is that T_{regs} mediate tolerance in an early phase around day 8, whereas other cells such as Kupffer cells are responsible for long-lasting tolerance induction.

The role of T_{regs} could be clarified by a time kinetics experiment of T_{reg} depletion: DEREG mice are pretreated with ConA on day 0, and on day 6, 8, 10, 12 and 14, T_{regs} are depleted before ConA restimulation in order to elucidate the role of T_{regs} in tolerance induction. A similar approach could be used to identify the role of Kupffer cells, in this case for instance by depleting these cells by injection of clodronate liposomes (Rooijen et al., 1990).

4.2 Conversion of T_{regs} with Rolipram?

The results of this study (Figures 3.4 and 3.5) also touch upon a possible conversion of effector T cells into T_{regs} upon Rolipram treatment. T_{regs} were characterised by expression of Foxp3 in this study, as Foxp3 is the major transcription factor of T_{regs} in the murine system (Hori et al., 2003). However, a variety of T_{reg} cell types with different marker expression exists (de Lafaille & Lafaille, 2009). Accordingly, it might also be possible that Rolipram had effects on Foxp3$^-$ T_{regs} that remain unobserved in the present study.

FACS analysis upon ConA and Rolipram treatment revealed a slight increase in the number of intrahepatic Foxp3$^+$ T_{regs} and MFI of Foxp3 expression per single T_{reg} cell. However, the characterisation of the origin of these Foxp3 cells poses an experimental challenge. The increase of T_{reg} cell number may reflect three different effects: Proliferation of existing nT_{reg} cells, conversion of effector T cells into iT_{regs}, and/or migration of T_{reg} cells into the liver. Further analyses are required to differentiate these three possibilities. Proliferation of T_{regs} can be measured by staining T_{regs} with a monoclonal antibody against Ki-67, a nuclear protein used as proliferation marker (Chen

et al., 2010). In order to adress the question of conversion of effector T cells into T_{regs}, a possible experimental approach is the adoptive transfer of effector T cells purified from $CD45.1^+$ congenic mice into $CD45.2^+$ animals before ConA pretreatment. During FACS analysis, the expression of Foxp3 in $CD45.1^+$ cells can be used to quantify a conversion effect caused by Rolipram. Accordingly, migration of T_{regs} can be measured by adoptively transferring $CD45.1^+$ T_{regs} into $CD45.2^+$ mice before ConA pretreatment, and determining the amount of $CD45.1^+Foxp3^+$ T_{regs} in the liver.

Both, a possible conversion of effector T cells to T_{regs} and the augmentation of Foxp3 expression per T_{reg} cell can be explained by increased intracellular cAMP levels. Cyclic AMP is increased in T_{regs} (Bopp et al., 2007), and the CREB (cAMP responsive binding protein) is involved in Foxp3 expression in T cells (Ruan et al., 2009). However, the observed effect of a 1.3-fold increase in number and a 1.2-fold increase of mean Foxp3 expression under Rolipram treatment is rather small. This might be explained by the fact that *in vivo* T_{reg} generation is a complex process controlled on multiple levels, both intra- and intercellular. The determining regulators of Foxp3 expression are poorly understood. But it seems convincing that not only cAMP-dependent transcription factors regulate Foxp3 expression. Indeed, a mechanism for enhanceosome-based Foxp3 induction that depends on c-Rel, p65, NFATc2, smad and phosphorylated CREB has been recently described by (Ruan et al., 2009). Only one of these five transcription factors, namely CREB, is influenced by cAMP. This might explain the low increase in Foxp3 expression per cell: in order to upregulate Foxp3 expression, several transcription factors have to be activated, of which Rolipram activates only one. In conclusion, the protective effect of Rolipram in ConA-hepatitis might not be the result of conversion of effector T cells into T_{regs}. However, as cAMP and CREB are involved in Foxp3 expression, other cAMP-elevating drugs with a better therapeutic range or preference for T cells could favour T_{reg} conversion. An example is VIP which acts by elevating cAMP via a G-protein coupled receptor (Gonzalez-Rey & Delgado, 2007). Interestingly, VIP pretreatment also ameliorated ConA-induced hepatitis by increased IL-10 levels (Luo et al., 2009). Further studies have to performed in order to elucidate the role of T_{regs} in VIP-mediated protection from ConA-hepatitis.

Treatment of animals with Rolipram generated T_{regs} that were able to suppress *in vitro* proliferation of $CD4^+$ effector cells more efficiently than T_{regs} from untreated animals. In addition, T_{regs} from Rolipram-treated animals demonstrated an increase in IL-10 production *in vitro*. Interestingly, this increase in the suppressive capacity of

T_{regs} was not only observable in ConA-tolerant Rolipram-treated animals, but also in Rolipram-treated non-tolerant animals. Thus, treatment with Rolipram alone was sufficient to improve T_{reg}-mediated suppression, according to the findings of Bopp et al. (2009). However, it is questionable whether the IL-2 production in co-cultures adequately represents the proliferative capacity of effector T cells. Furthermore, Treg concentration and suppression of IL-2 production correlated negatively, i.e. lower Treg numbers suppressed IL-2 production to a stronger degree than higher Treg numbers, which is contrary to the assumed improved suppression by higher Treg numbers. In order to measure the actual proliferation of effector T cells, these cells could be stained with CFSE before cultivation. Furthermore, it remains to be investigated whether the improved T_{reg}-mediated suppression is directly associated with an increased expression of Foxp3. RNA from cultured cells can be purified for subsequent qRT-PCR analysis of Foxp3 mRNA expression. Alternatively, protein expression of Foxp3 can be measured by staining cultured cells for Foxp3, and analyzing cells via FACS.

4.3 Effects and target cells of Rolipram

The two main effects of Rolipram in the mouse model of immune-mediated liver damage were increased plasma IL-10 levels and protection from ConA-mediated hepatitis. The hypothesis is that Rolipram rises IL-10 production mainly in T_{regs}, which is supported by results from the co-culture experiments in this study. Rolipram was able to increase *in vitro* IL-10 production by T_{regs} that were isolated not only from ConA-tolerant, but also from non-tolerant animals. The importance of T_{regs} with respect to IL-10 production has also been demonstrated by Erhardt et al. (2007). The observed protection from ConA-hepatitis might be caused by an increased IL-10 production by T_{regs}. A participation of T_{regs} in the protective effect of Rolipram is also indicated by the fact that Rolipram was not able to protect from hepatitis in T_{reg}-depleted mice.

However, other cell types have to be kept in mind with respect to IL-10 release, e.g. Foxp3$^-$ T_{regs} and Kupffer cells. Furthermore, although tolerance towards ConA hepatitis is mediated by IL-10 (Erhardt et al., 2007), there was not always a stringent correlation between plasma IL-10 levels and the severity of liver damage, especially in long-lasting tolerance. Moreover, in Rolipram-treated, ConA-tolerant mice, plasma IL-10 levels were significantly increased, but a reduction of further liver damage was not

achieved. A possible explanation is that IL-10 underlies a saturation effect: Above a certain level, IL-10 might not be able to further suppress inflammation.

Taking the mode of action of Rolipram into account, both increased IL-10 levels and protection from hepatitis can be caused by other cells than T_{regs}, and protection from hepatitis can be caused by mechanisms independent from IL-10. Rolipram rises intracellular cAMP levels by selectively inhibiting the phosphodiesterase-4 (PDE4) enzyme family, which is expressed in a variety of immune cells (Müller et al., 1996). For instance, T_{regs} and effector T cells express the same pattern of PDE4 subtypes, albeit featuring different kinetics of cAMP metabolism (Bazhin et al., 2010). Accordingly, inhibition of PDE4 has been demonstrated to have immunosuppressive effects not only in murine T_{regs} (Bopp et al., 2009), but also in human effector T cells (Jimenez et al., 2001). On the one hand, the increase of cAMP levels in effector T cells might explain a possible conversion of effector T cells into T_{regs} observed in the FACS analysis of cells isolated from Rolipram-treated, ConA-tolerant animals. On the other hand, an influence of Rolipram on effector T cells might be demonstrated by repeating the T_{reg}-depletion experiment in DEREG mice: Although Rolipram was not able to significantly improve hepatitis in T_{reg}-depleted mice upon a single ConA injection, the visible tendency towards protection might reach statistical significance when using bigger treatment groups. A similar approach can be used to clarify whether Rolipram requires T_{regs} in order to protect from ConA hepatitis in ConA-tolerant mice. After identifying the time point of T_{reg}-mediated ConA tolerance, Rolipram can be injected into T_{reg}-depleted mice before ConA restimulation in order to see whether it protects the liver from damage.

Apart from effector T cells, PDE4 inhibition inhibits immune responses in human and murine eosinophils (Hatzelmann & Schudt, 2001), murine neutrophils (Sousa et al., 2010), and more important in this context, in human Kupffer cells (Gobejishvili et al., 2008). ConA tolerance is mediated by Kupffer cells, and they also account for IL-10 production (Erhardt 2007). Furthermore, IL-10 production in macrophages is induced by a cAMP-dependent pathway (Avni et al., 2010). Thus, it has to be clarified to what extent Rolipram stimulates IL-10 production in Kupffer cells. An experimental approach is the isolation and cultivation of Kupffer cells from animals treated with ConA and Rolipram, and determination of IL-10 production *in vitro*.

Interestingly, Heystek et al. (2003) demonstrated that dendritic cells (DC) lose their ability to generate IFN-γ^+ Th1 cells when treated with Rolipram. Thus, the suppression of the pro-inflammatory Th1 response following Rolipram treatment could not only be

mediated by the direct suppressive effect of Rolipram on effector T cells, but also on other immune cells that favor activation of naive T cells into Th1 cells, such as DC and Kupffer cells. The generation of Th1 cells after ConA pretreatment is an important part in the generation of Foxp3$^+$ T$_{\text{regs}}$ which mediate tolerance. Accordingly, the lack of an adequate Th1 response might result in two observations: Firstly, the failure to induce tolerance within 8 days after a low-dose ConA pretreatment under protection with Rolipram (data not shown). Secondly, the absence of T-bet$^+$IL-10$^+$Foxp3$^+$ T$_{\text{regs}}$ 14 days after treatment with Rolipram and ConA. Hence, a threshold of immune activation is necessary for the generation of Foxp3$^+$ T$_{\text{regs}}$ after ConA challenge.

4.4 Translation of the results in mice to the human system

This study aimed at investigating the effect of Rolipram on T$_{\text{regs}}$. The murine model of ConA-induced hepatitis has been used in this study, as it reflects the process of human autoimmune diseases (Tiegs et al., 1992). Although ConA does not act as an autoantigen, there are several similarities between the murine model and human autoimmune diseases. These similarities include a good responsiveness to immunosuppressive drugs (Gantner et al., 1997), strain-related differences in susceptibility (Mizuhara et al., 1998), the prevalence of CD4$^+$ T cells (Tiegs et al., 1992), and the involvement of T$_{\text{regs}}$ in state of remission (Erhardt et al. (2007), Lohse & zum Büschenfelde (1993)). Hence, a better understanding of the role of immunosuppressive drugs on T$_{\text{regs}}$ in the model of ConA-hepatitis might lead to new therapeutic options for the treatment of human autoimmune diseases.

One aim of this study was to enrich Foxp3$^+$ T$_{\text{regs}}$ locally in the organism by inducing a possible conversion of effector T cells into T$_{\text{regs}}$. This *in vivo* conversion seeks to avoid disadvantages of strategies that aim at expanding nT$_{\text{regs}}$ or converting effector T cells into iT$_{\text{regs}}$ *in vitro*, for instance by stimulation with TGF-β, and subsequent adoptive retransfer of expanded/converted cells. Generally, the conversion of effector T cells into T$_{\text{regs}}$ leads to improved immunosuppression which is desirable in different clinical situations, including autoimmune diseases, allergies and transplant rejection. For instance, diseases like diabetes (Lindley et al., 2005), multiple sclerosis (Viglietta et al., 2004), and asthma (Hartl et al., 2007) have been associated with impaired T$_{\text{reg}}$

function and would benefit from such a possible conversion. However, one has to keep in mind that the goal of achieving a highly specific immunosuppression by recruting T_{regs} will be missed by unspecifically converting effector T cells into T_{regs}, as the generation of antigen-unspecific T_{regs} will probably result in general immunosuppression. General immunosuppression is associated with a high risk of side effects. Hence, therapies should achieve specific immunosuppresion by conversion of antigen-specific T_{regs}, for example through the identification of autoantigens in different autoimmune diseases, and subsequent induction of T_{regs} with subimmunogenic doses of (auto-)antigen (Daniel 2011). This process could be supported by immunosuppressive drugs that have a stronger T_{reg}-inducing effect than Rolipram, for example Rapamycin, as shown in renal transplant patients (Segundo et al., 2010), and VIP, as observed *in vitro* (Pozo et al., 2009). Interestingly, the model of ConA-hepatitis allows the investigation of T_{reg} induction, as T_{reg} frequencies are increased in the liver one day after ConA pretreatment (Erhardt et al., 2007). However, as ConA is not an autoantigen, the mechanism of T_{reg} induction might differ from that in human autoimmunity.

In order to demonstrate an application of the results obtained in this study to human T cells, Rolipram was tested in a model involving human cells. Rolipram inhibited proliferation of human $CD4^+$ T cells in an *in vitro* proliferation assay with human PBMC. The experiment was conducted with unsorted PBMC due to the easier experimental handling. Although the result suggests a general immunosuppressive activity of Rolipram, it is uncertain to what extent the inhibition of $CD4^+$ T cell proliferation depends on T_{regs}. In order to clarify the role of T_{regs} in this setting, the experiment should be repeated with a more differentiated setting: After PBMC are isolated from the blood of a healthy donor, cells can be sorted for $CD4^+CD25^-$ effector T cells and $CD4^+$CD25+ T_{regs}. Subsequently, effector T cells are stained with eFlour and cultured either alone or with T_{regs}, in the presence or absence of Rolipram (see Figure 4.1). If Rolipram exerts its effect on T_{regs}, the single-cultured T cells should be able to proliferate as strong as the Rolipram-free controls, whereas an inhibitory effect should be visible in co-cultures. Furthermore, the experiment should include T_{regs} from more than one donor in order to increase reliability.

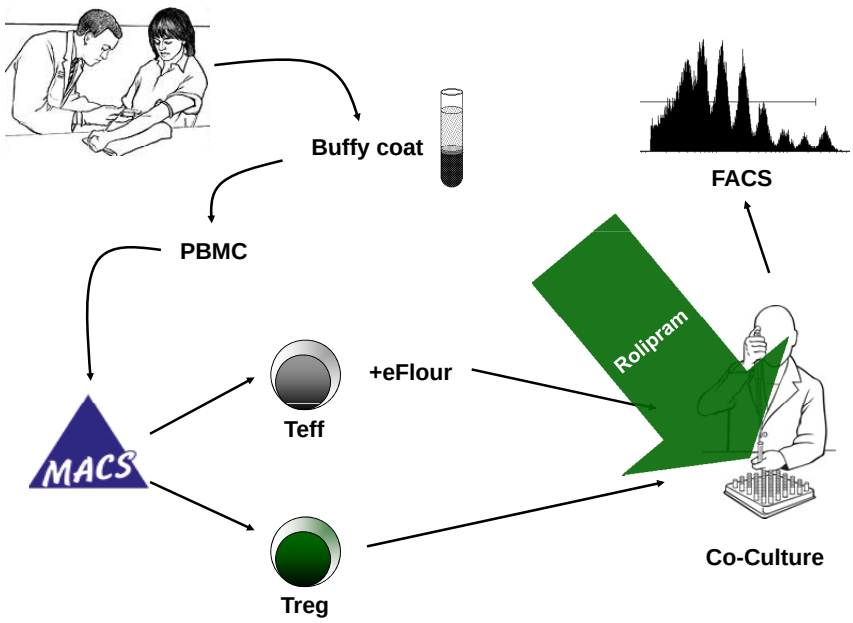

Figure 4.1: Experimental setting of a co-culture system containing Teff and T_{regs} in order to investigate whether T_{regs} participate in the immunosuppressive effect of Rolipram in humans
PBMC are isolated from the blood of a healthy donor. Cells are sorted for $CD4^+CD25^-$ effector T cells and $CD4^+CD25^+$ T_{regs} via magnetic assorted cell sorting (MACS). Effector T cells (Teff) are stained with eFlour and cultured either alone or with T_{regs}, in the presence or absence of Rolipram.

4.5 Conclusion and outlook

This study was intended to investigate the effect of Rolipram on T_{regs} in the model of ConA-mediated hepatitis with a focus on a possible conversion of effector T cells into T_{regs}. Rolipram had a protective effect on ConA hepatitis. It increased the frequency of T_{regs} and improved their *in vitro* suppressive capacity. In T_{reg}-depleted mice, the protective effect of Rolipram was abrogated. Moreover, injection of Rolipram prior to ConA pretreatment resulted in tolerance 14 days later. In an *in vitro* experiment with human cells, Rolipram inhibited the proliferation of human $CD4^+$ cells.

For further investigation, the role of T_{regs} in the model of ConA-mediated hepatitis should be clarified. The most important aspect in this context is the identification of

the time point when T_{regs} are the main mediators of ConA tolerance. Afterwards, it may be possible to investigate the synergistic effect of Rolipram treatment and ConA rechallenge. Furthermore, the necessity of a Th1 immune response for the generation of T-bet$^+$IL-10$^+$Foxp3$^+$ T_{regs}, and the participation of these T_{regs} in ConA tolerance should be clarified.

Rolipram increased the frequency of Foxp3$^+$ T_{regs} in the liver. Whether this increase is due to conversion, or due to migration and/or proliferation remains to be investigated. In conclusion, both the increase of the proportion of Foxp3$^+$ cells as well as the increased MFI of Foxp3 expression could be due to conversion of T_{regs}, but it is unlikely that these two effects are the main cause of the protective effect of Rolipram. However, a possible proliferation of T_{regs} can be investigated by adoptive cell transfer experiments.

The results suggest that Rolipram exerted its protective effect not only by influencing T_{regs}, but also other immune cells, mainly effector T cells and possibly Kupffer cells. The inhibition of effector T cells could be mediated directly by inhibition of PDE4 and subsequent induction of anergy, or indirectly by increased IL-10 production from Kupffer cells. Furthermore, Rolipram could influence dendritic cells which favour the differentiation of naive T cells into Th1 cells.

The ConA model features several characteristics of human autoimmune diseases. Hence, a better understanding of the effect of immunosuppressive drugs on T_{regs} might lead to new therapeutic applications. As the transferability of results from mouse to man is always questionable, the general inhibitory potential of Rolipram has been exemplified. A more differentiated *in vitro* proliferation assay with human-derived co-cultured T_{regs} and Teff can be conducted in order to further explore the effect of Rolipram on human T_{regs}.

5 Summary

This study was intended to investigate the effect of the immunosuppressive drug Rolipram in the model of Concanavalin A (ConA)-induced hepatitis in mice. The model of ConA-induced hepatitis resembles human autoimmune liver diseases. This work focused on the possible effect of Rolipram on regulatory T cells (T_{regs}) and a possible conversion of these cells.

T_{regs} are specialised in the suppression of extensive immune reactions. An impaired function of T_{regs} is related to a variety of human autoimmune diseases and allergies, such as diabetes, multiple sclerosis, and asthma. Hence, T_{regs} might be a promising target in the therapy of diseases caused by overshooting immune reactions.

The results of this study are:

1. Rolipram protected from ConA-induced hepatitis after a single ConA injection. The protection from hepatitis by Rolipram after a single ConA injection was confirmed, and was accompagnied by an increase of plasma IL-10 levels. However, there was no synergistic effect of Rolipram treatment and ConA-restimulation in Rolipram-treated, ConA-tolerant mice, even though these animals also had significantly increased IL-10 levels.

2. Rolipram increased the expression of Foxp3 in T_{regs}. There was a significant increase in the number of intrahepatic Foxp3$^+$ T_{regs} in Rolipram-treated, ConA-tolerant animals. The results are compatible with a conversion of effector T cells into T_{regs}.

3. *In vivo* treatment with Rolipram improved the *in vitro* suppressive capacity of T_{regs}. In a co-culture of effector T cells and T_{regs}, T_{regs} from Rolipram-treated animals inhibited the production of the proinflammatory cytokine IL-2 by effector T cells. At the same time, these T_{regs} produced higher levels of the anti-inflammatory cytokine IL-10.

4. The absence of T_{regs} decreased the effect of Rolipram. Depletion of T_{regs} in DEREG mice before treatment with Rolipram and ConA stimulation resulted in aggra-

vated liver damage. This liver damage was not diminished by Rolipram. The results suggest that T_{regs} play a role with respect to the effect of Rolipram.

5. Treatment with Rolipram prior to ConA pretreatment still allowed tolerance induction. The acute immune response after ConA pretreatment was considered essential for tolerance induction. However, this study demonstrated that a suppression of the immune response by injection of Rolipram prior to ConA-pretreatment lead to tolerance after 14 days.

6. Rolipram inhibited the proliferation of human $CD4^+$ T cells. An *in vitro* proliferation assay was performed with human PBMC of a healthy donor. In presence of Rolipram, $CD4^+$ T cells demonstrated less proliferative capacity.

In conclusion, Rolipram exerted its immunosuppressive effect in the model of ConA-induced hepatitis partly by $Foxp3^+$ T_{regs}. In this context, both the increased expression of Foxp3 as well as the increased production of IL-10 play an important role. The conversion of effector T cells into T_{regs} is compatible with the results, but does not fully explain the protective effect of Rolipram. Hence, Rolipram seems to influence other immune cells directly and indirectly, such as effector T cells and Kupffer cells. An immunosuppressive effect of Rolipram was exemplified for human $CD4^+$ T cells.

6 Zusammenfassung

Die vorliegende Arbeit hatte zum Ziel, den Effekt des Immunsuppressivums Rolipram am Modell der Concanavalin A (ConA)-induzierbaren Hepatitis in der Maus zu untersuchen. Die ConA-induzierte Hepatitis dient als Modell für immunvermittelte Lebererkrankungen beim Menschen. Der Fokus dieser Arbeit lag auf der Untersuchung eines möglichen Effekts von Rolipram auf regulatorische T-Zellen (T_{regs}) und eine mögliche Konversion dieser Zellen.

T_{regs} sind auf die Unterdrückung überschießender Immunreaktionen spezialisiert. Eine Beeinträchtigung der Funktion von T_{regs} wird mit einer Vielzahl von humanen Autoimmunerkrankungen und Allergien, wie z.B. Diabetes, Multiple Sklerose und Asthma in Verbindung gebracht. T_{regs} sind daher ein vielversprechendes therapeutisches Ziel bei Erkrankungen, denen eine erhöhte Aktivität des Immunsystems zugrunde liegt.

In dieser Arbeit wurden folgende Ergebnisse erzielt:

1. Rolipram schützte vor ConA-Hepatitis nach einmaliger ConA-Gabe. Der durch Rolipram vermittelte Schutz vor einem ConA-induzierten Leberschaden nach einmaliger ConA-Gabe konnte bestätigt werden. Dieser Schutz wurde von erhöhten Plasmaspiegeln von IL-10 begleitet. Es kam jedoch bei Rolipram-behandelten, ConA-toleranten Tieren nicht zu einem synergistischen protektiven Effekt von Rolipram-Gabe und ConA-Restimulation. Allerdings fand sich eine signifikante Erhöhung der Produktion von IL-10 auch in diesen Tieren.

2. Rolipram erhöhte die Expression von Foxp3 in T_{regs}. In Rolipram-behandelten, ConA-toleranten Tieren fand sich eine signifikante Erhöhung von Foxp3-exprimierenden T_{regs} in der Leber. Die Ergebnisse lassen sich mit einer Konversion von Effektor-T-Zellen in T_{regs} vereinbaren.

3. Die *in vivo*-Behandlung mit Rolipram verbesserte die immunsuppressive Aktivität von T_{regs} *in vitro*. In einer Ko-Kultur von Effektor-T-Zellen und T_{regs} konnte gezeigt werden, dass T_{regs} aus Rolipram-behandelten Tieren - unabhängig von der ConA-Vorbehandlung - die Produktion des proinflammatorischen Zytokins IL-2 durch

Effektor-T-Zellen hemmten und gleichzeitig höhere Mengen des anti-inflammatorischen Zytokins IL-10 produzierten.

4. Die Abwesenheit von T_{regs} verringerte die Wirkung von Rolipram. Die Depletion von T_{regs} in DEREG-Mäusen vor Gabe von Rolipram und Stimulation mit ConA resultierte in einem erhöhten Leberschaden. Dieser Leberschaden konnte durch Rolipram nicht verringert werden. Dies spricht für eine Beteiligung von T_{regs} am Effekt von Rolipram.

5. Bei Gabe von Rolipram vor der ConA-Vorbehandlung entwickelte sich trotz des anti-inflammatorischen Effekts von Rolipram Toleranz. Die akute Entzündungsantwort nach ConA-Vorbehandlung wurde bisher als Grundlage für die Entwicklung der Toleranz betrachtet. In dieser Arbeit konnte allerdings gezeigt werden, dass eine Unterdrückung der Entzündungsantwort durch Rolipram-Gabe vor der ersten ConA-Injektion trotzdem Toleranz nach 14 Tagen induzierte.

6. Rolipram verringerte die Proliferationskapazität humaner $CD4^+$ T-Zellen. Mit PBMC eines gesunden menschlichen Spenders wurde ein *in vitro*-Proliferationsassay durchgeführt. Hier fand sich eine verringerte Proliferationskapazität der $CD4^+$ Zellen in Anwesenheit von Rolipram.

Zusammenfassend lässt sich feststellen, dass Rolipram einen Teil seines immunsuppressiven Effekts im Modell der ConA-Hepatitis durch eine Wirkung auf $Foxp3^+$ T_{regs} erzielt. Hier ist insbesondere die erhöhte Foxp3-Expression sowie die vermehrte Fähigkeit zur Produktion von IL-10 von Bedeutung. Die Konversion von Effektor-T-Zellen in $Foxp3^+$ T_{regs} lässt sich mit den Ergebnissen vereinbaren, erklärt aber allein nicht den protektiven Effekt von Rolipram. Daher wird angenommen, dass Rolipram auch direkt und indirekt auf andere Immunzellen, wie z.B. Effektor-T-Zellen und Kupffer-Zellen wirkt. Eine generelle immunsuppressive Wirkung von Rolipram auf menschliche $CD4^+$ T-Zellen konnte exemplarisch bestätigt werden.

7 Abbrevations

ALT	alanine transaminase
AMP	adenosine monophosphate
AST	aspartate transaminase
BSA	bovine serum albumin
C57Bl6	C57 black 6
cAMP	cyclic adenosine monophosphate
CD	cluster of differenciation
CFSE	carboxyfluorescein succinimidyl ester
CNI	calcineurin inhibitor
COPD	chronic obstructive pulmonary disease
CP	crossing point
CREB	cAMP responsive binding protein
c-Rel	C-Rel proto-oncogene protein
CTLA	cytotoxic T-lymphocyte antigen
CXCR	Chemokine, CXC Motif, Receptor
DC	dendritic cell
DEREG	depletion of regulatory T cells
DMSO	dimethyl sulfoxide
DNAse	deoxyribonuclease
DT	diphtheria toxin
DX5	CD49b
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbend assay
FACS	fluorescence-activated cell sorting
FC	fragment crystallizable
FCS	fetal calf serum
FITC	fluorescein isothiocyanate

Foxp3	Forkhead-Box-Protein P3 (murine)
FOXP3	Forkhead-Box-Protein P3 (human)
FSC	forward scatter
G	gravitational constant
GFP	green flourescent protein
hATG	human anti-thymocyte globulin
HBSS	Hanks balanced salt solution
i.p.	intraperitoneal
i.v.	intravenous
IFN	interferon
IL	interleukin
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
iTregs	adaptive (induced) Tregs
IU	international units
Leuk.	leukocytes
LFA	lymphocyte function-associated antigen
LN	lymph nodes
mAb	monoclonal antibody
MACS	magnetic activated cell sorting
MFI	mean of fluorescence intensity
MMF	mycophenolate mofetil
mRNA	messenger ribonukleic acid
mTOR	mammalian target of Rapamycin
n	number
NFAT	nuclear factor of activated T-cells
NK	natural killer
NOD	non-obese diabetic
nTregs	natural Tregs
OKT3	Orthoclone OKT3
p	probability
PBMC	peripheral blood mononuclear cells
PBS	phosphat-buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase

PE	R-phycoerythrin
pH	potentia hydrogenii
qRT-PCR	quantitative real-time polymerase chain reaction
RA	rheumatoid arthritis
rATG	rabbit anti-thymocyte globulin
RNA	ribonukleic acid
rpm	rotations per minute
RPMI	Roswell Park Memorial Institute
Sal	saline
SCID	severe combined immunodeficiency
SDH	succinate dehydrogenase
SEM	standard error of the mean
smad	Sma and Mad related proteins
SSC	side scatter
Stat1	signal transducer and activator of transcription
T_{resp}	Responder cells
T-bet	T-box transcription factor TBX21
Teff	effector T cells
TGF	transforming growth factor beta
TGN1412	CD28-SuperMAB
Th1	T-helper cell
TMB	tetramethylbenzidine
TNF	tumor necrosis factor
Tr1	type 1 T regulatory cell
T_{regs}	regulatory T cells
U	enzyme unit
VIP	vasoactive intestinal peptide
WT	wild type

Bibliography

Allison, A. C. & Eugui, E. M. (2000). Mycophenolate mofetil and its mechanisms of action. *Immunopharmacology*, 47(2-3), 85–118.

Avni, D., Philosoph, A., Meijler, M. M., & Zor, T. (2010). The ceramide-1-phosphate analogue PCERA-1 modulates tumour necrosis factor-alpha and interleukin-10 production in macrophages via the cAMP-PKA-CREB pathway in a GTP-dependent manner. *Immunology*, 129(3), 375–385. PMID: 19922425.

Bazhin, A. V., Kahnert, S., Kimpfler, S., Schadendorf, D., & Umansky, V. (2010). Distinct metabolism of cyclic adenosine monophosphate in regulatory and helper CD4+ t cells. *Molecular Immunology*, 47(4), 678–684. PMID: 19939455.

Belghith, M., Bluestone, J. A., Barriot, S., Mégret, J., Bach, J., & Chatenoud, L. (2003). TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nature Medicine*, 9(9), 1202–1208. PMID: 12937416.

Bennett, C. L., Christie, J., Ramsdell, F., Brunkow, M. E., Ferguson, P. J., Whitesell, L., Kelly, T. E., Saulsbury, F. T., Chance, P. F., & Ochs, H. D. (2001). The immune dysregulation, polyendocrinopathy, enteropathy, x-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nature Genetics*, 27(1), 20–21. PMID: 11137993.

Beyersdorf, N., Gaupp, S., Balbach, K., Schmidt, J., Toyka, K. V., Lin, C., Hanke, T., Hünig, T., Kerkau, T., & Gold, R. (2005). Selective targeting of regulatory t cells with CD28 superagonists allows effective therapy of experimental autoimmune encephalomyelitis. *The Journal of Experimental Medicine*, 202(3), 445–455. PMID: 16061730.

Bielekova, B., Richert, N., Howard, T., Packer, A. N., Blevins, G., Ohayon, J., McFarland, H. F., Stürzebecher, C., & Martin, R. (2009). Treatment with the phos-

phodiesterase type-4 inhibitor rolipram fails to inhibit blood–brain barrier disruption in multiple sclerosis. *Multiple Sclerosis (Houndmills, Basingstoke, England)*, 15(10), 1206–1214. PMID: 19776093.

Bopp, T., Becker, C., Klein, M., Klein-Hessling, S., Palmetshofer, A., Serfling, E., Heib, V., Becker, M., Kubach, J., Schmitt, S., Stoll, S., Schild, H., Staege, M. S., Stassen, M., Jonuleit, H., & Schmitt, E. (2007). Cyclic adenosine monophosphate is a key component of regulatory t cell-mediated suppression. *The Journal of Experimental Medicine*, 204(6), 1303–1310. PMID: 17502663.

Bopp, T., Dehzad, N., Reuter, S., Klein, M., Ullrich, N., Stassen, M., Schild, H., Buhl, R., Schmitt, E., & Taube, C. (2009). Inhibition of cAMP degradation improves regulatory t cell-mediated suppression. *Journal of Immunology (Baltimore, Md.: 1950)*, 182(7), 4017–4024. PMID: 19299699.

Brunkow, M. E., Jeffery, E. W., Hjerrild, K. A., Paeper, B., Clark, L. B., Yasayko, S. A., Wilkinson, J. E., Galas, D., Ziegler, S. F., & Ramsdell, F. (2001). Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature Genetics*, 27(1), 68–73. PMID: 11138001.

Buckner, J. H. (2010). Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory t cells in human autoimmune diseases. *Nature Reviews. Immunology*, 10(12), 849–859. PMID: 21107346.

Changelian, P. S., Flanagan, M. E., Ball, D. J., Kent, C. R., Magnuson, K. S., Martin, W. H., Rizzuti, B. J., Sawyer, P. S., Perry, B. D., Brissette, W. H., McCurdy, S. P., Kudlacz, E. M., Conklyn, M. J., Elliott, E. A., Koslov, E. R., Fisher, M. B., Strelevitz, T. J., Yoon, K., Whipple, D. A., Sun, J., Munchhof, M. J., Doty, J. L., Casavant, J. M., Blumenkopf, T. A., Hines, M., Brown, M. F., Lillie, B. M., Subramanyam, C., Shang-Poa, C., Milici, A. J., Beckius, G. E., Moyer, J. D., Su, C., Woodworth, T. G., Gaweco, A. S., Beals, C. R., Littman, B. H., Fisher, D. A., Smith, J. F., Zagouras, P., Magna, H. A., Saltarelli, M. J., Johnson, K. S., Nelms, L. F., Des Etages, S. G., Hayes, L. S., Kawabata, T. T., Finco-Kent, D., Baker, D. L., Larson, M., Si, M.-S., Paniagua, R., Higgins, J., Holm, B., Reitz, B., Zhou, Y.-J., Morris, R. E., O'Shea, J. J., & Borie, D. C. (2003). Prevention of organ allograft rejection by a specific janus kinase 3 inhibitor. *Science (New York, N.Y.)*, 302(5646), 875–878. PMID: 14593182.

Chen, J., Gao, J., Zhang, D., Wang, Z., & Zhu, J. (2010). CD4+Foxp3+ regulatory t cells converted by rapamycin from peripheral CD4+CD25(-) naive t cells display more potent regulatory ability in vitro. *Chinese Medical Journal*, 123(7), 942–948. PMID: 20497692.

Chorny, A., Gonzalez-Rey, E., Ganea, D., & Delgado, M. (2006). Vasoactive intestinal peptide generates CD4+CD25+ regulatory t cells in vivo: therapeutic applications in autoimmunity and transplantation. *Annals of the New York Academy of Sciences*, 1070, 190–195. PMID: 16888164.

Clipstone, N. A. & Crabtree, G. R. (1992). Identification of calcineurin as a key signalling enzyme in t-lymphocyte activation. *Nature*, 357(6380), 695–697. PMID: 1377362.

de Lafaille, M. A. C. & Lafaille, J. J. (2009). Natural and adaptive foxp3+ regulatory t cells: more of the same or a division of labor? *Immunity*, 30(5), 626–635. PMID: 19464985.

Eastwood, D., Findlay, L., Poole, S., Bird, C., Wadhwa, M., Moore, M., Burns, C., Thorpe, R., & Stebbings, R. (2010). Monoclonal antibody TGN1412 trial failure explained by species differences in CD28 expression on CD4+ effector memory t-cells. *British Journal of Pharmacology*, 161(3), 512–526. PMID: 20880392.

Erhardt, A., Biburger, M., Papadopoulos, T., & Tiegs, G. (2007). IL-10, regulatory t cells, and kupffer cells mediate tolerance in concanavalin a-induced liver injury in mice. *Hepatology (Baltimore, Md.)*, 45(2), 475–485. PMID: 17256743.

Erhardt, A., Wegscheid, C., Claass, B., Carambia, A., Herkel, J., Mittrücker, H., Panzer, U., & Tiegs, G. (2011). CXCR3 deficiency exacerbates liver disease and abrogates tolerance in a mouse model of Immune-Mediated hepatitis. *Journal of Immunology (Baltimore, Md.: 1950)*, 186(9), 5284–5293. PMID: 21441449.

Faggioni, R., Jones-Carson, J., Reed, D. A., Dinarello, C. A., Feingold, K. R., Grunfeld, C., & Fantuzzi, G. (2000). Leptin-deficient (ob/ob) mice are protected from t cell-mediated hepatotoxicity: role of tumor necrosis factor alpha and IL-18. *Proceedings of the National Academy of Sciences of the United States of America*, 97(5), 2367–2372. PMID: 10681432.

Flynn, J. M. & Byrd, J. C. (2000). Campath-1H monoclonal antibody therapy. *Current opinion in oncology*, 12(6), 574–581. PMID: 11085457.

Fort, M. M. & Narayanan, P. K. (2010). Manipulation of regulatory t cell function by immunomodulators: a boon or a curse? *Toxicological Sciences: An Official Journal of the Society of Toxicology*. PMID: 20484381.

Gallagher, S. R. & Desjardins, P. R. (2008). Quantitation of DNA and RNA with absorption and fluorescence spectroscopy. *Current Protocols in Protein Science / Editorial Board, John E. Coligan ... [et Al*, Appendix 3, Appendix 4K. PMID: 18491298.

Gantner, F., Küsters, S., Wendel, A., Hatzelmann, A., Schudt, C., & Tiegs, G. (1997). Protection from t cell-mediated murine liver failure by phosphodiesterase inhibitors. *The Journal of Pharmacology and Experimental Therapeutics*, 280(1), 53–60. PMID: 8996181.

Gantner, F., Leist, M., Lohse, A. W., Germann, P. G., & Tiegs, G. (1995). Concanavalin a-induced t-cell-mediated hepatic injury in mice: the role of tumor necrosis factor. *Hepatology (Baltimore, Md.)*, 21(1), 190–198. PMID: 7806154.

Giembycz, M. A. & Field, S. K. (2010). Roflumilast: first phosphodiesterase 4 inhibitor approved for treatment of COPD. *Drug Design, Development and Therapy*, 4, 147–158. PMID: 20689641.

Gobejishvili, L., Barve, S., Joshi-Barve, S., & McClain, C. (2008). Enhanced PDE4B expression augments LPS-inducible TNF expression in ethanol-primed monocytes: relevance to alcoholic liver disease. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, 295(4), G718–724. PMID: 18687753.

Gonzalez-Rey, E. & Delgado, M. (2007). Vasoactive intestinal peptide and regulatory t-cell induction: a new mechanism and therapeutic potential for immune homeostasis. *Trends in Molecular Medicine*, 13(6), 241–251. PMID: 17467339.

Grütz, G. (2005). New insights into the molecular mechanism of interleukin-10-mediated immunosuppression. *Journal of Leukocyte Biology*, 77(1), 3–15. PMID: 15522916.

Hartl, D., Koller, B., Mehlhorn, A. T., Reinhardt, D., Nicolai, T., Schendel, D. J., Griese, M., & Krauss-Etschmann, S. (2007). Quantitative and functional impairment

of pulmonary CD4+CD25hi regulatory t cells in pediatric asthma. *The Journal of Allergy and Clinical Immunology*, 119(5), 1258–1266. PMID: 17412402.

Hartmann, G., Bidlingmaier, C., Siegmund, B., Albrich, S., Schulze, J., Tschoep, K., Eigler, A., Lehr, H. A., & Endres, S. (2000). Specific type IV phosphodiesterase inhibitor rolipram mitigates experimental colitis in mice. *The Journal of Pharmacology and Experimental Therapeutics*, 292(1), 22–30. PMID: 10604928.

Hatzelmann, A. & Schudt, C. (2001). Anti-inflammatory and immunomodulatory potential of the novel PDE4 inhibitor roflumilast in vitro. *The Journal of Pharmacology and Experimental Therapeutics*, 297(1), 267–279. PMID: 11259554.

Hebenstreit, G. F., Fellerer, K., Fichte, K., Fischer, G., Geyer, N., Meya, U., Sastre-y-Hernández, M., Schöny, W., Schratzer, M., & Soukop, W. (1989). Rolipram in major depressive disorder: results of a double-blind comparative study with imipramine. *Pharmacopsychiatry*, 22(4), 156–160. PMID: 2668980.

Heystek, H. C., Thierry, A., Soulard, P., & Moulon, C. (2003). Phosphodiesterase 4 inhibitors reduce human dendritic cell inflammatory cytokine production and th1-polarizing capacity. *International Immunology*, 15(7), 827–835. PMID: 12807821.

Hoffmann, P., Eder, R., Kunz-Schughart, L. A., Andreesen, R., & Edinger, M. (2004). Large-scale in vitro expansion of polyclonal human CD4(+)CD25high regulatory t cells. *Blood*, 104(3), 895–903. PMID: 15090447.

Hori, S., Nomura, T., & Sakaguchi, S. (2003). Control of regulatory t cell development by the transcription factor foxp3. *Science (New York, N.Y.)*, 299(5609), 1057–1061. PMID: 12522256.

Jimenez, J. L., Punzón, C., Navarro, J., Muñoz-Fernández, M. A., & Fresno, M. (2001). Phosphodiesterase 4 inhibitors prevent cytokine secretion by t lymphocytes by inhibiting nuclear Factor-kappaB and nuclear factor of activated t cells activation. *Journal of Pharmacology and Experimental Therapeutics*, 299(2), 753 –759.

Kaneko, Y., Harada, M., Kawano, T., Yamashita, M., Shibata, Y., Gejyo, F., Nakayama, T., & Taniguchi, M. (2000). Augmentation of valpha14 NKT cell-mediated cytotoxicity by interleukin 4 in an autocrine mechanism resulting in the development of

concanavalin a-induced hepatitis. *The Journal of Experimental Medicine*, 191(1), 105–114. PMID: 10620609.

Karppanen, H., Paakkari, P., Orma, A. L., & Paakkari, I. (1979). Central hypotensive effects of imidazole acetic acid and rolipram (ZK 62 711) in rats [proceedings]. *Agents and Actions*, 9(1), 84–86. PMID: 223420.

Keymeulen, B., Vandemeulebroucke, E., Ziegler, A. G., Mathieu, C., Kaufman, L., Hale, G., Gorus, F., Goldman, M., Walter, M., Candon, S., Schandene, L., Crenier, L., Block, C. D., Seigneurin, J., Pauw, P. D., Pierard, D., Weets, I., Rebello, P., Bird, P., Berrie, E., Frewin, M., Waldmann, H., Bach, J., Pipeleers, D., & Chatenoud, L. (2005). Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. *The New England Journal of Medicine*, 352(25), 2598–2608. PMID: 15972866.

Kühn, R., Löhler, J., Rennick, D., Rajewsky, K., & Müller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*, 75(2), 263–274. PMID: 8402911.

Krause, W. & Kühne, G. (1988). Pharmacokinetics of rolipram in the rhesus and cynomolgus monkeys, the rat and the rabbit. studies on species differences. *Xenobiotica; the Fate of Foreign Compounds in Biological Systems*, 18(5), 561–571. PMID: 3400274.

Küsters, S., Gantner, F., Künstle, G., & Tiegs, G. (1996). Interferon gamma plays a critical role in t cell-dependent liver injury in mice initiated by concanavalin a. *Gastroenterology*, 111(2), 462–471. PMID: 8690213.

Lahl, K., Loddenkemper, C., Drouin, C., Freyer, J., Arnason, J., Eberl, G., Hamann, A., Wagner, H., Huehn, J., & Sparwasser, T. (2007). Selective depletion of foxp3+ regulatory t cells induces a scurfy-like disease. *The Journal of Experimental Medicine*, 204(1), 57–63. PMID: 17200412.

Larsen, C. P., Pearson, T. C., Adams, A. B., Tso, P., Shirasugi, N., Strobert, E., Anderson, D., Cowan, S., Price, K., Naemura, J., Emswiler, J., Greene, J., Turk, L. A., Bajorath, J., Townsend, R., Hagerty, D., Linsley, P. S., & Peach, R. J. (2005). Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *American journal of transplantation: official journal*

of the American Society of Transplantation and the American Society of Transplant Surgeons, 5(3), 443–453. PMID: 15707398.

Lebwohl, M., Tyring, S. K., Hamilton, T. K., Toth, D., Glazer, S., Tawfik, N. H., Walicke, P., Dummer, W., Wang, X., Garovoy, M. R., & Pariser, D. (2003). A novel targeted t-cell modulator, efalizumab, for plaque psoriasis. *The New England journal of medicine*, 349(21), 2004–2013. PMID: 14627785.

Lindley, S., Dayan, C. M., Bishop, A., Roep, B. O., Peakman, M., & Tree, T. I. M. (2005). Defective suppressor function in CD4(+)CD25(+) t-cells from patients with type 1 diabetes. *Diabetes*, 54(1), 92–99. PMID: 15616015.

Liu, W., Putnam, A. L., Xu-Yu, Z., Szot, G. L., Lee, M. R., Zhu, S., Gottlieb, P. A., Kapranov, P., Gingeras, T. R., de St Groth, B. F., Clayberger, C., Soper, D. M., Ziegler, S. F., & Bluestone, J. A. (2006). CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ t reg cells. *The Journal of Experimental Medicine*, 203(7), 1701–1711. PMID: 16818678.

Liu, Z. X., Govindarajan, S., Okamoto, S., & Dennert, G. (2000). NK cells cause liver injury and facilitate the induction of t cell-mediated immunity to a viral liver infection. *Journal of Immunology (Baltimore, Md.: 1950)*, 164(12), 6480–6486. PMID: 10843705.

Lohse, A. W. & zum Büschenfelde, K. H. M. (1993). Remission of experimental autoimmune hepatitis is associated with antigen-specific and non-specific immunosuppression. *Clinical and Experimental Immunology*, 94(1), 163–167. PMID: 8403500.

Louis, H., Moine, O. L., Peny, M. O., Quertinmont, E., Fokan, D., Goldman, M., & Devière, J. (1997). Production and role of interleukin-10 in concanavalin a-induced hepatitis in mice. *Hepatology (Baltimore, Md.)*, 25(6), 1382–1389. PMID: 9185757.

Luo, Q., Wang, Y., Feng, D., Xu, Y., & Xu, L. (2009). Vasoactive intestinal peptide attenuates concanavalin a-mediated liver injury. *European Journal of Pharmacology*, 607(1-3), 226–233. PMID: 19222997.

Marco, R. D., Xiang, M., Zaccone, P., Leonardi, C., Franco, S., Meroni, P., & Nicoletti, F. (1999). Concanavalin a-induced hepatitis in mice is prevented by interleukin (IL)-10

and exacerbated by endogenous IL-10 deficiency. *Autoimmunity*, 31(2), 75–83. PMID: 10680745.

Miyara, M. & Sakaguchi, S. (2011). Human FoxP3(+)CD4(+) regulatory t cells: their knowns and unknowns. *Immunology and Cell Biology*. PMID: 21301480.

Mizuhara, H., Kuno, M., Seki, N., Yu, W. G., Yamaoka, M., Yamashita, M., Ogawa, T., Kaneda, K., Fujii, T., Senoh, H., & Fujiwara, H. (1998). Strain difference in the induction of t-cell activation-associated, interferon gamma-dependent hepatic injury in mice. *Hepatology (Baltimore, Md.)*, 27(2), 513–519. PMID: 9462651.

Müller, T., Engels, P., & Fozard, J. R. (1996). Subtypes of the type 4 cAMP phosphodiesterases: structure, regulation and selective inhibition. *Trends in Pharmacological Sciences*, 17(8), 294–298. PMID: 8810876.

Mohty, M. (2007). Mechanisms of action of antithymocyte globulin: T-cell depletion and beyond. *Leukemia: official journal of the Leukemia Society of America, Leukemia Research Fund, U.K*, 21(7), 1387–1394. PMID: 17410187.

Nicoletti, F., Marco, R. D., Zaccone, P., Salvaggio, A., Magro, G., Bendtzen, K., & Meroni, P. (2000). Murine concanavalin a-induced hepatitis is prevented by interleukin 12 (IL-12) antibody and exacerbated by exogenous IL-12 through an interferon-gamma-dependent mechanism. *Hepatology (Baltimore, Md.)*, 32(4 Pt 1), 728–733. PMID: 11003616.

Paintlia, A. S., Paintlia, M. K., Singh, I., & Singh, A. K. (2008). Combined medication of lovastatin with rolipram suppresses severity of experimental autoimmune encephalomyelitis. *Experimental Neurology*, 214(2), 168–180. PMID: 18775426.

Palacios, R. (1982). Concanavalin a triggers t lymphocytes by directly interacting with their receptors for activation. *Journal of Immunology (Baltimore, Md.: 1950)*, 128(1), 337–342. PMID: 6459373.

Pozo, D., Anderson, P., & Gonzalez-Rey, E. (2009). Induction of alloantigen-specific human t regulatory cells by vasoactive intestinal peptide. *Journal of Immunology (Baltimore, Md.: 1950)*, 183(7), 4346–4359. PMID: 19734220.

Prakken, B. J., Samodal, R., Le, T. D., Giannoni, F., Yung, G. P., Scavulli, J., Amox, D., Roord, S., de Kleer, I., Bonnin, D., Lanza, P., Berry, C., Massa, M., Billetta, R., & Albani, S. (2004). Epitope-specific immunotherapy induces immune deviation of proinflammatory t cells in rheumatoid arthritis. *Proceedings of the National Academy of Sciences of the United States of America*, 101(12), 4228–4233. PMID: 15024101.

Riley, J. L., June, C. H., & Blazar, B. R. (2009). Human t regulatory cell therapy: Take a billion or so and call me in the morning. *Immunity*, 30(5), 656–665.

Rooijen, N. V., Kors, N., vd Ende, M., & Dijkstra, C. D. (1990). Depletion and repopulation of macrophages in spleen and liver of rat after intravenous treatment with liposome-encapsulated dichloromethylene diphosphonate. *Cell and Tissue Research*, 260(2), 215–222. PMID: 2141546.

Ruan, Q., Kameswaran, V., Tone, Y., Li, L., Liou, H., Greene, M. I., Tone, M., & Chen, Y. H. (2009). Development of foxp3(+) regulatory t cells is driven by the c-Rel enhanceosome. *Immunity*, 31(6), 932–940. PMID: 20064450.

Sakaguchi, S. (2005). Naturally arising foxp3-expressing CD25+CD4+ regulatory t cells in immunological tolerance to self and non-self. *Nature Immunology*, 6(4), 345–352. PMID: 15785760.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., & Toda, M. (1995). Immunologic self-tolerance maintained by activated t cells expressing IL-2 receptor alpha-chains (CD25). breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *Journal of Immunology (Baltimore, Md.: 1950)*, 155(3), 1151–1164. PMID: 7636184.

Sakaguchi, S., Wing, K., & Miyara, M. (2007). Regulatory t cells - a brief history and perspective. *European Journal of Immunology*, 37 Suppl 1, S116–123. PMID: 17972355.

Sass, G., Heinlein, S., Agli, A., Bang, R., Schümann, J., & Tiegs, G. (2002). Cytokine expression in three mouse models of experimental hepatitis. *Cytokine*, 19(3), 115–120. PMID: 12242077.

Schümann, J., Wolf, D., Pahl, A., Brune, K., Papadopoulos, T., van Rooijen, N., & Tiegs, G. (2000). Importance of kupffer cells for t-cell-dependent liver injury in mice. *The American Journal of Pathology*, 157(5), 1671–1683. PMID: 11073826.

Schmidt, J., Elflein, K., Stienekemeier, M., Rodriguez-Palmero, M., Schneider, C., Toyka, K. V., Gold, R., & Hünig, T. (2003). Treatment and prevention of experimental autoimmune neuritis with superagonistic CD28-specific monoclonal antibodies. *Journal of Neuroimmunology*, 140(1-2), 143–152. PMID: 12864982.

Segundo, D. S., Fernández-Fresnedo, G., Gago, M., Beares, I., Ruiz-Criado, J., González, M., Ruiz, J. C., Gómez-Alamillo, C., Arias, M., & López-Hoyos, M. (2010). Number of peripheral blood regulatory t cells and lymphocyte activation at 3 months after conversion to mTOR inhibitor therapy. *Transplantation Proceedings*, 42(8), 2871–2873. PMID: 20970555.

Sehgal, S. N. (1998). Rapamune (RAPA, rapamycin, sirolimus): mechanism of action immunosuppressive effect results from blockade of signal transduction and inhibition of cell cycle progression. *Clinical biochemistry*, 31(5), 335–340. PMID: 9721431.

Serres, S. A. D., Sayegh, M. H., & Najafian, N. (2009). Immunosuppressive drugs and tregs: a critical evaluation! *Clinical Journal of the American Society of Nephrology: CJASN*, 4(10), 1661–1669. PMID: 19696218.

Siebler, J., Wirtz, S., Klein, S., Protschka, M., Blessing, M., Galle, P. R., & Neurath, M. F. (2003). A key pathogenic role for the STAT1/T-bet signaling pathway in t-cell-mediated liver inflammation. *Hepatology (Baltimore, Md.)*, 38(6), 1573–1580. PMID: 14647068.

Silva, A. J. d., Brickelmaier, M., Majeau, G. R., Li, Z., Su, L., Hsu, Y.-M., & Hochman, P. S. (2002). Alefacept, an immunomodulatory recombinant LFA-3/IgG1 fusion protein, induces CD16 signaling and CD2/CD16-Dependent apoptosis of CD2+ cells. *The Journal of Immunology*, 168(9), 4462–4471.

Sommer, N., Löschmann, P. A., Northoff, G. H., Weller, M., Steinbrecher, A., Steinbach, J. P., Lichtenfels, R., Meyermann, R., Riethmüller, A., & Fontana, A. (1995). The antidepressant rolipram suppresses cytokine production and prevents autoimmune encephalomyelitis. *Nature Medicine*, 1(3), 244–248. PMID: 7585041.

Sousa, L. P., Lopes, F., Silva, D. M., Tavares, L. P., Vieira, A. T., Rezende, B. M., Carmo, A. F., Russo, R. C., Garcia, C. C., Bonjardim, C. A., Alessandri, A. L., Rossi, A. G., Pinho, V., & Teixeira, M. M. (2010). PDE4 inhibition drives resolution of neutrophilic inflammation by inducing apoptosis in a PKA-PI3K/Akt-dependent and NF-kappaB-independent manner. *Journal of Leukocyte Biology*, 87(5), 895–904. PMID: 20103769.

Steinman, L. (2005). Blocking adhesion molecules as therapy for multiple sclerosis: natalizumab. *Nature reviews. Drug discovery*, 4(6), 510–518. PMID: 15931259.

Suntharalingam, G., Perry, M. R., Ward, S., Brett, S. J., Castello-Cortes, A., Brunner, M. D., & Panoskaltsis, N. (2006). Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *The New England Journal of Medicine*, 355(10), 1018–1028. PMID: 16908486.

Taams, L. S., Palmer, D. B., Akbar, A. N., Robinson, D. S., Brown, Z., & Hawrylowicz, C. M. (2006). Regulatory t cells in human disease and their potential for therapeutic manipulation. *Immunology*, 118(1), 1–9. PMID: 16630018.

Tagawa, Y., Sekikawa, K., & Iwakura, Y. (1997). Suppression of concanavalin a-induced hepatitis in IFN-gamma(-/-) mice, but not in TNF-alpha(-/-) mice: role for IFN-gamma in activating apoptosis of hepatocytes. *Journal of Immunology (Baltimore, Md.: 1950)*, 159(3), 1418–1428. PMID: 9233639.

Takeda, K., Hayakawa, Y., Kaer, L. V., Matsuda, H., Yagita, H., & Okumura, K. (2000). Critical contribution of liver natural killer t cells to a murine model of hepatitis. *Proceedings of the National Academy of Sciences of the United States of America*, 97(10), 5498–5503. PMID: 10792025.

Tiegs, G., Hentschel, J., & Wendel, A. (1992). A t cell-dependent experimental liver injury in mice inducible by concanavalin a. *The Journal of Clinical Investigation*, 90(1), 196–203. PMID: 1634608.

Turner, C. R., Andresen, C. J., Smith, W. B., & Watson, J. W. (1994). Effects of rolipram on responses to acute and chronic antigen exposure in monkeys. *American Journal of Respiratory and Critical Care Medicine*, 149(5), 1153–1159. PMID: 8173755.

Uhlig, H. H., Coombes, J., Mottet, C., Izcue, A., Thompson, C., Fanger, A., Tannapfel, A., Fontenot, J. D., Ramsdell, F., & Powrie, F. (2006). Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ t cells during cure of colitis. *Journal of Immunology (Baltimore, Md.: 1950)*, 177(9), 5852–5860. PMID: 17056509.

Viglietta, V., Baecher-Allan, C., Weiner, H. L., & Hafler, D. A. (2004). Loss of functional suppression by CD4+CD25+ regulatory t cells in patients with multiple sclerosis. *The Journal of Experimental Medicine*, 199(7), 971–979. PMID: 15067033.

Vincenti, F., Kirkman, R., Light, S., Bumgardner, G., Pescovitz, M., Halloran, P., Neylan, J., Wilkinson, A., Ekberg, H., Gaston, R., Backman, L., & Burdick, J. (1998). Interleukin-2-receptor blockade with daclizumab to prevent acute rejection in renal transplantation. daclizumab triple therapy study group. *The New England journal of medicine*, 338(3), 161–165. PMID: 9428817.

Wei, H., Chuang, Y., Li, B., Wei, H., Sun, R., Moritoki, Y., Gershwin, M. E., Lian, Z., & Tian, Z. (2008). CD4+ CD25+ foxp3+ regulatory t cells protect against t cell-mediated fulminant hepatitis in a TGF-beta-dependent manner in mice. *Journal of Immunology (Baltimore, Md.: 1950)*, 181(10), 7221–7229. PMID: 18981144.

Whitacre, C. C. (2001). Sex differences in autoimmune disease. *Nature Immunology*, 2(9), 777–780. PMID: 11526384.

Ye, F., Yan, S., Xu, L., Jiang, Z., Liu, N., Xiong, S., Wang, Y., & Chu, Y. (2009). Tr1 regulatory t cells induced by ConA pretreatment prevent mice from ConA-induced hepatitis. *Immunology Letters*, 122(2), 198–207. PMID: 19200442.

Danksagung

Danken möchte ich sehr herzlich Frau Prof. Dr. Gisa Tiegs für das aktuelle und interessante Thema sowie für die wissenschaftliche Hilfe, ihr stetes Interesse am Fortschritt dieser Arbeit und ihr Engagement für den wissenschaftlichen Nachwuchs als Sprecherin des Graduiertenkollegs „Entzündung und Regeneration“.

Ein ganz besonderer Dank gilt meiner Betreuerin Frau Dr. Annette Erhardt für die immer freundliche und geduldige Betreuung, ihre Hilfsbereitschaft, ihre vielen guten Ratschläge und technischen Hilfestellungen.

Darüber hinaus möchte ich auch meinen Kollegen Roja Barikbin, Sven Burghardt, Benjamin Claaß, Antonio Galante, Nicole Lange, Christine Loscher, Stefan Lüth, Annett Ostmann, Nicola Peters, Gabriele Sass, Julia Solomentsew, Elena Tasika, Andrea Tiedemann und Claudia Wegscheid für die herzliche Aufnahme in die Arbeitsgruppe, für ihre fortwährende Unterstützung und für spannende Diskussionen danken.

Weiterhin danke ich Anne Schramm und Frau Dr. Eva Tolosa für ihre Hilfe bei den Versuchen mit humanen Zellen, Herrn Prof. Dr. Nolte und Herrn Prof. Dr. Wenzel für die Ratschläge im Rahmen der Betreuungskommission sowie Benjamin Claaß und Andreas Nohn für Tipps zum Textverarbeitungsprogramm LaTeX.

Dem Graduiertenkolleg „Entzündung und Regeneration“ danke ich für die vielen lehrreichen Seminare, Vorlesungen und Workshops, das Berichtskolloquium in der Lüneburger Heide und für die Möglichkeit, meine Ergebnisse auf Kongressen in Marburg und Leipzig vorzustellen.

Diese Arbeit ist von der Deutschen Forschungsgemeinschaft (Teilprojekt Z3 des Sonderforschungsbereichs 841) und der Medizinischen Fakultät der Universität Hamburg finanziell gefördert worden; auch diesen Institutionen gilt mein Dank.

Curriculum Vitae

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

Eidesstattliche Versicherung

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

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