A microRNA network in regulatory T cell differentiation

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Erklärung:
Hiermit erkläre ich, Sebastian Johannes Claudius Warth, dass die vorliegende Dissertation mit dem Titel “A microRNA network in regulatory T cell differentiation" von mir eigenständig und ohne unerlaubte Hilfsmittel angefertigt wurde und ich mich dabei nur der ausdrücklich bezeichneten Quellen und Hilfsmittel bedient habe. Diese Arbeit wurde weder in der jetzigen noch in einer veränderten Form einer anderen Prüfungskommission vorgelegt.

Sebastian Warth München, den 9.9.2013

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1 Zusammenfassung

Regulatorische T-Zellen (Treg-Zellen) sind unverzichtbar, um die periphere Immuntoleranz aufrecht zu erhalten. Sowohl die Entwicklung als auch die Funktion von Treg-Zellen ist abhängig von der Anwesenheit von MicroRNAs. Um MicroRNAs mit Bedeutung für die Treg-Zelldifferenzierung zu identifizieren, haben wir 147 T-Zell-exprimierte MicroRNAs kloniert und funktionell getestet. Unter diesen MicroRNAs konnte eine größere Anzahl identifiziert werden, die die Differenzierung naiver $CD4^+$ T-Zellen in Treg-Zellen fördern oder beeinträchtigen. Aus diesen Befunden lässt sich ein hypothetisches Netzwerk von MicroRNA-Interaktionen ableiten, das der Treg-Zelldifferenzierung zugrunde liegt. Wir haben uns für die weitere exemplarische Untersuchung der MikroRNAs miR-99a, miR-100 und miR-10b entschieden, weil diese den stärksten Effekt auf die Treg-Zelldifferenzierung, jedoch keinen Effekt auf die Th17-Zelldifferenzierung hatten.

Durch die Analyse von potentiellen Ziel-mRNAs der Kandidaten-MicroRNAs konnten wir eine direkte Regulation der 3' untranslatierten Region (3'UTR) der Mechanistic target of rapamycin (Mtor) Kinase durch die miR-99a zeigen während der 3'UTR von RAR-related orphan receptor alpha (Rora) durch miR-10b reprimiert wurde. Interessanterweise enthält sowohl der Mtor 3'UTR als auch der Rora 3'UTR zusätzlich eine Zielsequenz für miR-150. Entsprechend konnte miR-150 Überexpression diese 3'UTRs reprimieren und führte ebenfalls zu einer verstärkten Treg-Zelldifferenzierung. Während miR-150 über die ganze Treg-Zelldifferenzierung hinweg stark exprimiert war, konnte die eigentlich geringe Expression von miR-10b sowie von miR-99a und miR-100 durch die Behandlung von T-Zellen mit Retinsäure stark induziert werden. Retinsäure ist bekannt dafür, direkt und indirekt die Treg-Zelldifferenzierung stark zu stimulieren.

Wir schlagen daher ein Modell vor, in dem die Kooperation von hochexprimierten sowie induzierbaren MicroRNAs die notwendige Herunterregulation von kritischen Inhibitoren der Treg-Zelldifferenzierung erreichen kann.

2 Abstract

Regulatory T cells (Treg) are essential in the maintenance of peripheral immune tolerance. Their development and function requires the presence of mature microRNAs. To identify microRNAs important in Treg cell differentiation, we functionally screened a set of 147 T cell-expressed microRNAs. We determined a larger number of microRNAs to promote or interfere with naive $CD4^+$ T cell to Treg cell conversion, suggesting a network of microRNA interaction, which underlies Treg cell differentiation. We chose to further investigate miR-99a, miR-100 and miR-10b, which had the strongest effect on Treg and no effect on Th17 differentiation.

Testing potential targets of these candidates we determined a direct negative regulation of the Mechanistic target of rapamycin (mTOR) 3' untranslated region (3'UTR) by miR-99a as well as the repression of the RAR-related orphan receptor alpha (Rora) 3'UTR by miR-10b. Interestingly, both Mtor and Rora 3'UTRs additionally possess target sites for miR-150. Consistently, these 3'UTRs were repressed by miR-150 overexpression, which also effectively promoted Treg differentiation. While miR-150 was highly expressed throughout Treg cell differentiation, the low expression of miR-10b, miR-99a and -100 was strongly induced upon retinoic acid treatment, a known direct and indirect inducer of Treg cell differentiation.

We therefore propose a model in which the cooperation of highly expressed as well as inducible microRNAs can achieve sufficient downregulation of critical inhibitors of Treg cell differentiation.

3 Directory

4 Introduction

4.1 T helper cells in protective immunity and tolerance

The mammalian immune system confers protection against harmful pathogens through the interplay of innate and adaptive immune cells and molecules. The innate immune system is activated by general molecular patterns of pathogens and provides a first line of defense mechanisms (Hoffmann et al., 1999). These involve activation of the complement system, opsonization and phagocytosis of pathogens as well as activation of antigen presenting cells. The latter link the innate immune response with adaptive immunity that provides huge antigen receptor diversity in order to target unique antigens of a particular pathogen with high affinity.

The adaptive immune system is mainly constituted of B cells, which produce antibodies, and of T cells, that differentiate into $CD8^+$ (Cluster of differentiation 8 positve) cytotoxic T cells and $CD4^+$ helper T cells (Murphy et al., 2008). T cells leave the thymus as naive cells and migrate with the blood stream or lymph vessel and enter lymph nodes. Naive $CD4^+$ T cell are activated when they encounter an activated dendritic cell that presents a peptide:MHCII (Major Histocompatibility Complex II) complex recognized by the T cell receptor (TCR). Different types of pathogens evoke specific cytokine milieus that direct the course of T helper cell differentiation following activation into the various effector T cell lineages or transitional states, such as Th1, Th2, Th17, Th9 and follicular T helper cells (Tfh) (O'Shea and Paul, 2010; Lu et al., 2011). Each lineage secretes specific effector cytokines that act on cytotoxic T cells or B cells and that feed back to the innate immune system to enforce immune responses in the control of a pathogen.

All T cells undergo combinatorial rearrangements of gene segments of the TCR in the thymus. This process results in an enormous diversity of receptor variants including self-reactive receptors that may elicit autoimmunity (Pancer and Cooper, 2006). TCRs with a high affinity for self-peptide:MHC complexes undergo negative selection in the thymus resulting in apoptosis of cells bearing those TCRs (Kyewski et al., 1986). The control of the emerging T cell repertoire by this process in the thymus is called central tolerance and was believed for a long time to be sufficient to suppress autoimmunity (Mathis and Benoist, 2007). However, this concept was challenged by experiments with mice that were thymectomized at day 3 after birth. These mice develop fatal autoimmunity with uncontrolled $CD4^+$ T cell activation,

which can be rescued by transfer of spleen cells, particularly of $CD4^+$ $CD25^+$ T cells (Sakaguchi et al., 1982; Asano et al., 1996). These experiments not only show a thymic output of potentially self-reactive T cells, but also prove the existence of a T cell subpopulation with the propensity to suppress these autoreactive T cells in the periphery. These so-called regulatory T cells or Treg cells are now well-accepted to constitute one essential column of the immune system that exerts suppression of constantly activated self-reactive T cells. This process is referred to as dominant tolerance or peripheral tolerance (Piccirillo and Shevach, 2004). The peripheral tolerance also achieves the adaptation of suppressive functions to newly emerging non-harmful antigens such as food and environmental antigens, commensal bacteria or paternal antigens during pregnancy and thus extends the definition of self-antigens (Chen, 2003; Zenclussen et al., 2006; Round and Mazmanian, 2010).

Taken together, the discovery of regulatory T cells has extended our understanding and led to a much more diversified view on the immune system. It has an enormous potential to recognize and neutralize a huge variety of foreign antigens, but this is kept in check by powerful tolerance mechanisms resulting in a delicate equilibrium of the protective and the destructive forces of adaptive immunity in healthy individuals.

4.2 Regulatory T cells

4.2.1 The significance of Foxp3 in Treg cells

The experiments by Sakaguchi and Asano proved the existence of a suppressive type of T cell that is characterized by expression of $CD4^+$ $CD25^+$ (Sakaguchi et al., 1982; Asano et al., 1996). Yet, these markers are not uniquely expressed by Treg cells, since they show similar expression on activated effector T cells. The first evidence for a lineage specific marker of Treg cells was found in mice with the *scurfy* phenotype that is characterized by uncontrolled $CD4^+$ T cell activation resulting in systemic autoimmunity. This phenotype could be attributed to a frame-shift mutation in the gene encoding for the transcription factor forkhead box P3 (Foxp3). The mutation resulted in a truncated Foxp3 protein lacking the forkhead DNA-binding domain (Brunkow et al., 2001). The similarities of the *scurfy* phenotype with the phenotype resulting from $CD4^+$ $CD25^+$ T cell ablation led to studies that identified Foxp3 as the lineage-specifying transcription factor of Treg cells. Foxp3 was essential for thymic Treg cell generation and could confer suppressive functions on $CD4^+$ $CD25^-$ cells upon ectopic expression mice (Hori et al., 2003; Fontenot et al., 2003). In addition, these studies established Foxp3 as a highly specific marker for Treg cells. Induced ablation of Foxp3 in

mature regulatory T cells resulted in loss of their suppressive capacity and gain of effector functions. It highlighted a role of Foxp3 expression not only for the induction but also for the maintenance of Treg cell identity (Williams and Rudensky, 2007). Characterization of the transcriptional signature of Treg cells revealed hundreds of differentially expressed genes in Treg compared to conventional T cells. Among these were relatively few direct targets of Foxp3, including upregulated genes such as *CTLA-4* or *CD25* and downregulated genes such as *IL-7R* (Gavin et al., 2007; Hill et al., 2008). Thus, Foxp3 is now seen as a merely late-acting transcription factor that stabilizes a transcriptional program pre-established during T cell activation (Samstein et al., 2012a).

Stable expression of Foxp3 is dependent on epigenetic modifications in conserved enhancer regions of the Foxp3 gene, namely the conserved non-coding sequences CNS1, CNS2 and CNS3 (Floess et al., 2007; Zheng et al., 2010). Particularly, DNA demethylation of CNS2, which is also called Treg-specific demethylated region (TSDR), is essential for Treg cell stability as its deletion results in a progressive loss of Foxp3 expression with cell division.

4.2.2 The origin of Treg cells

4.2.2.1 Thymic Treg cell differentiation

A major source for regulatory T cells is the thymus where $CD4^+$ $CD8^-$ thymocytes can differentiate into Treg cells. Recruitment into the Treg cell lineage is dependent on TCR signals (Jordan et al., 2001). $CD4^+$ $CD8^-$ thymocytes that have productively rearranged their TCR gene segments are selected based on the affinity of their TCR to self-peptide:MHCII complexes (Roberts et al., 1990). Different from negatively selected cells with a high affinity TCR, T cells with weak self-peptide:MHCII affinity become conventional $CD4^+$ T cells. In the current model, T cells that bear a TCR with moderate self-affinity are recruited into the Treg cell lineage (Jordan et al., 2001). Expression of Foxp3 in this process is not sufficient, but epigenetic chromatin modifications in the *Foxp3* locus are required to establish a stable Treg identity (Floess et al., 2007; Zheng et al., 2010). Along that line recent results show that Treg-specific DNA methylation patterns are imposed on developing thymocytes as a function of the time of TCR triggering, independent from Foxp3 expression (Ohkura et al., 2012).

In our current understanding, the role of thymic Treg cells in the immune system is to establish dominant immune tolerance via suppression of immune responses by self reactive T cells that would, if unrestrained, lead to fatal autoimmunity (Haribhai et al., 2011).

4.2.2.2 Peripheral Treg cell induction

While a major source for Treg cells is thymic differentiation, Treg cells can also differentiate from naive $CD4^+$ T cells in the periphery. This was already implicated by the initial transfer experiments in thymectomized mice (Asano et al., 1996). In an *Ovalbumin* (*OVA)-TCR* transgenic mouse model Zhang et al. showed *in vivo* generation of $CD4^+$ $CD25^+$ T cells upon oral administration of Ova-peptide (Zhang et al., 2001). These cells conferred suppressive functions *in vivo* and *in vitro* partly in an antigen-specific way. The antigen-specific response could only be elicited from newly differentiated Treg cells because these mice lacked Ova-specific thymic Treg cells. Other experiments confirmed the *de novo* generation of $CD4^+$ $CD25^+$ T cells from naive $CD4^+$ T cells in a transfer model of T cells with an antigen-specific-TCR (*Rag-2$^{-/-}$/TCR-HA*) into mice with systemic expression of the respective antigen (*Rag-2$^{-/-}$ /HA*-transgenic) or *Rag-2$^{-/-}$* control hosts (Apostolou et al., 2002). These experiments established the antigen-specific induction of Treg cells from naive T cells in the periphery. These studies also showed that the transforming growth factor β (TGFβ) promotes differentiation into Treg cells (Cobbold et al., 2004; Kretschmer et al., 2005).

Peripheral induction of Treg cells can be observed under multiple other conditions *in vivo* such as tolerance induction by commensal bacteria, establishment of feto-maternal tolerance during pregnancy, in autoimmune conditions like experimental autoimmune encephalomyelitis or during graft-versus-host disease in adoptive T cell transfer (Atarashi et al., 2011; Round and Mazmanian, 2010; Samstein et al., 2012b; Korn et al., 2007; Haribhai et al., 2011). Peripheral Treg cells are also generated in immune responses against pathogens or parasites and limit excessive immune-mediated tissue damage (Grainger et al., 2010; Brincks et al., 2013). In addition to that, peripherally induced Treg cells receive a lot of attention as they suppress beneficial immune responses against cancer cells (Nishikawa and Sakaguchi, 2010).

The generation of peripheral Treg cells can be recapitulated *in vitro* by stimulation using anti-CD3 and anti-CD28 antibodies in the presence of TGFβ and IL-2 (Chen, 2003; Zheng et al., 2002, 2004). Importantly, TGFβ-induced Treg cells generated *in vitro* suppress conventional $CD4^+$ T cell proliferation *in vivo* in an antigen-specific manner after transfer and also suppress pathology in a dust-mite asthma model over four weeks in a non-antigen-specific manner (Chen, 2003).

Regardless of equal functionality of Treg cells induced *in vitro* and *in vivo*, the overlap in their transcriptional signature is low compared to the extensive overlap between thymic Treg cells and *in vivo*-induced Treg cells (Haribhai et al., 2011). A likely explanation may be

differences of the characteristic epigenetic modifications found in Treg cells generated *in vivo*, which are essential to establish the full transcriptional program in thymic Treg cells. Particularly, demethylation of CNS2 is missing in Treg cells generated *in vitro* (Floess et al., 2007). Since CNS2 is crucial for a stable expression of Foxp3, these Treg cells progressively lose their identity and potentially become effector cells, which is a major drawback for therapeutic approaches using Treg cells. However, there are hints that prolonged *in vitro* culture in Treg-polarizing conditions can partially establish CNS2 demethylation (Ohkura et al., 2012).

Recent findings may adjust our view on the origin of peripheral T cells. One study shows that the ability of thymocytes to differentiate into Treg cells decreases with maturation (Wirnsberger et al., 2009). Interestingly this is also true for naive $CD4^+$ T cells that have recently emigrated from the thymus and can be discriminated by expression of CD24 and Qa-2 (Paiva et al., 2013). Notably, this may also have implications in regard to the stability of the Treg cell program induced *in vitro* with respect to the importance of epigenetic programming imparted in the thymus.

The TCR repertoire of Treg cells generated in the periphery has only a small overlap with the TCR repertoire of thymic Treg cells. This indicates that peripheral Treg cells cover unique aspects of dominant tolerance (Haribhai et al., 2011). These may on the one hand cover auto-antigens that were not presented in the thymus to provide central tolerance. On the other hand induction of dominant tolerance to foreign antigens provides an ability of the immune system to adapt to newly arising conditions throughout lifetime.

4.2.3 Molecular mechanisms involved in TGFβ-induced Treg cell differentiation

Induction of Foxp3 expression is dependent on the binding of several transcription factors to *cis*-regulatory elements (Merkenschlager and von Boehmer, 2010; Rudensky, 2011). Different signaling cascades are triggered by T cell activation under Treg-polarizing conditions. Activation with anti-CD3 and anti-CD28 antibodies mimicking TCR ligation and co-stimulation, respectively, results in the activation of the phosphoinositide 3-kinase (PI(3)K) pathway triggering activation of mechanistic target of rapamycin (mTOR), the activation of the NFκB pathway with activation of c-Rel, signaling via the MAP kinase pathway resulting in AP-1 activation and the Ca^{2+} influx-dependent activation of NFAT (Sauer et al., 2008; Long et al., 2009; Mantel et al., 2006; Wu et al., 2006). IL-2 receptor ligation induces mainly Stat5 activation and TGFβ receptor signals induce activation of SMAD2 and SMAD3 as well as the TGFβ-activated kinase (Burchill et al., 2007; Gu et al., 2012). These and other factors

like Runx1, CREB, ATF, Foxo1 and Foxo3 and also Foxp3 itself bind to the *Foxp3* promoter as well as to the CNS elements to establish the *Foxp3* expression and Foxp3-dependent transcription (Josefowicz et al., 2012).

4.2.3.1 NFAT binding to Foxp3 or AP1 direct divergent differentiation programs

During productive effector T cell activation, the MAP kinase pathway is activated or enhanced through co-stimulatory molecules ultimately leading to the expression of c-Jun and c-Fos (Jain et al., 1992). They dimerize to form the transcription factor AP1, which can then form heterodimers with NFAT to bind to composite binding sites (Jain et al., 1992; Rooney et al., 1995). NFAT can also form heterodimers with Foxp3 and in this way bind to composite binding sites in promoters of Treg cell signature genes such as *CTLA-4* (Wu et al., 2006). In this model, Foxp3 and AP-1 compete for NFAT to direct either an effector cell or a Treg cell transcriptional program. The model is in agreement with data on the chromatin accessibility in activated conventional T cells compared to mature Treg cells suggesting that the Treg cell transcriptional signature reflects a stabilized enhancer landscape of the activated state (Samstein et al., 2012a). NFAT:Foxp3 complexes would then occupy loci to stabilize the activation-induced gene expression in a Treg cell-specific manner while AP-1 would guide NFAT to effector cell-associated genes such as *IL-2* (Wu et al., 2006).

4.3 Reciprocal programs control the differentiation into Treg and Th17 cells

4.3.1 RORγt and RORα synergize in Th17 and antagonize Treg cell differentiation

The discovery of Th17 cells and the unraveling of signaling events that promote differentiation of naive $CD4^+$ T cells into Th17 cells uncovered a common feature with induced Treg differentiation. The induction of Th17 cells depends on the presence of TGFβ, as does the induction of Treg cells. The current model suggests that TGFβ promotes Treg cell differentiation in a dose dependent manner, which is counteracted by the pro-inflammatory cytokine IL-6 that directs Th17 differentiation (Veldhoen et al., 2006; Bettelli et al., 2006). In Th17 cells, IL-6 induces expression of IL-21 receptor via Stat3, which further amplifies Stat3 signaling and induces the lineage-specifying transcription factor RORγt as well as RORα. These synergize with Stat3 to activate expression of IL-17A and IL-17F, the hallmark cytokines of Th17 cells (Yang et al., 2008). On the transcription factor level, RORγt and RORα in the case of Th17 cells and Foxp3 in the case of Treg cells, seem to mutually repress each other's capacity to *trans*-activate gene expression (Ivanov et al., 2006; Zhou et al., 2008a; Ichiyama et al., 2008).

4.3.2 Mtor in Treg and Th17 cell differentiation

Another pathway involved in reciprocal lineage differentiation is the PI(3)K/ Akt/ mTOR pathway. In a simplistic view, activation of this pathway during T cell activation promotes Th17 differentiation while its inhibition promotes Treg cell differentiation. As a multitude of factors are involved and integrate signals from activation signals, cytokine cues and metabolic conditions, a much more complex situation arises (Powell et al., 2011). Signaling through receptors like CD28 or common gamma chain receptors such as IL-2R or IL-7R induces activation of PI(3)K which phosphorylates phosphatidylinositol (4,5)-bisphosphate to form phosphatidylinositol (3,4,5)-triphosphate. This serves as a docking site for PDK-1 via its SH2 domain that allows its association with receptor-associated tyrosine kinases. Activated PDK-1 finally phosphorylates Akt on Thr308 and thereby partially activates Akt allowing phosphorylation of target genes among them the tuberous sclerosis complex (TSC) (Laplante and Sabatini, 2012). Such phosphorylation inactivates the small GTPase activity of the TSC complex that catalyzes hydrolysis of Rheb-GTP to Rheb-GDP. In its GTP-bound form Rheb ultimately activates the mTORC1. It is composed of mTOR, Raptor, mLST8, PRAS40 and DEPTOR. mTORC2 is constituted of mTOR, mSIN1, PROTOR, DEPTOR and the scaffolding protein RICTOR. The activation of mTORC2 is less well understood (Gamper and Powell, 2012).

The catalytic function of both complexes is conferred by the serine/threonine protein kinase mTOR. mTORC1 phosphorylates the p70 ribosomal S6 kinase and inactivates the EiF4E-binding protein by phosphorylation, which facilitates and enhances protein translation and promotes cell growth and division (Laplante and Sabatini, 2012). Phosphorylation of EiF4E-binding protein also releases HIF1α, which promotes a glycolytic metabolic state, cooperates with RORγt to drive the Th17 differentiation program and represses Treg cell differentiation (Shi et al., 2011; Dang et al., 2011).

mTORC2 activity is required for phosphorylation of Akt on Ser473, which enables the inactivation of Foxo transcription factors by phosphorylation through Akt (Jacinto et al., 2006). Since Foxo proteins are required for Treg cell differentiation, their inactivation by mTORC2 supposedly promotes Th17 and inhibits Treg cell differentiation (Kerdiles et al., 2010).

In agreement with the described functions of the mTOR complexes in reciprocal lineage differentiation, deletion of mTOR in $CD4^+$ T cells leads to a profound increase of Treg cell differentiation upon T cell activation and completely abrogated Th17 cell differentiation (Delgoffe 2009).

4.3.3 Retinoic acid in Treg and Th17 cell differentiation

Retinoic acid is a metabolite of vitamin A and is active in its 9-cis or all-trans retinoic acid (ATRA) isoforms (Chambon, 1996). It is bound in the cell by heterodimers of the nuclear retinoid X receptor (RXR) and retinoic acid receptor (RAR). Dendritic cells at mucosal sites in the gut can induce expression of gut-homing receptors on activated $CD4^+$ T cells by secretion of the vitamin A metabolite retinoic acid (Iwata et al., 2004). These dendritic cells were also found to be immunosuppressive and this prompted studies on the effect of retinoic acid on differentiation of Treg and Th17 cells (Mucida et al., 2007). Retinoic acid strongly induced differentiation of $CD4^+$ $CD25^-$ T cells into Treg cells while it strongly interfered with differentiation into the Th17 lineage even in Th17-polarizing conditions. This effect was mediated in part through inhibition of effector cytokine secretion in bystander memory T cells (Hill et al., 2008). Yet, retinoic acid was also active in the absence of effector cytokines and was dependent on expression of retinoic acid receptor α (RARα), whose expression in naive $CD4^+$ T cells was strongly reduced by IL-6 (Mucida et al., 2009; Nolting et al., 2009). The inhibitory effect on Th17 differentiation, however, was rather dependent on expression of the retinoic acid receptor α1. Recently, retinoic acid was shown to induce expression of microRNA-10a in TGFβ-induced regulatory T cells (Jeker et al., 2012; Takahashi et al., 2012).

4.4 Post-transcriptional gene regulation by microRNAs

4.4.1 MicroRNAs

MicroRNAs (microRNAs) are small non-coding RNAs of 21-23 nucleotides (nt) of length that recognize target messenger RNAs (mRNAs) by complementary base pairing. This results in translational repression or degradation of the target mRNA. Mello and Fire founded the microRNA field with studies on RNA-interference (RNAi) in *C. elegans* (Fire et al., 1998). MicroRNAs were found throughout the multicellular organisms and until today, genome sequencing data and prediction algorithms led to the annotation of 1186 precursors / 1908 mature microRNAs in mouse and 1872 precursors / 2578 mature microRNAs in man (miRBase release 20). Estimations based on microRNA target sequence conservation across species predicted that more than one third of human genes are direct microRNA targets (Lewis et al., 2003, 2005). In the following I will mainly focus on microRNA biology in animals and particularly in mammals.

4.4.2 MicroRNA biogenesis

MicroRNAs are transcribed from polymerase II promoters as primary precursor microRNAs (pri-miR) with several kilobases (kB) of length (Lee et al., 2004). They are either encoded monocistronically in intergenic loci under the control of their own promoters or as polycistronic transcriptional units in an intragenic manner lying in introns, in the coding sequence or in the untranslated region of mRNAs (Cai et al., 2004).

A pri-miR contains a bulged hairpin structure that is the substrate of the so-called microprocessor complex (Figure 1). It is a multiprotein complex with a heterodimeric core composed of the DiGeorge syndrome critical region gene 8 (DGCR8) and the RNaseIII enzyme Drosha, which precisely cleaves the pri-miR resulting in a 65-70nt long hairpin structure called precursor microRNA (pre-miR) (Lee et al., 2003; Gregory et al., 2004; Han et al., 2006). This cleavage by Drosha leaves the pre-miR with a 2nt 3' overhang that is recognized by exportin 5, which mediates nuclear export in a Ran-GTP-dependent manner (Yi et al., 2003). The maturation of the pre-miR is completed in the cytoplasm by cleavage of the hairpin adjacent to the loop by the RNaseIII enzyme Dicer (Bernstein et al., 2001; Knight and Bass, 2001; Hutvagner et al., 2001). This cleavage by Dicer results in a ~22nt mature microRNA (miR) duplex having 2nt 3' overhangs on either strand which is bound by a Dicer-associated Argonaute (Ago) protein (Elbashir et al., 2001). One strand of the mature

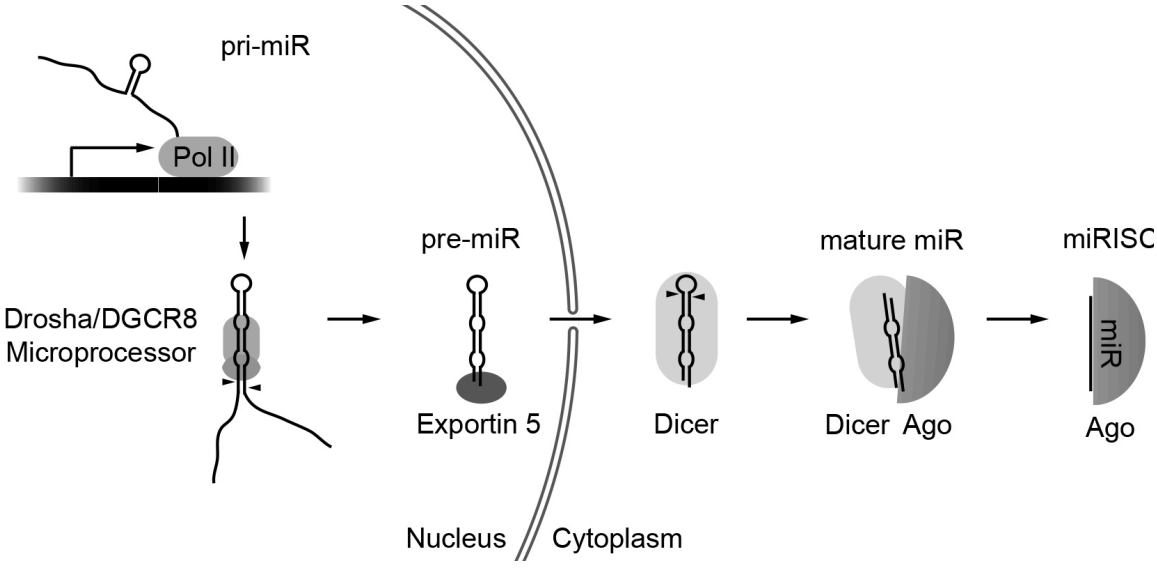

Figure 1: Schematic of microRNA biogenesis.
MicroRNAs are transcribed from polymerase II promotors as primary microRNAs (pri-miRs). Cleavage by the microprocessor complex generates precursor microRNAs (pre-miRs), which are exported to the cytoplasm, where they are further cut by Dicer to form mature microRNAs (miRs). One strand is loaded on an Argonaute (Ago) protein to form the microRNA-induced silencing complex (miRISC).

microRNA is transferred onto Ago in a defined orientation to ensure correct base pairing when a target is recognized (Hammond et al., 2001). The thermodynamic stability of the microRNA duplex usually leads to preferential binding of one strand to Ago, which is called the microRNA strand, while the other strand called microRNA* strand undergoes rapid degradation (Khvorova et al., 2003). The suffix -5p or -3p indicates that the used microRNA single strand was closer to the 5' or to the 3'end of the pre-miR hairpin. The microRNA:Ago complex is called microRNA-induced silencing complex (miRISC) and is the functional unit of microRNA-mediated post-transcriptional gene regulation. Some microRNAs, such as miR-451, have a particular pre-miR-structure that are nor Dicer substrates but are directly bound by Ago2.

4.4.3 MicroRNA function

The miRISC binds a target mRNA through complementary base pairing between the loaded microRNA and the target mRNA (Figure 2) (Liu et al., 2005). This mediates translational silencing alone or, in addition, the degradation of the target transcript. Prediction algorithms based on species conservation of microRNA sequences together with experimental evidence established that nucleotides 2-7 in the microRNA are crucial for target regulation in most cases and are called microRNA 'seed' (Lai, 2002; Lewis et al., 2003; Lim et al., 2003; Hafner et al., 2010). Most seed-matches in the target mRNA, which are complementary to the microRNA seed sequence, are found in the 3' untranslated region (3'UTR) of target mRNAs (Grimson et al., 2007).

Different mechanisms are discussed for how target mRNA expression is suppressed by the miRISC. The differences are based on findings that a microRNA affects target mRNA expression only on the protein level or in addition to that also on the mRNA level (Pillai et al., 2004). The first mechanism called translational silencing can act on translational initiation or on translation elongation. The initiation of translation is the rate-limiting step of translation and begins with binding of the eIF4E subunit of the eIF4F complex to the m^7-guanosyl in the 5' terminus of the mRNA (5'CAP) (Gingras et al., 1999). The eIF4F complex also mediates the adoption of a circular structure of mRNA by binding to poly(A) binding proteins (PABP) that decorate the 3' poly(A) tail of the mRNA which greatly enhances translation efficiency (Kahvejian et al., 2005).

MiRISCs were proposed to bind the eIF4F as well as the 5'CAP of the mRNA and could thereby interfere with translation initiation (Mathonnet et al., 2007). However, contradictory results show that abrogation of the CAP-binding residues in Ago also abrogates its interaction with GW182 thus leaving the mechanism partly elusive (Eulalio et al., 2008).

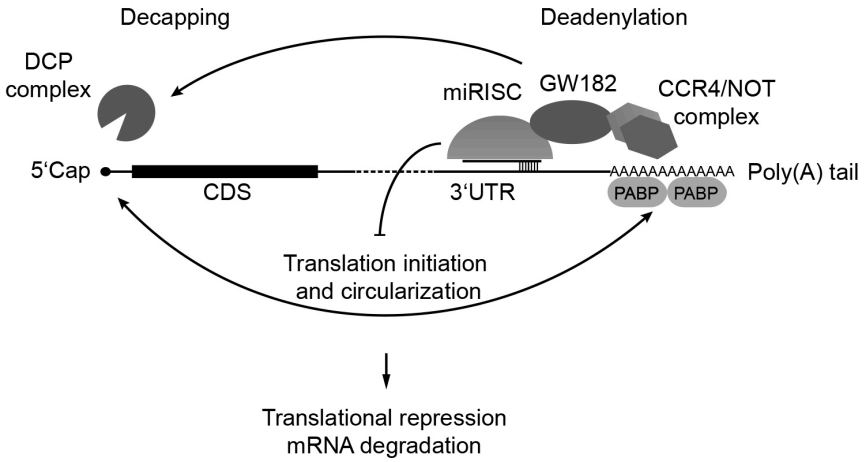

Figure 2: Schematic depiction of miRISC-mediated mechanisms of post-transcriptional regulation. Binding of the miRISC to a target mRNA via the microRNA 'seed' sequence interferes with translation initiation and circularization through eIF4-mediated interaction of the 5'CAP with PABP. MiRISC binding recruits deadenylation factors via GW182 and mediates mRNA decapping. This results in translational repression and potentially in mRNA degradation. miRISC: microRNA-induced silencing complex, CDS: coding sequence, 3'UTR: 3'untranslated region, PABP poly(A)-binding protein.

MiRISC can also promote deadenylation of the 3'end of the target mRNA by binding to GW182 proteins, which recruit the CCR4-NOT-deadenylation complex (Chekulaeva et al., 2011). This would interfere with PABP binding and mRNA circle formation and thereby interfere with efficient translation. Deadenylation and subsequent mRNA decapping provide mechanisms for destabilization and enhanced degradation of the target mRNA, which is also mediated in a miRISC- and CCR4-NOT-dependent manner (Behm-Ansmant et al., 2006; Braun et al., 2011). Translational repression can also take place in the absence of a poly(A) tail suggesting that both processes act in parallel and may be regulated independently, which is in agreement with the observation that some microRNAs regulate a target only on the protein level while others additionally reduce target mRNA levels.

It is a subject of debate whether one of these mechanisms is the primary mode of microRNA-mediated repression and the other mechanisms being secondary effects or whether all of these exist in parallel and depend on the particular microRNA and mRNA in the cellular context (Bartel, 2009).

4.4.4 Concepts of microRNA-mediated gene regulation

The significance of posttranscriptional gene regulations by microRNAs was best demonstrated by knock-out studies that targeted components of the microRNA biogenesis pathway. Deletion of *Dgcr8* or *Dicer* largely reduced the abundance of all mature microRNAs

and impaired embryonic development (Bernstein et al., 2003; Yang et al., 2005; Kanellopoulou et al., 2005; Wang et al., 2007). Other studies have addressed the impact of single microRNAs on post-transcriptional gene regulation. An evolutionary analysis uncovered that many microRNAs are highly conserved throughout species. The biological function of a microRNA depends on the co-expression of a seed-matched target. The 6-8bp (basepair) seed-matched target sequence should be stochastically distributed as long as it is not under selective pressure. Conservation of such a motif across species is a strong indication for microRNA-mediated regulation with biological relevance. However, microRNA and target conservation alone are not informative regarding the spatial and/or temporal disribution within an organism that determine its biological function. Consequently, only tissue-specific case studies of microRNA-target relations can unambiguously prove biological relevance.

MicroRNAs can influence target gene expression in three general modes, the classical switch mode, the tuning mode and the mode of neutral interaction (Bartel, 2009). The defining parameter is the effective target mRNA expression level that results in a substantial change of the cellular response.

In the classical switch mode, the target mRNA level is at a critical height. A moderate decrease by microRNA-mediated repression will switch the cellular response in this situation. An example for this situation is the fate decision of colon cancer stem cells between self-renewal and differentiation. Self-renewal is maintained by high Notch expression and characterized by low miR-34a expression. Differentiating daughter cells downregulate Notch1 through Numb, but high expression of microRNA-34a, which targets Notch1, enforces a clear decision towards differentiation (Bu et al., 2013).

In tuning interactions, an ideal target mRNA level will result in a physiologic response while higher or lower expression levels result in adverse effects. Here, microRNA-mediated repression helps to stabilize an appropriate expression level. An example for such a tuning interaction is the expression of the transcription factor c-Myb in B cell development (Thomas et al., 2005). Development of B1 cells in the peritoneal cavity is very sensitive to an appropriate expression level of c-Myb, which is a target of miR-150 (Xiao et al., 2007). Deletion of miR-150 results in elevated c-Myb expression, which strongly increases B1 cell numbers at the expense of B2 cells. Conversely, ectopic expression of miR-150, which represses c-Myb expression by no more than 30%, results in a strong decrease of B1 cells.

In neutral interactions, the microRNA reduces the target level to some extent but the effective target mRNA expression level has a broad range that is not affected by regulation of the

single microRNA. This can account for accidental seed matches that were not counter-selected by evolution due to a lack of selective pressure. However neutral interactions may be a more apt description of the moderate character of single microRNA:mRNA interactions. This is supported by a proteomic study analyzing microRNA-223 overexpression or deletion in neutrophil granulocytes. The great majority of miR-223 targets were repressed by less than 30% (Baek et al., 2008). However, hundreds of targets were repressed by miR-223 in that manner and given that one target can be regulated by multiple microRNAs, the idea of a network of microRNA-mRNA target interaction suggests itself (Lewis et al., 2005). Each node in this network, which is one microRNA-mRNA target interaction, may be classified as a neutral interaction but the outcome of a network decision would in the end be as clear as a switch interaction (Bartel, 2009). The redundancy in this system provides robustness of biological responses against failure of single nodes and leaves room for evolutionary adaption.

4.5 MicroRNA function in T cells

4.5.1 Phenotypes of mature microRNA ablation in the T cell compartment

The general impact of microRNAs in T cells was analyzed in genetic models with deletions of components of the microRNA biogenesis pathway, which prevents generation of almost all mature microRNAs. As germ-line knock-outs (ko) were lethal, conditional deletion approaches were pursued (Bernstein et al., 2003). Deletion of *Dicer* during early thymic T cell development widely decreased expression of mature microRNAs in αβ thymocytes. This resulted in apoptosis of T cell progenitors but had no significant effect on the thymic development of conventional $CD4^+$ and $CD8^+$ T cells (Cobb, 2005). Deletion of *Dicer* in $CD4^+$ expressing thymocytes resulted in a strong reduction of microRNA expression initiating with the CD4/CD8 double positive stage in thymocyte development (Muljo et al., 2005). In these mice thymic T cell output was normal for $CD4^+$ T cells but reduced for $CD8^+$ T cells. In the periphery, *Dicer*-deficient $CD4^+$ and $CD8^+$ T cells numbers were both reduced. Upon *in vitro* stimulation $Dicer^{-/-}$ $CD4^+$ T cells expanded slower than wildtype cells, which could be attributed to both, enhanced apoptosis and delayed proliferation (Muljo et al., 2005). Experiments performed to elucidate the differentiation capacity of $Dicer^{-/-}$ $CD4^+$ cells revealed a bias towards Th1 differentiation in non-polarizing conditions and an inability of Th2 cells to repress inappropriate IFNγ production. This was accompanied by enhanced expression of the Th1 lineage-specifying transcription factor Tbet. Conditional knock-out of

Drosha or *Dgcr8* in T cells exhibited a very similar phenotype indicating that the observed alterations indeed were dependent on the absence of mature microRNAs rather than on other individual functions of Dicer or DGCR8 (Chong et al., 2008; Steiner et al., 2011). In a reconstitution screen of single mature microRNAs in microRNA-deficient *Dgcr8*$^{-/-}$ T cells, the Th1 bias was rescued by expression of mature miR-29a or miR-29b alone. Both microRNAs directly target Tbet and Eomes, which are key transcription factors for IFNγ production (Steiner et al., 2011). In addition to the Th1 bias, ablation of Dicer in T cells also impairs differentiation of CD4^{+} T cells into the Th17 lineage suggesting, that the presence of mature microRNAs is essential for proper T helper lineage differentiation (Cobb et al., 2006).

4.5.2 Deficiency of mature microRNA in Treg cell development and function

Mice with T cells that are devoid of mature microRNAs develop severe autoimmunity characterized by lymphoproliferation, systemic inflammation and lymphocytic infiltrations into colon, liver and lung (Chong et al., 2008; Cobb et al., 2006). This could be attributed to markedly reduced numbers of Treg cells in spleen and lymph nodes. The generation of thymic Treg cells was strongly impaired as well as the TGFβ-induced differentiation of naive CD4^{+} T cells into Treg cells analyzed *in vitro*. Importantly, this highlighted the Treg cell-intrinsic importance of microRNAs (Cobb et al., 2006; Chong et al., 2008). Chong et al. further established that *Drosha*$^{-/-}$ and *Dicer*$^{-/-}$ Treg cells have a reduced capacity to suppress effector T cell proliferation. Together with the reduced Treg cell compartment, this could explain the autoimmune phenotype observed in these mice. Interestingly, specific conditional deletion of *Dicer* or *DGCR8* in Treg cells resulted in accelerated autoimmunity resembling the *scurfy*-phenotype that results from a loss-of-function mutation of *Foxp3* (Chong et al., 2008; Liston et al., 2008; Zhou et al., 2008b). Furthermore, Zhou et al. found that microRNA-deficient Treg cells loose expression of the Treg cell-specific transcriptional signature including Foxp3 and instead in part produce IFNγ, which would contribute to enhanced autoimmunity. Other studies observed a loss of Treg cell suppressive capacity, but no loss of Treg cell identity (Chong et al., 2008; Liston et al., 2008).

Taken together these results show that mature microRNAs are required for various processes in CD4^{+} T cells such as proliferation, prevention of apoptosis and differentiation of T helper cell lineages. They are essential for Treg cell generation, function and lineage stability. Block of microRNA biogenesis is a rather crude measure to assess their biological functions. It alters the expression of thousands of genes at the same time and likely includes effects secondary to these perturbations. In order to understand the underlying molecular mechanisms

more precisely, the analysis of individual microRNAs will be the method of choice. Profiling experiments showed differential expression of a limited number of specific microRNAs in the hematopoietic system and also among T cell subsets and developmental stages. These data provide a basis for the analysis of attractive microRNA candidates in the differentiation of Treg cells (Monticelli et al., 2005; Cobb et al., 2006; Landgraf et al., 2007; Kuchen et al., 2010). To date a few of the differentially-regulated microRNAs were analyzed in more detail and are discussed in the following.

4.5.3 The impact of individual microRNAs on T cells and Treg cells

MiR-155

MiR-155 is widely expressed in cells of the immune system. Germ line deletion of *miR-155* perturbs germinal center formation, results in Th2-biased T cell differentiation and diminishes IL-2 production by $CD4^+$ T cells (Rodriguez et al., 2007; Thai et al., 2007). Furthermore, miR-155 deficiency impaired Th17 cell differentiation and protected mice from disease in an autoimmune encephalomyelitis model (O'Connell et al., 2010). MiR-155 expression was increased in CD8 T cells upon viral infections and provided accumulation of CD8 cells by an anti-apoptotic and pro-proliferative function (Dudda et al., 2013).

In Treg cells, miR-155 was found upregulated and its induction was Foxp3-dependent, supposedly under the control of a Foxp3 binding element in the *miR-155* locus (Cobb et al., 2006; Lu et al., 2009). Deletion of miR-155 reduced thymic and peripheral Treg cell numbers (Lu et al., 2009; Kohlhaas et al., 2009). This could be attributed to a lack of competitive fitness of miR-155-deficient T cells due to attenuated IL-2 signaling through derepression of the negative regulator SOCS1 (Lu et al., 2009). However, the functional capacity of Treg cells is not affected by miR-155 deficiency (Kohlhaas et al., 2009).

MiR-17~92

The *microRNA 17~92* cluster on chromosome 13 is a polycistronic unit that encodes hairpins for miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1. It is an oncomir that has transforming potential and is overexpressed in B cell lymphoma and other cancers (He et al., 2005). Deletion of *miR-17~92* leads to reduced B cell numbers due to a survival defect at the pro-B/ pre-B cell stage, which was attributed to a loss of microRNA repression of the pro-apoptotic factor Bim (Ventura et al., 2008). MiR-17~92 expression reconstituted proliferation and survival of *Dgcr8*-deficient $CD4^+$ T cells and in another study ectopic expression promoted increased survival of $CD4^+$ and $CD8^+$ T cells via targeting of Bim and PTEN

(Steiner et al., 2011; Xiao et al., 2008; Khan et al., 2013). Overexpression in $CD4^+$ T cells further resulted in overall activation of T cells accompanied by lymphoproliferation and autoimmunity. Dissection of the contribution of individual microRNAs from the cluster revealed that miR-17 and miR-19b directly targeted PTEN, facilitated T cell proliferation and were essential for IFNγ production in Th1-polarizing conditions. Moreover, they suppressed induction of Treg cells (Jiang et al., 2011). Recently, two studies showed that the microRNAs of the 17~92 cluster promoted development Tfh cells, which were essential to establish proper B cell responses in germinal centers (Baumjohann et al., 2013; Kang et al., 2013). Overexpression resulted in higher numbers of follicular Tfh cells, while the knock-out prevented migration of Tfh cells into B cell follicles and compromised antibody responses in viral infection models.

MiR-146a

TCR stimulation induced miR-146a expression via induction of the NFκB transcription factors p50 and c-Rel in naive and memory T cells (Rusca et al., 2012; Yang et al., 2012). MiR-146a itself negatively fed back on NFκB activity, possibly by targeting Traf6 and IRAK1. MiR-146 knock-out established a central memory T cell phenotype in human and mouse T cells and resulted in spontaneous T cell activation and autoimmunity in mice. However, autoimmunity could also be mediated by miR-146a deficiency in Treg cells only. While their suppressive capacity *in vitro* was unaffected, miR-146-deficient Treg cells seemed to have a selective loss in the ability to regulate IFNγ production in Th1 responses, since blockade of IFNγ rescued the phenotype (Lu et al., 2010). Since miR-146a targeted Stat1 mRNA downstream in IFNγ-receptor signaling, it was proposed that Treg cell-mediated suppression of Th1 responses required a miR-146a-modulated IFNγ signaling and expression of the Th1 lineage-specifying transcription factor Tbet within the suppressing Treg cell (Lu et al., 2010). This would represent a transcriptional symmetry between the suppressor cell and the suppressed effector cell (Koch et al., 2009).

MiR-10a

Two studies reported a selective expression of miR-10a in Treg cells, but not in conventional T cells (Jeker et al., 2012; Takahashi et al., 2012). *In vitro*, miR-10a contributed to Foxp3 protein expression levels, but deletion of miR-10a did not interfere with Treg cell induction and was therefore considered to be no more than a marker of Treg cells (Jeker et al., 2012). The identification of Bcl-6 as a direct target of miR-10a provided a link to Tfh cell

differentiation. In an adoptive T cell transfer model induced Treg cells differentiated into Tfh cells, which was promoted by miR-10a overexpression and reduced by miR-10a knockdown. In addition, miR-10a also interfered with Th17 induction (Takahashi et al., 2012).

MiR-181a

MicroRNA-181a is highly expressed during thymic T cell development. Its expression enhances TCR signaling by targeting several inhibitory phosphastases like SHP2 and DUSP6 and knockdown of miR-181a impairs positive and negative selection (Li et al., 2007).

MiR-182

MicroRNA-182 targets Foxo1, whose inactivation in T cells is required to allow T cell proliferation (Stittrich et al., 2010). In early T cell differentiation, Foxo1 is inactivated by TCR-dependent phosphorylation. IL-2 secreted in the course of T cell activation induces miR-182 that in turn suppresses Foxo1 expression in the late stages of differentiation and thereby promotes T cell expansion. Inhibition of miR-182 ameliorated the disease score in a rheumatoid arthritis model.

MiR-326

MiR-326 was overexpressed in tissue samples derived from multiple sclerosis patients (Du et al., 2009). In line with these results, transgenic miR-326 expression in a mouse model of multiple sclerosis promoted disease and increased the frequency of disease-promoting Th17 cells, while knockdown of miR-326 had an opposing effect. These results could also be recapitulated *in vitro* and were attributed to targeting of Ets-1, a negative regulator of Th17 cell differentiation. Remarkably, the expression of miR-326-insensitive Ets-1 rescued the enhanced disease in miR-326 overexpressing mice.

MiR-301

MiR-301 was selectively upregulated in T cells in responses against the myelin antigen in a mouse model of multiple sclerosis (Mycko et al., 2012). In vitro studies revealed that miR-301 promoted Th17 differentiation via direct regulation of the inhibitor of IL-6/STAT3 signaling PIAS3 and thereby increased pro-inflammatory IL-6 signaling. A knockdown of PIAS3 as well as the overexpression of miR-301 *in vivo* resulted in more severe disease symptoms. Notably, miR-301 knockdown had no effect on Treg cell differentiation.

Altogether, the analyses of individual microRNAs that were analyzed in various T cell conditions and disease models exhibited some aspects observed in animals with deficiency of mature microRNAs in T cells or Treg cells. The collective data so far suggest that there is no single master regulator microRNA for Treg cell differentiation. More likely, multiple microRNAs may act in concert and exert different effects in different T cell subsets or conditions.

4.6 Control mechanisms for microRNA regulation

The effectiveness of microRNA-mediated repression can be regulated on several layers. In the following, regulations on the level of microRNA expression as well as on the level of target site abundance in 3'UTRs will be introduced.

4.6.1 Regulation of microRNA turnover

As for all other RNA species, the balance between transcription, processing and degradation determines the expression level of each microRNA within the cell. A study of microRNA expression of dark/light adaption of retinal neurons showed changes in the expression levels of particular microRNAs within 90minutes upon adaption to either situation (Krol et al., 2010). This highlighted that microRNA expression levels can be efficiently controlled by regulated transcription and degradation. A global analysis of microRNA expression in murine embryonal fibroblasts in an inducible Dicer deletion system inferred microRNA half-lives between 28 h and >200 h (Gantier et al., 2011). These differences in the turnover of different microRNAs in the same cellular system imply further control mechanisms that act on particular microRNAs. Cell type-specific differences in degradation may be responsible for greatly varying turnover rates observed between fibroblasts and retinal neurons.

Another possibility to influence the expression levels of mature and functionally active microRNAs is to regulate one or more of the steps that are involved in their biogenesis (Hoefig and Heissmeyer, 2008; Bronevetsky and Ansel 2013). One prominent example is the maturation of let-7 microRNAs in embryonic stem cells. Here the processing of the primary let-7 transcript by Drosha/Dgcr8 was blocked by the binding of the RNA-binding protein Lin-28 (Viswanathan et al., 2008).

MicroRNA activity can also be regulated on the miRISC level. Human AGO2 can be phosphorylated on Y393 under hypoxic stress-inducing condition. This reduced the binding of AGO2 to Dicer and led to decreased processing of a large set of pre-miR that contained a particularly small loop (Shen et al., 2013). Other phosphorylations of Ago were described and could provide additional regulatory mechanisms for Ago activity (Rüdel et al., 2011; Zeng et al., 2008).

More generally, the expression level of Ago correlated with expression of mature microRNAs (Diederichs and Haber, 2007). This implicated that microRNAs are stable as long as they are loaded onto Ago proteins. In support of this, downregulation of Ago in activated T cells heavily reduced the half-life of microRNAs to less than two hours and could be considered a reset mechanism for the expressed microRNAs (Bronevetsky et al., 2013).

To date, mechanisms for the degradation of mature microRNA are largely unknown. A potential effector could be the Exoribonuclease 1 (Eri1) protein, since conditional deletion of this 3' exonuclease in T cells or NK cells resulted in a moderate but global increase of microRNA expression independent of the microRNA sequence (Thomas et al., 2012).

4.6.2 Variation of 3'UTR lengths by alternative polyadenylation

Polymerase II-transcribed mRNA transcripts in the cytoplasm are composed of a 5'UTR, an intron-free coding region between the start and the stop codons and the 3'UTR. The 5' and 3'ends are post-transcriptionally stabilized by a 5' cap structure and a 3' poly(A) tail. The 3'UTR of an mRNA is the main substrate for post-transcriptional regulation via *cis*-acting elements, such as microRNA target sites and binding sites for RNA-binding proteins, which determine the stability and translation efficiency of mRNAs (Glasmacher et al., 2010; Grimson et al., 2007; Myer et al., 1997). The generation of alternative mRNA 3'UTRs can lead to 3'UTR shortening and in consequence to the evasion from post-transcriptional gene regulation by microRNAs and RNA-binding proteins. This constitutes another layer for control of post-transcriptional gene regulation. 3'UTRs are encoded on the most 3' located exon or alternative 3' exons of a gene. Tian et al. demonstrated in a database analysis of expressed sequence tags that more than 30% of mouse genes show alternative polyadenylation resulting in transcripts with alternative 3'ends (Tian et al., 2005). At first this was assumed to be a consequence of imprecise 3'end processing but tissue-specific expression patterns of transcript variants with specific 3'UTRs rather suggested regulation (Zhang et al., 2005). A more general shortening of 3'UTRs is observed in rapidly cycling cells, for example activated T cells or cancer cells (Kumar et al., 2007; Lu et al., 2005;

Sandberg et al., 2008). Other reports related regulated 3'UTR shortening of individual transcripts to T and B cell differentiation (Chuvpilo et al., 1999; Takagaki et al., 1996). The 3'end of a nascent mRNA during transcription is not defined by the stop of mRNA synthesis by polymerase II. Instead it depends on cleavage and polyadenylation of the nascent transcript, which is determined by a polyadenylation signal (PAS) 10-30 nucleotides upstream in the sequence, downstream GU-rich elements as well as by UGUA motifs (Figure 3; Mandel et al., 2008; Nevins and Darnell, 1978). The distribution of 3'end variants of expressed sequence tags in various tissues suggests that particular PAS are more abundantly selected across tissues and are called strong PAS. Comparison of 3'UTRs from annotated human expressed sequence tags show that among the observed alternative PAS the strongest PAS tend to have the canonical sequence AAUAAA and to be situated most 3' in the UTR

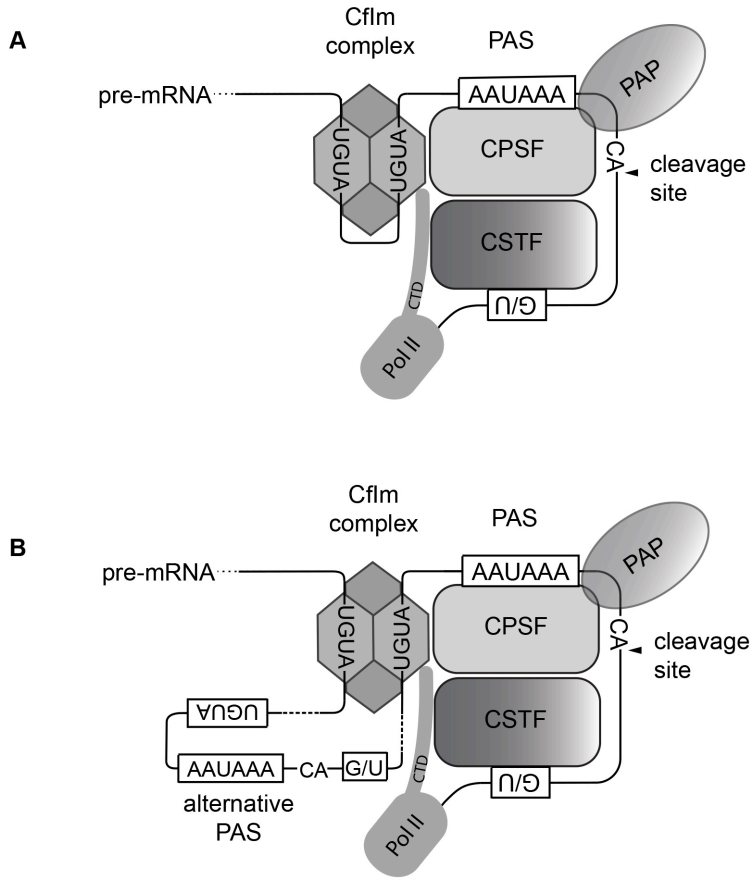

Figure 3: Schematic interactions of the cleavage and polyadenylation machinery with the pre-mRNA. A) Proteins of the cleavage and polyadenylation machinery are co-transcriptionally assembled by the polymerase II (Pol II) C-terminal domain (CTD) and bind to sequence elements in the pre-mRNA. This promotes cleavage and polyadenylation of the nascent transcript. Anti-parallel binding of the UGUA motif by the CfIm complex was suggested to be involved in the regulation of alternative polyadenylation, since it could loop out alternative PAS (B) and thereby may determine the PAS used for cleavage and polyadenylation. PAS: polyadenylation signal, CPSF: cleavage and polyadenylation stimulating factor, CSTF: cleavage stimulating factor, CfIm: cleavage factor Im, PAP: poly(A) polymerase.

(Beaudoing et al., 2000). However, strong sites may have biased annotation of expressed sequence tags and longer 3'UTRs may therefore be underrepresented in databases.

Efforts to identify the mechanisms that could control 3'UTR shortening by regulation of alternative polyadenylation focus on the mRNA 3'end processing machinery, which comprises cleavage and polyadenylation stimulating factor (CPSF), cleavage stimulating factor CstF, cleavage factors Im and IIm (CfIm, CFIIm), poly(A) polymerase (PAP) and poly(A) binding protein II, which act together to cleave the mRNA and provide 3'end polyadenylation (Mandel et al., 2008; Martin et al., 2012).

In addition, alternative 3'UTRs can also result from alternative splicing events that provide a different 3' exon to encode an alternative 3'UTR (Sandberg et al., 2008; Takagaki et al., 1996).

4.7 Aim of this study

Deletion of components of the microRNA biogenesis pathway largely prevents the formation of mature microRNAs. Mice with a conditional deletion of these components in $CD4^+$ T cells exhibit a marked reduction of Treg cell generation in the thymus and the differentiation of naive $CD4^+$ T cells into Treg cells *in vitro* is severely impaired (Chong et al., 2008; Cobb et al., 2006). These mice develop severe T cell-mediated autoimmunity. Moreover, a conditional deletion in mature Treg cells leads to the loss of regulatory function and also results in autoimmunity (Chong et al., 2008; Liston et al., 2008; Zhou et al., 2008b). These findings lead to the conclusion that microRNAs are essential for Treg cell differentiation and function. The contribution of individual microRNAs to the differentiation into Treg cells is completely unknown.

The first objective of this study was to identify microRNAs that effectively modulate Treg cell differentiation. To that end, a functional screening approach was pursued to test overexpression of individual T cell-expressed microRNAs in an *in vitro* Treg cell differentiation model. As a prerequisite for a successful screen, the timing of effective post-transcriptional gene regulation during Treg cell differentiation needed to be determined. Importantly, the experimental setup that allowed testing of microRNA function in the determined time frame had to be established.

The second objective was to validate these microRNA candidates and to determine the critical targets that can mediate the observed microRNA effects on Treg cell differentiation. Ultimately, the cooperation of identified microRNAs in repression of identified target genes during Treg cell differentiation should be addressed.

5 Materials

5.1 Mice

*DO11.10*tg; *CARΔ1*tg mice were purchased from Taconic Farms, Inc. These mice express a truncated version of the coxsackie adenovirus receptor (*CARΔ1*tg) with deletion of the cytoplasmic domain (Wan et al., 2000a). It is expressed under the lymphocyte-specific proximal Lck promoter/CD2 enhancer and renders mouse T cells infectable with type 5 adenovirus. DO11.10 is a transgenic TCR specific for MHC II-loaded ovalbumin OVA323-339 peptide. It can be used for antigen specific T cell activation, but that feature was not utilized in this study.

Eri1-deficient mice ($Eri1^{fl/fl}$) were created and provided by M. Ansel (Ansel et al., 2008). *CD4-Cre* mice were used for loxP site recombination (Lee et al., 2001).
All animals were housed in a specific pathogen-free barrier facility in accordance with the Helmholtz Zentrum München institutional, state and federal guidelines.

5.2 Cell culture

Cell lines

The human A549 cell line was purchased from ATCC and is a lung adenocarcinoma epithelial line. The human embryonal kindney carcinoma cell line HEK293A was purchased from Invitrogen. Mouse embryonal fibroblasts (MEF) were generated by K. Hoefig as described (Hoefig et al., 2013).

Table 1: Cell culture ingredients

Dulbecco's Modified Eagle Medium (DMEM)	Gibco, Invitrogen
RPMI 1640, without L-glutamine	BioWhittaker, Lonza
Fetal bovine serum (FBS)	PAN BIOTECH GmbH
HEPES (1M)	Gibco, Invitrogen
PenStrep (10,000U/ml)	Gibco, Invitrogen
β-Mercaptoethanol	Sigma-Aldrich
GIBCO™ L-Glutamine-200 mM (100x), liquid	Gibco, Invitrogen
100x NEAA	BioWhittaker, Cambrex
GIBCO™ MEM Vitamin Solution (100x), liquid	Gibco, Invitrogen
Sodium pyruvate (100mM)	BioWhittaker

5.3 Antibodies and cytokines

The antibodies in the following tables were against mouse antigens and of rat origin.

Table 2: Anti-mouse antibodies and cytokines for T cell polarization

Anti-CD3 (145-2C11)	In-house production
Anti-CD28 (37N)	In-house production
Anti-IFN-γ (XMG1.2)	In-house production
Anti-IL-2 (JES6-5H4)	Miltenyi Biotec
Anti-IL-4 (11B11)	In-house production
Anti-IL-12 (C17.8)	In-house production
Recombinant human IL-2 (ProleukinS)	Novartis
Recombinant human TGF-β1	R&D Systems
Recombinant mouse IL-6	R&D Systems

Table 3: Antibodies for western blot

Anti-actin (I-19), polyclonal goat anti-mouse	Santa Cruz
Anti-tubulin (B-5-1-2)	Santa Cruz
Anti-panAgo (11G1)	In-house production, (Bronevetsky et al., 2013)
Anti-Eri1 (5G8)	In-house production, (Ansel et al., 2008)
Anti-mTOR (7C10)	Cell signaling
Anti-Foxp3, polyclonal rabbit anti-mouse	Gift from Steve Ziegler

Table 4: Antibodies and cell labeling reagents

Anti-CD44 (IM7)	eBioscience
Anti-CD62L (MEL-14)	eBioscience
Anti-CD90.1 (Thy1.1, clone OX-7)	eBioscience
Anti-Foxp3 (FJK-16s)	eBioscience
Anti-IFN-γ (XMG1.2)	eBioscience
Anti-CD69 (H1.2F3)	eBioscience
Anti-CD127 (A7R34)	eBioscience
Anti-CD5 (53-7.3)	eBioscience
Anti-IL-17A (eBio17B7)	eBioscience
Anti-CD25 (PC61.5)	eBioscience
Anti-CD90.1 (HIS51)	eBioscience
LIVE/DEAD® Fixable Dead Cell Stain Kit	Invitrogen
Cell Proliferation Dye eFluor®	eBioscience

5.4 Primer

Table 5: Primer, entry and destination vectors of the microRNA library

Cloning primers were purchased from Metabion.

miR-146a	Fwd	CACCCATGCCCAGCATGTTTAATG
	Rev	TGATCAGCTGTGACCTGGAA
miR-142	Fwd	CACCGGAGTGGAGGGAAGAAGGTT
	Rev	CCAAGTATCAGGGGTCAGGA
miR-let7b	Fwd	CACCTGAGTACAGCCTGCAGATGG
	Rev	GCCAGTCTCCGTATTTCCAA
miR-106b	Fwd	CACCTGACTACATCACCGCAGCAT
	Rev	TACCTGCACGAACAGCACTT
miR-16-2	Fwd	CACCTGGTTTTTGATTTTTGGCTTG
	Rev	CCCAGAGTAAACCTTTATGCAA

let-7a-2	Fwd	CACCTCAAAGGTACCAGACACCCATA
	Rev	TGACCCAAAGAGACCAAACA
miR-17	Fwd	CACCTTTTTAAGGCTTACATGTGTCCAA
	Rev	TGCCAGAAGGAGCACTTAGG
miR-21	Fwd	CACCTTGTACTCCGGCTTTAACAGGT
	Rev	TGACGACTACCCCAATTTCC
let-7f-1	Fwd	CACCTGCATTCATGGGGTCTCATA
	Rev	GGCCTGGTCCTAGATACTTACTTTT
miR-15b	Fwd	CACCATCCAGAACCGCCTACAGAG
	Rev	CCTGTCACACTAAAGCAGCA
miR-24-1	Fwd	CCACCCAGGTGCATCAAGGAAACT
	Rev	CCTGCTTCTAGGGAATGCAA
miR-let-7d	Fwd	CACCCAGTGGGCTGAGTGTTAGAGA
	Rev	TCCAAAACTTCCCAGTTACCC
miR-23a, miR-24-2, miR-27a	Fwd	CACCTCCAGAGGTAGAGGCAGGAA
	Rev	CTATCTGCTTTGGGGAACCA
miR-222	Fwd	CACCGCTTTGGGGATAGCATTTGA
	Rev	GGGGGAAAGAGGAAGACAAG
miR-181a, miR-181-b	Fwd	CACCTACATGCGTCCTTGCAGTTC
	Rev	CCGAGAAACGGCGTTAATAC
miR-155	Fwd	CACCTGAACCGTGGCTGTGTTAAA
	Rev	CGAGAATGGCCGTCCTGAAT
miR-101a	Fwd	CACCGGTGCATAGGTGTGAGATTGGG
	Rev	ACCACCCAACAGTGAAGGAC
miR-150	Fwd	CACCAGGGGAAGTGCTAGGCTCTC
	Rev	GTTGGAGTGATGGGAACACCC
miR-19b-1, miR-20a, miR-92a-1	Fwd	CACCCTGATGGTGGCCTGCTATTT
	Rev	CCGTTTTACACACCAACGAA
miR-26a-1	Fwd	CACCGCGCTGGTTGTTGTGTCTAA
	Rev	AACACTCGTTGTTGCTGGTG
miR-29a	Fwd	CACCCCACCCTGCTTACCTCTGTG
	Rev	ATTGGTTTGGCCCTTTATCC
miR-146b	Fwd	CACCCCATAGGCTGTGATCTCTCTCTCT
	Rev	ATGTTTGGAGCCTGAAGGTG
miR-29b-1	Fwd	CACCTGTAAGCCTCGTGCTCACTG
	Rev	GGAGTCCACTTCCAGAGAAGG
miR-93	Fwd	CACCTGCTGGGACTAAAGTGCTGA
	Rev	TGTCAGACCGAGACAAGTGC
miR-223	Fwd	CACCCCGTTTTTGTTTGGAGCATT
	Rev	CAGTCCATGGCATTTTCACA
miR-25	Fwd	CACCCCTCCCCTCTTGGACCTTAG
	Rev	AGCCTAAGGGGAAGGCATAG
miR-17, miR-18a, miR-19a	Fwd	CACCCTTCTGGCTATTGGCTCCTC
	Rev	CGAGCAAACACGAAAATGAA
miR-125b-1	Fwd	CACCCAGGGCTGTATGGAGACAGAA
	Rev	GGGTCACCTGATCCCATCTA
miR-221	Fwd	CACCAGCATGATGAATGACCACCA
	Rev	ACAAATGCTGAGGTCGGAAG
miR-15, miR-16a	Fwd	CACCTGGCTTTGAAAGATGTGCTG
	Rev	TGCTAGCAAGAAGCACTTGG
miR-30e	Fwd	CACCCATTCTCCCCATGTTGACCT
	Rev	AATGCCTGACTGCATCACAG
miR-140	Fwd	CACCGTGGGGCTGTCATCTGACTT
	Rev	CCTCCCTTCACCCTAGAACC
miR-30d	Fwd	CACCCTGCCAGCAAAACTTGTGTC
	Rev	GGGTGGGTGGTAGACATGAA
miR-26b	Fwd	CACCGCACTACACCCAGGTTCCTC
	Rev	GCTTAGGGGTGATCCACAAA
Let-7g	Fwd	CACCCCACCGATGCTTTTGGATAA

	Rev	AAGCCTCTCAAATGACAACCA
miR-652	Fwd	CACCCAGGTGCTCCTGAACCTCTT
	Rev	CCCCCTGCTCTCTCTCTCTT
miR-191	Fwd	CACCGGACTCACAGGGCTAATCCA
	Rev	TTCAGAGAGAGGACCCAGGA
miR-374, miR-421	Fwd	CACCGATGTTTGGGGAATGGTCAC
	Rev	CCTTCCACACCCTCAGGATA
miR-425	Fwd	CACCTCCTGGGTCCTCTCTCTGAA
	Rev	CGGCACCTTTGGTTCTTTAG
miR-342	Fwd	CACCCTTCCCAGGACTCTTGGTTG
	Rev	TCTCACTCTGCTGGTCATGG
miR-126	Fwd	CACCAGCACTGTTGTGTGGCTGAG
	Rev	CTAGTCAGGGAGGGGAGCTT
miR-214	Fwd	CACCTCCTTGTTAGCAACATTTGGA
	Rev	AGGCTTCATAGGCACCACTC
miR-23b, miR-27b, miR-24-1	Fwd	CACCGCAATTGGAGAACAGGGTGT
	Rev	CAGGCATTCTCACTGCTCAA
miR-22	Fwd	CACCTCTGTAAAGGGGCACAAAGC
	Rev	ATTTCTTCCCACTGCCACAC
miR-125a	Fwd	GAGCTGGGGTGTCTTCTCTG
	Rev	CCCTGAAGATCAGCAGGAAC
miR-290, miR-291a, miR-292, miR-291b	Fwd	CACCGTGAAGTTTGGGGACCAGAA
	Rev	TAGACTCACCACCCCTGGAC
miR-207	Fwd	CACCGGCTCGGCTACAAAAGAGGT
	Rev	CGCTGCCTTTGTACATCTGA
miR-154	Fwd	CACCCTCCATCACCAGACCCTTGT
	Rev	TATGTCCCTCCCTGAGTCCA
miR-188	Fwd	CACCCAAGTGACTTTCCTGCCTGT
	Rev	TCCTTAGCTATGCAGGGAGTTC
miR-293, miR-294, miR-295	Fwd	CACCCTCATGAGGGCTGGGATTAC
	Rev	TTCATGTTTGGAGGCTGAGG
miR-103-1	Fwd	CACCCAGGGCTATACAGCCTTTGC
	Rev	ACTGAGAGCAAACCCCAGAA
miR-32	Fwd	CACCTTGCATGGCATACAGAAGTG
	Rev	TGGGTGAAGAAGTAGAACATGG
miR-30b	Fwd	CACCGAGGGAAAGGGTGGAGAAAG
	Rev	CAATGCAATTCAGCTGACAAA
miR-31	Fwd	CACCCCTGTGCATAACTGCCTTCA
	Rev	GCTGGGCACATGTAAGGTTT
miR-30a	Fwd	CACCTTTCTTATGGCCAACAGTAATGG
	Rev	TTTTGGTGTGTGTGAATTGACC
miR-30c-1	Fwd	CACCGAGCATTGAGCAGGTGAGAA
	Rev	GTCTACCCCAGCAGAGCATC
miR-29c	Fwd	CACCAGCAAGGAAGGGGTAAGAGC
	Rev	CATTGCCATAGAATTATGAATGAAA
cluster-18b-106a	Fwd	CACCAACACAACCCCATTTGCACT
	Rev	CTTGCGCTGTAGTTGCTTCA
miR-27b	Fwd	CACCGCAGCACGTAAGTCTTGGTTC
	Rev	TTGGTGTGTGTGAATTGACCTAA
let-7c-1	Fwd	CACCTCGCTGCTAATGGAAGTGTG
	Rev	AACAGCCCGTGAGAAATAGC
miR-186	Fwd	CACCGAGGGGTAATGGAGCACTGA
	Rev	GACATGGCCCAGAAAAAGAA
miR-195, miR-492	Fwd	CACCATCCCCAGAGCTGAATTCCT
	Rev	CTGAGCCTTCCACCTCTGAC
miR-19b-2, miR-20b, miR-92a-2, miR-363	Fwd	CACCTCCCCTGGTTTCTGCATAGT
	Rev	GAGGCAGGCAGATTTCTGAG
miR-378	Fwd	CACCAGCAGAAGCAGTGCAGTGAA
	Rev	AGCTCACATGCAAACACAGG

miR-139	Fwd	CACCGCTTCTCCTCCTTCCCACTC
	Rev	AAAGATCCCCAAGGAGAGGA
miR-99b, let-7e, miR-125a	Fwd	CACCCTCAGGCCTGGGATATGAAA
	Rev	CCCTTCAAGCTCATTTCTGC
miR-339	Fwd	CACCGCCCAGTCTCCTGTTTCTCA
	Rev	AGAGGGAGAGACCTGGAAGG
miR-193	Fwd	CACCGCGTCGTGTAAACCTTCGAG
	Rev	CAGGCCGGTACAGAAGAAAA
miR-297a-1	Fwd	CACCGATGAACCCCAAACTCCTGA
	Rev	TAGCTGGGCACTGGAGAGAT
miR-144, miR-451	Fwd	CACCCCTGCCTCACAACTTCGTTT
	Rev	CCAGCCTCGGATGCTAATAA
miR-9	Fwd	CACCTCTTTTCCTCGCCTTTCTGA
	Rev	TCTTGCTTTCTTCCCAGGTG
miR-148a	Fwd	CACCTGTGACATTGCCACCAGAGT
	Rev	TGTGGTCCTTCTCTCCTTGC
miR-10a	Fwd	CACCGAGTCCCTTTGCACAACAGC
	Rev	GAGTGAACAAGGACCCAAGC
let-7i	Fwd	CACCGCCAGCCTCTTTAAGGGATCT
	Rev	CGAAACCCAACAACAGACAA
miR-196a-1	Fwd	CACCTGCTGAGAGGCCAAGTAGGT
	Rev	CCTACAACCCAAAGGCTTGA
miR-322, miR-502	Fwd	CACCAGACTTTGGAGCTGGCAAGA
	Rev	CAAGTGAGGCGCTAACAACA
miR-130a	Fwd	CACCGAAAGGTTGCAGGCAGAGAG
	Rev	TGGGCTCAGGATAGAGCAGT
miR-299	Fwd	CACCCTCTGCCTTGGGAACTCTTG
	Rev	AGCATCCCTTGATGATGAGC
miR-215, miR-194-1	Fwd	CACCAGACTCTCGTCCAGGAAGCA
	Rev	GCAGTCGGTGAGTGAGATGA
miR-181c, miR-181d	Fwd	CACCTTGTGAATGCATCCCTTGAA
	Rev	AGCCCCCTTATCTGACACCT
miR-181a, miR-181b	Fwd	CACCAGCACAAAGTGGAGGTTTGC
	Rev	CCCAGGGCTACACAGTTGAT
miR-669a-2	Fwd	CACCACACCAATGCCACTCATCAA
	Rev	TGCATGTACACTTAAGGCATCAC
miR-376a	Fwd	CACCTGTTTCAGATGAGCCAAGCA
	Rev	GAAGCCGACTCCAGAAAACA
miR-296	Fwd	CACCCATCAAGGTAGCTGGT
	Rev	TTTCCCAGCCAAGGATACAG
miR-680-1	Fwd	CACCGGGGACCACAAACCTGTAAG
	Rev	GAGTCGATCACCACCCCTAA
miR-703	Fwd	CACCGAGGATCCATTGATGCGTCT
	Rev	AATGGAATGAGCCCAATCAG
miR-320	Fwd	CACCTAGCTTTGGACTCCGTCACC
	Rev	AGCTATTGGCTGCTCTGTCC
miR-431	Fwd	CACCATGAAGTTGTGGCCCTTCAC
	Rev	ACCCTCATTGCGAGTAGTGG
miR-669c, miR-297c	Fwd	CACCCGCACACACACAGAGGAATG
	Rev	TGGGCGATATCACATGAATAC
miR-100	Fwd	CACCTGAAGCTCACTTCATCAAGCA
	Rev	ACCAGTCAGAGGCAATACGG
miR-340	Fwd	CACCGTGAGATCTATTCACTCTTGTCCTCC
	Rev	CATCTGCCTCTGGTGAGTGAG
miR-491	Fwd	CACCAAGCAGTGGTGGTTTCTATCTGC
	Rev	CCTTGCTACTTCATTCCATAGCCAG
miR-10b	Fwd	CACCCGATGAGGGAACTCATTGCT
	Rev	AAACCTGGCTCTCTGGCTTT
miR-34a	Fwd	CACCCTCCAGCTGAATCCCGACTA

	Rev	GTACCCCGACATGCAAACTT
miR-99a	Fwd	CACCAATCCTCATGCTTGTAACCCTA
	Rev	GGCTCTGCTACAAATCCTTCC
miR-132, miR-212	Fwd	CACCCCTGCATGCTCCACACAC
	Rev	GCTCTGTATCTGCCCAAACC
miR-151	Fwd	CACCGCCAGACTCTACGCACATGA
	Rev	TGTGACATGTTGCTCATGGA
miR-152	Fwd	CACCCTGAAGTTCTGGGGAACTCG
	Rev	AAAGTGGACACCGAGTCAGG
miR-197	Fwd	CACCTGGGGACTTTTGAAGTTGGA
	Rev	TGCTTCTGGATTCTGACATGA
miR-210	Fwd	CACCGGGTCGCAGGTGAAATAGAA
	Rev	CAGGTGCAGGACAGAGAAGC
miR-324-3p	Fwd	CACCTGCTGATCTACTCCTCCAACC
	Rev	ACCACAGTGGGGTAACATGC
miR-326	Fwd	CACCCCGCTGGAAGGATCTTCTCT
	Rev	CTAGCCCAGGGCCATATACA
miR-328	Fwd	CACCAGAGAACCTGCGTTCAGGAC
	Rev	CTGCCCTCTCGTCTGTAGAA
miR-346	Fwd	CACCCCCAGGCTTCTGTGAAGGAT
	Rev	GCCCATCTTCAGTCATGTCC
miR-365-1	Fwd	CACCCTTCCGCCTAGGGACACAT
	Rev	TTACCTCTGGAGGGCAAAAA
miR-370	Fwd	CACCGGTGGAGCTGTGTGGGTATG
	Rev	GCCTCTCTGTGCTCTGTTCC
miR-423	Fwd	CACCAAGAAGCCAGGGAGACTGAG
	Rev	CGCCCAAGAATAAATTTCCA
miR-505	Fwd	CACCACTTTTCCGAGGTCAATAAGC
	Rev	AGTGTGCTTGCTATGTGGAAT
miR-325	Fwd	CACCTTGGGTTCTGCTGGGTTTTA
	Rev	GCTCACTGCCTTAACCCTTG
miR-184	Fwd	CACCGGAGAGAATGGGAGTGGTCA
	Rev	AGGATGCAACTTTCGGCTTA
miR-744	Fwd	CACCGTGGGCAGATAATGGCAGTT
	Rev	TACAGAGGCTCGCTGGGTAT
miR-182	Fwd	CACCGGGAATGTCAGGAAGGGATT
	Rev	CTGTCTCTCCCTCACCAAGC
miR-484	Fwd	CACCCCCAGGCCCTTTATTTTGAT
	Rev	TATCACGTGACGAGGCAGAG
miR-183, miR-96	Fwd	CACCTGGGAGTAGGTGAGGTCCAG
	Rev	AGGATGCAGGAAACCAACAC
miR-130b, miR-301	Fwd	CACCTCAGCTCCCTCCTTGGAGTA
	Rev	TGCAGCAGACTCCCCTATCT
miR-185	Fwd	CACCATGGCTAGGGTTTGCTCTCA
	Rev	TATCAGCTGCTGGTGTCAGG
miR-720	Fwd	CACCCTAGCGACCCCTCTTCAGTG
	Rev	TCAGAAATCCACCTGCCTCT
miR-98	Fwd	CACCTTCTTCTGCCCTTGAATTGG
	Rev	AGGCTGTCCTCGAATGTTTG
miR-141, 200	Fwd	CACCCGCAGTAAATGGGTGTGTTG
	Rev	GTTCCCAGGGTGAAAAGACA
miR-500	Fwd	CACCGGAGTGGCATCTCCATGTTT
	Rev	TCTGGCCCTTATTGTCCATC
miR-700	Fwd	CACCCTCACTGAGCCATCGCATTA
	Rev	CTGGCCGAATCTGCTACTTC
miR-674	Fwd	CACCGAGGCATCATTGGGTTCACT
	Rev	GCAGGAGCTGGAGAACAAAC
miR-361	Fwd	CACCGGGCAAGAATGAGGCTAACA
	Rev	GGAGTGGGGATCTGTGAAGA

miR-33	Fwd	CACCCACCTGTGGAGCAGTCTCAA
	Rev	CAAGGGAGACCAACAGGAAG
miR-99b, let-7e, miR-125a	Fwd	CACCCTCAGGCCTGGGATATGAAA
	Rev	CCCTTCAAGCTCATTTCTGC
miR-339	Fwd	CACCGCCCAGTCTCCTGTTTCTCA
	Rev	AGAGGGAGAGACCTGGAAGG
miR-193	Fwd	CACCGCGTCGTGTAAACCTTCGAG
	Rev	CAGGCCGGTACAGAAGAAAA
miR-297a-1	Fwd	CACCGATGAACCCCAAACTCCTGA
	Rev	TAGCTGGGCACTGGAGAGAT
miR-144, miR-451	Fwd	CACCCCTGCCTCACAACTTCGTTT
	Rev	CCAGCCTCGGATGCTAATAA
miR-9	Fwd	CACCTCTTTTCCTCGCCTTTCTGA
	Rev	TCTTGCTTTCTTCCCAGGTG
miR-148a	Fwd	CACCTGTGACATTGCCACCAGAGT
	Rev	TGTGGTCCTTCTCTCCTTGC
miR-10a	Fwd	CACCGAGTCCCTTTGCACAACAGC
	Rev	GAGTGAACAAGGACCCAAGC
let-7i	Fwd	CACCGCCAGCCTCTTTAAGGGATCT
	Rev	CGAAACCCAACAACAGACAA
miR-196a-1	Fwd	CACCTGCTGAGAGGCCAAGTAGGT
	Rev	CCTACAACCCAAAGGCTTGA
miR-322, miR-502	Fwd	CACCAGACTTTGGAGCTGGCAAGA
	Rev	CAAGTGAGGCGCTAACAACA
miR-130a	Fwd	CACCGAAAGGTTGCAGGCAGAGAG
	Rev	TGGGCTCAGGATAGAGCAGT
miR-299	Fwd	CACCCTCTGCCTTGGGAACTCTTG
	Rev	AGCATCCCTTGATGATGAGC
miR-215, miR-194-1	Fwd	CACCAGACTCTCGTCCAGGAAGCA
	Rev	GCAGTCGGTGAGTGAGATGA
miR-181c, miR-181d	Fwd	CACCTTGTGAATGCATCCCTTGAA
	Rev	AGCCCCCTTATCTGACACCT
miR-181a, miR-181b	Fwd	CACCAGCACAAAGTGGAGGTTTGC
	Rev	CCCAGGGCTACACAGTTGAT
miR-669a-2	Fwd	CACCACACCAATGCCACTCATCAA
	Rev	TGCATGTACACTTAAGGCATCAC
miR-376a	Fwd	CACCTGTTTCAGATGAGCCAAGCA
	Rev	GAAGCCGACTCCAGAAAACA
miR-296	Fwd	CACCCATCAAGGTAGCTGGT
	Rev	TTTCCCAGCCAAGGATACAG
miR-680-1	Fwd	CACCGGGGACCACAAACCTGTAAG
	Rev	GAGTCGATCACCACCCCTAA
miR-703	Fwd	CACCGAGGATCCATTGATGCGTCT
	Rev	AATGGAATGAGCCCAATCAG
miR-320	Fwd	CACCTAGCTTTGGACTCCGTCACC
	Rev	AGCTATTGGCTGCTCTGTCC
miR-431	Fwd	CACCATGAAGTTGTGGCCCTTCAC
	Rev	ACCCTCATTGCGAGTAGTGG
miR-669c, miR-297c	Fwd	CACCCGCACACACACAGAGGAATG
	Rev	TGGGCGATATCACATGAATAC
miR-100	Fwd	CACCTGAAGCTCACTTCATCAAGCA
	Rev	ACCAGTCAGAGGCAATACGG
miR-340	Fwd	CACCGTGAGATCTATTCACTCTTGTCCTCC
	Rev	CATCTGCCTCTGGTGAGTGAG
miR-491	Fwd	CACCAAGCAGTGGTGGTTTCTATCTGC
	Rev	CCTTGCTACTTCATTCCATAGCCAG
miR-10b	Fwd	CACCCGATGAGGGAACTCATTGCT
	Rev	AAACCTGGCTCTCTGGCTTT
miR-34a	Fwd	CACCCTCCAGCTGAATCCCGACTA

	Rev	GTACCCCGACATGCAAACTT
miR-99a	Fwd	CACCAATCCTCATGCTTGTAACCCTA
	Rev	GGCTCTGCTACAAATCCTTCC
miR-132, miR-212	Fwd	CACCCCTGCATGCTCCACACAC
	Rev	GCTCTGTATCTGCCCAAACC
miR-151	Fwd	CACCGCCAGACTCTACGCACATGA
	Rev	TGTGACATGTTGCTCATGGA
miR-152	Fwd	CACCCTGAAGTTCTGGGGAACTCG
	Rev	AAAGTGGACACCGAGTCAGG
miR-197	Fwd	CACCTGGGGACTTTTGAAGTTGGA
	Rev	TGCTTCTGGATTCTGACATGA
miR-210	Fwd	CACCGGGTCGCAGGTGAAATAGAA
	Rev	CAGGTGCAGGACAGAGAAGC
miR-324-3p	Fwd	CACCTGCTGATCTACTCCTCCAACC
	Rev	ACCACAGTGGGGTAACATGC
miR-326	Fwd	CACCCCGCTGGAAGGATCTTCTCT
	Rev	CTAGCCCAGGGCCATATACA
miR-328	Fwd	CACCAGAGAACCTGCGTTCAGGAC
	Rev	CTGCCCTCTCGTCTGTAGAA
miR-346	Fwd	CACCCCCAGGCTTCTGTGAAGGAT
	Rev	GCCCATCTTCAGTCATGTCC
miR-365-1	Fwd	CACCCTTCCGCCTAGGGACACAT
	Rev	TTACCTCTGGAGGGCAAAAA
miR-370	Fwd	CACCGGTGGAGCTGTGTGGGTATG
	Rev	GCCTCTCTGTGCTCTGTTCC
miR-423	Fwd	CACCAAGAAGCCAGGGAGACTGAG
	Rev	CGCCCAAGAATAAATTTCCA
miR-505	Fwd	CACCACTTTTCCGAGGTCAATAAGC
	Rev	AGTGTGCTTGCTATGTGGAAT
miR-325	Fwd	CACCTTGGGTTCTGCTGGGTTTTA
	Rev	GCTCACTGCCTTAACCCTTG
miR-184	Fwd	CACCGGAGAGAATGGGAGTGGTCA
	Rev	AGGATGCAACTTTCGGCTTA
miR-744	Fwd	CACCGTGGGCAGATAATGGCAGTT
	Rev	TACAGAGGCTCGCTGGGTAT
miR-182	Fwd	CACCGGGAATGTCAGGAAGGGATT
	Rev	CTGTCTCTCCCTCACCAAGC
miR-484	Fwd	CACCCCCAGGCCCTTTATTTTGAT
	Rev	TATCACGTGACGAGGCAGAG
miR-183, miR-96	Fwd	CACCTGGGAGTAGGTGAGGTCCAG
	Rev	AGGATGCAGGAAACCAACAC
miR-130b, miR-301	Fwd	CACCTCAGCTCCCTCCTTGGAGTA
	Rev	TGCAGCAGACTCCCCTATCT
miR-185	Fwd	CACCATGGCTAGGGTTTGCTCTCA
	Rev	TATCAGCTGCTGGTGTCAGG
miR-720	Fwd	CACCCTAGCGACCCCTCTTCAGTG
	Rev	TCAGAAATCCACCTGCCTCT
miR-98	Fwd	CACCTTCTTCTGCCCTTGAATTGG
	Rev	AGGCTGTCCTCGAATGTTTG
miR-141, 200	Fwd	CACCCGCAGTAAATGGGTGTGTTG
	Rev	GTTCCCAGGGTGAAAAGACA
miR-500	Fwd	CACCGGAGTGGCATCTCCATGTTT
	Rev	TCTGGCCCTTATTGTCCATC
miR-700	Fwd	CACCCTCACTGAGCCATCGCATTA
	Rev	CTGGCCGAATCTGCTACTTC
miR-674	Fwd	CACCGAGGCATCATTGGGTTCACT
	Rev	GCAGGAGCTGGAGAACAAAC
miR-361	Fwd	CACCGGGCAAGAATGAGGCTAACA
	Rev	GGAGTGGGGATCTGTGAAGA

miR-33	Fwd	CACCCACCTGTGGAGCAGTCTCAA
	Rev	CAAGGGAGACCAACAGGAAG

Table 6: Cloning primers for other adenoviral constructs

Cloning primers were purchased from Metabion.

Mtor-3'UTR_fwd	CACCTGAGGCCTGGAAAACCACGTC
Mtor-3'UTR_rev	TGATGAACAGAAAGCCAGTCATACACAT
Rora 3'UTR-F	CACCTAAATGTCGCGCCCGAGCAC
Rora 3'UTR-R	ACAGCAGCATAAATACCTCCCAACG
hFoxp3 F	CACCCCTATGCCCAACCCTAGGCC
hFoxp3 R	TCAGGGGCCAGGTGTAGGG

Table 7: Genotyping primer

Genotyping primers were purchased from Metabion.

Car for	CAGGAGCGAGAGCCGCCTAC
Car rev	CAGCCACTCGATGTCCAGCGGTC
DO11.10 F	GAGCAGCTTCCTTCCATCCTGAGAG
DO11.10 R	TGGCTCTACAGTGAGTTTGGTGCCA
CD4Cre for	ACGACCAAGTGACAGCAATG
CD4Cre rev	CTCGACCAGTTTAGTTACCC
Mexo A (Eri1 F)	GGGTGGTATATCCTCAGTTACTTTTG
Mexo B (Eri1 R floxed)	GCCATAACCTTGAACCTGCA
Mexo C (Eri1 R wt)	GCAACCCGAGGTAAAAGGAG

Table 8: Quick change primer

Quick change primers were purchased from Metabion and were HPLC purified

QC MTOR 99A MUT F	ACAGAAGATGGGTAACTGAGAAATACGACCTTTGACTTAACTTACAAGAAAACTCAT
QC MTOR 99A MUT R	ATGAGTTTTCTTGTAAGTTAAGTCAAAGGTCGTATTTCTCAGTTACCCATCTTCTGT
QC MTOR 150 MUT F	TAATCCTTCAGAAGCCAAGCCTTGGATTTCTCTGGAACAGAAGATGGGTAACTGAGAAATAC
QC MTOR 150 MUT R	GTATTTCTCAGTTACCCATCTTCTGTTCCAGAGAAATCCAAGGCTTGGCTTCTGAAGGATTA

Table 9: qPCR primer for detection of mature microRNA

For quantitative PCR of mature microRNAs the following kits (based on hairpin primers) from Applied biosystems were used.

TaqMan MicroRNA Assay hsa-miR-99a	Applied Biosystems
TaqMan MicroRNA Assay hsa-miR-10b	Applied Biosystems
TaqMan MicroRNA Assay hsa-miR-150	Applied Biosystems
TaqMan MicroRNA Assay hsa-miR-155	Applied Biosystems
TaqMan MicroRNA Assay snoRNA202	Applied Biosystems

Table 10: qPCR primer for detection of mRNAs

All qPCR analyses for mRNAs were performed using the following primers (HPLC-purified)

Name	Sequence	Probe
Eri1_CDS F92	TGAAGTCAAAGGAGTTGGGAAC	#92
Eri1_CDS R92	GCACTGGATGCTCAGGAAC	#92
Eri1UTR_short F64	TGGTGAGACTAATGGAAAGTTAGAAAA	#64
Eri1UTR_short R64	TGGCTTCAGATTCACCTATGC	#64
Eri1UTR_int F3	GCCTACGTTGTGTGTAAAGATACTG	#3
Eri1UTR_int R3	CAAACACTAGCACCAGTGTAAGC	#3
Eri1UTR_long F4	CGTCCTTAGTGTTGACCCTCAT	#4
Eri1UTR_long R4	TGTGTGCATTGATGCTGTGT	#4
mTOR F70	AGAAGACAGCGGGGAAGG	#70
qmTOR R70	GCATCTTGCCCTGAGGTTC	#70
qRora CDS F63	CAGATAACGTGGCAGACCTTC	#63
qRora CDS R63	AGCTGCCACATCACCTCTCT	#63

5.5 Instruments

Table 11: Instruments

BD FACS Calibur Flow Cytometer	BD Biosciences
BD LSRFortessa Cell Analyzer	BD Biosciences
BD LSRII Flow Cytometer	BD Biosciences
Blotting chamber	BioRad
Fluorescence microscope	Axiovert 200M (Zeiss) Olympus DP72
Light cycler LC480	Roche
Orion Microplate luminometer	Berthold

5.6 Chemicals, enzymes and kits

Table 12: Chemicals

Ampicillin	Roche
Biozym DNA Agarose	Biozym Scientific GmbH
Brefeldin A	Sigma-Aldrich
BSA (Albumin Fraktion V)	Roth
Deoxynucleotide (dNTP) set	Fermentas
DEPC	Roth
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich
DNA Ladder 2-Log (0.1–10.0 kb)	New England BioLabs
Dynabeads M450 Tosylactivated	Invitrogen
ECL plus western blotting reagent	GE healthcare
Ethidium bromide 1% (w/v)	Serva
Ionomycin, Free Acid, Streptomyces conglobactus	Calbiochem
JetPEI	Polyplus transfection
Kanamycin sulfate	Roth
Milk powder	Roth
Paraformaldehyde	Sigma-Aldrich
Phorbol-12-myristate-13-acetate (PMA)	Calbiochem
All-trans retinoic acid	Sigma
Saponin	VWR International GmbH
Tri®Reagent (Trizol)	Invitrogen
Trypsin 0.05% / EDTA 0.02% in PBS	Pan biotech GmbH
Tween 20	Applichem
β-Mercaptoethanol 99%	Sigma-Aldrich

Table 13: Enzymes

Gateway LR Clonase II Enzyme Mix	Invitrogen
proteinase K	Invitrogen
Restriction enzymes	New England BioLabs
T4 DNA Ligase (10,000 U/ml)	New England BioLabs
Taq Polymerase (5,000 U/ml)	New England BioLabs

Table 14: Kits

DETACHaBEAD® Mouse CD4	Invitrogen
Dynabeads® M-450 Tosylactivated	Invitrogen
Dynabeads®Mouse CD4 (L3T34) Kit	Invitrogen
iProof™ High-Fidelity PCR Kit	Bio-rad
Light cycler480 Probes master	Roche
miRNeasy Mini Kit	Qiagen
Naive CD4+ CD62L+ T cell Isolation Kit II, mouse	Miltenyi Biotec
Nucleobond®Xtra Maxi Kit	Macherey-Nagel GmbH & Co. KG
pENTR™ 11 Dual Selection	Invitrogen
pENTR™/D-TOPO Cloning Kit	Invitrogen
PureYield™ Plasmid Miniprep System	Promega
QIA®quick gel extraction Kit	Qiagen
Quantitect reverse transcription kit II	Qiagen
TaqMan microRNA reverse transcription kit	Applied Biosystems
TaqManMicroRNA Assay	Applied Biosystems
Universal Probe Library	Roche
XL QuickChange	Stratagene

Table 15: Solution and Buffers

APS	10% APS w/v in H2O
Lysis buffer	20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.25% (v/v) Nonidet-P40, 1.5 mM MgCl2, 1 mM DTT, protease inhibitor mix without EDTA (Roche) in H2O
PBS	137 mM NaCl, 10 mM phosphate, 2.7 mM KCl
SDS (10%)	10% SDS (w/v) dissolved in H2O
SDS sample buffer (4x)	200 mM Tris/HCl (pH 6.8), 8% w/v SDS, 4% glycerol, 0.1% w/v bromophenol blue, 10% v/v β-mercaptoethanol in ddH2O
SDS-PAGE running buffer (5x)	25 mM Tris-Base, 200 mM glycine, 10% (w/v) SDS in H2O
TAC lysis buffer	13 mM Tris, 140 mM NH4Cl (pH 7.2) in H2O
TBE (5x)	45 mM Tris-HCl, 1 mM EDTA (pH 8.0) in H2O
TBS (1x)	20 mM Tris-Base, 137 mM NaCl, 3.8 ml 1M HCl, dissolved in ddH2O
TBS-T (1x)	20 mM Tris-Base, 137 mM NaCl, 3.8 ml 1M HCl, 0.1% (v/v) Tween 20, dissolved in H2O
TE (10x)	100 mM Tris/HCl (pH 8.0), 10 mM EDTA (pH 8.0) in H2O
Western blot buffer	25 mM Tris-Base, 192 mM glycin, 20% v/v methanol (pH 8.4) in H2O

5.7 Vectors

5.7.1 Entry vectors

pENTR/D-TOPO

This vector was purchased from Invitrogen and was provided in a linearized form with topoisomerase coupled to the strand ends (Figure 4). Thus it was suitable for blunt end directional cloning of PCR products generated with a forward primer that begins with a CACC motif. The cloning cassette was flanked by AttL recombination sites that allow λ-recombination into destination vectors.

pENTR11-AscI

pENTR11 was purchased from Invitrogen and the multiple cloning site was modified to contain restriction sites for the *Asc1, Not1* and *EcoRV* restriction endonucleases, which allows reversion of the pENTR/D-TOPO cloning cassette (Figure 4). The restriction sites for *Asc1* and *Not1* restriction endonucleases were flanked by AttL recombination sites that allow λ-recombination into destination vectors.

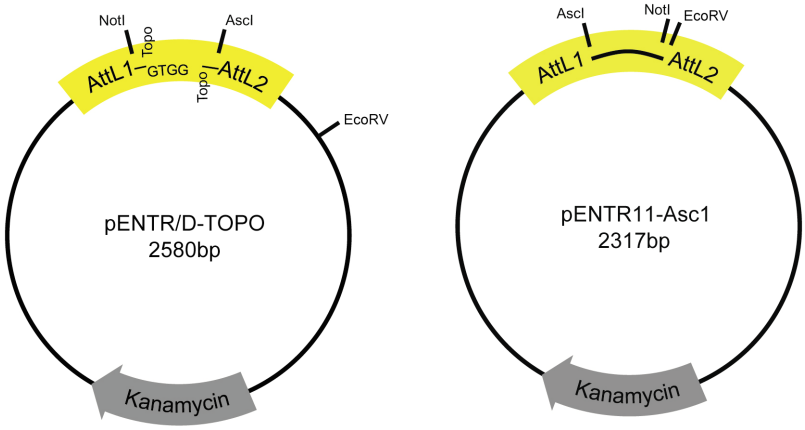

Figure 4: Vector maps of pENTR/D-TOPO and pENTR11-Asc1.
pENTR/D-TOPO was supplied as a linearized vector with Topoisomerase II (TOPO) coupled to both ends to facilitate blunt end cloning of PCR products. The GTGG motif should provide directionality when combined with a CACC motif in the forward primer. pENTR11-Asc1 was equipped with *Asc1* and *Not1* restriction sites and used for reversion of *Not1/Asc1* restriction fragments from pENTR/D-TOPO. The AttL recombination sites flanking the respective inserts in entry vectors can be used for λ-recombination into destination vectors. (Modified from Invitrogen vector maps).

5.7.2 Adenoviral vectors

pCAGAdDu

The pCAGAdDu plasmid contains the human type 5 adenovirus genome with E1 and E3 genes deleted to render recombinant adenoviruses replication-incompetent (Figure 5, Russell, 2000). The adenoviral producer cell line HEK293A complements replication deficiency as it has been immortalized through stable integration of sheared adenovirus genome (Graham et al., 1977). Since adenoviral vectors are large (~40 kb) and consequently not well suited for traditional restriction enzyme-mediated cloning, we employed the Gateway® system. The gene of interest is initially cloned into a smaller entry vector, from which it can be easily transferred into the adenoviral destination vector via lambda recombination reaction (LR) (Landy, 1989). The pCAGAdDu vector combines the CAG promoter (chicken actin promoter and CMV enhancer) with an expression cassette containing LR sites (AttL1, AttL2) flanking the procaryotic ccdB selection marker (Figure 5, Bernard and Couturier, 1992). This expression cassette is fused to an internal ribosome entry site (IRES) element that allows co-expression of the eukaryotic infection marker enhanced green fluorescent protein (eGFP), which is fused to a sequence containing the bovine growth hormone poly(A)-signal. The pCAGAdDu-Thy1.1 variant encodes the surface marker molecule Thy1.1 instead of eGFP. We chose the CAG cis-regulatory sequences, since the prototypic CMV promoter was found to be highly activation-dependent and therefore unfavorable for gene expression in naive T cells. pCAGAdDu lacking the recombination cassette was used as a control virus.

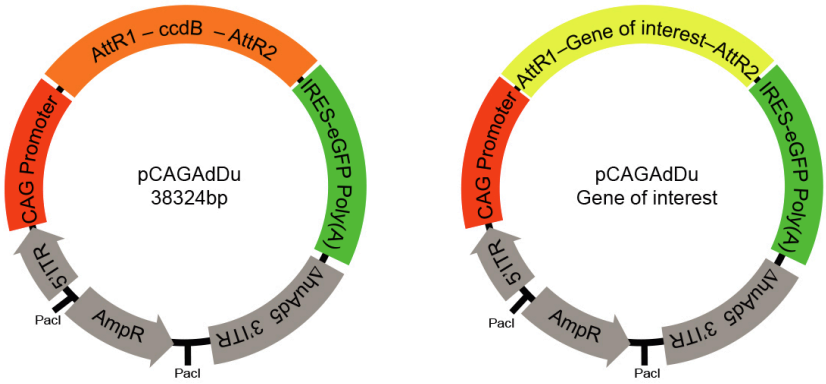

Figure 5: pCAGAdDu+ccdB (left side) and pCAGAdDu containing a gene of interest after λ-recombination (right side). The AttR sites in destination vectors are the target sites for the AttL flanked inserts from entry vectors. The bacterial toxin ccdB gene (ccdB) is removed by λ-recombination and selects against non-recombined clones. IRES: internal ribosomal entry site; eGFP: enhanced green fluorescent protein; Poly(A): bovine growth hormone poly(A)-signal; ΔhuAd5: human adenovirus type5 genes with deleted E1 replication genes; ITR: inverted repeats.

5.8 pAdsiCheck

This vector was based on pCAGAdDu. The CAG-expression cassette was replaced by the luciferase reporter cassette of the psiCHEK2 (Promega). Briefly, this is composed of a firefly luciferase gene under the HSV-TK promotor and a renilla luciferase gene under control of the SV40 promotor, which is followed by LR sites (AttL1, AttL2) flanking the procaryotic ccdB selection marker. This allowed insertion of 3'UTRs by Gateway recombination to assess their effect on renilla luciferase activity relative to firefly luciferase activity.

6 Methods

6.1 Modulation of Treg cell differentiation using adenoviral transduction

We published parts of the following protocol under the title "Adenoviral Transduction of Naive $CD4^+$ T Cells to Study Treg Differentiation" in the Journal of Visualized Experiments (JoVE), Warth and Heissmeyer 2013 (http://www.jove.com/video/50455). It provides a workflow for the generation of infectious adenoviral particles to transduce a gene or microRNA of interest into naive T cells to study Treg cell differentiation.

6.1.1 Cloning of a gene or microRNA of interest into an entry vector

1.1.) Clone your gene or microRNA of interest into an entry vector. You may use PCR amplification followed by blunt-end ligation into a topoisomerase-coupled vector (e.g. with the pENTR™/D-TOPO® Cloning Kit) or restriction enzyme-mediated cloning. In this work, the iProof™ High-Fidelity PCR Kit™ was used according to the manufacturers instructions. The primers used in this study are listed in the Material section. Generally, for cloning of microRNAs, primers should be designed to generate constructs of at least 270nt that contain the mature microRNA and 125nt of genomic sequence on either side of the microRNA (Chen et al., 2004). C57/BL6J genomic DNA was used as a template. Plasmids with inverted inserts were reverted by restriction digest using *Not1/Asc1* and ligation of the insert into pENTR11 equipped with *Not1/Asc1* restriction sites in an inverted order.

6.1.2 Transfer of the gene or microRNA of interest into the destination vector

2.1) Transfer your gene of interest from the entry vector into the destination vector pCAGAdDU by LR recombination (e.g. Gateway® LR Clonase™ II Enzyme Mix). This will create the adenoviral expression vector.

2.2) Linearize 10 µg of the adenoviral expression vector in a *PacI* restriction digest, precipitate the DNA and resuspend it in water at a concentration of 3 µg per 100 µl. Linearization liberates the viral inverted repeats (ITR), which are required for replication and encapsidation of the viral DNA into virus particles.

6.1.3 Transfection of HEK293A cells to generate the primary virus lysate

3.1) Seed 1x10^5 HEK293A cells in 2 ml DMEM media (supplemented with 10% FBS, 5% PenStrep, 10 mM HEPES) in one well of a 6-well plate suitable for adherent cells and incubate the cells for 6-14 h at 37 °C in a 10% CO_2 incubator to allow them to adhere.

3.2) Lipofection: Transfer 6 µl of jetPEI reagent into 94 µl of 50 µM NaCl, vortex briefly. Add the mixed solution to 100 µl linearized adenovirus vector while vortexing and incubate this transfection mix for 15-30 min at room temperature (RT).

Perform all following steps under the appropriate biosafety conditions for adenovirus infection (which was S2 in this work)!

3.3) Dispense the solution dropwise on the HEK293A cell-containing well and incubate the cells at 37 °C and 10% CO_2. With GFP as a fluorescence marker, you can evaluate the transfection efficiency visually after 12-36 h using an inverted fluorescence microscope. Add 0.5ml of fresh medium every 3 days.

3.4) Check every 2-3 days with a light or fluorescence microscope for cytopathic effects (CPE), which are areas with enlarged and rounded cells that start to detach. This is indicative of efficient virus generation. Upon occurrence of broader zones of CPE (Figure 6), it will take 24-72 h before all cells are infected.

3.5) When all cells show signs of CPE, but before an overall detachment of cells occurrs, detach the cells by gentle pipetting and transfer cells with supernatant (SN, 3-5 ml) to a 15 ml polystyrene tube.

3.6) Freeze the cells in the SN on dry ice for 15-20min and thaw them quickly at 37 °C afterwards to rupture the cells. Repeat this freeze-and-thaw-cycle (F/TC) two more times. Keep the primary virus lysate on ice for usage within a day or freeze it at -80 °C for long-

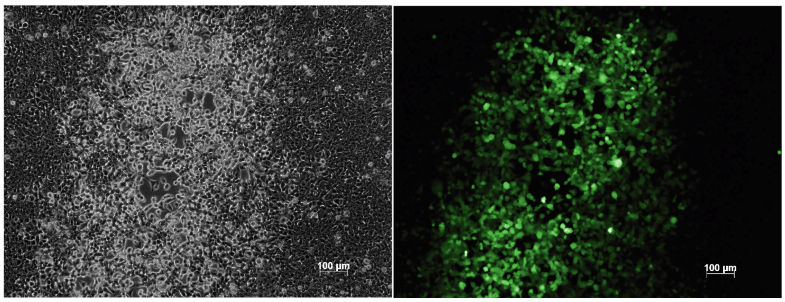

Figure 6: Cytopathic effects (CPE) in adenovirus-producing HEK293A cells.

Occurrence of CPE at day ten after transfection of 10^5 HEK293A cells with linearized pCAGAdDu vector. The left panel shows the phase contrast image, the right panel shows the green fluorescence indicative of infection.

term storage. Any additional F/TC will reduce the virus titer by 30-50%.

6.1.4 Amplification of the primary virus lysate

4.1) Seed and grow HEK293A cells to 90% confluence on a 14 cm tissue culture dish.

4.2) Infect the cells with half of the primary virus lysate (1.5-2.5 ml) and incubate the cells for at least 36 h. Nearly all cells should be infected then, which can be determined by fluorescence microscopy. For the re-amplification of an already amplified (i.e. more concentrated) virus stock, infect HEK293A at a multiplicity of infection (MOI, see 6.3) of 50.

4.3) Cells should be harvested when all of them show CPE but before detachment. With efficient virus production this state is reached within 48 h after infection. (If it takes up to one week, consider another round of amplification by increasing the amount of adenovirus stock used for the infection described in 4.2).

4.4) Detach the cells by gentle pipetting and transfer cells and SN to a 50 ml polystyrene tube. Spin cells down at 300 *g* for 10 min at 4 °C.

4.5) Remove the SN and resuspend the pellet in a suitable volume of medium or SN (ca. 1ml).

4.6) Perform 3 F/TC to disrupt the cells and centrifuge at 800*g* for 15 min at 4 °C. Take off the SN that contains the virus particles (i.e. the concentrated virus lysate), and aliquot the virus lysate to store it at -80 °C.

6.1.5 Determination of the adenovirus titer in concentrated virus lysate

5.1) Seed 10^5 A549 cells per well into 5 wells of a 12-well plate in 1ml medium and let cells adhere for 6 h.

5.2) Use 1 µl of concentrated adenovirus (thawed on ice) to perform a serial dilution in medium (1:5,000/ 1:10,000/ 1:50,000/ 1:100,000) and add 10 µl per well. Leave one well uninfected to adjust the gating in flow cytometry.

5.3) After 36 h, take off the SN, wash with PBS and detach cells (e.g. by trypsinization). For biohazard precautions, it is recommended to fix cells in 100 µl 4% paraformaldehyde in PBS for 10 min at room temperature and wash with PBS one time.

5.4) Perform a FACS analysis of infection marker expression. Plot ‘µl viral lysate applied’ against the absolute number of infected cells (Figure 7). Determine the linear range of infection and calculate the titer per ml of undiluted virus from the standard curve over the linear range using x=1000 µl. In the example, the titer for x=1 µl is 30016535/µl ≈ $3x10^{10}$/ml.

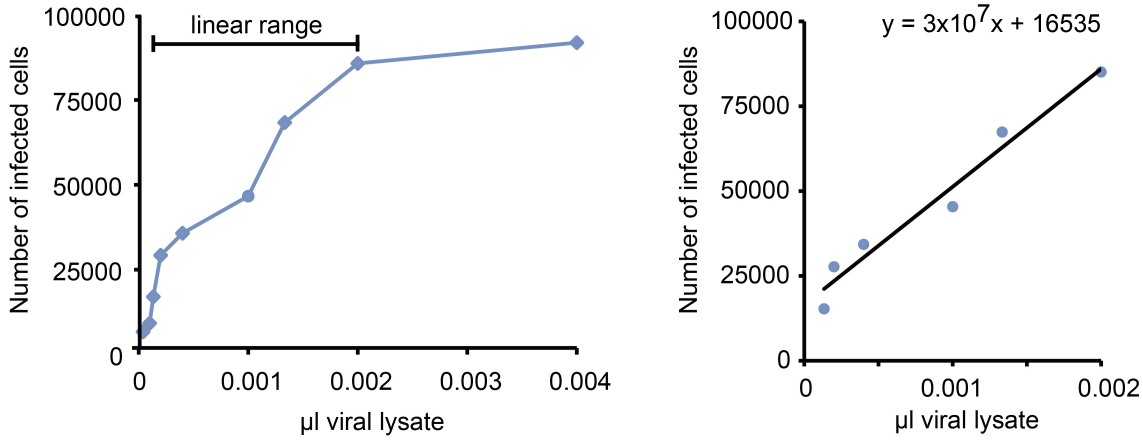

Figure 7: Determining the titer of adenovirus lysates by infection of A549 cells.

10^5 A549 cells were infected with the indicated virus dilution, incubated for 48h and analyzed for expression of the infection marker GFP by flow cytometry. The number of GFP^+ cells is plotted against the amount of virus used. The titer of undiluted virus is calculated from the standard curve over the linear range using x=1000 µl. The graph shows a representative example.

6.1.6 T cell infection

6.1) Isolate naive/resting $CD4^+$ T cells from DO11.10 tg; CARΔ1 tg mice using MACS (Naive $CD4^+$ T Cell Isolation Kit II) or FACS sorting ($CD4^+$ $CD25^-$ $CD62L^+$ $CD44^-$).

6.2) For small-scale experiments, pipet an appropriate volume of viral lysate to achieve an MOI of 50 into one well of a 96-well round bottom plate.

6.3) Add up to $4x10^5$ T cells in a final infection volume of 50 µl in T cell medium (RPMI1640, 10% FBS, 5% PenStrep, 5% Sodiumpyruvate, 1x non-essential amino acids, 100x MEM vitamin solution, 1x L-Glutamine, 1:250000 β–Mercaptoethanol, 10mM HEPES).

Example:

An MOI of 50 shall be used to infect $3x10^5$ T cells; the viral titer is 3×10^9 /ml

Virus volume = MOI x T cell number / viral titer; $50 \times 3x10^5 / 3 \times 10^9$/ml=0.005 ml

Note: for infection of larger cell numbers, scale up using an MOI of 50 in an infection volume of 165 µl per 10^6 naive T cells in a polystyrene tube with loose cap (up tp 3ml per tube).

6.4) Incubate cells for 90min at 37 °C in a 5% CO_2 incubator.

6.5) Spin down cells at 300g for 5 min at room temperature, take off SN, resuspend in 200 µl PBS. Centrifuge again and take off SN.

Optional: you may wash cells again if you want to remove the virus more efficiently.

6.6) Resuspend cells in 200 µl T cell medium without stimulating antibodies and without IL-2 or other cytokines and rest them for 40 h at 37 °C in a 5% CO_2 incubator to allow expression of the gene of interest before activation.

6.1.7 Activation of T cells and Treg- or Th17-polarization

7.1) Pipet a volume of anti-CD3- and anti-CD28-coupled beads that equals your cell number (e.g. $4x10^5$) into a small reagent cup, add the 10-fold volume of PBS and put them on a magnet for 2 min. Take off the supernatant and resuspend the beads in 200 µl polarizing medium.

Treg-polarizing medium: T cell medium +1 ng/ml recombinant human TGFβ, 100 U/ml IL-2 (that is 1000 IE/ml of ProleukinS)

Th17-polarizing medium: 1ng/ml recombinant human TGFβ, 5 ng/ml ecombinant mouse IL-6, 10 µg/ml anti-IL-12 (clone C17.8, E. Kremmer), 5 µg/ml anti-IFNγ (clone XMG1.2,E. Kremmer), 10 µg/ml anti-IL-4 (clone 11B11, E. Kremmer) and 2.5 µg/ml anti-IL-2 (clone JES6-5H4).

7.2) Centrifuge the rested cells as before, take off the SN and resuspend cells in 200 µl polarizing medium containing anti-CD3- and anti-CD28-antibody-coupled beads, incubate for 72 h at 37 °C in a 5% CO_2 incubator without changing medium.

For Th17 cell differentiation:

Prepare restimulation medium (T cell medium containing 20 nM PMA and 1 µM ionomycin)

After 72 h, spin down cells at 300 *g* for 5 min at room temperature, take off SN, and resuspend them in 150 µl restimulation medium and incubate the cells for 2.5 h at 37 °C in a 5% CO_2 incubator. After 2.5 h, add 50 µl restimulation medium supplemented with 40 µg/ml BrefeldinA (final concentration 10 µg/ml) and incubate another 2.5 h at 37 °C in a 5% CO_2 incubator.

6.1.8 Treg cell fixation protocol

8.1) Wash cells: Spin down cells at 300g for 5 min at room temperature, take off SN, resuspend in 200 µl PBS, centrifuge again and take off SN, perform all following washing steps accordingly.

8.2) Resuspend the cells in 100 µl fixable dead cell staining solution and incubate for 30 min at 4 °C.

8.3) Wash cells, resuspend in 2% paraformaldehyde in PBS, incubate 15 min at RT.

8.4) Wash cells, resuspend them in 200 µl ice-cold 70% methanol in PBS and incubate for 30 min on ice.

Note: If cells had been infected with adenovirus, they can be treated without biohazard precaution from now on!

8.5) Prepare 60 µl master-mix of 60 µl PBS+ 10 µg/ml Fc-block (anti-F_C receptor 3 to block unspecific binding). Wash cells, resuspend them in 40 µl of PBS+anti-FCR3 and incubate 15 min at RT in the dark.

8.6) Add 20 µl of PBS+anti-FCR3 containing 1 µg PE-coupled anti-Foxp3 antibody, mix well and incubate at 4 °C over night in the dark.

8.7) Wash cells twice in PBS and analyze cells on a flow cytometer.

6.1.9 Th17 cell fixation protocol

9.1) Wash cells: Spin down cells at 300 *g* for 5 min at room temperature, take off SN, resuspend in 200 µl PBS. Centrifuge again and take off SN, perform all following washing steps accordingly.

9.2) Resuspend the cells in 100 µl fixable dead cell staining solution and incubate for 30 min at 4 °C.

Wash cells, resuspend in 4% paraformaldehyde in PBS, incubate 10 min at RT.

9.3) Wash cells in PBS.

Note: Cells can be treated from now on without biohazard precaution! They may be stored over night at 4 °C in the dark.

9.4) Resuspend the cells in PBS/ 0,5% Saponin/ 1% BSA and spin cells down.

Prepare 60 µl master-mix of 60 µl in PBS/ 0,5% Saponin/ 1% BSA + 10 µg/ml Fc-block.

9.5) Resuspend the cells in 40 µl of PBS/ 0,5% Saponin/ 1% BSA + Fc-block and incubate for 10 min at RT in the dark.

9.6) Add 20 µl of PBS/ 0,5% Saponin/ 1% BSA + Fc-block containing 1 µg PE-coupled anti-IL-17 antibody and 1 µg APC-coupled anti-IFNγ, mix well and incubate for 30 min at room temperature in the dark.

9.7) Wash cells twice in PBS/ 0,5% Saponin/ 1% BSA

9.8) Wash cells 1-2 times until cells are no longer soapy and analyze cells on a flow cytometer.

6.1.10 Surface staining

Single cell suspensions at $<10^6$ cells/ml were washed and then incubated with staining antibody at a suitable dilution in PBS for 20 min on ice in the dark. Cells were washed again and then analyzed on a flow cytometer.

6.1.11 Proliferation dye analyses

Naive $CD4^+$ T cells were washed 2-3x in PBS to remove remaining FBS. Cells were then labeled with 10 µM of cell proliferation dye in PBS and incubated for 15 min at room temperature in the dark. After that, 10x the volume of cold T cell medium containing 10% FBS was added and cells were incubated on ice for 5 min. Cells were washed two more times with T cell medium containing 10% FBS and then further treated as non-labeled cells.

6.2 Luciferase reporter assays

Luciferase reporter assays were performed using murine embryonal fibroblast (MEF) cells. $5x10^4$ cells were plated and incubated over night at 37 °C in a 10% CO_2 incubator. Cells were infected with an adenovirus generated from pAdsiCheck with insertion of the respective 3'UTR behind the renilla luciferase gene. Cells were co-infected with a microRNA or control virus andwere incubated for 48 h at 37 °C in a 10% CO_2 incubator. Cells were washed once in 500 µl PBS and then 250 µl of 'passive lysis buffer' were added followed by 30 min incubation under gentle rocking and shaking. Cell lysates were homogenized by pipeting up and down and 300 µl lysate were transferred to a 96-well plate and frozen at -80 °C. Lysates were thawed and spun down at 5000 *g*. 20 µl lysate were transferred to a light-protected 96-well plate avoiding the pellet of aggregates that emerged during lysis. All further steps were performed according to the manufacturers instructions.

Mutations of the microRNA binding sites in the 3'UTR of mTOR were inserted using the QuickChange® II XL Gold Kit (Stratagene) according to the manufacturer's instructions with the primers indicated in Table 8.

6.3 Quantitave PCR (qPCR)

RNA was obtained using the Trizol method or the miRNeasy Mini Kit for T cells that had been cultured with stimulation beads, which interfered with the Trizol method. Reverse transcription was performed with the Quantitect reverse transcription kit II for mRNAs based on oligo(dT) primers or, for microRNAs, using the TaqMan microRNA reverse transcription

kit with a specific first strand synthesis (TaqManMicroRNA or snoRNA202 Assay). The qPCR was performed with Probes Master LC480 using the primers indicated in Table 10 and the universal probe library probes (mRNAs) or with microRNA-specific primers (TaqManMicroRNA Assay, Table 9).

6.4 Statistical analysis

Statistical analyses were performed using Microsoft Excel 2008 for Mac Version 12.3.6 or GraphPad Prism 4.0c. P-values were calculated by Student's t-test or one-way analysis of variance (ANOVA).

6.5 Molecular biology standard procedures

6.5.1 PCR for cloning and genotyping

Cloning PCR was performed using the iProof™ High-Fidelity PCR Kit as indicated above using the primers described in Table 5 and 6. Genotyping PCR was performed using mouse tail tips that were lysed overnight at 55 °C in 200 µl genotyping buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 0.2 mg/ml proteinase K). After inactivation of the proteinase K at 95 °C for 15 min, 300 µl of water were added and 0.5 µl were used as template for PCR using NEB-TAQ PCR kit with the primers listed in Table 7.

6.5.2 Preparation and purification of DNA

Plasmid DNA was purified with the PureYield™ Plasmid Miniprep System (Promega). For higher amounts of DNA, the Nucleobond®Xtra Maxi Kit (Macherey-Nagel GmbH & Co. KG) was used. Both kits were applied according to the manufacturer's protocol.

6.5.3 Restriction digest

Restriction digest of plasmid DNA or PCR products were performed using NEB reagents and the indicated restriction enzymes. Typically, 1-10 µg DNA were digested for 1 h at 37 °C using 10 U of restriction enzymes in a 25 µl digest.

6.5.4 Gel electrophoresis and DNA extraction from agarose gels

DNA was loaded on 1-2% agarose gels containing 20 µg/ml of ethidium bromide and subjected to electrophoresis in the electric field. If required, the desired PCR product was cut

out under 254 nm UV light and purified using the QIAquick PCR Purification Kit according to the manufacturer's protocol (Qiagen).

6.5.5 Immunoblot

For immunoblot lysates, cells were washed three times in PBS at 4 °C and the dry pellett was shock frozen in liquid nitrogen. Pellets were thawed on ice and immediatedly lysed at 5-10x10^6 cell/10 µl of lysis buffer. Lysates were incubated for 15 min on ice, vortexed every 2 min and then centrifuged at 10,000 *g*, 4 °C for 10 min. The amount of protein in the cleared supernatant was determined by the Bradford protein assay. Protein samples were boiled in SDS sample buffer for 5 min at 95 °C and loaded into the slots of the gel. In addition, a protein size maker (Precision Plus Protein All Blue Standards, Biorad) was loaded. The gels consisted of a lower separating and an upper stacking part. The separating part consisted of 8–12% acrylamide, 0.1% SDS, 0.1% APS and 0.06% TEMED in 375 mM Tris-HCl (pH 8.8). The stacking part contained 5% acrylamide, 0.1% SDS, 0.1% APS and 0.1% TEMED in 126 mM Tris-HCl (pH 6.8). After loading the samples, the electrophoresis was first run for 15 min at 80 V and then for 1.5 h at 120 V. The proteins were blotted on nitrocellulose membranes at 40 V at 4 °C over night in Western blot buffer using a wet gel blotting chamber (Biorad). For protein detection, membranes were first blocked in 5% milk dissolved in TBS for 2 h at room temperature. The blocked membranes were washed with TBS-T (3 x 10 min) and the diluted primary antibody was applied for 2 h at RT or over night at 4 °C. The membranes were washed again and incubated with an anti-mouse-Ig antibody, conjugated with horseradish peroxidase, for 1 h at RT. After three more washing steps (TBST-T, TBS, H_2O for 10 min each) the blots were developed with ECL plus Western blotting reagent (GE healthcare).

7 Results

7.1 The kinetics of Treg cell differentiation

The conditions commonly used to *in vitro* differentiate naive $CD4^+$ T into Treg cells require activation via cross-linking of the T cell receptor complex combined with co-stimulation via CD28 in the presence of the cytokines TGFβ and Interleukin-2 (IL-2) (Chen et al., 2003; Zheng et al., 2004). Treg cell differentiation is typically determined by the expression of the Treg cell-specific transcription factor Foxp3 around 72 h after induction. A few kinetic studies addressed the question, at which time during induction the Treg cell fate is actually established. The concept of a window of opportunity was developed, in which the cell fate decision is most sensitive to variations in the differentiation conditions (Sauer et al., 2008).
The first goal of this thesis' work was to define such a window of opportunity for post-transcriptional gene regulation of Treg cell differentiation under the conditions applied in this study. To that end, we analyzed the kinetics of several parameters of Treg cell differentiation and post-transcriptional gene regulation during Treg cell induction.

7.1.1 Expression of *Foxp3* mRNA and protein during Treg cell induction

Expression of the transcription factor Foxp3 determines commitment to the Treg cell lineage (Fontenot et al., 2003; Hori et al., 2003). In order to characterize the kinetics of Treg cell differentiation, Foxp3 expression was analyzed by intracellular anti-Foxp3 staining and flow cytometry every 12 h during 72 h of Treg cell induction (Figure 8A). Foxp3-positive cells emerged in small quantities at 24 h of Treg cell induction with a steady increase of the percentage of Foxp3-positive cells until 48 h. From then on, the fraction of Foxp3-positive cells remained constant over the observed time frame. The analysis of Foxp3 protein expression levels by immunoblots closely reflected these kinetics. It revealed hardly detectable Foxp3 protein levels at 24 h that increased strongly towards 36 h and reached a maximum between 48-72 h (Figure 8B). Transcription of *Foxp3* mRNA intimately reflected the described protein expression kinetics, as determined by real-time PCR quantification. The *Foxp3* mRNA expression initiated between 24 h and 36 h after activation and steadily increased towards 48-72 h (Figure 8C). Altogether these kinetic studies suggested a scenario, in which the levels of Foxp3-encoding mRNA transcripts determine Foxp3 protein expression without obvious signs of post-transcriptional gene regulation of Foxp3 itself.

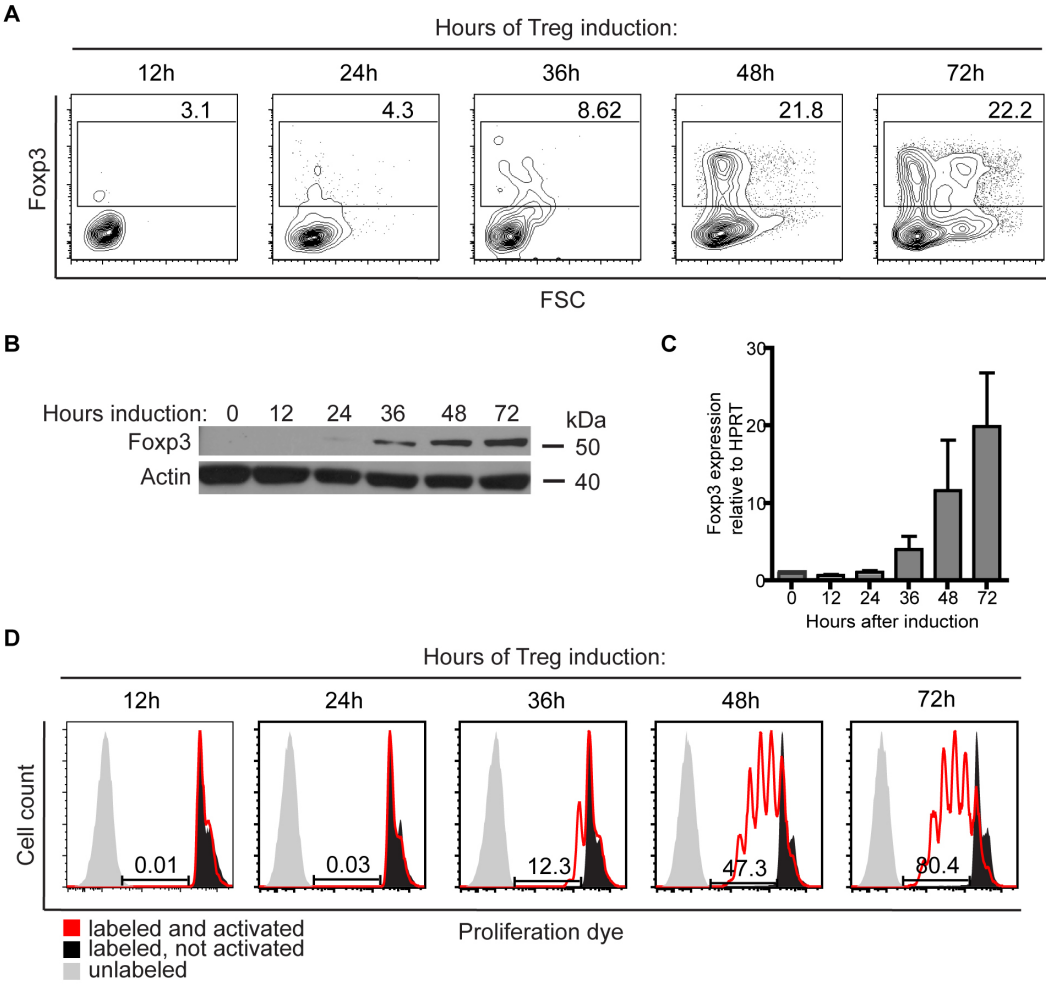

Figure 8: Kinetic studies of Treg cell induction.
Naive CD4$^+$ T cells purified from *DO11.10*tg; *CARΔ1*tg mice were stimulated in Treg-polarizing conditions or left unstimulated and were analyzed at the indicated time points. Treg cell differentiation was assessed by Foxp3 protein expression analyzed by flow cytometry (A) or by immunoblot (B). Foxp3 mRNA expression was determined by qPCR relative to HPRT and normalized to the 0 h time point (C). Proliferation was analyzed by labeling cells with a cell proliferation dye before activation as in A). Dilution of proliferation dye was analyzed using flow cytometry. Unlabeled cells (grey area) or unactivated cells (black area) served as controls. (D). Data are representative of two or more independent experiments (A, B, D) or represent means ± SD of three independent experiments (C).

7.1.2 Kinetics of proliferation during Treg cell differentiation

It was unclear to what extent and at what time point the T cells would proliferate under the Treg cell differentiation conditions applied in this study. Moreover, uncoupling of post-transcriptional gene regulation has been suggested to depend on T cell proliferation (Sandberg et al., 2008). Therefore, the proliferation kinetics during Treg cell induction was recorded by analyzing the dilution of a cell proliferation dye due to cell division in flow cytometry (Figure 8D). The first few cells that underwent cell division could be observed 36 h after Treg cell induction. In the following, cells expanded rapidly with over 80% of cells having gone

through one to four cell divisions within 72 h after induction. Thus, in the chosen differentiation conditions, cell proliferation initiates already after 36 h and occurred at the same time and with similar kinetics as Foxp3 expression. This may suggest a regulatory link between cell fate commitment and cell proliferation.

7.2 A window of post-transcriptional gene regulation during Treg cell induction

Post-transcriptional gene regulation by microRNAs is regulated on several layers that together determine microRNA specificity and efficiency (Hoefig and Heissmeyer, 2008). Kinetic studies were performed to define the time frame during which microRNAs may effectively influence Treg cell differentiation.

7.2.1 Ago proteins are downregulated at 48 h of Treg cell induction

Ago proteins are an essential and limiting component of the miRISC that confers microRNA-mediated repression of gene expression (Diederichs and Haber, 2007; O'Carroll et al., 2007). Ago proteins were shown to be heavily downregulated in Th1 cell differentiation after 48h of activation resulting in a global decrease of microRNA expression (Bronevetsky et al., 2013). To find out, whether Ago protein regulation also occurs during Treg cell differentiation, we analyzed expression of Ago proteins during Treg cell induction over 72 h by immunoblot. To that end, we used a monoclonal antibody that our lab has established against a shared peptide in all four mouse Ago proteins and that recognizes Ago1-4 equally well (pan-Ago, Figure 9A) (Bronevetsky et al., 2013). While Ago proteins were strongly expressed during the first 36 h of Treg cell induction, they were almost completely downregulated at 48 h. This implicates the potential shutdown of microRNA activity at a time, when Treg cell identity has been established, as evident from the robust expression of the lineage-specifying transcription factor Foxp3 (Figure 8A-C).

7.2.2 3'UTR shortening of Eri1 mRNA occurred early during Treg cell induction

The 3'UTR of an mRNAs contains the information for post-transcriptional regulation via *cis*-regulatory elements such as microRNA target sites, which determine the stability and translation efficiency of mRNAs (Grimson et al., 2007; Myer et al., 1997). The use of alternative 3'UTRs provides a mechanism for evasion from post-transcriptional gene regulation and may result in enhanced translation efficiency. So like the control of Ago expression, the 3'UTR length may limit microRNA-mediated gene silencing. Sandberg et al. investigated global 3'UTR length distribution in 48 h-activated compared to resting primary murine $CD4^+$ T cells and reported a shift towards transcripts with shortened 3'UTRs in activated T cells (Sandberg et al., 2008). Whether this process also takes place during Treg

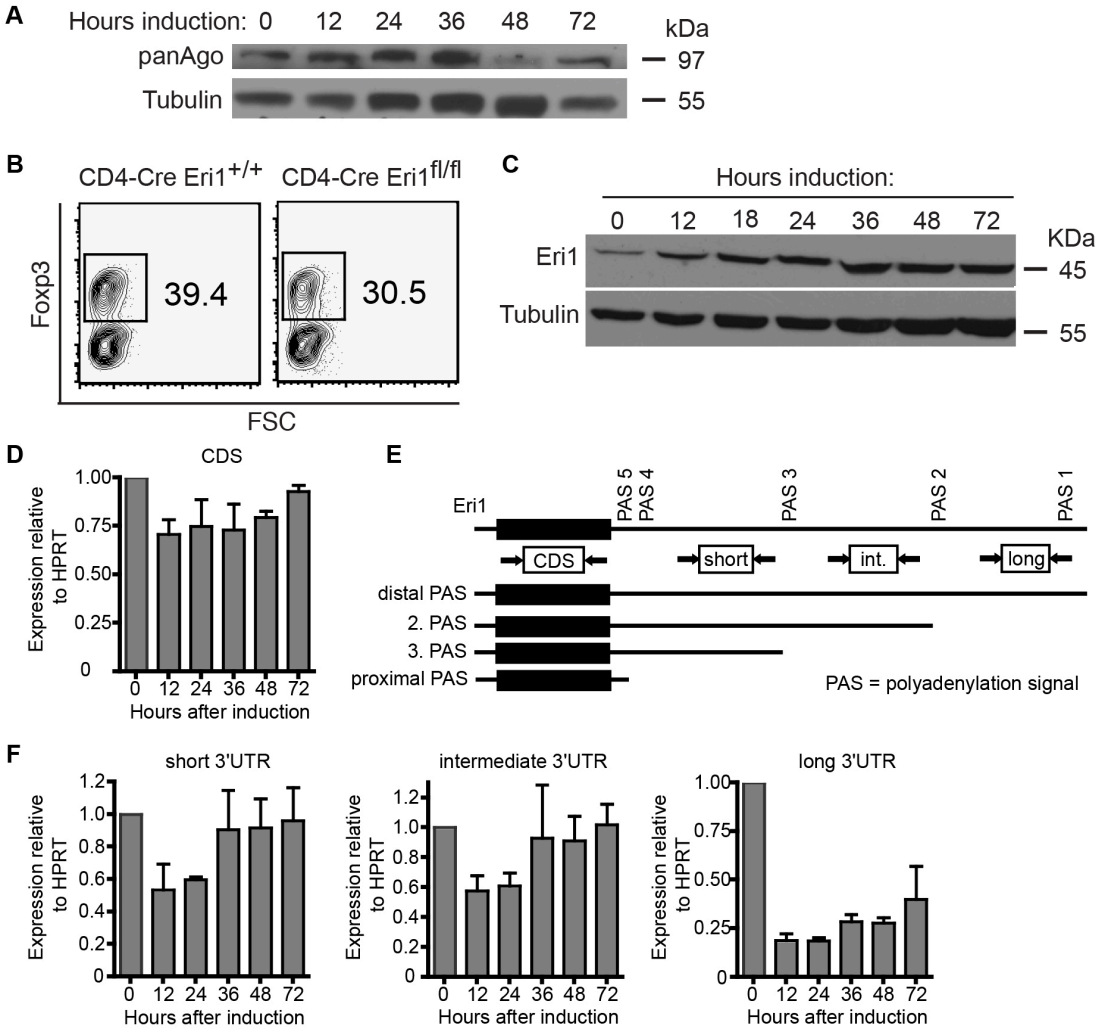

Figure 9: Kinetics of post-transcriptional gene regulation during Treg cell induction.
A) Naive CD4$^+$ T cells purified from *DO11.10*tg; *CARΔ1*tg mice were stimulated in Treg-polarizing conditions for the indicated times. Expression of Ago1-4 (panAgo) protein expression was analyzed by immunoblot. B) Naive T cells purified from CD4-Cre Eri1+/+ or CD4-Cre Eri1fl/fl mice were stimulated in Treg-polarizing conditions for 72 h and differentiation was analyzed by Foxp3 expression by flow cytometry; data are representative of three independent experiments. C) Cells were treated as in (A) and expression of Eri1 protein was analyzed by immunoblotting. D, F) Cells were treated as in (A) and Eri1 mRNA variants were determined by qPCR using the primers depicted in (E) relative to HPRT and normalized to the 0 h time point. E) Schematic representation of Eri1 mRNA with alternative polyadenylation signals (PAS, top) and primer pairs (below) that were designed to detect mRNAs with different 3'UTRs resulting from alternative PAS usage (bottom). Data are representative of two (A, C), or three (B) independent experiments or represent means ± SD of two independent experiments (D, F).

cell differentiation remained to be elucidated. As a first step in finding an answer to this question, we analyzed expression of variants of the Eri1 3'UTR during Treg cell induction. Eri1 is an ubiquitously expressed 3'-to-5' processive exoribonuclease (Ansel et al., 2008). Its deletion resulted in impaired differentiation of naive T cells into Treg cells (Figure 9B). Eri1 protein was induced upon and expressed throughout Treg cell induction (Figure 9C). Interestingly, the protein expression did not at all reflect mRNA expression as determined by qPCR analyses with primers that generate an amplicon in the coding region of Eri1 (CDS,

Figure 9D-E). The sequence of this amplicon is contained in all known isoforms of the Eri1 mRNA and the expression of mRNAs containing this sequence was reduced by 25% upon Treg cell induction (Figure 9D). This could be explained by increased Eri1 protein stability after activation. However, another possible explanation would be the higher translation efficiency after Treg cell induction, which could result from the usage of an alternative 3'UTR.

Based on a global mapping of conserved polyadenylation signals (PAS) in various tissues and several species by Poly(A)-sequencing, the Eri1 mRNA can exist in at least five isoforms with different 3'UTR lengths (Figure 9E, Derti et al., 2012). QPCR primers were designed to detect three of the 3'UTR variants (Figure 9E). Already 12 h after Treg cell induction, the detection of amplicons in the in the long 3'UTR showed a reduction of more than 75%, while short and intermediate 3'UTR isoforms showed a reduction by approximately 50% as compared to the detection of an amplicon in the CDS, which was reduced by only 25% (Figure 9F). This suggests the preferential expression of Eri1 mRNA isoforms at 12 h and 24 h with very short 3'UTRs and a smaller proportion of Eri1 mRNA with intermediate or long 3'UTRs. Although other possible explanations have not been experimentally addressed, these findings are consistent with the use of a more proximal PAS after Treg cell induction. Following that concept, the predominant PAS 1 resulting in expression of the long 3'UTR would be used in cells before activation, which would then switch towards the PAS 2 (intermediate 3'UTR) or to the PAS 4 or 5 (very short 3'UTRs) already 12 h after activation.

Although post-translational regulation of Eri1 protein during T cell activation and differentiation has not been tested in this study, the increased Eri1 protein that was observed 12 h after T cell stimulation is likely to be explained by increased translational output from Eri1 mRNA isoforms with short 3' UTRs. At 36 h, expression of all 3'UTR-containing Eri1 mRNAs was upregulated and was potentially correlated with commitment to the T cell lineage and beginning of Foxp3 expression as well as with initiation of cell proliferation.

Taken together the results suggest a window opportunity for post-transcriptional gene regulation by microRNAs. It initiates with T cell activation and is counter-regulated by shortening of 3'UTRs already 12 h after activation and finally by downregulation of Ago between 36 h and 48 h of activation.

7.3 Manipulation of Treg cell differentiation by adenoviral gene transfer

The window of opportunity during which microRNAs could affect the outcome of T cell differentiation decisions implied that the first hours after activation are critical. Therefore, experimental approaches to study the effect of gene products that influence this process should ideally by present from the moment of T cell activation on. We employed an adenoviral gene transduction system that allowed expression of a gene of interest in naive T cells. The tropism of type 5 adenoviruses for human cells is determined by expression of the coxsackie adenovirus receptor on the host cell (Zhang and Bergelson, 2005). Wan and colleagues generated mice with transgenic expression of the coxsackie adenovirus receptor delta 1 gene (*CARΔ1*) under control of a T cell-specific promoter (Wan et al., 2000a). CARΔ1 has no signaling capacity due to a deletion of intracellular domains but retains transmembrane and extracellular domains. This enabled efficient infection of mouse T cells with type 5 adenoviruses at the naive state without conferring or requiring activation. This key property of adenoviral gene transduction in regard to the window of opportunity prompted us to further evaluate the adenoviral system in T cell infection and Treg cell differentiation.

7.3.1 Adenoviral gene transfer did neither influence T cell viability nor Treg cell differentiation and allowed microRNA overexpression in naive T cells

To assess a potential dose-effect, adenoviral gene transduction was performed at different multiplicities of infection (MOIs). In this experiment a control adenovirus that encoded GFP was used as a marker of infection and analyzed by flow cytometry (Figure 10A, upper panel). Virus titers were established as described in the Methods section and used to calculate the amount of viral supernatant that, when combined with the appropriate T cell numbers, resulted in different MOIs ranging from 1 to 50. Naive $CD4^+$ T cells were isolated from *DO11.10*tg; *CARΔ1tg* mice, infected with control adenovirus or left uninfected and were rested 40 h followed by 40 h of activation. Under these conditions, an MOI of 1 that represents the incubation of one infectious viral particle with one T cell for 90 min resulted in 36% of infected cells and increasing the MOI to 50 augmented the infection efficiency to 83% (Figure 10A, upper panel). For subsequent experimental readouts on the single cell level, an infection of 50% of the cells was considered ideal, while for biochemical analyses infection rates of 80% or more were attempted. At an MOI of 1-50, the cell viability appeared unaffected by adenoviral infection (Figure 10A, middle panel) and, most importantly, there was no significant effect of adenoviral infection itself on Treg cell differentiation, as determined by flow cytometry (Figure 10A, lower panel).

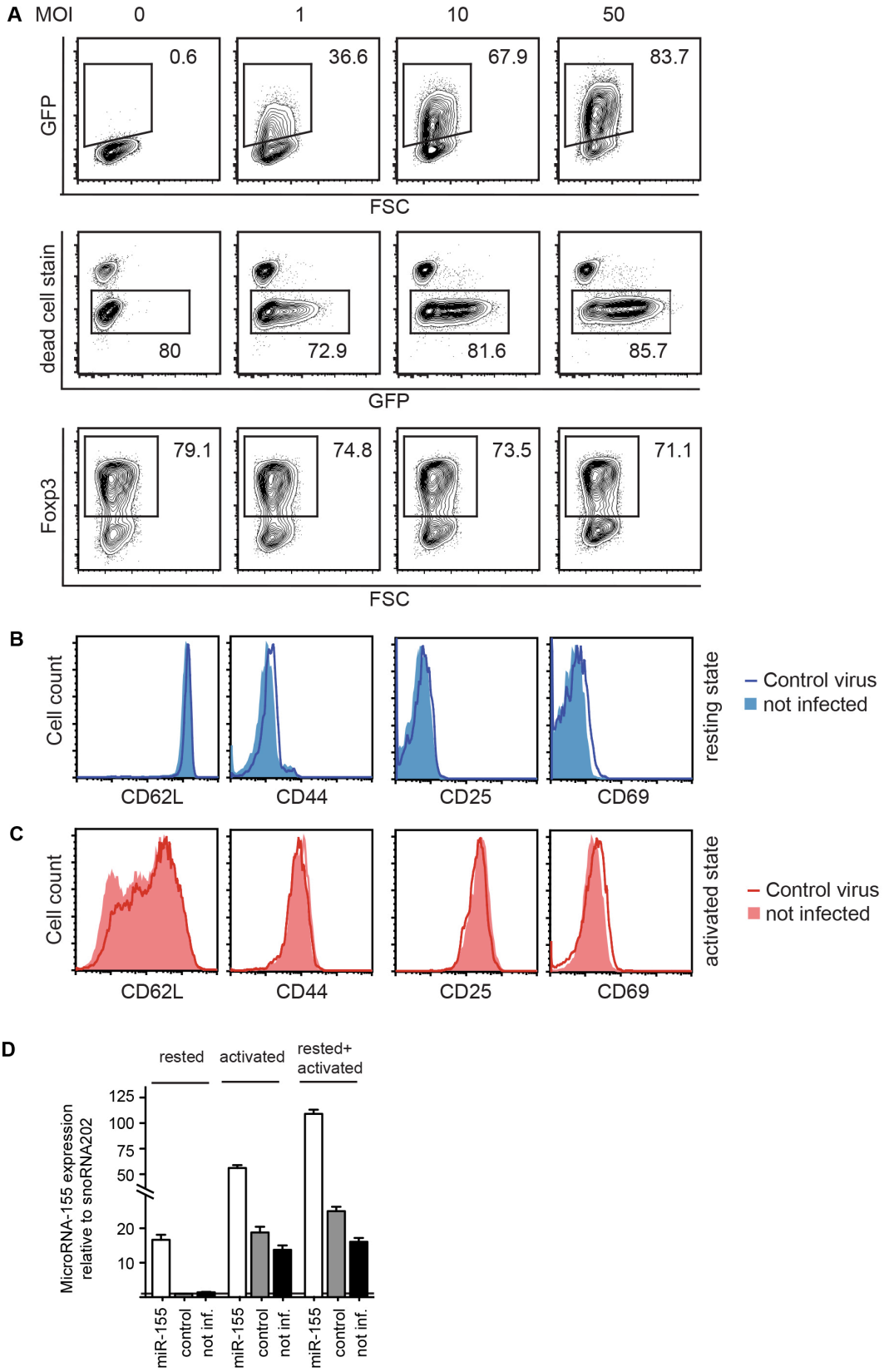

Figure 10: Evaluation of adenoviral gene transfer.

Figure 10: Evaluation of adenoviral gene transfer.
Naive CD4$^+$ T cells purified from *DO11.10*tg; *CARΔ1*tg mice were infected with control adenovirus at the indicated MOI or at an MOI of 50 or were left uninfected. A) Cells were rested for 40 h and then activated in Treg-polarizing conditions. After 72 h expression of the infection marker GFP, exclusion of the dead cell stain and the expression of the differentiation marker Foxp3 was analyzed by flow cytometry. B) Cells were rested in T cell medium and after 40 h naive cell and activation markers were analyzed by flow cytometry. C) Cells were treated as in B and were then activated in Treg-polarizing conditions for 40 h before analysis of naive cell and activation markers. D) Naive CD4$^+$ T cells purified from *DO11.10*tg; *CARΔ1*tg mice were infected with a miR-155 expressing or control adenovirus at an MOI of 50 or were left uninfected. After infection cell were rested for 40 h, activated for 40 h or rested for 40 h followed by activation for 40 h. Then relative expression of miR-155 was determined by qPCR and normalized to naive control cells. All data are representative of three or more independent experiments; error bars were calculated form technical replicates.

To demonstrate infection of T cells at a naive state, naive CD4$^+$ T cells were purified from *DO11.10*tg; *CARΔ1*tg mice and were infected with a control adenovirus at MOI 50 or left uninfected. The expression of the naive T cell marker CD62L and of the activated T cell markers CD44, CD25 and CD69 was analyzed by flow cytometry. The marker expression was indistinguishable between infected (Figure 10B, line) and non-infected (Figure 10B, area) cells and clearly indicated the naive state of the T cells with high expression of CD62L and low expression of CD44, CD25 and CD69. Upon T cell activation, a similar downreguation of CD62L and an upregulation of CD44, CD25 and CD69 occurred in infected (Figure 10C, line) and non-infected (Figure 10C, area) cells, which excluded an influence of adenoviral infection itself on T cell activation at the applied MOIs.

To analyze the achieved level of overexpression, naive CD4$^+$ T cells were infected with an adenovirus that expresses miR-155. Cells were either rested for 40 h or activated for 40 h or rested for 40 h followed by activation for 40 h (Figure 10D). Analysis of mature miR-155 showed a 17-fold overexpression in naive T cells infected with miR-155 adenovirus compared to control virus or non-infected cells. This matched the level of miR-155 upregulation induced by T cell activation in control virus or non-infected cells. The result illustrated that overexpression of miR-155 could provide microRNA levels of the activated state in naive cells. It also showed that this microRNA level was present at the moment of T cell activation.

Taken together, these data demonstrated that adenoviral gene transfer is a very suitable system to overexpress a gene or microRNA of interest in naive T cells.

7.3.2 Overexpression of human FOXP3 in naive T cells promoted Treg cell induction

The evaluation experiments established the feasibility of adenoviral gene transfer in Treg cell induction without facing any significant adverse effects of the infection with control virus. We next wanted to test functionally, whether adenoviral overexpression of a transcription

factor with a Treg cell-promoting function could influence Treg cell induction. Foxp3 is necessary and sufficient to establish Treg cell identity. We reasoned that overexpression of Foxp3 may promote Treg cell differentiation and took advantage of a construct expressing human FOXP3 (hFOXP3) protein, which does not express the epitope that is recognized by the anti-mouse Foxp3 monoclonal antibody FJK-16 in intracellular flow cytometry analysis. So human FOXP3 or empty vector was adenovirally transduced and mouse Foxp3 was analyzed after resting and Treg-polarization of the T cells. The two parameters GFP as infection marker and mouse Foxp3 as the differentiation marker were plotted against each other (Figure 11A). To display the result of Foxp3 overexpression in a more simple way, the parameters of the quadrant plot were separated into histograms showing GFP expression

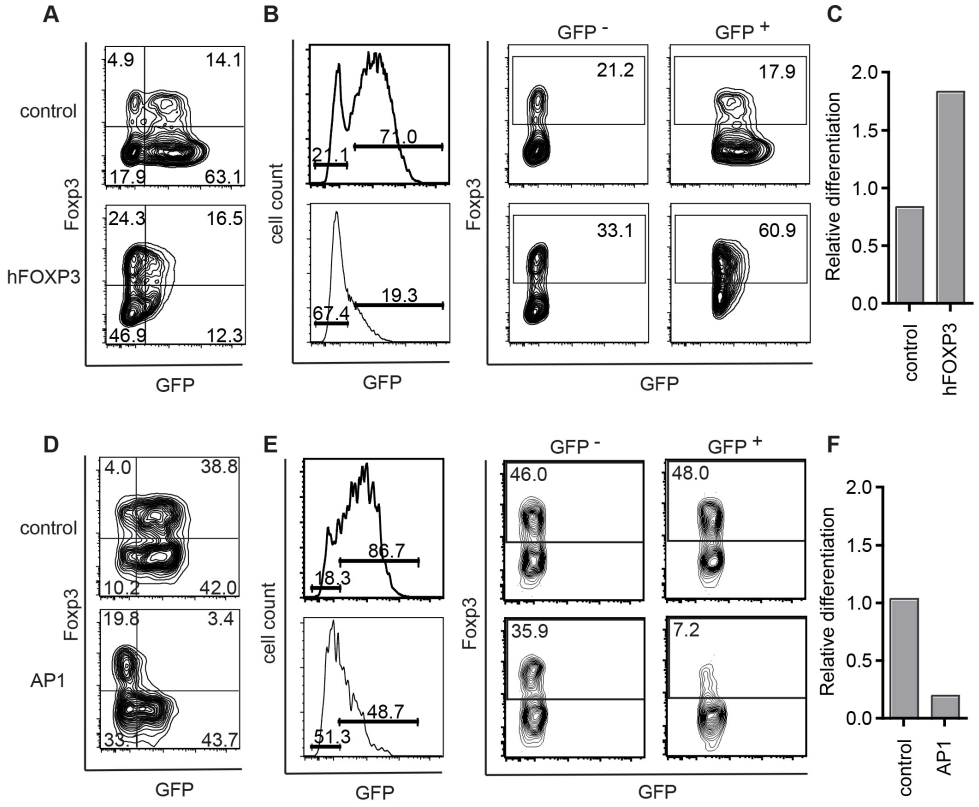

Figure 11: Manipulation of Treg cell differentiation by adenoviral overexpression of transcription factors during Treg cell induction.
Naive CD4$^+$ T cells purified from *DO11.10*tg; *CARΔI*tg mice were infected with human FOXP3 (A-C) or AP-1 (D-E) expressing adenovirus or empty virus control and rested for 40 h. Cells were then stimulated in Treg-polarizing conditions. After 72 h Treg cell differentiation (mouse Foxp3) in correlation with infection (GFP) was analyzed by flow cytometry. The Quadrant plots (A, D) from one experiment were divided into a histogram that defines GFP gating for the contour plots containing the Foxp3 gate (B, E). The bar diagrams (C, F) were calculated by division of the percentage of Foxp3 positive cells in the GFP$^+$ gate by the percentage of Foxp3 positive cells in the GFP$^-$ gate (17.9 / 21.2 for 'control' in B). This value was termed 'relative differentiation'. The shown results are representative of four or more independent experiments.

(Figure 11B, left panels) and Foxp3 expression in the GFP^- (Figure 11B, middle panels) and GFP^+ gate (Figure 11B, right panels). It became obvious that overexpression of hFOXP3 was associated with reduced levels of GFP expression, but strongly promoted Treg cell induction compared to non-infected cells (GFP^-) from the same sample. In addition, there were also more Foxp3-positive cells in the non-infected cells from the hFOXP3 sample in comparison with the control virus sample. These results suggested that hFOXP3 promoted Treg cell differentiation in a cell-intrinsic, but also in a cell-extrinsic manner. In order to have a measure for the cell intrinsic effect of an adenovirally transduced gene on Treg cell differentiation, relative differentiation (RD) was calculated by dividing the percentage of $Foxp3^+$ cells of the GFP^+ gate by the percentage of $Foxp3^+$ cells of the GFP^- gate, which generated the values in the bar diagrams of Figure 11C.
RD is calculated as follows when applied to the quadrant plot that comprises the populations [GFP^+ and $Foxp3^+$]; [GFP^+ and $Foxp3^-$]; [GFP^- and $Foxp3^+$]; [GFP^- and $Foxp3^-$]:

$$\frac{[GFP^+ \text{ and } Foxp3^+ / (GFP^+ \text{ and } Foxp3^+ + GFP^+ \text{ and } Foxp3^-)]}{[GFP^- \text{ and } Foxp3^+ / (GFP^- \text{ and } Foxp3^+ + GFP^- \text{ and } Foxp3^-)]}$$

Altogether, these results demonstrated the capacity of adenoviral gene transduction to increase the Treg cell differentiation by the promoting effect of hFOXP3.

7.3.3 Adenoviral overexpression of AP-1 in naive CD4 T cells suppressed Treg cell induction

T cell activation triggers expression of c-Fos that then forms the heterodimeric transcription factor AP-1 by binding to c-Jun. AP-1 binds in conjuction with NFAT to composite transcription factor binding sites, which has been shown to induce activation-associated genes during productive T cell activation (Wu et al., 2006). We reasoned that enforced expression of AP-1 during Treg induction may interfere with Treg-specific transcription mediated by heterodimeric Foxp3:NFAT binding to composite promoter binding sites. To provide stoichiometric amounts in an overexpression construct, *c-Jun* coding sequence was separated from *c-Fos* by the *2A-like* peptide sequence (P2A, coding forAsp-Val/Ile-Glu-X-Asn-Pro-Gly-Pro). This sequence results in cleavage of the nascent peptide by ribosomal skipping of hydrobond formation between last two amino acids (Gly-Pro). The ribosome therefore releases the upstream peptide while continuing synthesis of the downstream peptide (Osborn et al., 2005). Indeed, AP-1 overexpression largely reduced Treg cell differentiation as

expected (Figure 11D-F). Thus the results demonstrated the capacity of adenoviral gene transduction to downmodulate a T cell differentiation program. The strong increase or strong impairment of Treg cell differentiation by overexpression of hFOXP3 or AP-1, respectively, may mark the lower and upper end of the dynamic range that can be achieved in this system.
Altogether, adenoviral gene transduction enabled ectopic expression of microRNAs or transcription factors preceding CD4 T cell differentiation at the naive state. It allowed the testing of ectopic gene expression effects during initial signaling from the TCR to the Treg cell lineage commitment with a broad dynamic range.

7.4 A functional microRNA overexpression screen in Treg cell induction

MicroRNAs were shown to be essential for Treg cell differentiation (Chong et al., 2008; Cobb et al., 2006). We set out to identify individual microRNAs that play a role in Treg cell differentiation. To that end we chose the *in vitro* model of TGFβ-induced Treg cell differentiation to perform a functional screen. We used an arrayed overexpression approach in which single microRNAs were overexpressed in parallel in wildtype cells. The adenoviral overexpression system was used to infect naive T cells of *DO11.10tg*; *CARΔ1*tg mice to ensure a high level of micro-RNA overexpression at the time when the T cells are activated in Treg-polarizing conditions.

7.4.1 Generation of an adenoviral pri-microRNA library

We reviewed microRNA expression data from available expression analyses of various T cell lineages as well as developmental stages to come up with a list of T cell-expressed microRNAs (Landgraf et al., 2007; Monticelli et al., 2005, K. Kretschmer, unpublished data, M. Ansel, unpublished data). Chen et al. established a protocol for ectopic microRNA expression in which constructs of at least 270nt that contain the mature microRNA and 125nt of genomic sequence on either side of the microRNA were used. These constructs were effective for pri-microRNA processing by Drosha/DGCR8 and yielded efficient ectopic expression of mature microRNAs (Chen et al., 2004). Accordingly, we cloned a set of 147 T cell-expressed microRNAs as pri-microRNAs from C57BL/6 genomic DNA into the pENTR/D-TOPO vector. The microRNA clusters were cloned by including the whole endogenous cluster sequence or, if feasible, also as single microRNAs. For example, the 17~92 cluster on chromosome 13, which encodes hairpins for the microRNAs 17, 18a, 19a, 20a, 19b-1, 92a-1 was cloned as a construct containing miR-17 to miR-19a, as a second construct containing miR-20a to miR- 92a-1 and as a third construct containing only miR-17,

whereas for the other individual microRNAs no straightforward cloning strategy was available (Olive et al., 2010). A few microRNAs were cloned, but had to be transferred into the pENTR11 vector in order to correct the construct orientation. The whole entry vector library was tested for the correct sequence as well as orientation of the insert by restriction

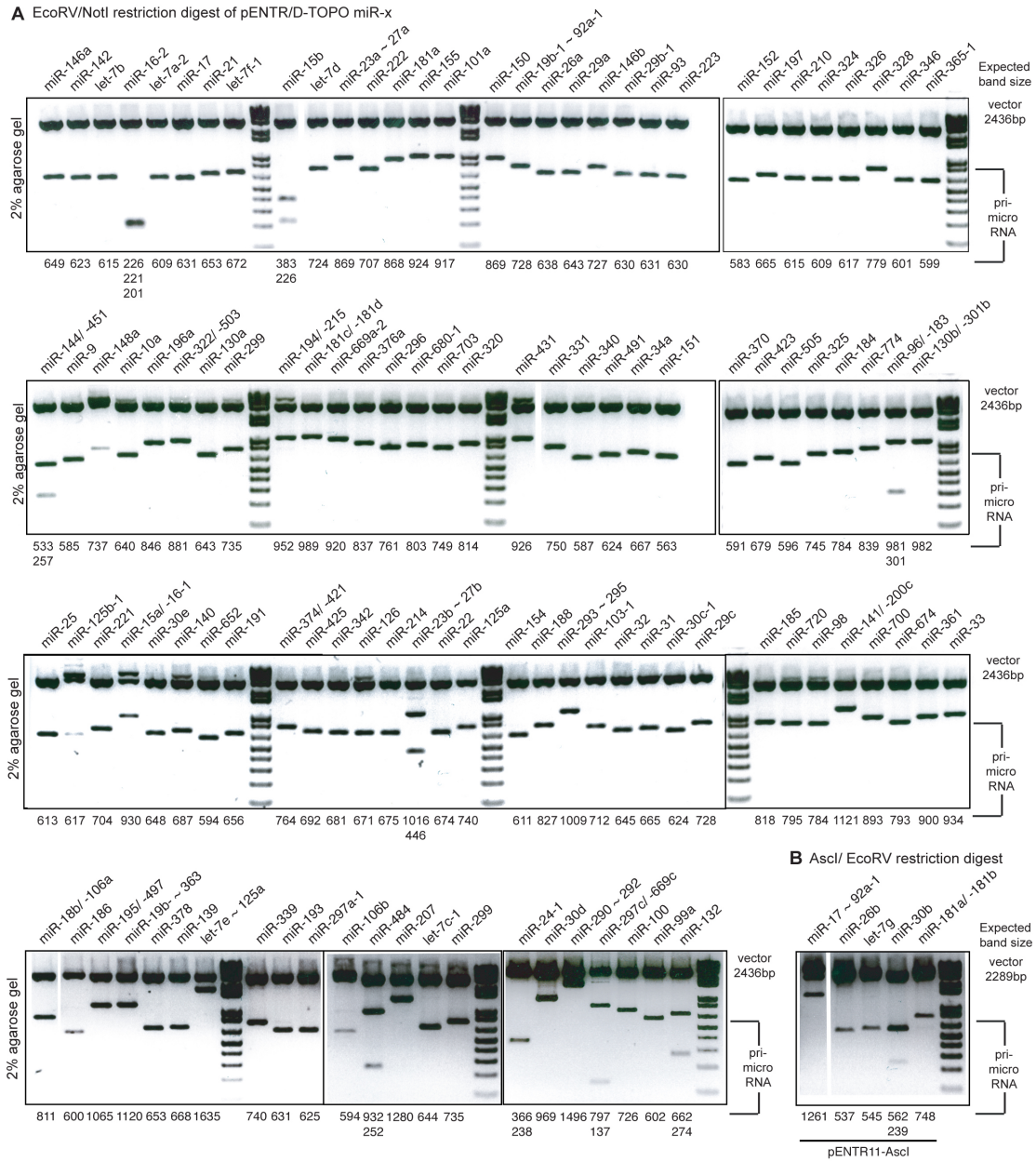

Figure 12: Restriction digest of the entry vector library containing 147 T-cell-expressed microRNAs. 1 µg of each microRNA-Entry vector was digested with EcoRV and NotI in case of pENTR/D-TOPO (A) or with *AscI* and *EcoRV* in case of pENTR11 (B, bottom right) for 90 min and run in a 2% agarose gel. Digestion of pENTR/D-TOPO or pENTR11-*AscI* (bottom panel) resulted in a vector band of 2436 bp or 2289 bp, respectively. The band sizes indicated below each lane comprised the microRNA insert. In addition, all clones were sequence-verified. A complete list of all constructs is included in the Material section under „Table 5: Primer, Entry vector and Destination vectors of the microRNA library“.

digest and sequencing (Figure 12). The correct pri-microRNA-inserts were then transferred into the adenoviral expression vector pCAGAdDu by λ recombination, which was used to generate adenovirus as previously described (Warth and Heissmeyer, 2013). Each virus was amplified one or more times in order to obtain viral cell lysates with comparable virus titers. A complete list of all microRNA construct used for the library is included in the Material section of this thesis.

7.4.2 A functional microRNA overexpression screen in Treg cell induction

The design of the screen is depicted in Figure 13A. In a 96-well format, naive T cells were infected in parallel with the adenovirus library using the same volume of viral cell lysates. This procedure was chosen to account for possible influences of components that are present in the HEK293A cell lysates, which contained the infectious particles in a raw lysate preparation. In addition, the cells were washed after infection and then rested for 40 h in T cell medium without supplemented cytokines to enable the overexpression of the transduced microRNA. Only then, cells were activated under sub-optimal Treg-polarizing conditions. These were chosen to allow for a good dynamic range in the read-out to similarly detect positive and negative effects of the overexpressed constructs. After 72 h, cells were intracellularly stained and protein expression of the differentiation marker Foxp3 and the infection marker GFP were analyzed by flow cytometry. This allowed the discrimination of differentiation in infected and differentiation in non-infected cells within the same well. To ensure a reliable analysis, samples with too small cell numbers or insufficient infection were excluded. Figure 13B shows representative FACS plots of a microRNA that strongly interfered with Treg cell differentiation (Figure 13B, left plot), of a microRNA that strongly promoted Treg cell differentiation (Figure 13B, central plot) and of a control virus (Figure 13B, right plot). For this control virus, infected cells showed Treg cell differentiation of about 50% in GFP+ cells [41.5 / (41.5+42.7)] as well as in GFP- cells [7.2/ (7.2+8.6)]. This results in a ‘relative differentiation’ (RD) of 1.08, which means that there was no difference in Treg cell differentiation between infected and non-infected cells. 15 control virus replicates at random positions throughout the screening plates served to calculate the mean RD = 1.006 ± 0.060 (Figure 13C). The 95% confidence interval ranged from 0.973 to 1.04. RD values above 1.04 were considered as a non-random positive effect, meaning that overexpression of the respective microRNA intrinsically promoted Treg cell differentiation. Accordingly, RD values below 0.973 were considered as a non-random negative effect, meaning that overexpression of the respective microRNA intrinsically interfered with Treg cell differentiation. In total, the microRNA overexpression screen identified 62 microRNAs with a

negative effect and 35 microRNAs with a positive effect on Treg cell induction. While most candidates had a moderate effect, the top positive candidate promoted by nearly 50% (RD = 1.5) the expression of the Foxp3 differentiation marker in infected cells while the top negative candidate reduced Treg cell differentiation by almost 50% (RD = 0.5) (Figure 13D).

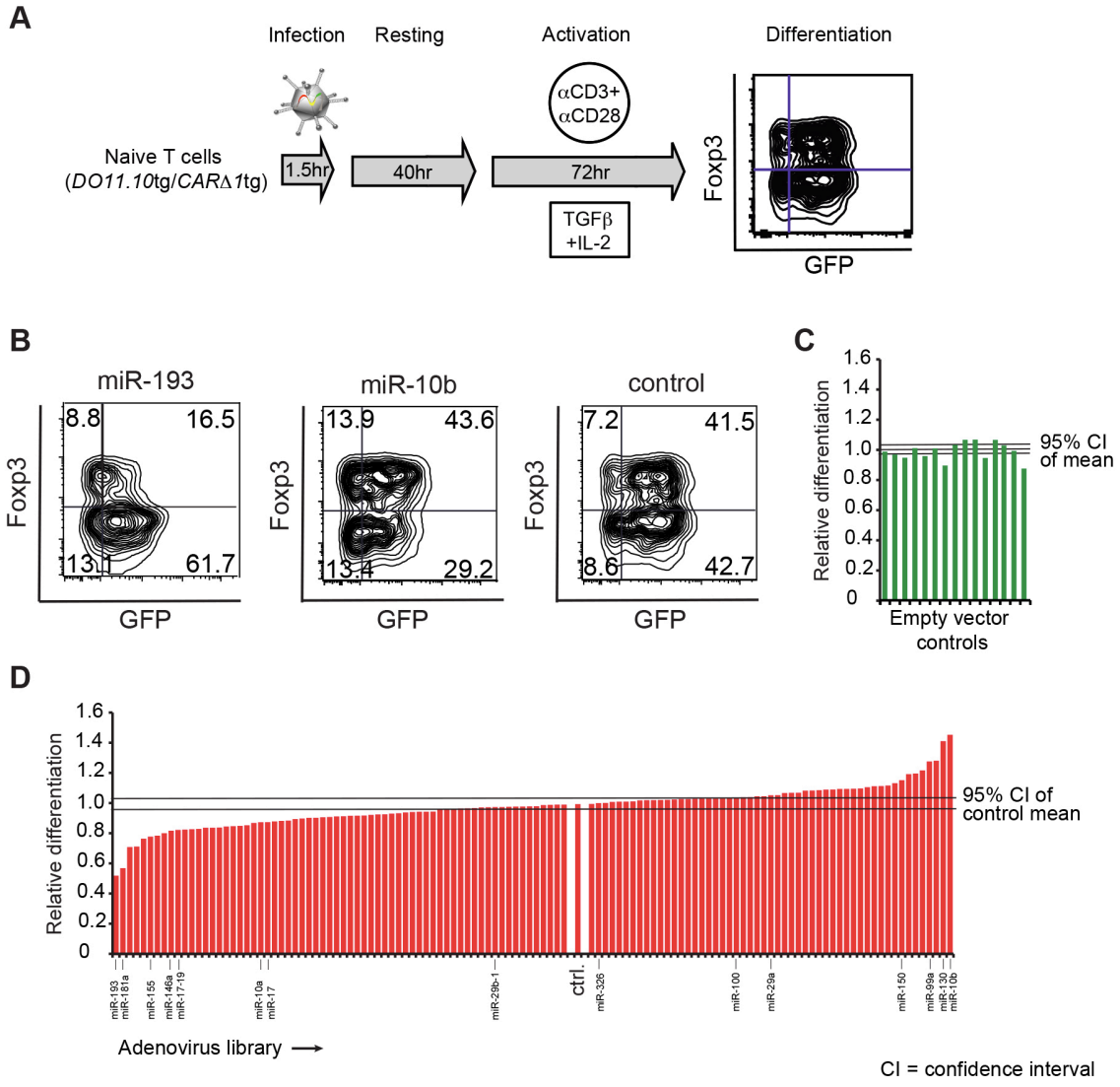

Figure 13: A functional adenoviral overexpression screen in Treg cell induction.
A) Scheme of the screen workflow. B-D) Naive CD4$^+$ T cells purified from *DO11.10*tg; *CARΔ1*tg mice were infected in parallel with the adenovirus library of T cell-expressed microRNAs or control viruses. Cells were rested for 40 h and stimulated subsequently for 72 h with anti-CD3/ anti-CD28-coupled beads in Treg-polarizing conditions. B) FACS plots display expression of the infection marker GFP versus the differentiation marker Foxp3 of the most positive (left plot) or most negative (central plot) candidates or control virus (right plot). C) 15 control virus replicates were used to calculate mean ± SD of relative differentiation of well-to well variance. Relative differentiation is a straight measure for cell intrinsic effects of microRNAs (see Section A functional microRNA overexpression screen in Treg cell induction). D) Relative differentiation results of all candidates in the screen. Candidates with a difference in relative differentiation greater or smaller than the 95% confidence interval (CI, horizontal lines in the bar graph) were considered as significantly different from control virus. A complete list of all constructs is included in the Material section under „Table 5: Primer, Entry vector and Destination vectors of the microRNA library“.

7.5 Identification and validation of microRNAs with selective effects in Treg cells

The microRNA candidates identified in the screen must not necessarily be causally involved in Treg cell differentiation, but may affect general cellular processes like metabolism, proliferation or cell death. To identify candidates with a selective effect on Treg cell differentiation, several selection and validation experiments were subsequently conducted.

7.5.1 Overexpression of selected microRNA candidates in Th17 differentiation

The identified microRNA candidates were retested in the reciprocal differentiation programs of naive T cells into the Treg or Th17 lineage to uncover Treg-selective effects (Figure 14). Most microRNA candidates with strong effects in the Treg cell differentiation screen could be reproduced and showed significant effects in repeat experiments (Figure 14, red bars). Th17 differentiation conditions were somewhat less stable than those for Treg cells (Figure 14, blue bars). Nevertheless, a clear picture emerged for microRNAs such as miR-181c, d with a similar effect in Treg and Th17 cells, or microRNAs with a selective positive effect on Treg cell differentiation such as miR-10b and no significant effect on Th17, or microRNAs with a selective negative effect on Treg cell differentation, such as miR-140, which may also have a reciprocal influence on Th17 differentiation. Following the initial assumption that the presence of post-transcriptional gene regulation by microRNAs is essential for Treg cell generation, we then focused on microRNAs that selectively promoted Treg cell differentiation, namely miR-99a, miR-100 and miR-10b. None of these affected Th17 differentiation in a similar extent or direction.

7.5.2 T cell proliferation is not affected by miR-99a, miR-100 and miR-10b overexpression

An obvious explanation for the positive effect of miR-99a, miR-100 and miR-10b could be that they promote proliferation particularly in Treg cells. To exclude that, a Treg cell differentiation experiment was designed using naive T cells that were labeled with a proliferation dye, which is diluted with every cell division. Cells were infected with control virus expressing only the Thy1.1 marker and were co-cultured with cells that were infected either with GFP-miR-99a, or GFP-miR-100, GFP-miR-10b virus or GFP-control virus. After 40 h of resting and additional 72 h Treg cell induction the samples were analyzed by flow cytometry for the infection markers GFP and Thy1.1, the differentiation marker Foxp3 as well as for the proliferation dye. Despite independent infection, double positive cells emerged, which must have resulted from secondary infection with residual adenovirus during the resting phase (Figure 15A). To analyze the effect of the individual constructs, differentiation

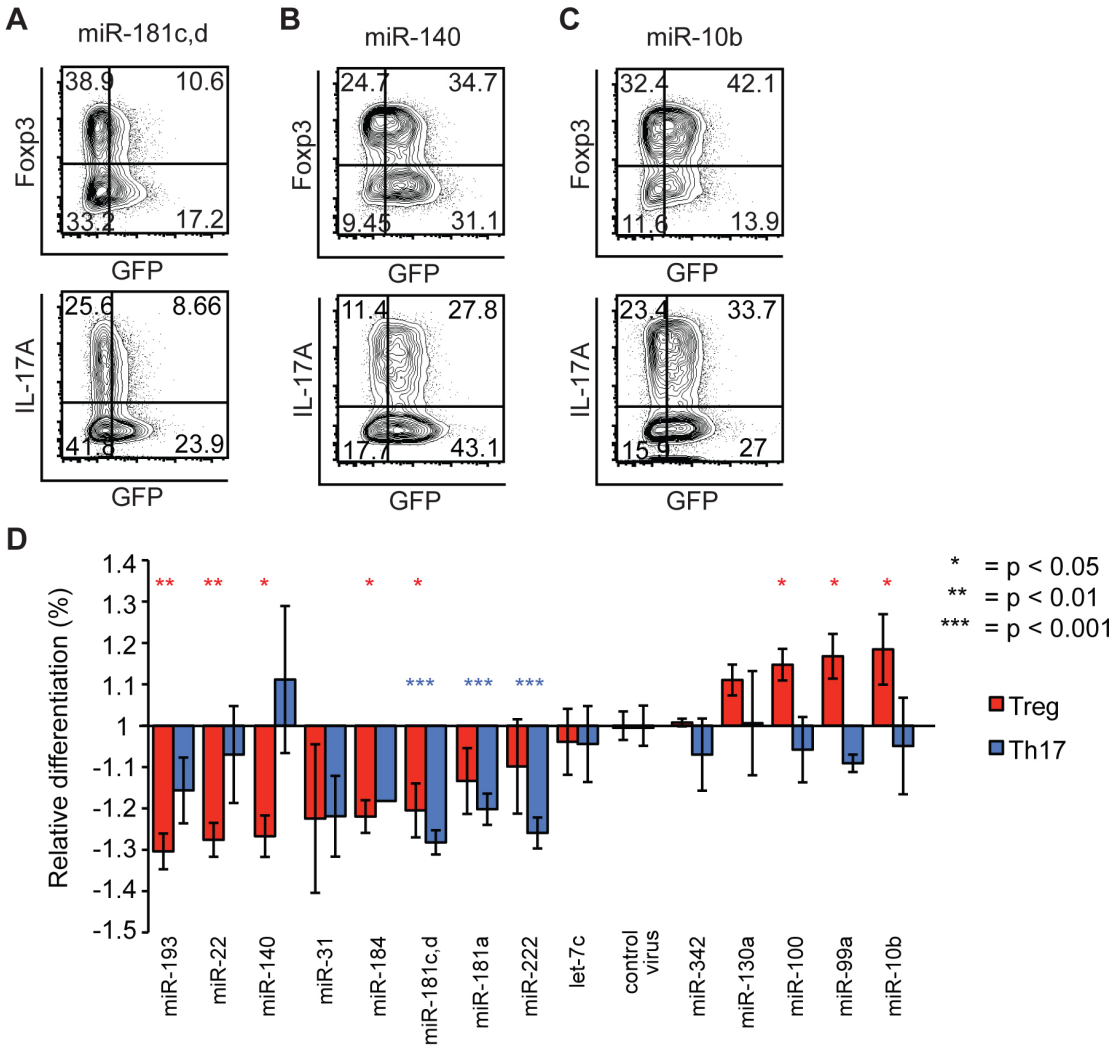

Figure 14: Overexpression of selected microRNA candidates in Th17 differentiation.
Naive CD4$^+$ T cells purified from *DO11.10*tg; *CARΔ1*tg mice were infected with the indicated adenovirus, rested for 40 h and were subsequently stimulated in polarizing conditions for Treg or Th17 cells for 72 h. Th17 cells were then restimulated with PMA/ionomycin for 5 hours with BrefeldinA present for the last 2.5 h. Cells were fixed and stained before expression of differentiation marker Foxp3 (Treg) or IL-17A (Th17) was analyzed by flow cytometry. Representative FACS plots of a common effect (A) or Treg cell-selective negative (B) or positive (C) microRNA overexpression effect on differentiation. The relative differentiaton bar diagram (D) shows means ± SD of three independent experiments; p-values were calculated using Student's t-test.

and proliferation in single positive cells were further analyzed by gating on Thy1.1$^+$/ GFP$^-$ (control) or Thy1.1$^-$/GFP$^+$ (microRNA or control) cells (Figure 15B).

The percentage of Foxp3$^+$ cells was very similar in cells infected with Thy1.1 or with GFP control virus (Figure 15B, control virus, upper versus lower panel). Compared to that, the percentage of Foxp3$^+$ cells was markedly increased by overexpression of miR-99a, miR-100 and miR-10b both in a cell intrinsic manner (Figure 15B, miR-99a, miR-100, miR-10b, lower panels compared to control lower panel) as well as in bystander cells from the same well infected with control virus (Figure 15B, miR-99a, miR-100, miR-10b, upper panels compared

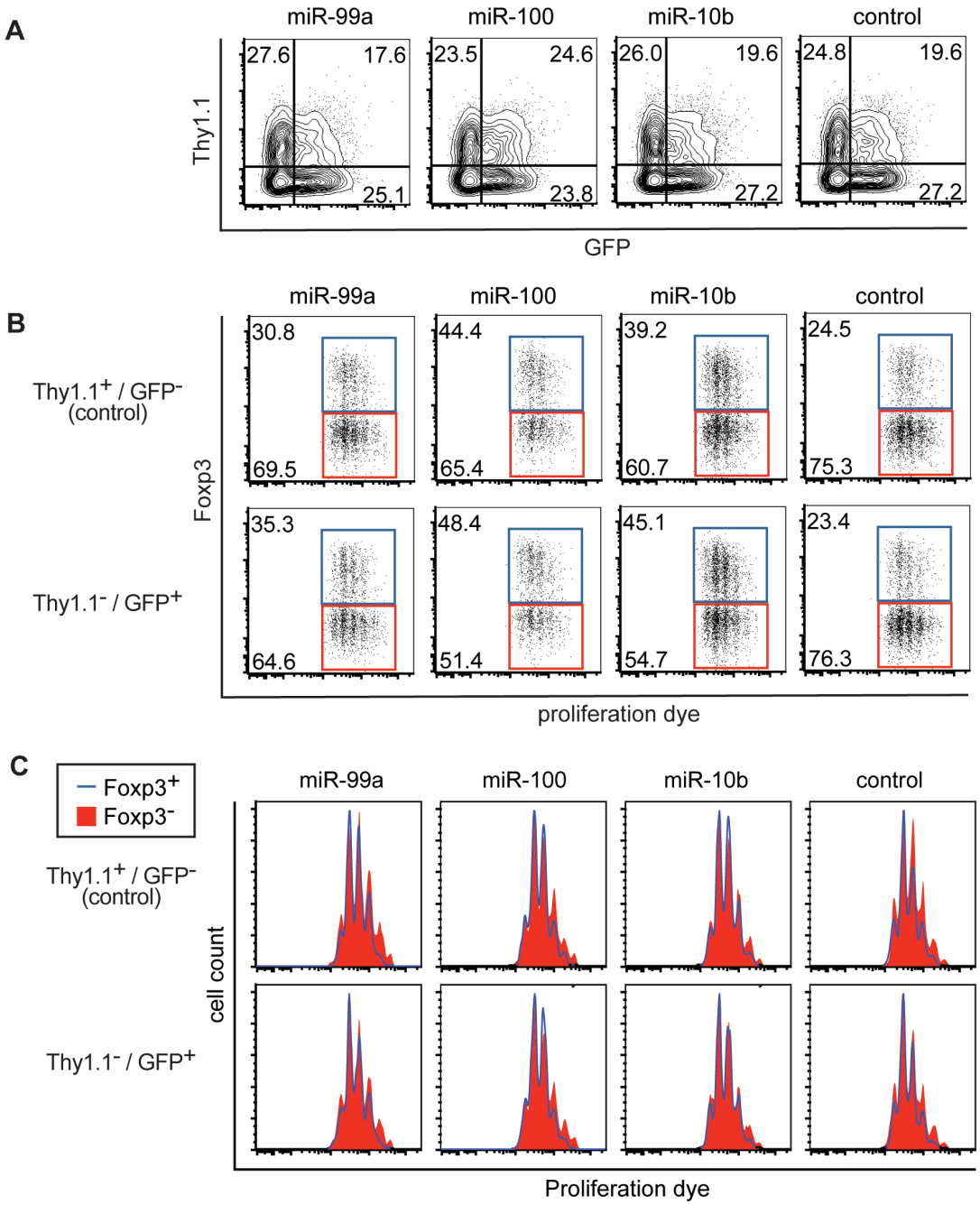

Figure 15: T cell proliferation is not affected by miR-99a, miR-100 and miR-10b overexpression. Naive CD4+ T cells purified from DO11.10tg; CARΔ1tg mice were labeled with proliferation dye. Then, cells were infected with one control adenovirus expressing the Thy1.1 marker or a second adenovirus expressing the marker GFP together with the indicated microRNA or control. Cells were mixed and rested for 40 h and stimulated subsequently with anti-CD3/ anti-CD28-coupled beads in Treg-polarizing conditions. After 72 h, expression of Foxp3 or infection marker expression as well as dilution of the proliferation dye was analyzed by flow cytometry. A) Contour plots showing the gating on Thy1.1+/ GFP- (control, upper left gate) or Thy1.1-/GFP+ (microRNA or control, lower right gate) populations. B) Dot blots show differentiation against proliferation markers of the Thy1.1-control virus infected cells and the GFP-microRNA virus or GFP-control virus-infected cells. C) Each histogram plot shows the overlay of Foxp3- (red areas) and Foxp3+ (blue lines) gates in the indicated populations. Data are representative of three independent experiments.

to control upper panel). These data confirm once again the cell-intrinsic promoting effects of miR-99a, miR-100 and miR-10b observed in the screen and reveal an additional cell-extrinsic effect on cells within the same well.

Importantly, the dilution of the proliferation dye was very similar between Foxp3$^+$ and Foxp3$^-$ cells within each plot (Figure 15B and C). Moreover, the dilution was also similar when comparing Thy1.1$^-$/ GFP$^+$, microRNA-expressing cells to control cells (Figure 15C, lower panels) as well as when comparing Thy1.1$^-$/ GFP$^+$ to Thy1.1$^+$/ GFP$^-$ cells (Figure 15C, lower versus upper panels). In conclusion, this suggest that miR-99a, miR-100 and miR-10b had no effect on T cell proliferation in Treg-polarizing conditions and, in particular, they did not have any effect on Treg cell proliferation. Hence, the promoting effects of these microRNAs could not be attributed to an altered cell proliferation.

7.5.3 T cell viability under miR-99a, miR-100 and miR-10b overexpression

MiR-99a and miR-100 have been associated with the induction of apoptosis in human esophageal squamous cell carcinoma (Sun et al., 2013). Therefore, viability was studied by analysis of dead cell stains in flow cytometry on miR-99a, miR-100 and miR-10b or control virus infected cells (Figure 16). There were no significant differences between microRNA or control virus-treated cells in the percentages of viable cells after 72 h in the standard Treg-polarizing conditions. Of note, the analysis at one time point could not provide a history of events as, for example, a previous effect on viability that was later on compensated by proliferation. However, the observed result of very similar proportions of viable cells at 72 h together with the observation of a very homogenous proliferation of microRNA-expressing

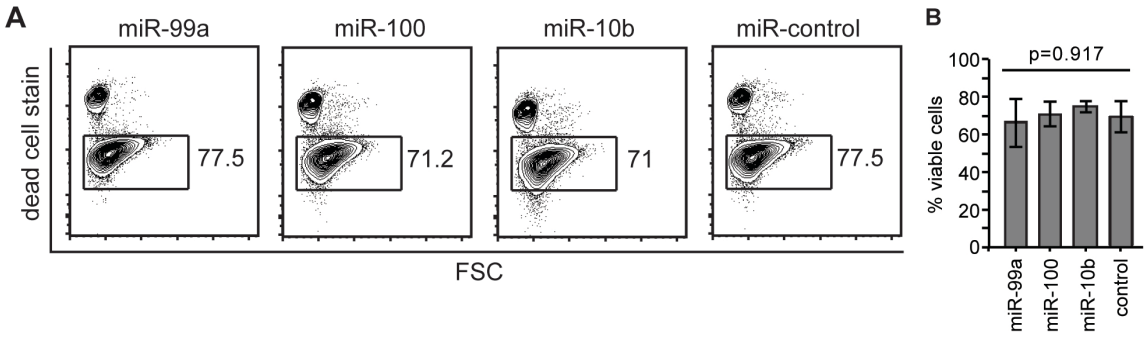

Figure 16: miR-99a, miR-100 and miR-10b do not affect viability during Treg cell induction.
Naive CD4$^+$ T cells purified from *DO11.10*tg; *CARΔ1*tg mice were infected with the indicated adenovirus. Cells were rested for 40 h and stimulated subsequently with anti-CD3/ anti-CD28-coupled beads in Treg polarizing conditions. After 72 h cell viability was analyzed using a dead cell staining dye. Representative FACS plots (A) and bar graph showing means ± SD (B) of four independent experiments and the p-value was calculated using one-way ANOVA.

and non-expressing cells strongly suggests, that there was no previous effect on viability that was compensated during differentiation.

7.5.4 T cell quiescence and activation in the presence of miR-99a, miR-100 or miR-10b overexpression

The Treg cell differentiation protocol used in this study involved resting of naive $CD4^+$ T cells after adenoviral gene transduction to allow overexpression of the transduced microRNA before activating the cells in Treg-polarizing conditions. The questions arose whether overexpression of the microRNA in naive T cells would have an influence on the naive state itself and whether activation of these cells was affected by the microRNA. To answer those questions, naive $CD4^+$ T cells were infected with miR-99a, miR-100, miR-10b or control adenovirus. The naive state was analyzed by flow cytometry after 40 h resting while the activated state was analyzed after cells had been rested for 40 h and subsequently been subjected to activation conditions for 18 h. All three microRNAs did not affect the naive state as defined by $CD62L^{high}/CD44^{low}$ expression compared to control (Figure 17A). Upon activation, downregulation of CD62L and upregulation of CD44 occurred similarly in microRNA or control virus-infected cells (Figure 17A). Judged by the expression of the activation marker CD25 as well as the early activation marker CD69, which were both not expressed in naive T cells but were highly upregulated in activated cells, there was also no difference between microRNA-overexpressing and control cells (Figure 17B). Expression of IL-7R, which was high in naive T cells and downregulated upon activation, and expression of the T cell receptor-induced protein CD5, which was low in naive T cells and increased upon stimulation showed again very similar patterns in microRNA-overexpressing and control samples (Figure 17C). Altogether, these data strongly suggested that miR-99a, miR-100 or miR-10b overexpression in naive T cells did neither alter the naive state nor the capacity or sensitivity of the T cells to become activated under our Treg differentiation-inducing conditions.

7.6 Target identification of miR-99a, -100 and -10b in Treg cell differentiation

MicroRNAs mediate their biological effects through repression of their target mRNA translation or by decreasing their stability. To further elucidate the microRNA function it was necessary to identify the respective mRNA targets.

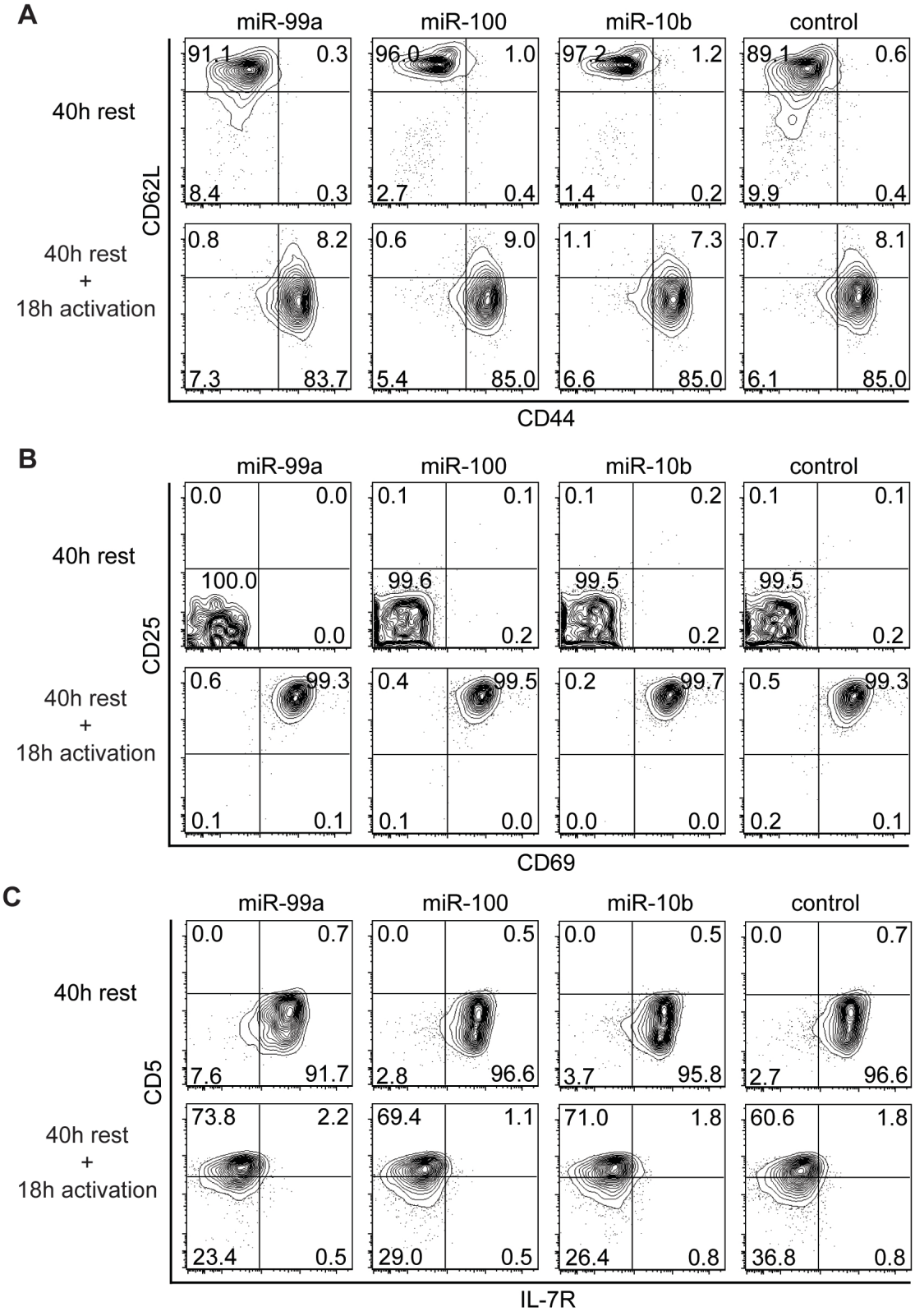

Figure 17: T cell quiescence and activation under miR-99a, miR-100 and miR-10b overexpression. Naive CD4$^+$ T cells purified from *DO11.10*tg; *CARΔ1*tg mice were infected with the indicated adenoviruses. Cells were rested for 40 h (upper panels) or rested for 40 h and stimulated subsequently for 18 h with anti-CD3/ anti-CD28-coupled beads in Treg-polarizing conditions (lower panels). A-C) Expression of surface markers was analyzed by flow cytometry. The data are representative of two or more independent experiments.

7.6.1 MicroRNA target prediction

The binding characteristics of microRNAs to their target sites within the 3'UTR of the target mRNA have been intensively studied and these published findings constitute the basis for

various microRNA target prediction algorithms that search for matches with annotated mouse 3'UTRs. To predict mRNA targets of miR-99a, miR-100 and miR-10b, the most common online tool TargetScan Release 6.2 and MicroCosm Targets Version 5 were used that base their search on the UCSC whole-genome alignment or the Ensemble annotation, respectively. Together, these tools cover a maximum of annotated transcripts. The prediction resulted in a long list of potential targets irrespective of their plausibility such as expression or function in T cell development. The search for an mRNA target of the identified microRNA candidates was narrowed down following the consideration, that a microRNA that promoted Treg cell differentiation should target an mRNA of a gene that itself is known to interfere with Treg cell differentiation.

7.6.2 MiR-99a directly targets Mtor mRNA

MiR-99a and -100 belong to the same microRNA family and share most of their sequence. Since they both promoted Treg cell differentiation in the screen, had significant effects in repeat experiments and had no significant effect but shared an adverse tendency on Th17 differentiation we focused on these microRNAs. Sun et al. already proposed mTOR as a target for miR-99a and miR-100 in human esophageal squamous cell carcinoma (Sun et al., 2013). Furthermore, mTOR was described as a promoter of Th17 differentiation as part of mTORC1 while deletion of mTOR in T cells abrogates effector T cell differentiation into several lineages and promotes Treg cell differentiation (Delgoffe et al., 2009; Shi et al., 2011). Indeed, TargetScan as well as Microcosm predicted conserved seed matches for miR-99a and miR-100 in the Mtor mRNA 3'UTR (Figure 18A). To confirm direct microRNA-target regulation, the Mtor 3'UTR was tested in a dual luciferase assay. To that end, the 3'UTR of Mtor was cloned just 3' to the renilla luciferase coding sequence into an adenoviral dual luciferase reporter construct that coexpressed the firefly luciferase reporter from an independent promoter. A wildtype mouse embryonal fibroblast (MEF) cell line was adenovirally infected with the dual luciferase reporter and co-infected with miR-99a, miR-10b or control virus. Preparing cell lysates and measuring dual luciferase activities, the miR-99a co-expression strongly reduced the ratio of renilla to firefly luciferase activities indicating repression of Mtor 3'UTR by miR-99a compared to control co-infections (Figure 18B). MiR-10b, which was not predicted to bind to Mtor 3'UTR, accordingly did not reduce the relative

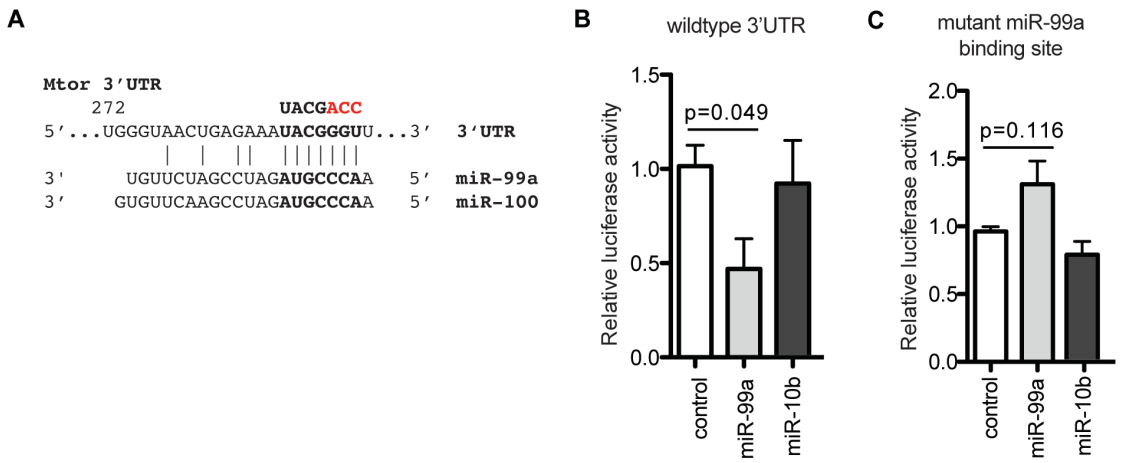

Figure 18: Mtor is a direct target of miR-99a/100.
A) Base pairing of the microRNA with its target sequence based on the TargetScan mouse 6.2 prediction. The seed sequence is displayed bold, seed mutations used in luciferase assays are displayed in red, the numbers indicate the position within the Mtor-3'UTR. B, C) MEF cells were infected with adenovirus encoding a wildtype (B) Mtor 3'UTR dual luciferase reporter construct or one with mutated miR-99a/100 target site (C). Cells were co-infected with adenovirus encoding the indicated microRNA or control adenovirus. After 48 h the cells were lysed and relative luciferase activities was determined. The values are means ± SD from four independent experiments, the p-values were calculated using the Student's t-test.

renilla luciferase activity. In order to prove direct regulation, the miR-99a binding site was mutated such that the seed sequence of miR-99a between position 2-4 was no longer complementary to the mutated Mtor 3'UTR (Figure 18A). As expected, miR-99a no longer repressed the luciferase activity placed under the control of the mutated 3'UTR, which proved that Mtor was in fact a direct target of miR-99a (Figure 18C).

7.6.3 Expression of Mtor mRNA and mTOR protein during Treg cell induction

A microRNA regulation can only exert biological effects if the mRNA target containing the microRNA target site is actually expressed in the respective cell. Therefore, we analyzed expression of Mtor mRNA and mTOR protein during Treg cell induction (Figure 19A–B). Mtor mRNA was downregulated several folds within 12-24 h of Treg cell induction and re-induced from 36 h to 72 h. Astonishingly, mTOR protein expression displayed a partly opposite kinetics with a constant increase after 24 h. This uncoupling of protein expression from mRNA abundance is reminiscent of the dynamic regulation of post-transcriptional regulation mechanisms described in the first part of this study. These observations suggested that post-transcriptional gene regulation of Mtor by miR-99a/100 was gradually relieved after 24 h of Treg cell induction and would rather influence differentiation early after induction. This is consistent with the proposed window of opportunity for post-transcriptional gene regulation in Treg cell differentiation.

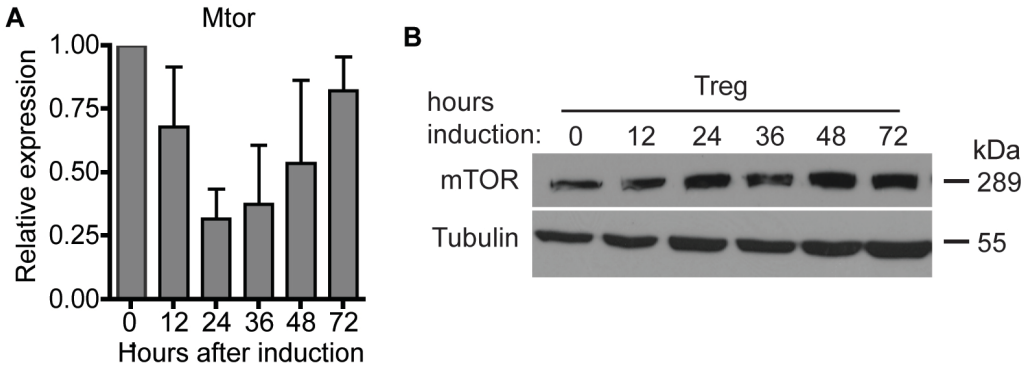

Figure 19: Expression of Mtor mRNA and mTOR protein during Treg cell induction.
Naive CD4$^+$ T cells purified from *DO11.10*tg; *CARΔ1*tg mice were stimulated in Treg-polarizing conditions for the indicated time. A) Expression of Mtor mRNA was determined by qPCR relative to HPRT. Data were normalized on the 0 h time point and represent means ± SD of three independent experiments. B) mTOR protein expression was analyzed by immunoblot at the indicated time points. Data are representative of two independent experiments.

7.6.4 MiR-99a overexpression reduced mTOR protein but not mRNA expression

Next we sought to evaluate, whether overexpression of miR-99a/100 during standard Treg cell induction conditions could influence expression of its target mRNA or protein levels in T cells as well. Although high levels of overexpression for miR-99a, miR-100 and miR-10b were determined by qPCR analysis (Figure 20A), the detection of Mtor mRNA expression in these samples did not show significant changes (Figure 20B). So we asked if the direct regulation by miR-99a/100 that was observed in the luciferase assays might rather be detectable on the protein level. Indeed, we found a strong reduction of mTOR protein expression 18h after activation when miR-99a was overexpressed compared to control virus or miR-10b infected cells (Figure 20C). Thus we obtained proof that mTOR is regulated by miR-99a in T cells and thereby uncovered one possible mechanism for the promoting effect of miR-99a overexpression on Treg cell induction.

7.6.5 Rora as a target mRNA for miR-10b

MiR-10b had no effect on the Mtor mRNA 3'UTR as concluded from the dual luciferase assay, so it must exert its effect on Treg cell differentiation via different targets. The Microcosm target search tool was used to predict mRNAs with 3'UTRs containing seed matches for miR-10b. After filtering the target list for plausibility requirements that demanded for a target that interfered with Treg cell differentiation, we proposed Rora mRNA

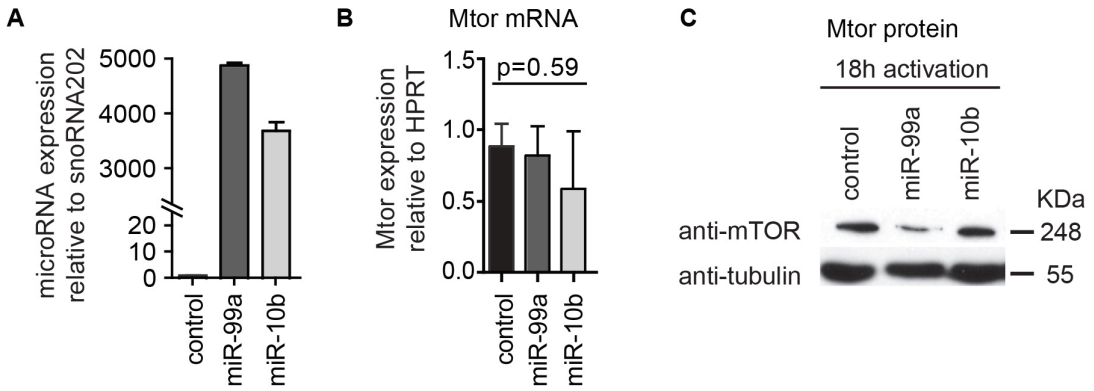

Figure 20: MiR-99a overexpression inversely correlated wih mTOR protein expression.
Naive CD4$^+$ T cells were purified from *DO11.10*tg; *CARΔ1*tg mice and transduced with the indicated adenovirus, then rested for 40 h and activated in Treg-polarizing conditions for 18 h (A and B). A) microRNA overexpression of the indicated microRNA was determined by qPCR relative to SnoRNA202. Data were normalized to control virus-infected samples and are representative of two or more independent experiments. B) qPCR analysis of Mtor mRNA expression, data were normalized on a control virus infected sample and represent means ± SD of three independent experiments and the p-value was calculated using one-way ANOVA.C) CD4 T cells were purified from *DO11.10*tg; *CARΔ1*tg mice and transduced with the indicated adenovirus, then rested for 24 h and activated in Treg-polarizing conditions for 18 h. Western blot analysis of mTOR and tubulin protein expression, the shown data are representative of two independent experiments. Immunoblot analyses were performed in collaboration with Christine Wolf.

as a potential miR-10b target (Figure 21A). Moreover, the Mirocosm algorithm predicted Rora 3'UTR to be targeted also by miR-99a/100, which involved a possible cooperativity in the Treg-promoting effects of miR-99a/100 and miR-10b. The *Rora* gene encodes for RORα, which is highly expressed in Th17 cells and its expression can be induced by TGFβ and IL-6, the cytokines that promote Th17 differentiation (Yang et al., 2008). Overexpression of Rorα promoted Th17 differentiation in Th17-polarizing conditions and conversely interfered with Treg cell differentiation in Treg-polarizing conditions. Interestingly, RORγt overexpression did not interfere with Treg cell differentiation under these circumstances (Yang et al., 2008). In order to see whether the predicted microRNAs would target Rora, the 3'UTR of the gene was cloned and tested in dual luciferase assays in MEF cells together with miR-10b, mir-99a or control (Figure 21B). MiR-10b co-expression markedly reduced the relative renilla luciferase activity indicating that the Rora 3'UTR is repressed by miR-10b. MiR-99a co-expression also resulted in a significant, yet moderate reduction in luciferase activity. The formal proof for direct regulation through the predicted target site by testing the 3'UTR with a binding site mutation still has to be obtained. Nevertheless, these results show the downregulation of the Rora 3'UTR by miR-10b and to a lesser extent by miR-99a and thereby suggest Rora as a new microRNA target in the context of Treg cell differentiation.

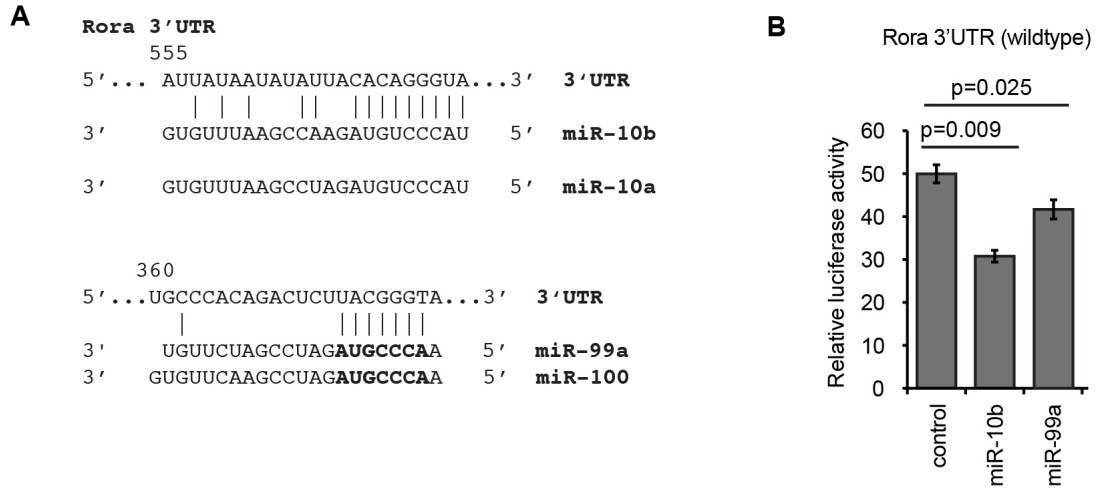

Figure 21: Rora is a target of miR-10b.
A) Base pairing of miR-99a, miR-100 and miR-10b with its target sequence in Rora-3'UTR based on microcosm target search output. The seed sequence is displayed bold, and the numbers indicate the position within the respective 3'UTR. B) MEF cells were infected with adenovirus encoding a wildtype Rora 3'UTR dual luciferase reporter construct together with adenovirus encoding the indicated microRNA or control adenovirus. Cells were lysed after 48 h and relative luciferase activity was determined. The values are representative of three independent experiments. P values were calculated using Students t-test on replicates.

7.7 Mtor and Rora are both targets of miR-150

Surprisingly, the examination of the 3'UTR of Mtor and Rora identified a predicted binding site for miR-150 in both of them (Figure 22A). Intriguingly, miR-150 had also been identified as a Treg-promoting microRNA in my screen (see Figure 13D). To analyze, whether the predicted targets were actually regulated by miR-150, the 3'UTRs of Mtor and Rora were tested in luciferase assays. Co-transduction of renilla luciferase fused to the Mtor-3'UTR construct with miR-150 reduced the relative luciferase activity by 50% compared to co-transduction with control virus (Figure 22B). The repression was in a similar range as with miR-99a co-transduction. Mutation of the miR-150 binding site reverted the regulation by mir-150 but not by miR-99a indicating direct regulation of Mtor mRNA by miR-150 (Figure 22C). For Rora, we observed a reduction of the relative luciferase activity by almost 50% when co-expressing miR-150, which was very similar to the effects observed with miR-10b expression (Figure 22D). Altogether, these results show that miR-99a and miR-10b share mRNA targets with each other and with miR-150 and raised the question, how these microRNAs cooperate during Treg cell induction.

7.8 MiR-99a/100 and miR-10b can be induced by all-trans retinoic acid

To elucidate, how miR-99a/100, miR-10b and miR-150 may cooperate in Treg cell-induction, we studied the relative expression of miR-99a/100, miR-10b and miR-150 over the period of

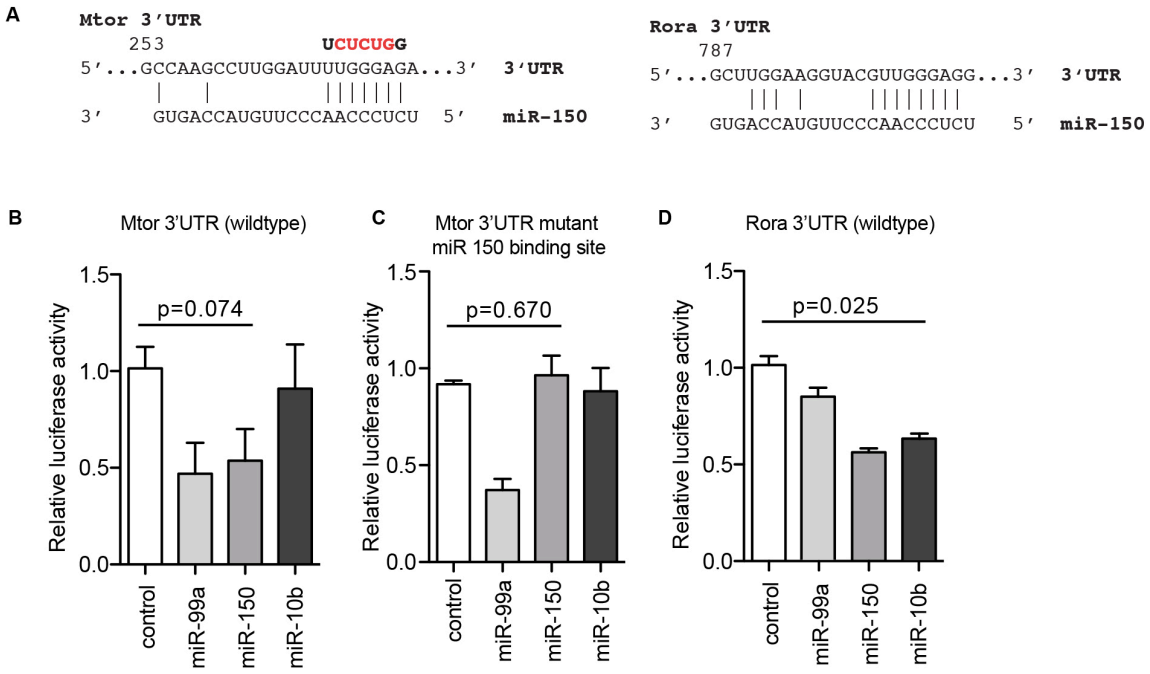

Figure 22: Mtor and Rora are targets of miR-150.
A) Base pairing of miR-150 with its target sequence in Mtor or Rora 3'UTR based on TargetScan mouse 6.2 or Microcosm prediction. The seed mutation used in luciferase assays is displayed in red, and the numbers indicate the position within the respective 3'UTR.
B, C) MEF cells were infected with adenovirus encoding a wildtype mTOR 3'UTR dual luciferase reporter construct or a similar construct with a mutated miR-99a/-100 target site. Cells were co-infected with adenovirus encoding the indicated microRNA or control adenovirus. After 48 h cells were lysed and relative renilla luciferase activity was determined. The values are means ± SD from four independent experiments; P-values were calculated using Student's t-test. D) MEF cells were infected with adenoviruses encoding a wildtype Rora 3'UTR dual luciferase reporter construct together with adenovirus encoding the indicated microRNA or control adenovirus. Cells were lysed after 48 h and relative luciferase activity was determined. The values are representative of three independent experiments. P-values were calculated using Student's t-test on replicates.

72 h of Treg cell differentiation (Figure 23A-D). MiR-99a/100, which were both recognized by the same qPCR assay, were detected in naive T cells, but downregulated to about 50% within the first 24 h of activation. MiR-10b was merely unchanged after Treg cell induction. MiR-150 was expressed at higher levels in naive T cells and during the first 24 h of Treg cell induction and became strongly reduced afterwards. This implied, that microRNA-99a/100, miR-10b and miR-150 could post-transcriptionally affect the window of opportunity of Treg cell induction. Comparing the expression levels of miR-99/-100, miR-10b and miR-150 relative to snoRNA202 without additional normalization, revealed extreme differences in the abundance of the different microRNAs (Figure 23D). MiR-150 was much more abundant compared to miR-99a/100, whose expression was three orders of magnitude lower. MiR-10b expression in turn was even 100-fold lower than miR-99a/100 expression levels. Given the high expression of miR-150 compared to miR-99a/100 and miR-10b we asked, whether

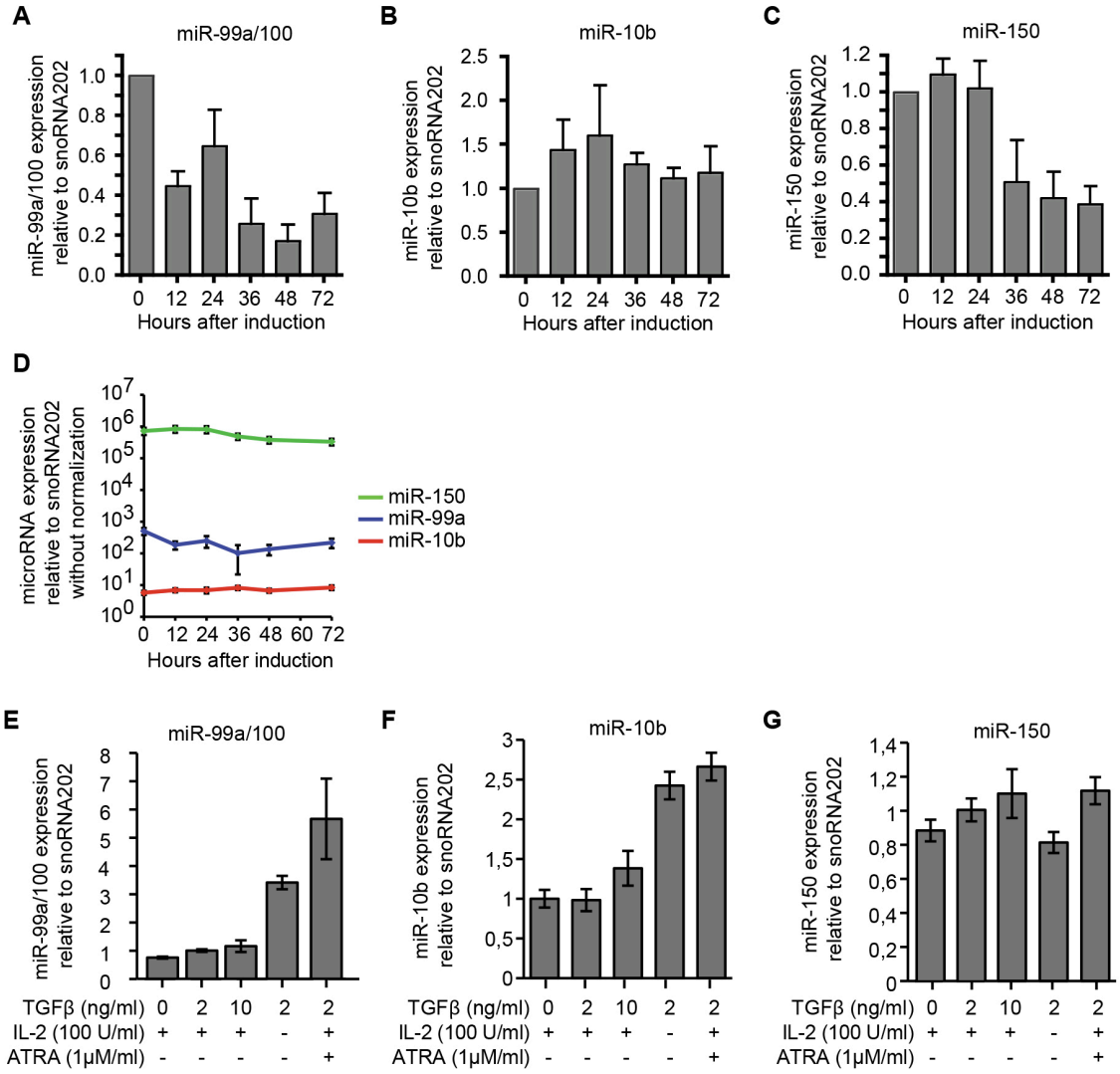

Figure 23: Mir-99a/100 and miR-10b expression are induced by *all-trans* retinoic acid during Treg cell induction.
Naive CD4$^+$ T cells purified from *DO11.10*tg; *CARΔ1*tg mice were stimulated in Treg-polarizing conditions for 72 h. Expression of the indicated microRNA was determined by qPCR relative to SnoRNA202. A–C) Relative levels of microRNA expression normalized to the 0 h value. Data are representative of two or more independent experiments. D) Relative expression levels of microRNAs without normalization. Data are representative of two or more independent experiments. E-G) Naive CD4$^+$ T cells purified from DO11.10tg; CARΔ1tg mice were stimulated for 18 h in Treg-polarizing conditions as indicated and microRNA expression was analyzed by qPCR. Data were normalized on standard conditions and represent means ± SD of two (0 and 10 ng/ml TGFβ), three (no IL-2), to five (2 ng/ml TGFβ, 0.1 and 1 μM ATRA) independent experiments. ATRA, *all-trans* retinoic acid.

expression of the latter could be induced under condition that are known to induce Treg cell differentiation. To explore potential inducing conditions, we first tested different TGFβ concentrations, which promoted Treg cell differentiation in a dose-dependent manner, but this

did not alter the expression levels of miR99a/miR100, miR-10b and miR-150 (Figure 23E-G). Unexpectedly, withdrawal of IL-2 from the Treg cell-induction cocktail led to a 3.5-fold induction of miR-99a/100 and a 2.5-fold induction of miR-10b. *All-trans* retinoic acid (ATRA) had already been implicated in regulation of miR-10b (Foley et al., 2011; Meseguer et al., 2011). Furthermore, ATRA promoted Treg cell differentiation in a both intrinsic and extrinsic manner (Hill et al., 2008; Mucida et al., 2007). When testing supplementation of the Treg cell differentiation conditions with 1 µM ATRA, a 6-fold induction of miR-99a/100 as well as a 2.5-fold induction of miR-10b became evident.

Collectively, these data suggest that the highly expressed miR-150 alone may suppress critical Treg-inappropriate target genes to promote Treg cell differentiation, which may be markedly augmented by a collaboration with miR-150 with miR99a/100 and miR-10b upon their induction with retinoic acid.

8 Discussion

8.1 The kinetics of Treg cell differentiation

We set out to identify microRNAs that could promote Treg cell differentiation in a functional screen. To be able to conduct such a microRNA overexpression screen in an *in vitro* model of Treg cell differentiation, the kinetics of several parameters had to be evaluated.

First, we established the expression of Foxp3, which is the hallmark feature of the Treg cell transcriptional program and the marker of the Treg cell lineage. The expression of Foxp3 in the *in vitro* Treg cell differentiation model initiated already at 36 h after activation and reached its full extent at 48 h. Notably, Foxp3 showed no obvious signs of post-transcriptional gene regulation as the protein expression levels closely matched the mRNA expression levels. The percentage of $Foxp3^+$ cells remained constant after 48 h and was the result of equal proliferation in $Foxp3^+$ and $Foxp3^-$ cells (Figure8 A-C, Figure 8C). Together, these kinetics justified the intracellular staining of Foxp3 at 72 h and the analysis of differentiation after the cells had vigorously proliferated.

Second, it was important to determine the time frame during which microRNA regulation can be effective during Treg cell differentiation. The Ago proteins are essential and limiting, since overexpression of Ago proteins increased the abundance of microRNAs and enhanced microRNA-mediated regulation (Winter and Diederichs, 2011). Ago proteins were shown to be strongly downregulated 48 h after T cell activation in Th1 conditions (Bronevetsky et al., 2013). MicroRNA expression in cells with Ago1 or Ago2 deletion is markedly decreased, thereby suggesting that the microRNA half-life is reduced, when Ago proteins are downregulated (Bronevetsky et al., 2013; Wang et al., 2012).

My results show that downregulation of all Ago proteins also occurs during Treg cell differentiation 48 h after T cell activation similar to Th1 cells. This led us to assume that a global microRNA expression control on the level of Ago regulation may be also active during Treg cell induction. Accordingly, microRNA overexpression would best affect Treg cell differentiation within 48 h after activation.

The expression of transcripts with shortened 3'UTRs that no longer contain distal microRNA target sites is another limiting factor for microRNA-mediated silencing. Consequently, microRNAs will be most effective to regulate Treg cell differentiation before the onset of this

process. To take that into account for our microRNA screen, we exemplarily analyzed the expression kinetics of Eri1 mRNA isoforms. We observed a shortening already at 12 h and re-expression of the long isoform at 36 h after Treg cell induction. A study that globally compared 3'UTR lengths in 6 h or 48 h-activated T cells to that in naive T cells describes significant shortening of 3'UTRs for 99 different transcripts, which resulted from alternative PAS, at 48 h but not at 6 h after T cell activation (Sandberg et al., 2008). Considering the different time points of analyses, this 3'UTR shortening overall follows a similar kinetics as Eri1 3'UTR shortening. Yet the re-expression of longer Eri1 isoforms suggests a somewhat earlier initiation of the kinetics in our experiments. This could be a consequence of different activation and polarization conditions. Alternatively, 3'UTR length variation could also be regulated in a transcript-specific manner with particular kinetics for different transcripts.
Sandberg et al. also found transcripts with a switch of the terminal exon after activation, which implicates alternative splicing as another, potentially independent mechanism for alternative 3'end formation. However, for Eri1 only one splice variant is annotated and thus alternative PAS usage likely accounts for the observed 3'end shortening. Future northern blot experiments will finally provide a comprehensive picture of length and quantity of all Eri1 transcript variants generated during Treg cell differentiation.
The shortening of the Eri1 3'UTR again suggests that the effect of microRNA on Treg cell differentiation may be most pronounced early after T cell activation or even concomitantly with initiation of TCR signaling. Regardless of the kinetics of individual transcripts, these findings strongly argued for an experimental set-up that allowed the modulation of microRNA expression in naive T cells at the very moment of T cell activation and throughout differentiation.

Notably, the Eri1 3'UTR shortening after 12 h correlated with the observed induction of cleavage stimulating factor 64 (CstF64) 12 h after T cell activation (Chuvpilo et al., 1999). CstF64 is part of the 3'end processing machinery that forms the mRNA 3'end during transcription. It has a high affinity for an alternative PAS, which was suggested to promote the use of a different terminal exon by alternative splicing. The knock-down of another 3'end processing factor, CfIm68, was shown to induce 3'UTR shortening resulting from preferential use of proximal PAS (Martin et al., 2012). Taken together this may suggest common mechanisms for PAS selection that can result in alternative splicing or 3'UTR shortening.

Several studies suggested a connection between 3'UTR shortening and cell proliferation implying that microRNAs repress proliferation in certain conditions (Bava et al., 2013; Kumar et al., 2007; Sandberg et al., 2008). Our analyses of cell proliferation kinetics during Treg cell differentiation detected the first divided cells 36 h after activation. This is clearly delayed compared to the 3'UTR shortening of Eri1, which initiated already after 12 h of T cell activation. The shortening may be a mechanism to shift cellular programs from differentiation, which required microRNA regulation, to cell proliferation that occurred in conditions with reduced microRNA regulation. In line with this concept, the marked downregulation of Ago proteins in the course of T cell activation may further diminish microRNA regulation. These mechanisms may exist in parallel or even in a successive order and could be regulated in an interdependent manner, for example by mTOR. As discussed below, Mtor may escape post transcriptional regulation by 3'UTR shortening similar to Eri1 and was shown to contribute to the downregulation of Ago after T cell activation (Bronevetsky et al., 2013).

8.2 The feasibility of adenoviral gene transfer to modulate Treg cell differentiation

The window of opportunity for microRNA regulation of the Treg cell differentiation program opens with the activation of cells by TCR triggering. Therefore, for perfoming an overexpression screen we sought an experimental system that provides overexpression at the time or even before activation of naive T cells. Current methods for ectopic gene expression in naive T cells have different disadvantages. For example, electroporation causes membrane depolarization and Ca^{2+}-influxes that are reminiscent of T cell activation and lentiviral approaches require cytokine stimulation (Circosta et al., 2009; Patel and Muthuswamy, 2012). The adenoviral transduction system can circumvent these disadvantages, if the experimental setup allows using naive $CD4^+$ T cells from CARΔ1 tg mice (Wan et al., 2000b). We described the setup, application and validation of adenoviral transduction of naive $CD4^+$ T cells in our recent manuscript (Warth and Heissmeyer, 2013). The validation experiments demonstrate the efficient infection of naive $CD4^+$ T cells isolated from commercially available *DO11.10*tg *CARΔ1*tg mice, the maintenance of the naive state after infection at an MOI of 50 and unaltered differentiation into Treg cells (Figure 10). Exemplary infection with a pri-miR-155 expressing adenovirus resulted in highly elevated levels of mature miR-155 overexpression on top of the activation-induced expression of miR-155. Taken together, the

adenoviral overexpression system was an indispensible prerequisite for a microRNA overexpression screen that can impact Treg cell differentiation from the very beginning and throughout the “window of opportunity”.

The potency of adenoviral manipulation of Treg cell differentiation was demonstrated by overexpression of the transcription factors human FOXP3 and AP1. HumanFOXP3 overexpression strongly promoted Treg cell differentiation. This was in agreement with reports showing that Foxp3 overexpression promoted Treg cell features in CD4 T cells and that Foxp3 could promote its own expression by binding to the CNS2 enhancer element in the Foxp3 locus (Fontenot et al., 2003; Hori et al., 2003; Zheng et al., 2010).

Conversely, an overexpression of AP1 had a strong inhibitory effect on Treg cell differentiation consistent with the described role of AP1 in the induction of prototypic effector T cell genes such as IL-2 (Jain et al., 1992). This is in contrast to a report that claimed a positive effect of AP1 on Foxp3 induction, because pharmacologic inhibition of c-JUN by an N-terminal Jun kinase inhibitor strongly reduced Treg cell differentiation as well as the activity of a Foxp3 reporter construct (Nguyen et al., 2010). These differences could result from different binding characteristics of the canonical AP1 dimer composed of c-JUN and c-FOS and of a c-JUN homodimer. The abundance of c-Fos was found to be the limiting factor for IL-2 production in activated human memory T cells (Bendfeldt et al., 2012). Therefore the expression level and the stoichiometry of c-JUN homo- or heterodimers may direct different outcomes that will either induce an effector or a regulatory T cell differentiation program.

The strong modulation of Treg cell differentiation by overexpression of transcription factors that are either promoting or inhibiting illustrates the power of adenoviral gene transduction into naive T cells. It is also a very evident example of the concept of ‘relative differentiation’ that was applied to analyze screen data. The dynamic range of 0.25-1.8 achieved with overexpression of these transcription factors was stronger than that obtained with microRNAs overexpression in my screen, which ranged from 0.5 and 1.5. In my experimental setup, an overexpressed microRNA acts in conjunction with endogenously expressed microRNAs. It is therefore surprising that this network of indirect modulation by post-transcriptional gene regulation almost reached the effect of very potent transcription factors, which directly trans-activate gene expression programs.

8.3 A functional screen identified microRNAs effective in Treg cell differentiation

We set out to identify the individual microRNAs that direct the process of Treg cell differentiation. To that end a functional screening approach was projected in order to test overexpression of T cell-expressed microRNAs in a model of Treg cell differentiation.

Our screen identified a multitude of microRNAs that affected Treg cell differentiation. To further strengthen the confidence in the screening results we compared our results with published data.

In our screen, the overexpression of each miR-17~92 cluster construct containing either miR-17 alone or miR-17, 18a and 19a or miR-20a, 19b-1 and 92a-1 interfered with Treg differentiation (Figure 13D). This was consistent with a reconstitution study in miR-17~92 cluster knock-out T cells, where reconstitution of miR-17 or miR-19b alone blocked Treg cell differentiation and promoted Th1 cell differentiation (Jiang et al., 2011). Noteworthy, that study applied retroviral transduction to overexpress the microRNA in late Treg differentiation. This suggests that the miR-17~92 cluster can influence Treg cell differentiation at early and late stages. MiR-10a was recently shown to be highly expressed in mature Treg cells and suggested to contribute to Treg cell lineage stability, whereas its deletion had no influence on differentiation of Treg (Jeker et al., 2012; Takagaki et al., 1996). In our screen, miR-10a overexpression interfered with Treg cell differentiation showing that microRNAs with a role in mature Treg cells can, upon overexpression, have an opposing role in the differentiation of Treg cells. Like miR-10a, miR-146a, which is important for Treg cell function, showed an inhibitory effect on differentiation in my screen (Lu et al., 2010). O'Connell et al. have shown that miR-155 contributes to Th17 cell differentiation (O'Connell et al., 2010). Consistently, miR-155 overexpression exhibited an opposing effect on Treg cell differentiation in our screen suggesting a potential role of miR-155 in the reciprocal Th17/Treg cell lineage decisions. For miR-326, Du et al. found a promoting effect selectively on Th17 differentiation and no effect on Treg cell differentiation (Du et al., 2009). This was similarly observed in our screen. Altogether, the comparison of microRNA effects in our screen largely overlapped with published effects of those microRNAs in T cell differentiation. This consolidated the screening data and provided a basis to further analyze the newly identified microRNAs.

We then focused mainly on the candidates with the strongest effects on Treg cell differentiation. A secondary screen with selected candidates in Th17 differentiation was pursued to exclude those microRNAs with a general effect. Indeed, microRNAs like miR-181, which interfered with Treg cell differentiation, also interfered with Th17 cell induction. MiR-181 was actually shown to target the mRNAs of phosphatases involved in regulation of TCR signaling like PTEN and Shp-1, which negatively regulate TCR signaling and thus may affect common pathways in Treg and Th17 cell differentiation (Li et al., 2007).
Several microRNAs like miR-140 or miR-99a that were effective in Treg cell differentiation showed either no effect or even a reciprocal tendency in Th17 cell differentiation. We concluded that these microRNAs conferred selective effects that were either promoting or interfering with Treg cell induction. To address the initial question, which microRNAs are required for proper Treg cell differentiation, we followed up on the microRNAs miR-10b, miR-99a and miR-100 that selectively promoted Treg cell differentiation. MiR-10b expression was found associated with tumor cell migration and metastasis (Ma et al., 2007; Tian et al., 2010). Notably, miR-10b expression could be induced by the transcription factor Twist1, which is a negative regulator of Th1 cell induction and IFNγ production (Ma et al., 2007; Pham et al., 2012). It is therefore tempting to speculate that Twist1 and miR-10b may synergize in Treg cell induction. In contrast to miR-10b, miR-10a had a strong negative effect on Treg cell differentiation. This is astonishing as these microRNAs only differ by one base pair outside of the seed region. Apart from many shared targets, the Microcosm algorithm predicted several targets for miR-10b, like Rora, which were not predicted for miR-10a. We demonstrated Rora targeting by miR-10b and further 3'UTR reporter experiments with miR-10a will be performed to confirm the predicted selective targeting.

MiR-99a and miR-100 belong to the same microRNA family and differ by only two bases outside of the seed sequence (Figure 18). MiR-99a and miR-100 were downregulated in various cancer cells and there is good evidence for mTOR, which promotes cell growth and is required for proliferation, to be a target of both microRNAs (Nagaraja et al., 2010; Sun et al., 2013). MiR-99a was upregulated in a radiosensitive cancer cell line upon irradiation and targets the chromatin remodeling factor SNF2H, which interfered with DNA damage repair as part of the SWI/SNF complex (Mueller et al., 2012). This complex can differentially regulate chromatin accessibility in T cells and directly induced the expression of AP1 (Mueller et al., 2012). Consequently, since AP1 activity strongly impaired Treg cell induction, its putative downregulation by miR-99a or miR-100 may be another attractive mechanism to explain the

promoting effects of miR-99a and miR-100 on Treg cell differentiation (Figure 11D-F). The selective effect of miR-10b, miR-99a and miR-100 on Treg cell but not on Th17 cell differentiation already implied that these microRNAs had no general effect on cellular processes. Further validation experiments specifically excluded an influence on proliferation or cell viability. Importantly, also the quiescent state of naive T cells prior to activation was not altered by miR-10b, miR-99a or miR-100 overexpression.

8.4 MTOR and RORα are central players in reciprocal lineage differentiation

MiR-99a and miR-100 had promoting effects on Treg cell differentiation and showed a tendency to inhibit Th17 cell differentiation in our experiments. We and others showed that Mtor mRNA is a direct target of miR-99a and miR-100 and, in addition, we established Mtor as a target for miR-150 (Nagaraja et al., 2010; Sun et al., 2013). MTOR is a central protein in reciprocal lineage differentiation (Delgoffe et al., 2009). It inhibits Treg and promotes Th17 cell differentiation and is thus an attractive target. Deletion of mTOR results in strongly increased Treg cell differentiation upon activation (Delgoffe et al., 2009). The existence of mTOR in the two different complexes mTORC1 and mTORC2 complicates the dissection of individual contributions. Deletion of Rheb resulting in mTORC1 inactivation does not influence Treg cell induction while mTORC1 activity is essential for Th17 differentiation (Delgoffe et al., 2009, 2011; Kim et al., 2013). Thus it is conceivable, that either mTORC2 activity alone is sufficient to interfere with Treg cell differentiation or that mTORC1 and mTORC2 act redundantly. MTORC2 but not mTORC1 activity is required for phosphorylation of Akt on Ser473, which enables the inactivation of Foxo transcription factors by phosphorylation through Akt (Jacinto et al., 2006). Since Foxo proteins are instrumental for Treg cell differentiation, it is suggestive that mTORC2 alone can repress Treg differentiation (Kerdiles et al., 2010). Yet this is in contrast with data on a central role of the mTORC1 target HIF1α in promoting Th17 and inhibiting Treg differentiation (Dang et al., 2011; Shi et al., 2011). Altogether, mTOR function in reciprocal lineage differentiation is beyond a simple switch as the different functional complexes regulate independent aspects of Treg inhibition or Th17 promotion. This is consistent with a strong promoting effect of miR-99a and miR-100 in Treg cell differentiation but a rather mild inhibitory effect on Th17 cell differentiation.

During Treg cell differentiation, the expression of Mtor mRNA continuously decreased during the first 24 h of Treg cell differentiation whereas the mTOR protein levels increased

(Figure 19). This could be explained with a higher translational efficiency of the Mtor mRNA after activation, potentially by escaping microRNA-regulation through shortening of the 3'UTR, as observed for Eri1 mRNA during Treg cell induction. 3'end sequencing data in various mouse tissues did not find alternative lengths of the 3'UTR encoded in the most 3' exon (Derti et al., 2012). Yet it identified two mTOR isoforms that may result from alternative splicing, since at least one of them ends in intronic canonical PAS. Further qPCR analyses or northern blot experiments will show, whether alternative splicing modulates the sensitivity of Mtor for post-transcriptional gene regulation during Treg cell differentiation.
The assumed increase in translational efficiency of Mtor suggests that miR-99a, miR-100 and miR-150 repression of Mtor could be most effective in early Treg cell differentiation. This is in line with data showing that pharmacologic inhibition of mTOR with rapamycin promoted Treg cell differentiation and had the strongest effect 18h after Treg cell induction (Sauer et al., 2008). Consistently, miR-99a overexpression resulted in a strong reduction of mTOR protein levels 18h after Treg cell induction in my experiments (Figure 20).
Taken together, mTOR regulation by miR-99a, miR-100 and miR-150 levers at a critical and central player to promote the Treg cell lineage decision.

We established Rora as a target of the Treg cell promoting microRNAs miR-10b, miR-150 and miR-99a. Whereas RORα and RORγt synergized in the induction of Th17 differentiation, only RORα was required to suppress the Treg cell differentiation program (Yang et al., 2008). A knock-out of RORα in T cells led to differentiation of naive T cells into Treg cells upon activation, even in Th17-polarizing conditions. Conversely, in Treg-polarizing conditions, overexpression of RORα alone repressed Treg cell differentiation by 50% whereas RORγt alone had no effect on differentiation (Yang et al., 2008). In the human Jurkat cell line, Foxp3 directly interacted with RORα in overexpression experiments and thereby inhibited transcription of RORα target genes (Du et al., 2008). This could provide a molecular basis for the reciprocal function of RORα in Treg versus Th17 cell differentiation.
A recent study showed a negative role of RORα in the development of Tfh cells, where RORα promoted transcriptional upregulation of *Ccr6, Il1r2, Il1r1* and *Il22* in a dose-dependent manner (Baumjohann et al., 2013). All these genes were associated with Th17 differentiation and function and were therefore termed 'Tfh-inappropriate' genes (Hirota et al., 2007; Liang et al., 2006; Shaw et al., 2012). Interestingly, in Tfh cells Rora is repressed by miR-17 and miR-92 from the miR-17~92 cluster. In analogy to that, miR-10b and miR-150 likely suppress the expression of RORα and its downstream-induced 'Treg-inappropriate'

genes during Treg cell differentiation. Interestingly, RORα has been demonstrated to interact with HIF1α, which is induced by mTORC1 activity (Kim et al., 2008; Shi et al., 2011). In cell line experiments, RORα induced HIF1α expression and synergized with HIF1α in the trans-activation of a reporter linked to target gene promoter sequences (Kim et al., 2008). Conversely, HIF1α was a direct transcriptional activator of RORα in HepG2 cell lines (Chauvet et al., 2004). This cross talk between the Th17-promoting functions of RORα and HIF1α downstream of mTOR suggests a feed forward loop of these pathways in Th17 differentiation. The microRNAs identified in our screen may therefore provide a suppressive network repressing one or both players in this signal amplification loop in the Th17 differentiation program.

8.5 A cooperative microRNA network to repress inappropriate gene expression during Treg cell induction.

Most of the non-random candidates we identified in our screen affected Treg cell differentiation moderately in a graded manner and can be classified as tuning interactions. This suggests that these microRNAs work together and are embedded in a network of microRNA:target interactions. It is woven by convergence of several microRNAs on one target, like miR-10b, miR-99a and miR-150 on Rora, as well as by pleiotropic effects where one microRNA targets several mRNA targets, like miR-150 targeting of Mtor, Rora and potentially others. In T cell differentiation, such a network may buffer differentiation decisions under weak polarizing signals. Sufficient stimuli or induction of key factors may then condition the network, for example by cooperation, to act in a switch like manner and promote lineage commitment by the suppression of inappropriate gene expression.

In that manner, ectopic miR-150 expression on top of the endogenously expressed microRNAs moderately promoted Treg cell differentiation. The network hypothesis implicates, that the loss of a single node can be buffered and does not abolish the network function. In agreement with that, deletion of the highly expressed miR-150 did not exhibit obvious changes in the mature T cell compartment, although Treg cell induction from naive T cells has not been analyzed in particular (C. Xiao and M. Ansel, personal communication).

The screen also identified a few microRNAs that markedly induced Treg cell differentiation resulting in about 50% increased Treg cell differentiation, which were miR-99a and miR-10b. These microRNAs may be the key factors that help to switch the microRNA network towards lineage commitment. Consistently, we showed that miR-10b and miR-99a/100 expression was induced by retinoic acid, which has been shown to markedly enhance Treg cell induction

(Mucida et al., 2007; Schlenner et al., 2012). Retinoic acid facilitated binding of pSMAD3 downstream of TGFβ signaling to the Foxp3 enhancer, which is indispensable for Foxp3 expression (Xu et al., 2010). In addition to that, already a moderate induction of miR-10b and miR-99a/100 by retinoic acid may be sufficient to strongly promote Treg cell differentiation, if target regulation occurs in a cooperative manner. Cooperative binding of two microRNAs to neighboring target sites within one target 3'UTR was shown to promote a much more efficient mRNA silencing than a single target site (Grimson et al., 2007; Sætrom et al., 2007). To prove the cooperation of miR-99a/miR-100 with miR-150 in target regulation, it will be interesting to study whether the overexpression of miR-99a in miR-150-deficient cells is still similarly effective. In addition, we prepare a reconstitution experiment that will be able to express individual Dicer-independent synthetic microRNAs in *Dicer*-deficient cells that lack almost all other microRNAs (Cifuentes et al., 2010).

Taken together our screen has identified microRNAs that may act together in a repressive post-transcriptional network. They include key microRNAs that are inducibly regulated and that can cooperate with constitutively expressed microRNAs. These microRNA networks target central signaling pathways and transcription factors. They are therefore interconnected in networks of transcriptional activation and epigenetic modification. Together they suppress inappropriate and promote appropriate gene expression to finally determine lineage commitment.

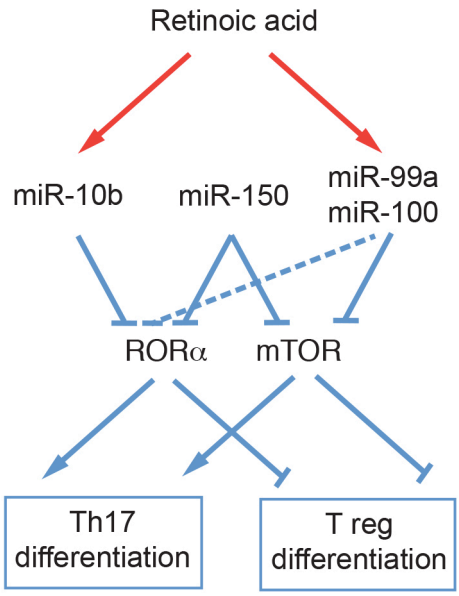

Figure 24: A model for microRNA cooperation in Treg cell differentiation.
The highly expressed miR-150 represses Mtor und Rora, which are critical inhibitors of Treg cell differentiation. Retinoic acid can induce miR-10b and miR-99a/100 that cooperate with miR-150 in target repression and thereby promote Treg cell differentiation

8.6 Conclusion

In this study we defined a window of opportunity for effective microRNA regulation during Treg cell differentiation. We developed an experimental system to test the effect of microRNA overexpression on Treg cell differentiation within this window of opportunity. A functional microRNA overexpression screen identified a number of microRNAs that interfered with or promoted Treg cell differentiation. This suggested the concept that not a single microRNA, but a network of microRNA:target interactions underlies Treg cell differentiation. Further studies on four candidate microRNAs with Treg cell-promoting effects and their targets established a core network, in which the cooperation of highly expressed as well as inducible microRNAs can achieve sufficient downregulation of critical inhibitors of Treg cell differentiation. Future experiments will assess the effectiveness of this core network in the absence of specific microRNAs and link it with the regulation of Treg cell differentiation by transcription factor and epigenetic modifications.

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10 Abbreviations

-/-	knock-out
A	Adenine
AP1	activator protein 1
ATRA	All-trans retinoic acid
bp	base pairs
C	Cytosine
CAR	Coxsackie adenovirus receptor
CD	Cluster of differentiation
CDS	coding sequence
CMV	Cytomegalovirus
CNS	Conserved non-coding sequence
CPE	Cytopathic effects
CPSF	Cleavage and polyadenylation specific factor
CstF	Cleavage stimulating factor
eGFP	Enhanced green fluorescent protein
Eri1	Exoribonuclease 1
F/TC	freeze-and-thaw-cycle
Foxp3	forkhead box P3
G	Guanine
GFP	Green fluorescent protein
HEK293	Human Embryonic Kidney 293 cells
hFOXP3	human FOXP3
h	hour, hours
IFNγ	Interferon γ
IL-2	Interleukin-2
IRES	Internal ribosomal entry site
ITR	Inverted repeat
kB	kilo bases
kDa	kilo Dalton
ko	knock-out
MEF	Murine embryonal fibroblasts

MHCII	Major Histocompatibility Complex II
min	Minutes
miR	MicroRNA
miRISC	MicroRNA-induced silencing complex
MOI	Multiplicity of infection
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin, protein
Mtor	Mechanistic target of rapamycin, mRNA
NFAT	Nuclear factor of activated T cells
nt	Nucleotides
Ova	Ovalbumin
PAS	Polyadenylation signal
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RD	Relative differentiation
Rora	RAR-related orphan receptor alpha, mRNA
RORα	RAR-related orphan receptor alpha, protein
RT	Room temperature
SN	Supernatant
T	Thymine
TCR	T cell receptor
Tfh	Follicular T helper cell
TGFβ	Transforming growth factor β
Treg cell	Regulatory T cell
TSDR	Treg-specific demethylated region
U	Uracil
UTR	Untranslated region

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