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Improving adoptive T cell therapies of cancer by ectopic expression of miR181a to repress inhibitory phosphatases

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by

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Abstract

Adoptive T cell therapy using antigen-specific T lymphocytes is a powerful immunotherapeutic approach against cancer. Nevertheless, many T cells against tumor-antigens exhibit only weak anti-tumoral response. To overcome this barrier it is necessary to improve the potency and anti-tumoral efficacy of these T cells. Activation and activity of T cells are tightly controlled to inhibit unwanted T cell responses and to reduce the risk of autoimmunity. Both are regulated by extrinsic signals and intrinsic mechanisms which suppress T cell activation. The intrinsic mechanisms include the expression of phosphatases that counteract the activation-inducing kinases. Modifying the expression of these phosphatases allows the targeted modulation of T cell reactivity. MicroRNAs (miRNAs) are regulatory small noncoding RNA molecules that control gene expression by targeting messenger RNAs in a sequence specific manner. Gene-specific silencing plays a key role in diverse biological processes, such as development, differentiation, and functionality. miR181a has been shown to be highly expressed in immature T cells that recognize low-affinity antigens.

The present study successfully shows that ectopic expression of miR181a is able to enhance the sensitivity of both murine and human T cells. In $CD4^+$ T helper cells as well as in $CD8^+$ cytotoxic T cells the overexpression of miR181a leads to downregulation of multiple phosphatases involved in the T cell receptor signaling pathway. Overexpression of miR181a in human T cells achieves a co-stimulatory independent activation and has an anti-apoptotic effect on $CD4^+$ T helper cells. Additionally, increasing the amount of miR181a enhances the cytolytic activity of murine $CD8^+$ TCR^{tg} T cells in an antigen-specific manner.

To test miR181a overexpressing T cells *in vivo*, a mouse tumor model using a B cell lymphoma cell line (A20-HA) expressing the Influenza hemagglutinin (Infl.-HA) antigen was established. The expression of model antigens in tumor cell lines enables targeted elimination of tumors using TCR^{tg} T cells. The transfer of miR181a overexpressing Infl.-HA TCR^{tg} $CD8^+$ T cells alone has no positive effect neither on tumor control nor on survival of A20-HA tumor-bearing mice. In contrast, the co-transfer of miR181a overexpressing Infl.-HA TCR^{tg} $CD8^+$ and $CD4^+$ T cells leads to improved tumor control and prolongs survival of A20-HA tumor-bearing mice. This effect is characterized by higher amounts of effector T cells and the expansion of Infl.-HA TCR^{tg} $CD8^+$ T cells.

All effects were achieved by changes in expression of several genes including molecules involved in T cell differentiation, activation, and regulation, cytotoxic effector molecules, and receptors important for the homing process of T cells in miR181a overexpressing T cells. The present study demonstrates that miR181a is able to enhance the anti-tumoral response of antigen-specific T cells and is a promising candidate for improving adoptive cell therapy.

Zusammenfassung

Der adoptive Transfer von antigen-spezifischen T Lymphozyten ist ein vielversprechender Therapieansatz zur Behandlung von Krebs. Dennoch weisen viele T Zellen, welche gegen Tumorantigene gerichtet sind, nur eine geringe anti-tumorale Wirkung auf. Um dieses Hindernis zu überwinden, ist es notwendig die Potenz und anti-tumorale Effizienz dieser T Zellen zu erhöhen. Die Aktivierung und Aktivität von T Zellen wird streng kontolliert, um unerwünschte T Zell Antworten zu inhibieren und das Risiko von Autoimmunerkrankungen zu minimieren. Dies wird durch extrinsische Signale und intrinsische Mechanismen, welche die T Zell Aktivierung unterdrücken, reguliert. Die Aktivierung von Phosphatasen, welche den aktivierungsinduzierten Kinasen entgegenwirken, gehört zu den o.g. intrinsischen Mechanismen. Die Veränderung der Expressionslevel dieser Phosphatasen ermöglicht die zielgerichtete Modifikation der T Zell Reaktivität. MicroRNAs (miRNAs) sind kleine, regulatorische und nichtkodierende RNA-Moleküle, welche die Expression von Genen kontrollieren, indem sie deren messenger RNA sequenzspezifisch binden und deren Translation inhibieren. Diese Herunterregulation von Genen spielt eine wichtige Rolle bei verschiedenen biologischen Prozessen wie der Entwicklung, Differenzierung und Funktionalität. Frühere Studien konnten zeigen, dass miR181a stark in unreifen T Zellen exprimiert ist, welche niedrig-affine Antigene erkennen.
Die vorliegende Arbeit zeigt deutlich, dass die ektope Expression von miR181a eine erhöhte Sensitivität von murinen und human T Zellen ermöglicht. In $CD4^+$ T Helferzellen als auch $CD8^+$ zytotoxischen T Zellen führt die Überexpression von miR181a zu einer Herunterregulation von mutiplen Phosphatasen, welche in der Signalkette der T Zell Rezeptoren involviert sind. Durch die Überexpression von miR181a in humanen T Zellen wird eine Aktivierung unabhängig vom Kostimulus erreicht. Gleichzeitig hat miR181a einen anti-apoptotischen Effekt auf humane T Helferzellen. In murinen TCR^{tg} T Zellen führt die Erhöhung der miR181a-Menge zu einer verstärkten zytolytischen Aktivität.
Um miR181a überexprimierende T Zellen *in vivo* zu testen, wurde ein Tumormodel mit einer B Zell Lymphom Zelllinie (A20-HA), welche das Influenza Hemagglutinin (Infl.-HA) Antigen exprimiert, etabliert. Die Expression von Modelantigen in Tumorzelllinien erlaubt die gezielte Bekämpfung des Tumors durch antigen-spezifische TCR^{tg} T Zellen. Der Transfer von miR181a überexprimierenden Infl.-HA TCR^{tg} $CD8^+$ T Zellen allein hat keinen positiven Effekt, weder auf die Tumorkontrolle noch hinsichtlich des Überlebens der A20-HA tumortragenden Mäuse. Im Gegensatz dazu führt der Kotransfer von miR181a überexprimierenden Infl.-HA TCR^{tg} $CD8^+$ und $CD4^+$ T Zellen zu einer verbesserten Tumorkontrolle und verlängert das Überleben der A20-HA tumortragenden Tiere. Dieser Effekt ist durch eine erhöhte Anzahl von Effektorzellen und die Expansion der Infl.-HA TCR^{tg} $CD8^+$ T Zellen gekennzeichnet.

Alle diese Effekte sind durch Änderungen der Expression zahlreicher Gene, z.B. von Molekülen der T Zell Differenzierung, Aktivierung und Regulierung, sowie von zytotoxischen Effektormolekülen und Rezeptoren, welche für den "Homing" Prozess von T Zellen von Bedeutung sind, in miR181a überexprimierenden T Zellen charakterisiert. Die vorliegende Arbeit zeigt, dass miR181a in der Lage ist, die anti-tumorale Wirkung antigen-spezifischer T Zellen zu erhöhen und stellt daher einen aussichtsreichen Kandidaten zur Verbesserung der adoptiven T Zelltherapie dar.

Contents

Abbreviations

%	percent
°C	degree centigrade
μ	micro
aa	amino acid
ACT	adoptive T cell therapy
ADAP	adhesion- and degranulation- promoting adaptor protein
ADCC	antibody-dependent cell mediated cytotoxicity
AP-1	activator protein 1
APC	allophycocyanin
APCs	antigen presenting cells
ATP	adenosine triphosphate
bp	basepair
BTLA	B and T lymphocyte attenuator
BIM	BCL-2-interacting mediator of cell death
CAR	chimeric antigen receptor
CD	cluster of differentiation
CDC	complement-dependent cytotoxicity
CLP	committed lymphoid progenitor
CTA	cancer testis antigen
CTLA4	cytotoxic T lymphocyte associated 4
Da	dalton
DAG	diacylglycerol
DC	dendritic cell
DGCR8	DiGerge syndrome critical region gene 8
DMSO	dimethyl sulfoxide
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
DUSP	dual specific phosphatase
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
EmGFP	emerged green fluorenscence protein
ELISA	enzyme linked immunosorbent assay
ERK	extracellular-signal regulated kinase
FACS	fluorenscent associated cell sorting
FCS	fetal claf serum
FITC	fluorescein-5-isothiocyanate
Fig.	Figure
FSC	forward scatter
GADS	GRB2-related adaptor protein
GRB2	growth factor receptor-bound protein 2
GM-CSF	granulocyte-macrophage colony stimulating factor

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h	hour(s)
HA	hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazin-ethanesulfonacid
HIV	human immunodeficiency virus
iDC	immature dendritic cell
IFNγ	interferon gamma
IL	interleukin
Infl.-HA	influenza hemagglutinin
$InsP_3$	inositol-1,4,5-trisphosphate
ITAM	immunoreceptor tyrosine based activation motif
ITK	interleukin-2-inducible T cell kinase
i.v.	intravenous(ly)
k	kilo
L	liter
LAT	linker for activation of T cells
LB	lysogeny broth
LCK	lymphocyte cell-specific protein-tyrosin kinase
LCMV	lymphocytic choriomeningitis virus
LFA-1	leukocyte function-associated antigen 1
LN	lymph node
m	milli
M	molar
MACS	magnetic cell sorting
MALT	Mucosa-associated lymphoid tissue
MAPK	mitogen activated protein kinase
mDC	mature dentritc cell
MDSC	myeloid-derived suppressor cell
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
min	minute(s)
miRNA	micro RNA
mRNA	messenger RNA
n	nano
NEAA	non-essential aminoacids
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor-κB
NK	natural killer
NTP	nucleotid triphosphate
nt	nucleotide(s)
OD	optical desity
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline

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PCR	polymerase chain reaction
PD-1	programmed death 1
PE	phycoerythrin
PerCP	peridinin chlorophyll a protein
PFA	paraformaldehyde
PI	propidium iodide
PI3K	phosphatidylinositol 3-Kinase
PKC	protein kinase C
PLCγ1	phospholipase Cγ1
PMA	phorbol myristrate acetate
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PTEN	phosphate and tensin homologue
PtdIns(4,5)P_2	phosphatidylinositol-4,5-bisphosphate
PTPN	protein tyrosine phosphatase non-receptor
RASGRP1	RAS guanyl-releasing protein 1
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
rev	reverse
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	room temperature
s.c.	subcutaneous(ly)
sd	standard deviation
SFK	SRC family kinase
SH2	SRC homology 2
SHP	SRC homology 2 domain containing protein-tyrosine phosphatase
shRNA	short hairpin RNA
SKAP55	SRC kinase-associated phosphoprotein of 55 kDa
SLP76	SH2 domain-containing leukocyte protein of 76 kDa
SMAC	supramolecular activation cluster
SOC	super optimal broth media
SP	single positive
SSC	sideward scatter
TAA	tumor associated antigen
Tab	tabular
TAE	tris-acetat-EDTA
TAP	transporter-associated with antigen processing
TCR	T cell receptor
TEMED	N,N,N',N'-tetramethyldiamin
TIL	tumor infiltrating lymphocytes
TNFα	tumor necrosis factor alpha

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T_{cm}	central memory T cell
T_{em}	effector memory T cell
T_{FH}	follicular helper T cell
T_H	helper T cell
T_{reg}	regulatory T cell
Tris	Tris(hydroxymethyl)-aminomethan
U	Unit
UV	ultraviolett
V	voltage
v/v	volume/volume
w/v	weight/volume
xg	x gravitational force
ZAP70	ζ-chain associated protein kinase, 70 kDa

1 Introduction

1.1 Cancer - a challenge for the immune system

Cancer is one of the leading causes of death today. Unrecognized cancers will grow progressively and finally destroy the host if not treated appropriately. Curing the disease requires the discrimination between malignant and normal cells. This is a huge challenge, because tumors often use a variety of mechanisms to evade, deceive, and suppress the host immune system.
Tumors can impair antigen recognition by reducing antigen processing or downregulation of major histocompartibility complex (MHC) molecules on their surface [Restifo et al., 1996]. It is supposed that between 70% and 95% of all human tumors downregulate MHC class I expression [Garrido and Algarra, 2001]. As a complete loss of MHC class I molecules would activate natural killer (NK) cells, most tumors express low level of MHC class I molecules to prevent NK-mediated cytotoxicity and simultaneously minimizing their recognition by T cells [Soloski, 2001].
Tumors can also secrete immunosuppressive cytokines to generate a microenvironment that renders tumor-specific T cells inefficient [Smyth et al., 2006], [Salazar-Onfray et al., 2007]. One of these immunosuppressive cytokines is TGFβ, that directly suppresses T cell-mediated cytotoxicity by downregulating the expression of perforin, granzymes, and IFNγ [Jarnicki et al., 2006]. Interleukin-10 (IL-10) is another important tumor-derived cytokine that downregulates MHC class I on the tumor cells. IL-10 can also inhibit the transporter associated with antigen processing (TAP) proteins that are involved in antigen presentation in both tumor cells and dendritic cells (DCs) [Matsuda et al., 1994], [Salazar-Onfray et al., 1997]. IL-10 also has direct effects on $CD4^+$ T cells. IL-10 inhibits the proliferation as well as the cytokine production, including IL-2, IFNγ, IL-4 and IL-5 [Del Prete et al., 1993], [Groux et al., 1996]. IL-10 has no direct inhibitory effects on $CD8^+$ T cells, it can be stimulatory or suppressive in a context depending manner [Moore et al., 2001]. It could be shown, that IL-10 was required for optimal $CD8^+$ T cell memory development in response to bacterial infection [Foulds et al., 2006]. But it is also known, that IL-10 rapidly suppresses $CD8^+$ T cell activity during chronic HIV, hepatitis C virus (HCV), and LCMV infections [Cacciarelli et al., 1996], [Rigopoulou et al., 2005], [Ejrnaes et al., 2006], [Brockman et al., 2009]. Thus, the stimulatory/inhibitory function of IL-10 on

T cells differ depending on the type of pathogen and the environment. Importantly, tumors lack adhesion and co-stimulatory molecules, which are necessary for full activation of T cells [Pardoll, 2003]. They can also express T cell inhibitory signals (e.g. PD-L1, PD-L2, B7x and other members of the B7 family) that directly inhibit the effector functions of tumor-specific T cells and thereby induce a state of unresponsiveness [Thompson et al., 2004], [Blank et al., 2005], [Hofmeyer et al., 2012].

Some tumors are associated with chronic inflammations. Mucosa-associated lymphoid tissue (MALT) lymphomas arise in the context of chronic inflammation caused by infectious agents, such as *Helicobacter pylori*, *Chlamydia psittacii* and *Borrelia burgdorferi* [Ferreri et al., 2009]. Also viral infections can cause cancer. The Epstein-Barr virus (EBV) can induce Burkitt's lymphoma and Hodgkin's lymphoma [Ferreri et al., 2009].

Traditionally, many of cancer types are treated with radiation and chemotherapy. Both treatments cause massive necrotic death of cancer cells and surrounding tissues, which provokes an inflammatory reaction [Zong and Thompson, 2006]. The outcome of therapy-induced inflammation is controversial. On the one hand, it can have tumor-promoting functions just like the necrosis which cause rapid tumor growth [Vakkila and Lotze, 2004], [Ammirante et al., 2010]. On the other hand, it can enhance the cross-presentation of tumor antigens and thereby induce anti-tumor immune responses leading to immunogenic cell death [Zitvogel et al., 2008]. It is important to notice that the majority of tumor-specific antigen targets are self or altered self. Because of their expression in normal tissues, there exists regulatory mechanisms that prevent autoimmunity. These regulatory mechanisms also restrain anti-tumor immune responses by tolerance. In contrast to radiation and chemotherapy, which also destroy healthy cells, leading to severe side effects in patients, immunotherapy aims to specifically target malignant cells and will be discussed in detail below.

1.2 Cancer Immunotherapy

The aim of cancer immunotherapy is to overcome tumor escape mechanisms and effectively and safely initiate or exploit and amplify existing anti-tumor responses. Therefore, cancer immunotherapy includes different strategies - some treatments boost the immune system in a general way (non-specific immunomodulation), whereas other treatments aim to target specific structures of cancer cells (antigen-targeting immunotherapy).

1.2.1 Non-specific immunomodulation

Non-specific immunomodulation aims to boost existing anti-tumor responses by administration of cytokines or antibody-mediated blockade of inhibitory molecules. One of the first cytokines, which was shown to have anti-tumor effects is interferon α (IFNα) [Gutterman, 1994]. IFNα inhibits tumor cell growth and enhances the maturation of antigen-presenting cells (APCs) and is now used for treatment of selected malignancies in the clinic. The administration of the T cell growth factor interleukin 2 (IL-2) can activate endogenous tumor-reactive cells leading to the regression of some human solid cancers [Rosenberg et al., 1985], [Lotze et al., 1986], [Rosenberg et al., 1998]. Another approach, the antibody-mediated blockade of a cell surface inhibitory molecule, cytotoxic T lymphocyte associated 4 (CTLA4), has resulted in objective clinical responses in 10–20% of patients with metastatic melanoma or renal cancer [Phan et al., 2003], [Attia et al., 2005]. Also the block of the inhibitory molecules programmed death 1 (PD-1) and transforming growth factor β (TGFβ) are used to inhibit suppressive effects on tumor-specific T cells. However, non-specific immmunomodulation requires the systemic administration of general immune modulators resulting in severe side effects.

1.2.2 Antigen-targeting immunotherapy

Antigen-targeted therapies sensitize immune cells to induce specific anti-tumor immune responses. The discovery of multiple tumor-associated antigens (TAAs) has facilitated several opportunities for immunotherapeutic concepts. TAAs are proteins expressed on tumor cells, which have no or only limited expression in normal cells, including differentiation antigens, overexpressed antigens, tumor-specific splice variants, mutated gene products, viral antigens, and cancer testis antigens (CTAs). CTAs are promising targets for immunotherapy because

they are activated in a wide spectrum of tumors, but they are not expressed in normal tissues of adults [Hofmann et al., 2008]. Induction of antigen-specific $CD4^+$ and $CD8^+$ T cells as well as humoral immune responses could be demonstrated for targeting several CTAs [Jager et al., 1998], [Sahin et al., 1995], [Valmori et al., 2000], [Ayyoub et al., 2002].

The aim of tumor vaccines is to induce tumor-specific immune responses by active immunization selectively targeting the tumor. Therapeutic vaccines are promising approaches for antigen-targeted immunotherapy because they are easy to use and have shown only low toxicities in preclinical and clinical trials [Wang et al., 1995], [Rosenberg et al., 2005]. Different antigen formats are used for tumor vaccination including whole cancer cells, proteins, peptides, RNA and DNA or viral vectors, which can be applied directly or indirectly after pulsing dendritic cells (DCs) followed by transfer into patients. DC vaccination aimed to induce tumor-specific effector T cells that can attack the tumor specifically and furthermore can generate immunological memory to control tumor relapse. Studies using *ex vivo* generated DCs showed that DC-based vaccines are safe and can induce the expansion of tumor-specific $CD4^+$ and $CD8^+$ T cells [Ueno et al., 2010]. The tumor antigens for loading DCs include mutated antigens and also non-mutated self-antigens [Gilboa, 1999], [Boon et al., 2006], [Parmiani et al., 2007]. Importantly, none of the cancer vaccines that have been tested in many hundreds of clinical trials could cure metastatic diseases, and their therapeutic benefit is modest and measured in months, not years [Restifo et al., 2012].

Antibody-based cancer therapies have been emphasized as one of the most promising therapeutics for cancer treatment. Antibodies targeting TAAs bind to cancer cells and initiate complement-dependent cytotoxicity (CDC) or antibody-dependent cell mediated cytotoxicity (ADCC) by natural killer (NK) cells or macrophages. They also can prevent tumor cell growth by obstructing survival or introducing apoptotic signals. By facilitating the uptake and presentation of tumor antigens by APCs, cancer specific antibodies could increase the immunogenicity of immune cells. Anti-tumor activity can be enhanced by adding radioisotopes or drugs to antibodies [Kaminski et al., 2005]. A special format are bispecific antibodies that bind tumor cells and receptors on immune cells (e.g. CD3 or Fc receptors) simultaneously, bringing both cell types into close proximity [Carter, 2001]. Another antigen-targeted immunotherapy is the adoptive T cell therapy, which will be explained in detail below (see 1.3).

1.3 Adoptive T cell therapy

In 1955, Mitchison et al. demonstrated in a murine model that the transfer of draining lymph node cells can target transplanted tumors [Mitchison, 1955]. Southam and colleagues showed in 1966 for the first time that the transfer of leukocytes into patients with established tumors inhibits tumor growth in about half of the patients [Southam et al., 1966]. The infusion of autologous tumor infiltrating lymphocytes (TILs) grown from tumor samples of patients with metastatic melanoma were first demonstrated to mediate the regression of melanoma in 1988 [Rosenberg et al., 1988]. The response rates could be increased by lymphodepletion with chemoradiotherapy to enhance engraftment and efficacy of adoptively transferred T cells [Dudley et al., 2002], [Laport et al., 2003] (see figure 1.1).

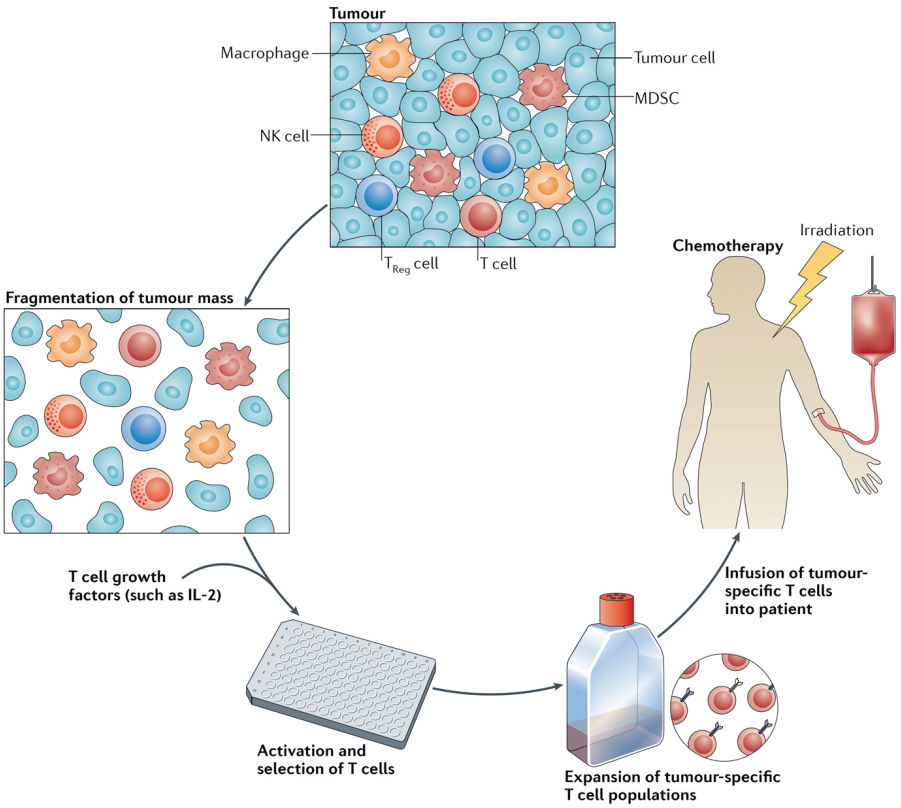

Figure 1.1: Isolation and expansion of tumor specific T cells.
Tumor masses are resected and fragmented, and the cells are placed in interleukin-2 (IL-2) containing medium. T cell populations with the desired T cell receptor (TCR) specificity are selected and expanded, and then adoptively transferred into patients with cancer. Prior to this adoptive transfer, hosts will be lymphodepleted by either chemotherapy alone or chemotherapy in combination with irradiation. Myeloid-derived suppressor cell (MDSC), natural killer (NK), regulatory T cell (T_{Reg}). (Figure obtained from [Restifo et al., 2012]).

It is thought that lymphodepletion enables T cell expansion and persistence through the generation of homeostatic space and the depletion of inhibitory cytokine producing immune cells [Dudley et al., 2002], [Wrzesinski and Restifo, 2005], [Muranski et al., 2006]. The combination of lymphodepletion with exogenous cytokine administration has been reported to enhance the persistence of adoptively transferred T cells [Yee et al., 2002] and thereby prolongs tumor elimination [Kochenderfer et al., 2010], [Kochenderfer et al., 2012]. The most commonly used cytokine is IL-2, which induces proliferation of effector $CD8^+$ T cells.

The major advantage of T cells for adoptive cell therapy (ACT) is their ability to recognize TAAs by their T cell receptors (TCRs). Further improvement of ACT could be achieved by reprogramming autologous T cells to express a tumor-reactive TCR of defined specificity. The transfer of specific TCRs into T cells offers the opportunity to redirect them toward any antigen of interest. The first clinical trials were using T cells engineered with a TCR specific for the melanoma antigen MART-1, which was isolated from TILs of a melanoma patient [Clay et al., 1999], [Duval et al., 2006], [Burns et al., 2009]. In these studies the response in patients was lower than that observed after ACT using whole TIL population [Dudley et al., 2005], [Morgan et al., 2006], [Dudley et al., 2008]. Because populations of TILs contain T cells with a variety of specificities, it could be that the use of TCR-engineered T cells of several specificities might be more effective against tumors with heterogeneous antigen expression than using a single TCR. In a study of metastatic melanoma using lymphodepletion following infusion of anti-MART-1 and anti-gp100 TCR-engineered lymphocytes, partial responses were observed in 6 of 20 (30%) patients. Importantly, there was some on-target / off-tumor toxicity in patients observed against normal melanocytes in the skin, ear, and eye, which required intervention with corticosteroid treatment [Johnson et al., 2009].

The majority of targeted tumor antigens are self-antigens, which are normally expressed during development and divergently expressed by tumors. CTAs are attractive targets because of their common expression in a broad range of tumors and their limited expression in normal tissues [Hofmann et al., 2008]. Tumors that express CTAs (e.g. NY-ESO1 [Robbins et al., 2011] or MAGEA3 [Chinnasamy et al., 2011]) include melanomas, lung carcinomas, and cancers of the head and neck, oesophagus and bladder [Simpson et al., 2005]. A clinical study targeting NY-ESO1 using autologous genetically TCR engineered T cells showed clinical responses in

8 out of 17 (47%) patients with metastatic melanoma and in 8 out of 10 (80%) patients with metastatic synovial sarcoma [Robbins et al., 2011]. There was no on-target / off-tumor toxicity against normal tissues observed, showing that NY-ESO1 is a safe and effective target. Another approach for providing specificity is the use of chimeric antigen receptors (CARs). CARs are composed of antibody binding domains fused to T cell signaling domains, enabling the recognition of MHC-nonrestricted structures on the surfaces of target cells [Alvarez-Vallina, 2001].
The majority of clinical trials use virus-based transduction to produce gene-engineered T cells (see figure 1.2). Virus-mediated gene transfer include vectors from gamma-retroviruses or more recently lentiviruses. These vectors integrate into the host genome and thereby drive long-term transgene expression. The first description of the engineering of human T cells with a specific TCR was by Clay et al. [Clay et al., 1999] using a gamma-retroviral vector. For integration into genomic host DNA gamma-retrovirus based transduction requires the replication of cells. Whereas lentiviral vectors can also integrate into minimally stimulated cells or non-dividing cells and can provide larger DNA sequences than retroviruses [Naldini et al., 1996].
Genetic modification of T cells for ACT is not limited to the generation of new antigen reactivity to T cells by transfer of TCRs and CARs. It can also be used to introduce genes for improving the efficacy of the transferred T cells. These include genes involved in co-stimulation [Krause et al., 1998], [Stephan et al., 2007], the prevention of apoptosis [Charo et al., 2005], the induction of inflammation [Kerkar et al., 2010], [Kerkar et al., 2011], [Pegram et al., 2012], or homeostatic proliferation [Cheng et al., 2002], as well as CARs encoding chemokine receptors that promote T cell homing [Moon et al., 2011].
Additionally, ACT can be combined with other anti-tumor therapies, such as therapeutic vaccination, checkpoint inhibition, agonistic antibodies, small molecule inhibitors of tumors, and targeting of tumor stroma and neo-vasculature in order to improve the clinical outcome.

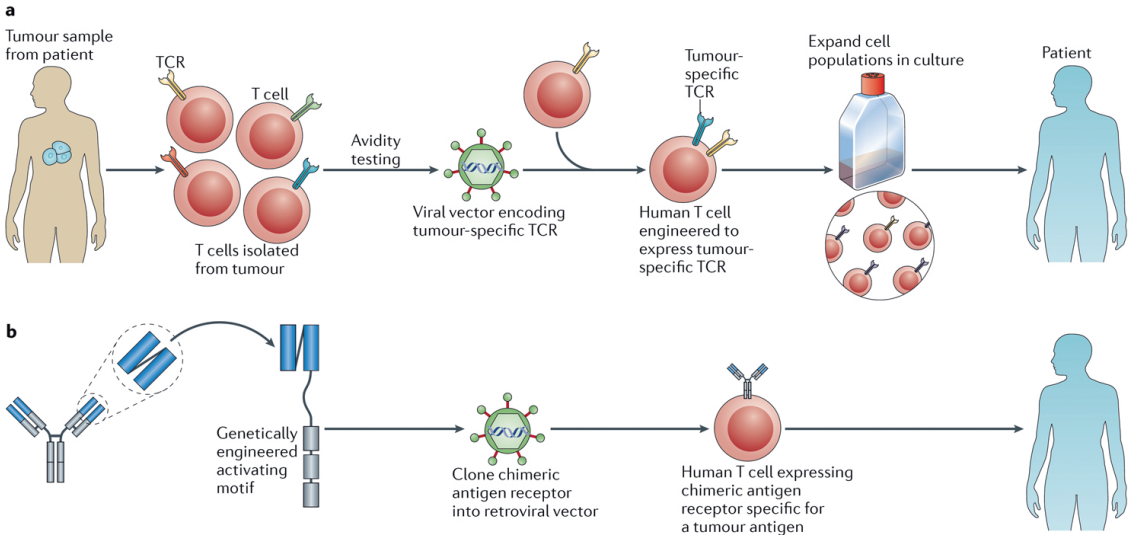

Figure 1.2: Genetically engineering of T cells to ensure specificity for tumor-associated antigens.
T cells can be genetically engineered to recognize tumor-associated antigens.
(a) T cells identified from patients with particularly good anti-tumor responses could be used to clone specific T cell receptors (TCRs). These TCRs can then be inserted into retroviruses or lentiviruses, which are used to infect autologous T cells from patients to be treated. **(b)** Chimeric antigen receptors (CARs) can be generated from sequences encoding the variable regions of antibodies recognizing tumor antigens. These sequences are engineered to encode a single chain, which is then genetically fused to the TCR intracellular domains that are able to activate T cells. CARs are able to recognize MHC-nonrestricted structures on the surfaces of target cells.
(Figure obtained from [Restifo et al., 2012]).

1.4 T cells in adaptive immunity

1.4.1 Development of T Lymphocytes

The development of T cells occurs in the thymus and depends on the specialized microenvironment that is provided by this organ (see figure 1.3). The thymic epithelium provides particular potent combinations of receptor ligands and growth factors to trigger and support T cell differentiation, proliferation and survival. Committed lymphoid progenitors (CLPs) from the bone marrow migrate via the blood into the thymus. In the thymus, they become $CD4^{-}CD8^{-}$ double negative (DN) committed T cell precursor cells. The heterogeneity of the DN thymocyte compartment has led to further subdivision of these cells by their surface expression of CD25 and CD44: DN1($CD25^{-}CD44^{+}$), DN2($CD25^{+}CD44^{+}$), DN3($CD25^{+}CD44^{-}$), and DN4($CD25^{-}CD44^{-}$) [Godfrey et al., 1993], [Hoffman et al., 1996]. With the transition from the DN1 to DN2 stage, the initiation of gene arrangement at the TCRβ, TCRγ, and TCRδ gene loci starts [Livak et al., 1999]. When T cells reach the DN3 stage, the expression of the pre-TCR

(TCRβ paired with a surrogate TCRα) or the $\gamma\delta$TCR results in signals that rescue cells from apoptosis and lead to thymocyte proliferation and further differentiation (β-selection) [Kruisbeek et al., 2000], [Michie and Zuniga-Pflucker, 2002].

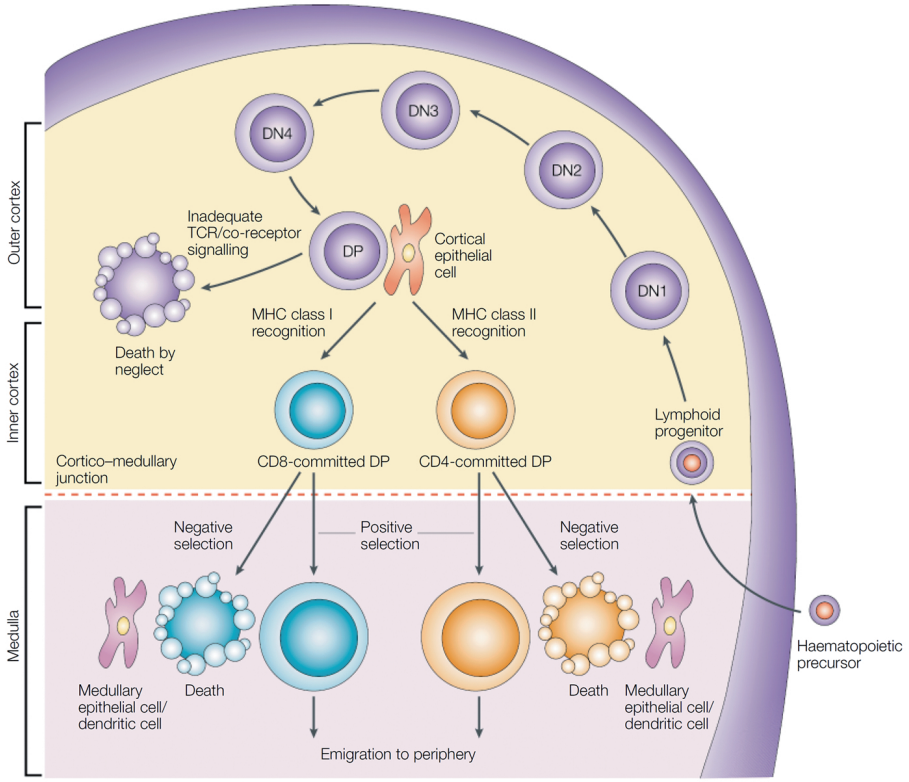

Figure 1.3: T cell development in the thymus.
Committed lymphoid progenitors arise in the bone marrow and migrate to the thymus. Lymphoid progenitors lack expression of T cell receptor (TCR), CD4 and CD8, and are termed double-negative (DN; no CD4 or CD8) thymocytes. DN thymocytes can be further subdivided into four stages of differentiation: DN1, DN2, DN3, and DN4. As cells progress through the DN2 to DN4 stages, they express the pre-TCR. Successful pre-TCR expression leads to substantial cell proliferation during the DN4 to double positive (DP). The DP thymocytes interact with cortical epithelial cells that express a high density of MHC class I and class II molecules associated with self-peptides undergoing negative and positive selection. This takes place in the medulla on encounter with strongly activating self-ligands on haematopoietic cells, particularly dendritic cells. Thymocytes that express TCRs that bind peptide–MHC-class-I complexes become $CD8^+$ single positive (SP) T cells, whereas those that express TCRs that bind peptide–MHC-class-II ligands become $CD4^+$ SP T cells. These cells can then exported from the medulla to peripheral lymphoid sites. (Figure obtained from [Germain, 2002]).

After β-selection (late DN3 and DN4) the recombination at the TCR-α locus produces the second component chain of the mature $\alpha\beta$ antigen receptor. At the same time, thymocytes also begin to express co-receptor proteins to form a large population of $CD4^+CD8^+$ double-

positive (DP) $\alpha\beta$-TCR-expressing immature cells. After completion of TCRα rearangements, DP thymocytes then undergo major histocompatibility complex (MHC)-mediated selection. The interaction of the TCR$\alpha\beta$ heterodimer with self-peptides complexed with MHC molecules that are expressed on thymic epithelial cells influences whether thymocytes are positively or negatively selected. DP thymocytes expressing TCRs which interact only poorly with the self-peptide–MHC ligands do not generate the intracellular signals which are required to sustain viability and die by neglect. Thymocytes that express high-affinity receptors for self-peptide-MHC could cause autoimmune pathology if they were permitted to leave the thymus and are thereby also deleted (negative selection). Thymocytes with TCRs that recognize self-ligands and generate signals that have an intensity between those resulting in neglect or negative selection are positively selected and can differentiate into either $CD4^+$ or $CD8^+$ single positive (SP) mature T cells.

1.4.2 Effector functions of T Lymphocytes

Naïve T cells are T cells that have survived positive as well as negative selection in the thymus. They circulate via the blood and the lymph stream and migrate to the T cell areas of lymph nodes and other secondary lymphoid organs, where they become activated. To achieve this, immature dendritic cells (iDCs) collect antigens in the periphery and present them complexed with MHC-molecules on their surface. Upon activation in inflamed tissues, they migrate to the lymph nodes and develop into mature DCs (mDCs), expressing co-stimulatory molecules, chemokines, and cytokines to initiate an immune response by acting as antigen presenting cells (APCs) [Banchereau et al., 2000]. Importantly, DCs remember their origins. DCs that migrate from the gut to the lymph nodes induce T cells to express gut-homing receptors (such as CCR9) [Kunkel et al., 2000], whereas DCs that being activated in damaged skin induce T cells to express skin-homing receptors ($\alpha4\beta1$ integrin, CCR4 and CCR10) [Mora et al., 2005], [Calzascia et al., 2005].

The primary T cell activation (priming) occurs after encounter of a peptide-MHC-complex that is recognized by the TCR as a first signal and the engagement of co-stimulatory molecules on the T cell surface with ligands expressed by mDCs as a second signal. After activation, antigen-specific T cells clonaly expand and differentiate into effector T cells, which migrate to inflamed tissues (see figure 1.4) or to the B cell areas in the lymph node.

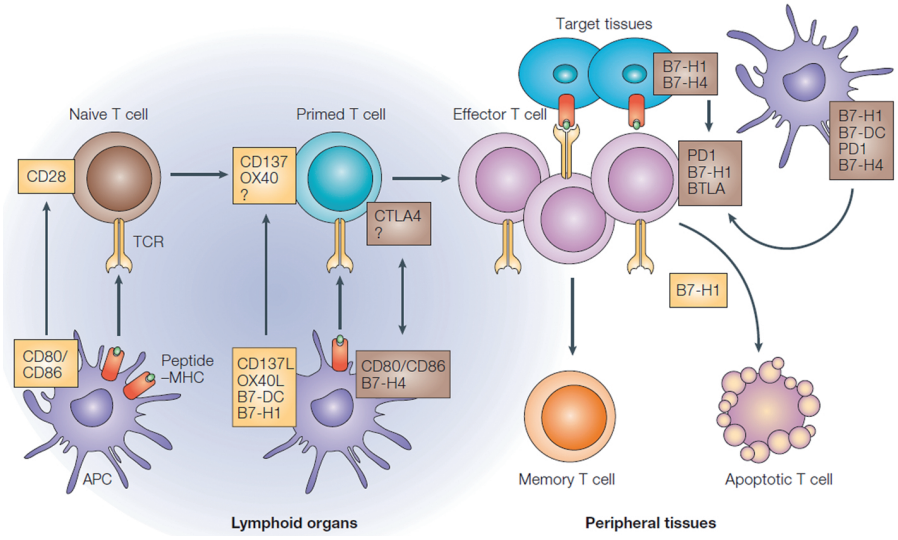

Figure 1.4: T cell activation and differentiation.
Activation of T cells occurs in lymphoid organs by antigen presenting cells (APCs) expressing co-stimulatory molecules and thereby promote T cell differentiation and maturation. Antigen-specific T cells expand and differentiate into effector T cells, which migrate into peripheral tissues. Most effector T cells are short-lived and die after antigen is cleared. A few antigen-experienced T cells survive as memory T cells.
B and T Lymphocyte attenuator (BTLA), programmed cell death 1 (PD1), cytotoxic T lymphocyte antigen 4 (CTLA4), (Figure obtained from [Brownlie and Zamoyska, 2013]).

$CD8^+$ T cells are able to directly kill target cells (virus-infected cells or tumor cells) by using lytic enzymes such as perforin and granulysin causing pores in the plasma membrane of the target cells. Consequently, Fas can be expressed and granzymes can be secreted by $CD8^+$ T cells and can flow through these pores into the target cells to initiate apoptosis [Kagi et al., 1994]. They also secrete cytokines such as Interferon-γ (IFNγ) and tumor necrosis factor (TNF). In contrast, $CD4^+$ T cells have only low cytotoxic capability, but could help to generate an efficient immune response through the secretion of different cytokines. There are different subsets of helper cells into which naïve $CD4^+$ T cells can differentiate. Previously it was believed that T_H1 cells exclusively provide help to $CD8^+$ T cells and T_H2 cells generally provide help to B cells. This assignment has changed by the description of additional subsets (T_H9, T_H17, T_H21, T follicular

helper [T_{FH}] cells and T regulatory cells [T_{reg}]). Each of them plays a distinct role in the overall immune response [Harrington et al., 2006]. T_H1 cells produce IL-2, IFNγ, TNFα leading to cell-mediated immunity by activation of macrophages and $CD8^+$ T cells. T_H2 cells mediate humoral immunity by secretion of IL-4, IL-5, IL-6, IL-10, and IL-13 leading to activation of antibody secreting B cells and the complement system. T_H17-type responses consist of T cells that produce IL-17. T_{FH} cells are a distinct subset of effector $CD4^+$ T cells that reside in germinal centers and help B cells to differentiate into antibody-secreting cells [Crotty, 2011]. T_{reg} cells suppress the function of other lymphocytes. There are several subsets of T_{reg} cells - natural and induced T_{reg} cells, thymic and peripheral T_{reg} cells and self-reactive and non-selfreactive T_{reg} cells. It could be shown that there are T_{reg} cells which were induced by the absence of co-stimulatory molecules and those induced in the presence of co-inhibitory molecules. There are T_{reg} cells that make TGFβ or IL-10 or neither [Shevach, 2006].

After activation, most of the effector T cells undergo a contraction phase wherein the majority of effector T cells die by apoptosis. A small pool of T cells survive as memory T cells, which carry the immunological memory. This memory T cells can efficiently proliferate following secondary antigen challenge [Kalia et al., 2010]. There are two different types of memory T cells - the central memory T cells (T_{cm}) and the effector memory T cells (T_{em}). T_{cm}, which carry the chemokine receptor CCR7, residing in secondary lymphoid organs have greater proliferative potential than T_{em} cells, which were CCR7-negative. T_{em} cells circulate in peripheral tissues and constitutively exhibit several effector functions (such as cytotoxicity) [Masopust et al., 2001].

A novel population of memory T cells with enhanced stem cell-like character (T_{scm}) compared to conventional T_{cm} cells were identified by Zhang et al. in 2005 [Zhang et al., 2005] in mice. In 2011, Gattinoni et al. described 2–3% of all circulating $CD8^+$ and $CD4^+$ T lymphocytes in healthy human donors as T_{scm} cells. They could show that T_{scm} cells have undergone clonal expansion, are long-lived, and that they rapidly acquired effector function following TCR stimulation [Gattinoni et al., 2011]. Like conventional memory T cells, they found persisting clonotypes in T_{scm} cells, representing a stable memory T cell population. Additionally, in adoptive transfer in NSG mice they demonstrated that T_{scm} cells have enhanced replicative and survival capabilities and also have enhanced anti-tumor activity and are more therapeutically effective compared to naïve and conventional memory subsets [Gattinoni et al., 2011].

1.4.3 TCR Signaling

Activation of T cells is initiated by ligation of TCRs by MHC molecules on antigen presenting cells (APCs) exposing peptides on MHC-complexes (peptide–MHC). The TCRs are complexed with CD3-chains, which carry intracellular signaling domains. The CD3-chains γ, δ, and ϵ of the TCR–CD3 complex each have a single extracellular domain, whereas CD3ζ has no extracellular domain. All of the CD3 chains have long cytoplasmic domains that contain immunoreceptor tyrosine based activation motifs (ITAMs). The SRC family kinase (SFK) members LCK and FYN are the first molecules which are activated following TCR clustering. The TCR them self has no intrinsic enzymatic activity and thereby depends on the kinase activity of the SFKs. LCK binds to the cytoplasmic domains of the TCR co-receptors CD4 and CD8 [Veillette et al., 1988]. After TCR binding to a peptide-MHC complex, these co-receptors are important for targeting of LCK into close proximity to the TCR-associated CD3 ITAMs [Artyomov et al., 2010]. Activated LCK phosphorylates the ITAMs in CD3γ-, CD3δ-, CD3ϵ-, and the CD3ζ-chains. This produces a structure for binding the paired SRC-homology 2 (SH2) domains of the SYK-family kinase ZAP70 (ζ-chain-associated protein kinase, 70 kDa). This results in conformational changes in ZAP70 [Deindl et al., 2007], which promotes its kinase activity, leading to the phosphorylation of its target molecules, including the linker for activation of T cells (LAT) [Zhang et al., 1998]. LAT is phosphorylated on several tyrosine residues, which act as docking sites for kinases and other adaptor molecules. These include the GRB2-related adaptor protein GADS and the SH2 domain-containing leukocyte protein of 76 kDa (SLP76). SLP76 interacts with phospholipase Cγ1 (PLCγ1) which interacts with interleukin-2-inducible T cell kinase (ITK; a kinase important for intracellular Ca^{2+} mobilization). The actin reorganization is also regulated by SLP76. This relies on bringing the RHO-family GTPase exchange factor VAV1, the adaptor protein NCK1 and actin nucleation-promoting factors, such as the Wiskott–Aldrich syndrome family proteins, into proximity.

The activation of PLCγ1 leads to an increase in the concentration of intracellular Ca^{2+}, which promotes the nuclear translocation of nuclear factor of activated T cells (NFAT) transcription factors and the activation of one or more mitogen activated protein kinase (MAPK) pathways leading to activation of nuclear factor-κB (NFκB) and activator protein 1 (AP-1) (see figure 1.5).

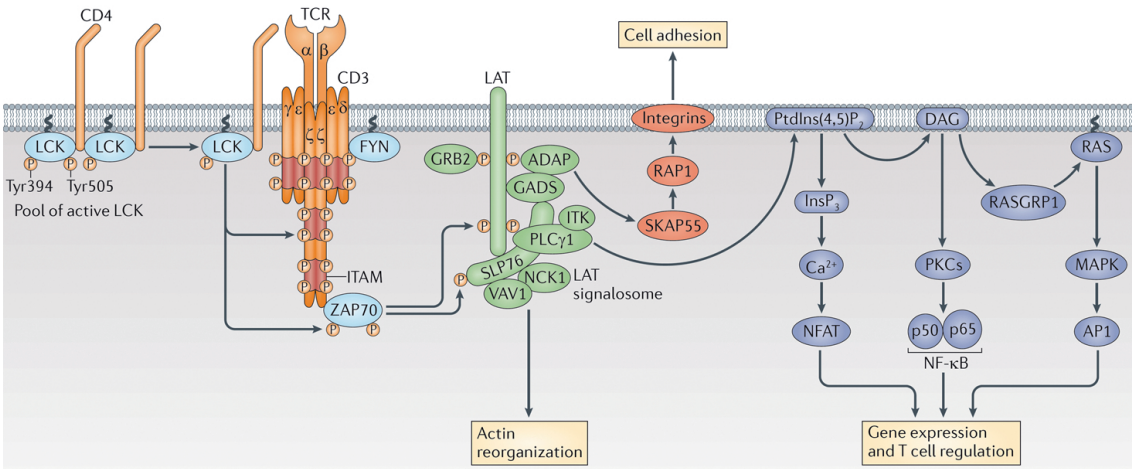

Figure 1.5: TCR signaling.
T cell receptor (TCR) signal transduction is initiated by the recognition of cognate peptide-MHC molecules on the surface of an antigen presenting cell. LCK is the first molecule which is recruited to the TCR-CD3 complex and phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3-chains. Phosphorylation of the ITAMs enables the recruitment of ZAP70, its phosphorylation by LCK and its activation. Activated ZAP70 (ζ-chain-associated protein kinase, 70 kDa) can then phosphorylate the linker for activation of T cells (LAT), which recruits numerous signaling molecules to form a multi-protein complex, termed the LAT signalosome. The LAT signalosome initiates different signaling pathways, including the Ca^{2+}, the mitogen activated protein kinase (MAPK) kinase and the nuclear factor-κB (NF-κB) signaling pathways, resulting in the mobilization of transcription factors.
Phospholipase Cγ1 (PLCγ1), growth factor receptor-bound protein 2 (GRB2), GRB2-related adaptor protein GADS, SH2 domain-containing leukocyte protein of 76 kDa (SLP76), adhesion- and degranulation-promoting adaptor protein (ADAP), interleukin-2 inducible T cell kinase (ITK), activator protein 1 (AP1), diacylglycerol (DAG), inositol-1,4,5-trisphosphate ($InsP_3$), nuclear factor of activated T cells (NFAT), protein kinase C (PKC), phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$), RAS guanyl-releasing protein 1 (RASGRP1), SRC kinase-associated phosphoprotein of 55 kDa (SKAP55). (Figure obtained from [Brownlie and Zamoyska, 2013]).

The signaling pathways that direct the events derived from TCR engagement ensure the formation of the immunological synapse (see figure 1.6). The immunological synapse is a specialized cell-cell junction between a T cell and an APC and can be divided into three supramolecular activation clusters (SMACs) [Kupfer et al., 1998]. The central SMAC (cSMAC) consists of the TCR-CD3 complex, CD4 or CD8 co-receptors, co-stimulatory (e.g. CD28) and co-inhibitory (e.g. cytotoxic T lymphocyte antigen 4 - CTLA4) molecules, CD2 and CD58 adhesion molecules, LCK and FYN kinases and down-stream signaling molecules. The cSMAC is surrounded by a peripheral ring enriched with the integrin leukocyte function-associated antigen 1 (LFA-1) and the cytoskeletal protein talin forming the peripheral SMAC (pSMAC). The transmembrane

molecules CD43, CD44, and CD45 are localized in a region distal to the synapse outside the pSMAC, known as distal SMAC (dSMAC).

The reorganization of the T cell surface molecules, after interaction of a T cell with an APC, are required for optimal functional interactions. CD45 is crucial for LCK activation by removing an inhibitory phosphate from LCK. After antigen binding, TCRs move to the center of the immunological synapse, where they initiate the signal transduction. Therefore, the formation of the immunological synapse enables productive TCR signaling.

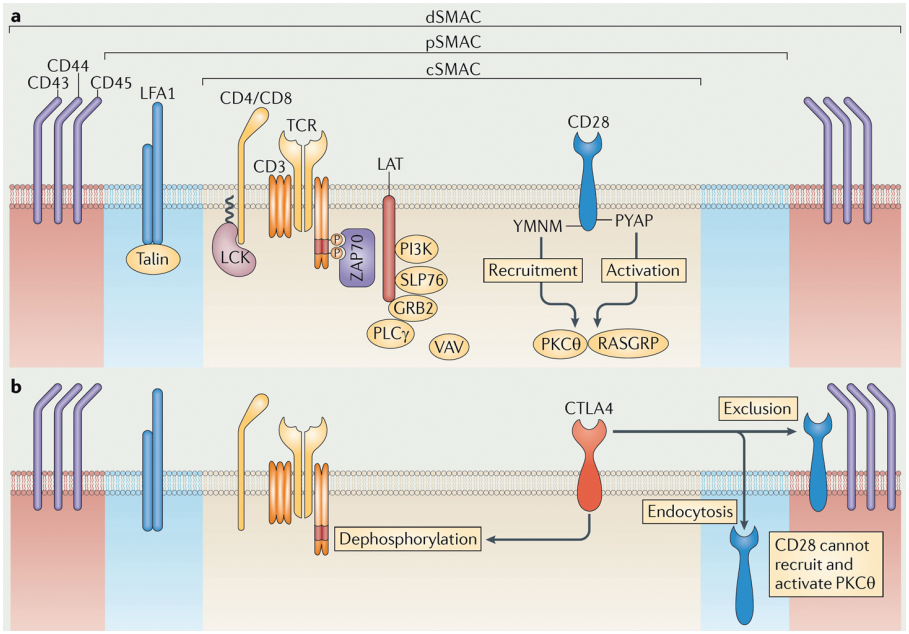

Figure 1.6: The immunological synapse.
The immunological synapse is composed of the central, peripheral and distal supramolecular activation complexes (cSMAC, pSMAC and dSMAC), which contain specific molecules including co-stimulatory molecules e.g. CD28 (part a) or co-inhibitory molecules e.g. cytotoxic T lymphocyte antigen 4 (CTLA4) (part b).
Protein kinase Cθ (PKCθ), RAS guanyl nucleotide-releasing protein (RASGRP), programmed cell death 1 (PD1), growth factor receptor-bound protein 2 (GRB2), linker for activation of T cells (LAT), lymphocyte cell-specific protein-tyrosine kinase (LCK), lymphocyte function associated antigen 1 (LFA1), phosphatidylinositol 3-kinase (PI3K), Phospholipase Cγ1 (PLCγ1), SH2 domain-containing leukocyte protein of 76 kDa (SLP76), ζ-chain-associated protein kinase, 70 kDa (ZAP70). (Figure obtained from [Chen and Flies, 2013]).

1.4.4 Regulation of T cell activation

One way to regulate the signaling in T cells is mediated by the phosphorylation of tyrosine residues in the active site of the SFK kinase domain. The phosphorylation of an activating tyrosine residue in their catalytic domain stabilizes an open conformation and promotes their full kinase activity. In contrast, the phosphorylation of a tyrosine residue in their carboxy-terminal domain results in a closed conformation of the protein and the downregulation of their kinase activity [Boggon and Eck, 2004], [Yamaguchi and Hendrickson, 1996]. The C-terminal Src kinase (Csk) negatively regulates SFKs by phosphorylating their inhibitory tyrosine residues [Okada et al., 1991]. The activating tyrosine residues could also be dephosphorylated by several phosphatases, including CD45 and the cytosolic phosphatases protein tyrosine phosphatase non-receptor type 6 (PTPN6, also known as SHP1) and PTPN22.

For SHP-1 many potential substrates have been identified in lymphocytes. These include the activating tyrosine residues of SFKs, ITAMs, ZAP70, and the adaptors LAT and SLP76. SHP-1 interacts with a variety of tyrosine-phosphorylated molecules through its SH2 domains. After T cell activation, SHP-1 is phosphorylated at a tyrosine in its C-terminal tail [Stefanova et al., 2003]. This phosphorylation causes the binding of SHP-1 to e.g. LCK, which leads to dephosphorylation and inactivation of LCK. SHP-2 is a relative of SHP-1 and has been shown to negatively regulate lymphocyte activity in a like manner [Frearson and Alexander, 1998], [Kwon et al., 2005], [Salmond et al., 2005].

PTPN22 is a potent negative regulator immediately downstream of TCR, which interacts with Csk through a proline-rich domain and dephosphorylates the activating Y394 residue of LCK, downregulating its activity [Cloutier and Veillette, 1999]. It has been shown that PTPN22 also acts by dephosphorylating the activating tyrosine residues of SFKs, as well as SFK substrates such as ZAP70, and the E3 ubiquitin ligase c-Cbl [Cloutier and Veillette, 1999], [Gjorloff-Wingren et al., 1999]. PTPN22 is upregulated in effector T cells to counteract increased sensitivity to activation and thereby prevents autoreactivity [Hasegawa et al., 2004], [Brownlie et al., 2012].

Another way to regulate the signaling in T cells is by targeting signaling molecules in the downstream pathway. The dual specificity phosphatases DUSP5 and DUSP6, which have different cellular localization, can specifically inactivate ERK1/2 by dephosphorylating the T202 and Y204 residues in the nucleus and cytosol [Theodosiou and Ashworth, 2002]. Both DUSP contain an N-terminal MAPK targeting domain and a C-terminal catalytic domain [Keyse, 2000]. The binding of their substrate causes a conformational change which results in rearrangement of DUSP active-site residues, resulting in a high-activity state of the phosphatase [Theodosiou and Ashworth, 2002].

These regulatory feedback loops exist to terminate or to limit T cell signaling to avoid autoreactivity. The regulation of T cell signaling cascades sets the threshold of activation, so that they are not fully activated by self-peptide-MHC complexes and only respond to foreign-peptide-MHC complexes.

1.5 microRNAs and Immunity

microRNAs (miRNAs) are $\sim$22 nt single-stranded RNAs that play essential roles in animals and plants [Ambros, 2004]. They function by direct binding to specific target messenger RNAs (mRNAs), leading to repression of protein expression and the promotion of target mRNA degradation [Ambros, 2003], [Bartel and Chen, 2004]. The first described miRNA found in 1993 is *lin*-4, which was identified as heterochronic gene controlling the developmental timing of cell fate at larval stages in *Caenorhabditis elegans* [Lee et al., 1993], [Wightman et al., 1993]. Until today $\sim$1100 different miRNAs have been identified in the human genome [microRNA, 2013]. Each miRNA can repress the expression of many, perhaps hundreds of, target genes [Selbach et al., 2008]. More than 100 miRNAs are expressed in immune cells, thereby having the potential to broadly influence the molecular pathways that control the development and function of innate and adaptive immune responses (see table 1.1) [Lindsay, 2008], [O'Connell et al., 2010].

Table 1.1: Selected miRNAs with roles in the immune system. (modified from [Lindsay, 2008])

miRNA	Function	Targets
miR17-5p	In combination with miR20a and miR106a inhibits monocyte proliferation, differentiation and maturation	AML-1
miR17∼92	Regulates pro- to pre-transition during B and T cell development	Bim, PTEN
miR20a	In combination with miR17-5p and miR106a inhibits monocyte proliferation, differentiation and maturation	AML-1
miR106a	In combination with miR17-5p and miR20a inhibits monocyte proliferation, differentiation and maturation	AML-1
miR125b	Expression downregulated by LPS and oscullations in expression after exposure to TNFα	TNFα
miR146	Expression induced in macrophages and alveolar/bronchial epithelial following activation	IRAK1, TRAF6
miR150	Increased expression leads to suppression of B cell formation by blocking in pro- to pre-B cells transition	
miR155	Required for normal production of isotype-switched, high-affinity IgG1 antibodies in B cells; determines T_H1 and T_H2 differentiation and positive regulator of antigen induced responses in T cells	PU.1, c-Maf
miR181a	Positive regulator of B and T cell development and $CD4^+$ T cell selection, activation and sensitivity	SHP2, PTPN22, DUSP5, DUSP6
miR223	Negative regulator of neutrophil proliferation and activation	Mef2c, IGFR

miRNAs are encoded by genomic DNA as single miRNA transcripts or in the introns of protein coding transcripts [Rodriguez et al., 2004]. Primary miRNAs (pri-miRNAs) containing 5'7-methylguanosine cap and 3'poly (A) tail are transcribed by the RNA polymerase II [Cai et al., 2004], [Lee et al., 2004]. The pri-miRNAs are then processed by the enzymes Drosha (RNAse III endonuclease) and DiGerge syndrome critical region gene 8 (DGCR8) [Lee et al., 2003] [Denli et al., 2004], [Gregory et al., 2004], [Han et al., 2004], [Landthaler et al., 2004], into a $\sim$70 nt intermediate with a stem-loop hairpin structure, the precursor miRNAs (pre-miRNAs). The pre-miRNAs are subsequently transported to the cytoplasm by the RanGTP-dependent dsRNA-binding protein Exportin 5 [Bohnsack et al., 2004], [Lund et al., 2004], where they are further processed into $\sim$22 nt miRNA duplex by the cytoplasmic RNAse III enzyme Dicer

[Ketting et al., 2001], [Lee et al., 2002]. The Dicer cleavage process is coupled with the integration of the guide strand (mature miRNA) into the RNA-induced silencing complex (RISC). The mature miRNA then guides the RISC to its target mRNA, which it recognizes through sequence complementarity, leading to inhibition of translation and/or decrease in mRNA stability [Chekulaeva and Filipowicz, 2009]. Whether target mRNAs are cleaved and degraded is determined by the complementarity between miRNAs and target mRNAs [Bartel, 2004], [Engels and Hutvagner, 2006]. Complete sequence complementarity leads to endonucleolytic cleavage and degradation of target mRNA, whereas less strict complementarity results in transcriptional repression [Cannell et al., 2008]. The miRNA biogenesis is depicted in figure 1.7.

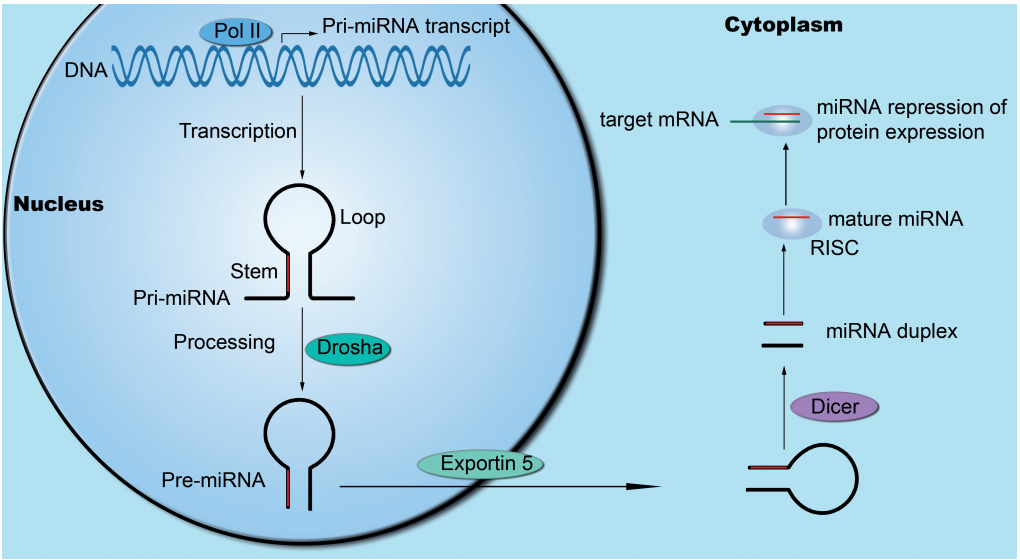

Figure 1.7: The biogenesis of miRNAs.
Primary miRNA (pri-miRNA) is transcribed by RNA polymerase II and then cleaved by the Drosha-DGCR8 complex to precursor miRNA (pre-miRNA). After export into the cytoplasm by Exportin 5, the pre-miRNA is further processed into $\sim$22 nt double miRNA duplex by the cytoplasmic RNAse III enzyme Dicer. The guide strand incorporates into the RNA-induced silencing complex (RISC), where the mature miRNA can bind to their target mRNA by base pairing, causing inhibition of protein translation or degradation of the mRNA according to the degree of homology between miRNA and target mRNA.

Many miRNAs are differentially regulated in hematopoietic lineages, some have been shown to play roles in controlling the development of immune cells [Chen, 2004], [Fazi et al., 2005]. Expression profiling of T cells has identified a broad range of miRNAs, which expression patterns vary between T cell subsets and stages of development [Monticelli et al., 2005], [Wu et al., 2007], [Merkerova et al., 2008]. The miR17$\sim$92 cluster affects the expression of pro-

apoptotic proteins, including BCL-2-interacting mediator of cell death (BIM) and phosphate and tensin homologue (PTEN). This miRNA cluster is expressed during the DN2 stage of the T cell development and is thought to increase T cell survival [Xiao et al., 2008]. Another important miRNA involved in the thymopoiesis is miR181a and will be further described in section 1.5.1. There is also evidence for a role of miRNAs in the differentiation of T cells into distinct T cell subsets. miR155 promotes differentiation into T_H2 cells by modulating c-Maf (musculoaponeurotic fibrosacroma) expression [Thai et al., 2007], [Rodriguez et al., 2007]. There are data showing that miR155 is also important for T_{reg} cell homeostasis and overall survival. This is thought to be achieved by direct targeting of *Socs*1 [Kohlhaas et al., 2009], [Lu et al., 2009]. The clonal expansion of activated helper T cells is driven by the IL-2 dependent expression of miR182. Here, miR182 directly represses *Foxo*1 [Stittrich et al., 2010]. These little insight shows that the development of T cells in the thymus and their activation in the periphery are controlled by miRNAs.

1.5.1 miR181a expression in T cells

Early studies by Chen et al. demonstrated that miR181a is selectively expressed in thymus-derived B cells. Therefore the authors identified a positive role for miR181a in B cell differentiation [Chen, 2004]. They also showed a reduction of $CD8^+$ T cells after ectopic expression of miR181a in mouse hematopoietic precursor cells, which suggested a potential role in the regulation in T cell differentiation. Subsequent studies demonstrated dramatic changes in miR181a expression during various stages of T cell development [Li et al., 2007]. The analysis of miR181a amounts in different DN (1-4) cells displayed a dynamic regulation of miR181a expression during T cell maturation. miR181a expression is higher in the early differentiation stages (DN1-DN3) than in the later stages (DN4, DP, $CD4^+$ SP, $CD8^+$ SP thymocytes), whereby the expression of miR181a in $CD4^+$ SP T cells is higher than in $CD8^+$ SP T cells [Li et al., 2007]. Li et. al showed that miR181a regulation of the positive and negative selection of T cells did not result from changes in the expression of surface receptors but involved the downregulation of multiple phosphatases. These included the tyrosine phosphatases SHP-2 and PTPN22 and the ERK-specific phosphatases DUSP5 and DUSP6. These phosphatases are all known to negatively regulate the TCR signaling pathway. Thereby, increased miR181a level caused reduced

phosphatase levels and finally lead to increased basal phosphorylation of protein kinases such as Lck and ERK [Li et al., 2007]. Li et al. could additionally show that treatment of thymocytes with antago-mir-181, which degrades its endogenous counterpart, reduces ERK activity and impairs positive selection of thymocytes in a fetal thymic organ culture system. This multi-target regulation (see figure 1.8) by miR181a was required for modulating T cell sensitivity, since the regulation of the T cell response by miR181a could not be fully recapitulated by knockdown of the single target protein levels with shRNAs [Li et al., 2007].

Another study also showed that miR181a was involved in positive selection of T cells. Conversely, they demonstrated that this was achieved by repressing the expression of BCL-2, CD69 and the TCR [Neilson et al., 2007]. Two years later, Ebert et al. demonstrated a miR181a-dependent transition from higher to lower TCR sensitivity as cells progress from the DP stage to the SP stage [Ebert et al., 2009]. DP thymocytes are more sensitive to TCR stimuli than SP, due at least in part to their higher miR181a expression. They also provided evidence that TCR signals directly feed back to modulate miR181a expression [Ebert et al., 2009].

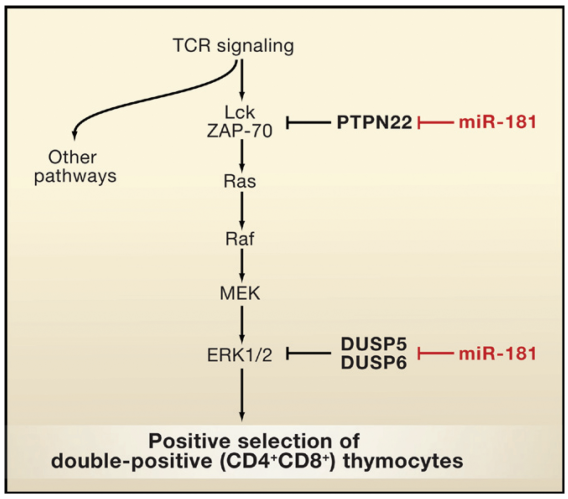

Figure 1.8: miR181a regulates TCR signaling by modulating multiple negative regulators.
In double positive ($CD4^+CD8^+$) thymocytes miR181a is highly expressed and is further downregulated upon T cell maturation. High expression of miR181a enables double positive thymocytes, but not mature T cells, to respond to self-antigens and are thereby positively or negatively selected. This process is characterized by downregulating the concentrations of several negative regulators of TCR signaling. (Figure obtained from [Xiao and Rajewsky, 2009]).

Li et al. reported in 2012 that naïve $CD4^+$ T cells from elderly individuals have a reduced signaling capacity of the ERK pathway [Li et al., 2012]. They could show that these results from increased protein expression of DUSP6 due to a decline in repression by miR181a. Li et al. conclude that the higher levels of DUSP6 enhance the threshold for full T cell activation by impairing the initial ERK signal after TCR stimulation [Li et al., 2012]. Consistent with previous studies [Li et al., 2007], they showed that only T cells with higher affinity to antigen are activated [Li et al., 2012].

In 2013, Henao-Mejia et al. demonstrated that miR181-deficient mice show defects in T cell development and homeostasis and defects in B cell development, as well as reduced organ size [Henao-Mejia et al., 2013]. miR181 deletion results in a complete absence of NKT cells in the thymus and periphery. They also proved that key components of the glycolytic pathway, pentose phosphate pathway, and nucleotide biosynthetic pathways were significantly down-regulated in miR181-deficient DP thymocytes. For instance, phosphatidylinositol 3-kinase (PI3K) signaling is regulated by miR181 through modulating the PTEN expression [Henao-Mejia et al., 2013]. All these findings demonstrate that miR181-mediated metabolic regulation has global physiological importance.

1.6 Aim of the thesis

The adoptive transfer of genetically engineered T cells is a promising immunotherapeutic approach for selective tumor elimination. Nonetheless, T cells against tumor-antigens exhibit only weak anti-tumoral responses. To overcome this barrier it is necessary to improve the potency and anti-tumoral efficacy of these T cells.

miR181a systematically reduces negative feedback at distinct steps of TCR signaling by down-regulating the protein level of multiple phosphatases that are negative regulators of the TCR signaling pathway [Li et al., 2007]. Thereby, increased miR181a results in reduced phophatase levels and higher basal phosphorylation of protein kinases (Lck and ERK1/2), which could increase TCR signaling and reduce the T cell activation threshold.

The aim of this thesis was to analyze whether miR181a overexpression in primary T cells could potentiate TCR signals in a way that the excitation threshold could be overcome, to generate high affinity T cells for adoptive T cell therapy. To study the role of miR181a in mature T cells

different *in vitro* assays were performed using primary human and murine T cells. These assays include cytokine expression analysis under different stimulation conditions, proliferation, apoptosis, and cytotoxicity. Finally an *in vivo* tumor model was generated to study the influence of miR181a overexpression in murine TCR^{tg} T cells on anti-tumor reactivity.

2 Materials and Methods

2.1 Materials

2.1.1 Hardware

Table 2.1: Hardware.

Hardware	Manufacturer
ABI Prism 7700 Sequence Detection System	Applied Biosystems, Foster City, USA
BioMark HD System	Fluidigm Corporation, San Francisco, USA
Bio-Plex 200	Bio-Rad, Munich, Germany
Centrifuge 5810	Heraeus instruments, Hanau, Germany
CO_2 Cell incubator	Heraeus instruments, Hanau, Germany
Digital Camera DCF420	Leica, Hamburg, Germany
Electrophoresis power supply ST 606	Gibco BRL/Invitrogen, Karlsruhe, Germany
FACS Aria	BD Biosciences, Heidelberg, Germany
FACS CantoII	BD Biosciences, Heidelberg, Germany
FACS Fortessa	BD Biosciences, Heidelberg, Germany
Gel documentation system Gel Jet Imager	Intas, Göttingen, Germany
Laminar flow bench herasafe	Heraeus instruments, Hanau, Germany
MACS Magnet	Miltenyi Biotec, Bergisch Gladbach, Germany
Microscope Wilovert S	Hund, Wetzlar, Germany
Microscope DM2000	Leica, Hamburg, Germany
Nanodrop 2000	Thermo Scientific, St. Leon-Rot , Germany
Neubauer Chamber	Carl Roth GmbH, Karlsruhe, Germany
Table centrifuge mikro 22R	Hettich, Tuttlingen, Germany
Thermocycler T3	Biometra, Göttingen, Germany
Thermomixer compact	Eppendorf, Hamburg, Germany
Tecan Infinite 200 reader	Tecan, Crailsheim, Germany
Vortexer VF2	IKA Labortechnik, Staufen, Germany

2.1.2 Consumables

Table 2.2: Consumables.

Consumables	Manufacturer
24 well plates	Costar, New York, USA
24 non tissue culture plates	BD Biosciences, Heidelberg, Germany
6 well plates	Costar, New York, USA
96 well plates (round bottom)	Costar, New York, USA
96 well plates (white flat)	Nunc, Wiesbaden, Germany
Cell strainer, 70 μm, Nylon	BD Biosciences, Heidelberg, Germany
Eppendorf tubes, 1.5 and 2 mL	Eppendorf, Hamburg, Germany
FACS tubes	BD Biosciences, Heidelberg, Germany
Falcon tubes, 15 and 50 mL	Greiner Bio-One GmbH, Frickenhausen, Germany
Filter tips, AvantGuard 100-1250 μL	Light Labs, Dallas, USA
Filter tips, Biosphere 10-100 μL	Sarstedt, Nümbrecht, Germany
Filter tips, Neptune 1-10 μL	Continental Lab Products, San Diego, USA
Glasslides	Carl Roth GmbH, Karlsruhe, Germany
Insulin syringes	BD Biosciences, Heidelberg, Germany
MACS separation columns	Miltenyi Biotec, Bergisch Gladbach, Germany
Micro haematocrit capillary tubes	Brand, Wertheim, Germany
PCR tubes, 0.5 mL	Molecular BioProducts, San Diego, USA
RNase-free tubes, 1.5 and 2 mL	Eppendorf, Hamburg, Germany
Serological Pipette, 5 mL, 10 mL, 15 mL	Greiner Bio-One GmbH, Frickenhausen, Germany
Syringe, 2 mL, 5 mL, 10 mL	BD Biosciences, Heidelberg, Germany
Tissue culture flasks	BD Biosciences, Heidelberg, Germany

2.1.3 Chemicals and enzymes

Table 2.3: Chemicals and enzymes.

Designation	Manufacturer
Adenosine-5'-triphosphatase (ATPase)	Sigma-Aldrich, Munich, Germany
Agar	Difco, Detroit, USA
Agarose	Sigma-Aldrich, Munich, Germany
Ampicillin	Sigma-Aldrich, Munich, Germany
APC Annexin V	BD Biosciences, Heidelberg, Germany
Bam*HI*	Fermentas, Thermo Scientific, St. Leon-Rot, Germany
BD Blood Lysis Buffer	BD Biosciences, Heidelberg, Germany
BD Permeabilization Solution 2	BD Biosciences, Heidelberg, Germany
BD Perm/Wash	BD Biosciences, Heidelberg, Germany
Bgl*II*	Fermentas, Thermo Scientific, St. Leon-Rot, Germany
Bovine serum albumin (BSA)	Serva, Heidelberg, Germany
Brefeldin A	Sigma-Aldrich, Munich, Germany
Calcium chloride	Carl Roth GmbH, Karlsruhe, Germany
Cell Proliferation Dye eFluor 670	eBioscience, Frankfurt, Germany
Citratbuffer	Carl Roth GmbH, Karlsruhe, Germany
Collagenase D	Roche, Mannheim, Germany
Di-nucleic acid tri-phosphate (dNTP)	Invitrogen, Life Technologies, Darmstadt, Germany
D-Luciferin	BD Biosciences, Heidelberg, Germany
Eosin	Carl Roth GmbH, Karlsruhe, Germany
Ethanol absolute p.A.	Applichem, Darmstadt, Germany
Ethidiumbromide	Sigma-Aldrich, Munich, Germany
Ethylendiamintetraacetate (EDTA)	Sigma-Aldrich, Munich, Germany
Ethylenglycol- bis (β-aminoethylehter)-N,N,N,N tetraacetic acid (EGTA)	Carl Roth GmbH, Karlsruhe, Germany
FACS Clean/Flow/Rinse	BD Biosciences, Heidelberg, Germany
FACS Lysing Solution	BD Biosciences, Heidelberg, Germany
Fetal calf serum (FCS)	PAA laboratories, Cölbe, Germany
Ficoll-Hypaque	Amersham Biosciences, Uppsala, Sweden
Fugene6	Roche, Mannheim, Germany
GeneRuler 50bp and 1kB DNA Ladder	Fermentas, Thermo Scientific, St. Leon-Rot, Germany

Designation	Manufacturer
Geneticin	Invitrogen, Life Technologies, Darmstadt, Germany
GM-CSF	PeproTech, London, UK
Haematoxylin	Carl Roth GmbH, Karlsruhe, Germany
HEPES	Invitrogen, Life Technologies, Darmstadt, Germany
Histofix	Carl Roth GmbH, Karlsruhe, Germany
Human serum albumin (HSA)	CSL Behring, Marburg, Germany
Indo-1	Invitrogen, Life Technologies, Darmstadt, Germany
Interleukines: IL-2, IL-4	Miltenyi Biotec, Bergisch Gladbach, Germany
Interleukin 7	PeproTech, London, UK
Interleukin 15	R&D System, Minneapolis, USA
Isofluoran	Abbott, Wiesbaden, Germany
Isopropanol p.a.	Merck, Darmstadt, Germany
Kanamycin	Sigma-Aldrich, Munich, Germany
LR clonase enzyme mix	Invitrogen, Life Technologies, Darmstadt, Germany
β-Mercaptoethanol	Invitrogen, Life Technologies, Darmstadt, Germany
Non-essential amino acids (NEAA)	Invitrogen, Life Technologies, Darmstadt, Germany
Parafin	Carl Roth GmbH, Karlsruhe, Germany
Paraformaldehyde (PFA)	Carl Roth GmbH, Karlsruhe, Germany
Phosphate buffered saline (PBS)	Invitrogen, Life Technologies, Darmstadt, Germany
PE Annexin V	BD Biosciences, Heidelberg, Germany
Penicillin / Streptomycin	Invitrogen, Life Technologies, Darmstadt, Germany
Phenol	Carl Roth GmbH, Karlsruhe, Germany
Polybrene	Sigma-Aldrich, Munich, Germany
Propidium iodide	Sigma-Aldrich, Munich, Germany
RPMI 1640 + GlutaMAX	Invitrogen, Life Technologies, Darmstadt, Germany
Retronectin	Takahara
Sodium acetate	Carl Roth GmbH, Karlsruhe, Germany
Sodium chlorid	Carl Roth GmbH, Karlsruhe, Germany
Sodium pyruvate	Invitrogen, Life Technologies, Darmstadt, Germany
Superscript II	Invitrogen, Life Technologies, Darmstadt, Germany
SYBR Green QuantiTect	Qiagen, Hilden, Germany
Stain Buffer (BSA)	BD Biosciences, Heidelberg, Germany
T4 Ligase	Fermentas, Thermo Scientific, St. Leon-Rot, Germany
Trishydroxymethylaminomethan (TRIS)	Applichem, Darmstadt, Germany
Triton-X100	Merck, Darmstadt, Germany

Designation	Manufacturer
Trypan Blue	Invitrogen, Life Technologies, Darmstadt, Germany
Trypton	Difco, Detroit, USA
Xylol	Carl Roth GmbH, Karlsruhe, Germany
Yeast extract	Difco, Detroit, USA

2.1.4 Antibodies

Table 2.4: Antibodies.

Designation	Manufacturer
Alexa Fluor 647 Mouse anti-LAT (pY226)	BD Biosciences, Heidelberg, Germany
Anti-Hamster IgG	Novus Biologicals, Littelton, USA
Anti-Human IFNγ PE-Cy7	eBioscience, Frankfurt, Germany
Anti-Human CD8a eFluor 650NC	eBioscience, Frankfurt, Germany
Anti-Human CD28	BioLegend, Eching, Germany
Anti-Mouse CD3e Functional Grade Purified	eBioscience, Frankfurt, Germany
Anti-Mouse CD28	Thermo Scientific, St. Leon-Rot , Germany
Anti-Mouse CD4 PE-Cy7	eBioscience, Frankfurt, Germany
Anti-Mouse CD8 PE-Cy7	eBioscience, Frankfurt, Germany
Anti-Mouse IgG2a	BioLegend, Eching, Germany
Anti-Mouse IFNγ PE-Cy7	eBioscience, Frankfurt, Germany
Anti-Mouse IL-2 PerCP-Cy5.5	eBioscience, Frankfurt, Germany
Anti-TNFα human	Miltenyi Biotec, Bergisch Gladbach, Germany
APC Mouse Anti-Human CD137	BD Biosciences, Heidelberg, Germany
APC Rat Anti-Mouse CD90.2	BD Biosciences, Heidelberg, Germany
APC-Cy7 Mouse Anti-Human CD4	BD Biosciences, Heidelberg, Germany
APC-Cy7 Mouse Anti-Human CD8	BD Biosciences, Heidelberg, Germany
APC-Cy7 Rat Anti-Mouse CD4	BD Biosciences, Heidelberg, Germany
APC-Cy7 Rat Anti-Mouse CD8a	BD Biosciences, Heidelberg, Germany
APC-Cy7 Rat Anti-Mouse CD62L	BD Biosciences, Heidelberg, Germany
Orthoclone OKT3 (Anti-Human CD3)	Janssen-Cilag, Neuss, Germany

Designation	Manufacturer
PE Anti-Human CD127	eBioscience, Frankfurt, Germany
PE Hamster Anti-Mouse CD154	BD Biosciences, Heidelberg, Germany
PE Mouse Anti-ERK1/2 (pT202/pY204)	BD Biosciences, Heidelberg, Germany
PE Mouse Anti-Human CD154	BD Biosciences, Heidelberg, Germany
PE Rat Anti-Mouse CD44	BD Biosciences, Heidelberg, Germany
PE-Cy7 Anti-Human IFNγ	eBioscience, Frankfurt, Germany
PE-Cy7 Rat Anti-Mouse TNFα	BD Biosciences, Heidelberg, Germany
PerCP-Cy5.5 Anti-Human IL-2	BioLegend, Eching, Germany
PerCP-Cy5.5 Rat Anti-Mouse CD25	BD Biosciences, Heidelberg, Germany
Purified anti-human CD28	BioLegend, Eching, Germany
V450 Mouse Anti-Human CD4	BD Biosciences, Heidelberg, Germany
V450 Mouse Anti-Human CD8	BD Biosciences, Heidelberg, Germany
V450 Rat Anti-Mouse CD44	BD Biosciences, Heidelberg, Germany
V500 Mouse Anti-Human CD3	BD Biosciences, Heidelberg, Germany

2.1.5 Peptides

Table 2.5: Peptides.

Designation	Sequence
Infl.-HA MHC class I	IYSTVASSL
Infl.-HA MHC class II	SFERFEIFPK

2.1.6 Buffer and Media

Table 2.6: Buffers.

Buffer	Composition
Annexin V Binding Buffer	0.1 M Hepes pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$
D-Luciferin Reaction Mix	1 μg/μL D-Luciferin, 50 mM Hepes, 0.4 mU/μL ATPase
EGTA Buffer	0.1 M Hepes pH 7.4, 1.4 M NaCl, 200 mM EGTA
Erythrocyte Lysis Buffer	2,89 g/L NH_4Cl, 1 g/L $KHCO_3$, 37,2 mg/L EDTA pH 7.5
FACS-Buffer	500 mL PBS, 5 % FCS_{hi}, 5 mM EDTA
Human Cell Culture Media	500 mL RPMI-medium containing Glutamax, 5 % Human serum-Type AB (heat inactivated), 1 % Sodium pyruvate (100 mM), 1 % Non-essential amino acids (NEAA), 0,5 % Penicillin (100 U/ml) / Streptomycin (100 μg/ml)
MACS-Buffer	500 mL PBS, 5 % HSA, 5 mM EDTA
Murine Cell Culture Media	500 mL RPMI-medium containing Glutamax, 10 % FCS (heat inactivated), 1 % Sodium pyruvate (100 mM), 1 % Non-essential amino acids (NEAA), 1 % HEPES (1 M), 500 μM β-Mercaptoethanol, 0,5 % Penicillin (100 U/ml) / Streptomycin (100 μg/ml)
LB-Agar	1 % Trypton (w/v), 0,5 % Yeast-Extract (w/v), 1 % NaCl (w/v), 1,5 % Agar-Agar (w/V) pH 7.5
LB-Media	1 % Trypton (w/v), 0,5 % Yeast-Extract (w/v), 1 % NaCl (w/v) pH 7.5
SOC-Medium	20 g/L Trypton, 5 g/L Yeast-Extract, 0.5 g/L NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM Glucose pH 7.0
TAE-Puffer, 50x (Tris-Acetat-EDTA)	2 M Tris-Base, 0.25 M NaOAc, 50 mM EDTA pH 7.8

2.1.7 Kits

Table 2.7: Kits.

Kit	Manufacturer
Cell Proliferation Dye eF670 Labeling Kit	eBioscience, Frankfurt, Germany
Fluidigm Immune Assay human	Applied Biosystems, Foster City, USA
Fluidigm Immune Assay murin	Applied Biosystems, Foster City, USA
Magnetic acitivated cell sorting (MACS) Kit $CD4^+$ and $CD8^+$ T cells (human and murin), $CD14^+$ Monocytes (human), $CD90.2^+$ cells (murine)	Miltenyi Biotec, Bergisch Gladbach, Germany
miRNeasy Mini Kit	Qiagen, Hilden, Germany
miScript Reverse Transcription Kit	Qiagen, Hilden, Germany
Pro Human Cytokines GroupI 7-plex (IL-2, IL-4, IL-10, IL-17, IFNγ, TNFα, RANTES)	Bio-Rad, Munich, Germany
NucleoSpin Kit	Macherey-Nagel, Düren, Germany
QiaQuick Gel Extraction Kit	Qiagen, Hilden, Germany
Qiaquick MinElute Gel Extraction Kit	Qiagen, Hilden, Germany
Qiaquick MinElute PCR Purification Kit	Qiagen, Hilden, Germany
Red Active Caspase Staining Kit	Abcam, Cambridge, UK

2.1.8 Primer

All oligos were purchased and synthesized from MWG operon.

Table 2.8: Primer.

Primer	Sequence (5′ ⟶ 3′)
dT18-tag	GAGATCTCGAGATCTCGATCGTACTTTTTTTTTTTTTTTT
hActin-s	AATGTGGCCGAGGACTTTGATTGC
hActin-as	AGGATGGCAAGGGACTTCCTGTAA
hDUSP5-s	TGTCAGCTACAGGCCAGCTTATGA
hDUSP5-as	TGCATGGTAGGCACTTCCAAGGTA
hDUSP6-s	TGGGAAAGACACCAAATCATGGGC
hDUSP6-as	TGCATTTGAGGTGACACTCCCTGA

Primer	Sequence (5′ ⟶ 3′)
hGAPDH-s	CCAGCCGAGCCACATCGCTCA
hGAPDH-s	CCATGGGTGGAATCATATTGG
hHPRT-s	TGACACTGGCAAAACAATGCA
hHPRT-s	GGTCCTTTTCACCAGCAAGCT
hPTPN22-s	TGGCTGTGGAAGGACTGGTGTTAT
hPTPN22-as	TAATGAAGGCCTCTGTGTCCGCAT
hSHP-2-s	ATGGAGCTGTCACCCACATCAAGA
hSHP-2-s	GACCAACTCAGCCAAAGTGGCAAA
miR181a	AACATTCAACGCTGTCGGTGAGT
mActin-s	TGTGATGGTGGGAATGGGTCAGAA
mActin-as	TGTGGTGCCAGATCTTCTCCATGT
mDUSP5-s	TGGTCTCTCCCAACTTTGGCTTCA
mDUSP5-as	TGCATATCAGGGCTCAGTGTCTGT
mDUSP6-s	AGTCGTCACACATCGAATCTGCCA
mDUSP6-as	ACCAGTGTTCTCATTCCAGTCGCT
mGAPDH-s	TCAACAGCAACTCCCACTCTTCCA
mGAPDH-s	ACCCTGTTGCTGTAGCCGTATTCA
mHPRT-s	AGGAGTCCTGTTGATGTTGCCAGT
mHPRT-s	GGGACGCAGCAACTGACATTTCTA
mPTPN22-s	AACACAGAGGCCTTCGCTAGTTCA
mPTPN22-as	TCAGCTTCTACCGTGAGGCTTTGT
mSHP-2-s	AGGTGGTTCCATGGTCACTTGTCT
mSHP-2-s	TCATGACGTGGGTCACTTTGGACT

2.1.9 Vectors and Bacteria

Table 2.9: Vectors and Bacteria.

Vector/Bacteria	Manufacturer
0906081-human-miR181a-pMA	GeneArt, Life Technologies, Darmstadt, Germany
0906082-mouse-miR181a-pMA	GeneArt, Life Technologies, Darmstadt, Germany
M620	Invitrogen, Life Technologies, Darmstadt, Germany
pCMVΔR8.91	Invitrogen, Life Technologies, Darmstadt, Germany
pENTR221-EmGFP-miR	Invitrogen, Life Technologies, Darmstadt, Germany
pENTR5´-EF1αp	Invitrogen, Life Technologies, Darmstadt, Germany
pIDE-CMVp	Invitrogen, Life Technologies, Darmstadt, Germany
pLenti6.4/R4R2/V5-Dest Gateway® Vector	Invitrogen, Life Technologies, Darmstadt, Germany
Stabl.3 (*E.coli*)	Invitrogen, Life Technologies, Darmstadt, Germany
TOP10 (*E.coli*)	Invitrogen, Life Technologies, Darmstadt, Germany

2.1.10 Cell lines

Table 2.10: Cell lines.

Cell line	Description
A20-HA	Mouse B cell lymphoma cell line (A20) expressing the Influenza hemagglutinin (HA) antigen
A20-Luc Cl.9	Mouse B cell lymphoma cell line (A20) expressing the enzyme luciferase from *photinus pyralis*
HEK293T	Human embryonic kidney cell line, used as production cell line for lentiviral particles
K562-Puro-Luc	Human leukemia cell line (A20) expressing the enzyme luciferase from *photinus pyralis*
PlatinumE	Retroviral packaging cells

2.1.11 Software

Table 2.11: Software.

Software	Manufacturer
ABI Prism 7700	Applied Biosystems, Foster City, USA
Bio-Plex Manager 6.0	Bio-Rad, Munich, Germany
FACSDiva	BD Biosciences, Heidelberg, Germany
FlowJo 7.6.3	FlowJo - Tree Star Inc., Oregon, USA
GraphPad Prism 6	GraphPad Software, San Diego, USA
Clone Manager 9	Scientific & Educational Software,Cary NC, USA
Photoshop CS6	Adobe Systems, Dubblin, Republic of Ireland

2.1.12 Animals

Age- and sex-matched mice were maintained under specific pathogen-free (SPF) conditions at the University of Mainz animal facility. All animal experiments were conducted in accordance to German animal experimentation regulations, permission number (Tierversuchsantrag) G10-1-016.

BALB/c

Wild type strain with H-2Kd haplotype.

BALB/c TCR CL-4

Mice transgenic for an influenza virus hemagglutinin (HA)518-526 peptide specific T cell receptor (TCR) in the context of the major histocompatibility complex (MHC) class I molecule H2-K^d. These mice were kindly provided by U. Hartwig (III. Medical Department, University of Mainz, Germany).

BALB/c TCR HA

Mice transgenic for an influenza virus hemagglutinin (HA)110-120 peptide specific T cell receptor (TCR) in the context of the major histocompatibility complex (MHC) class II molecule I-E^d. These mice were kindly provided by L. Klein (LMU, München, Germany).

2.2 Methods

2.2.1 Molecular Biological Methods

2.2.1.1 Plasmid vector constructs

The codon-optimized cDNA coding for human and mouse miR181a was obtained from Geneart (Regensburg, Germany) and cloned into the 3' UTR of EmGFP reporter gene in the Gateway pENTR vector. The pENTR vectors were recombined with a pENTR vector carrying the human EF1α promotor and a lentiviral destination vector for human miR181a (see figure 2.1) or a retroviral destination vector with a CMV promotor for murine miR181a. As control a *lacZ* targeting miRNA provided by the manufacturer was used and cloned into the same destination vectors.

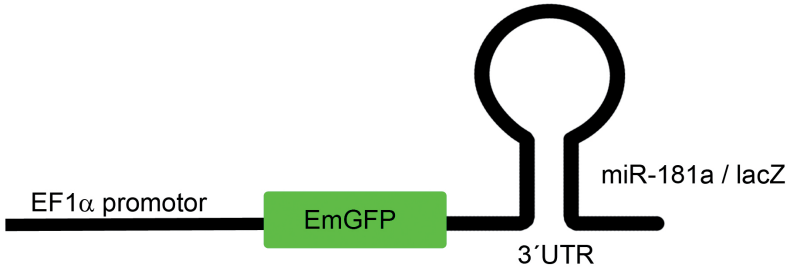

Figure 2.1: Design of vectors for expression of miR-181a/lacZ and EmGFP in human T cells under control of EF1α promotor.

2.2.1.2 Transformation

For the transformation of the plasmid vector of interest into competent bacteria, 5 μL of vector was added to 50 μL of freshly-thawed competent bacteria and incubated for 30 min on ice. The competent bacteria were then subjected to a heatshock for 45 sec at 42°C to facilitate the uptake of the DNA due to opening of pores on cell membrane. The heat-shock was followed by a recovery phase in 450 μL SOC-Medium for 1 h at 37°C in which the bacteria could build up the antibiotic resistance carried by the transformed vector. 50 μL of this were spread on LB-agar plates containing the antibiotic, either ampicillin (100 μg/mL) or kanamycin (25 μg/mL). The plates were incubated at 37°C overnight (upside down).

2.2.1.3 Preparation of plasmid DNA

For isolation of plasmid DNA, bacteria were inoculated in LB medium containing antibiotics and incubated over night under continuous shaking at 37°C. Bacteria were pelleted (15 min, 6.000 x g, 4°C) and plasmid DNA was isolated using the NucleoSpin Plasmid DNA Purification Kit (Machery Nagel) according to the manufacturer's instructions. The pelleted bacteria were lysed with a SDS containing alkaline buffer followed by neutralization of the lysate. After precipitation of proteins, genomic DNA and cell debris by centrifugation, the plasmid DNA containing supernatant was loaded onto an anion-exchange column and other contaminants such as soluble cell components and salts were removed by washing with an ethanol containing buffer. Finally, pure plasmid DNA was eluted with a slightly alkaline buffer or distilled water and stored at -20°C.

2.2.1.4 Sequencing

DNA sequencing was done by a commercial provider (MWG Biotech AG, Ebersberg, Germany).

2.2.1.5 Determination of nucleic acid concentration

For determination of nucleic acid concentrations, light absorption of DNA or RNA containing solutions was measured by spectrophotometry. Molecules absorb light at a distinct wavelength. The absorption maximum for purin and pyrimidine heterocyclic bases is 260 nm, whereas the aromatic site chains of different amino acids absorb light of about 280 nm. The concentration of RNA or DNA was calculated by the spectrometer directly according to the Lambert-Beer law. The purity of the DNA or RNA containing solutions was determined using the ratio of OD readings at 260 nm and 280 nm. Pure preparations have an OD_{260}/OD_{280} ratio of 1.8 to 2. Lower values indicate a contamination with proteins, while higher values indicate degradation of the nucleic acid. Only nucleic acids with a purity value between 1.8 to 2 were used.

2.2.1.6 RNA Extraction

Total cellular RNA was extracted from approximately $5x10^5$-$1x10^7$ T cells using the miRNeasy Mini Kit or RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The cells were first lysed and homogenized in a QIAzol Lysis reagent. The QIAzol Lysis Reagent is a monophasic solution of phenol and guanidine-thiocynanate, which immediately

inactivates RNases and removes most of the cellular DNA and proteins by organic extraction. After addition of Chloroform the homogenate is separated into aqueous and organic phases by centrifugation. RNA partitions to the upper, aqueous phase, while DNA and proteins partitions to the interphase and the organic phase. The upper, aqueous phase was extracted, and ethanol was added to provide appropriate binding conditions for all RNA molecules from 18 nucleotides (nt) upwards. The sample was then applied to the RNeasy Mini spin column, where the total RNA binds to the membrane and phenol and other contaminants were efficiently washed away. Highquality RNA was eluted in 50 μL RNase-free water.

2.2.1.7 Reverse Transcription of total RNA

For detection of miRNA the miScript Transcription Kit, including miScript Reverse Transcriptase and miScript RT Buffer, was used. miScript Reverse Transcriptase Mix comprised a poly(A) polymerase and a reverse transcriptase. The miScript Buffer enables maximum activity of both enzymes and contains dNTPs, oligo-dT primers and random primers. During the reverse-transcription step, miRNAs are polyadenylated. The oligo-dT primers have a universal tag sequence on the 5´end. This allows the amplification in the real-time PCR step using the miScript Universal Primer (provided in the miScript SYBR Green PCR Kit from Qiagen). The miScript Reverse Transcriptase and miScript RT Buffer were mix with up to 1 μg RNA and incubated 1 h at 37 °C. After reverse-transcription the samples were incubated for 5 min at 95 °C to inactivate miScript Reverse Transcriptase Mix.

For detection of mRNA from different proteins, total RNA (1-2 μg) was reverse transcribed with Superscript II according to the manufacturer's instructions and primed with a random hexamer oligonucleotide primer, including oligo-dT sequence (dT18-tag Primer see table 2.1.8). This primer binds to different sites of the whole RNA molecule and enables the reverse transcription of poly(A) tail negative RNA. Extracted total RNA (max. 2 μg) was mixed with dNTPs (10 mM) and random hexamer primer (5 μM), denatured for 2 min at 70 °C and stored on ice before the reverse-transcription mixture (including reverse transcriptase, 0.01 M DDT and first strand buffer) was added up to a final volume of 20 μL. The first strand cDNA-synthesis reaction was performed for 60 min at 42 °C. The reaction was stopped by denaturing for 15 min at 70 °C.

2.2.1.8 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a technique for amplifying DNA sequences *in vitro* by separating the DNA into two strands and incubating it with DNA polymerase and oligonucleotide primers. Primers are usually oligonucelotides of 18-25bp size, which are complementary to the nucleotides at both ends of the DNA fragment. For the primer design it was considered that guanosine-cytosine (G-C) content should be between 40-60 %.

2.2.1.9 Quantification of gene transcription by real-time RT PCR (RT-PCR)

Real-time quantitative analysis of the gene expression was performed using the ABI Prism 7700 Sequence Detection System instrument and software. PCR primers (see table 2.1.8) were designed and used for the specific amplification from first-strand cDNA stocks. To exclude the false positive PCR products due to contaminating genomic DNA in the RNA preparation or the existence of pseudogenes, the individual gene-specific primer sets were designed to span exon/intron boundaries and quality-controlled by PCR reactions using genomic DNA. Each PCR was done in triplicates using 5 μL of 1:10 diluted first-strand cDNA, 1 μL of gene-specific PCR primers (10 μM) and 15 μL QuantiTect SYBR Green in a 30 μL reaction for 40 cycles. In each experiment the template-free negative controls were included. The expression of housekeeping genes HPRT, GAPDH and β-Actin were used as an internal calibrator to normalize for the variances in the quality of RNA and the amount of input cDNA for each target analyzed. The threshold cycle (Ct) value, i.e. the cycle number at which the fluorescence exceeds baseline, was adjusted manually and a common value was determined for all runs.
Ct values were used to calculate the amount of sample PCR product in comparison to the internal housekeeping gene (ΔCt = $Ct_{targetgene}$ - $Ct_{housekeepinggene}$). The Ct calculation method was applied to compare relative expression values of different target genes. The Ct values were determined by subtracting the Ct value of a reference (ctrl miRNA transduced samples) from the Ct value obtained for the respective sample ($\Delta\Delta$Ct = ΔCt_{sample} - $\Delta Ct_{reference}$).

2.2.1.10 Real-Time PCR for Detection of miR181a

For detection of mature miRNA, the cDNA serves as the template for real-time PCR analysis using a miR181a-specific miScript Primer Assay in combination with the miScript SYBR Green PCR Kit. miR181a is amplified using the miScript Universal Primer (provided in the miScript SYBR Green PCR Kit), which primes from the universal tag sequence, together with the miScript Primer Assay which is specific for the mature miR181a (see table 2.1.8). Real-time quantitative analysis was performed using the ABI Prism 7700 Sequence Detection System instrument and software as described before (see section 2.2.1.9).

2.2.1.11 Gene Expression Analysis using Fluidigm

For gene expression analysis the BioMark System from Fluidigm Corporation (Fluidigm Corporation, San Francisco, USA) was used. This system enables microarray-like studies using qPCR. For paralell detection of different genes, TaqMan-probes are necessary. These are oligonucleotides consisting of a reporter dye and a quencher. They are blocked against an extension on their 3'-end. During the PCR-reaction, they are hydrolyzed resulting in a release of the fluorescent dye.

For gene expression analysis from transduced human T cells, a human immune panel designed by Arbel Tardor and provided from Applied Biosystems (Applied Biosystems, Foster City, USA) was applied. For gene expression analysis from transduced murine T cells, a murine immune panel designed by Mustafa Diken and provided from Applied Biosystems (Applied Biosystems, Foster City, USA) was used. Samples were prepared as described before (see section 2.2.1.6 and 2.2.1.7) and cDNA samples were utilized for specific target amplification (STA). In this step, a pool of all assays of the human immune panel allows the enrichment of target templates. After STA-reaction, samples and assays were loaded onto the Dynamic Array IFC (integrated fluidic circuits) and mixed using IFC Controler HX from the BioMark HD System. The real-time PCR and data collection was performed on the BioMark HD Reader. Data were analyzed with the help of the functional unit Computational Medicine (TRON, Mainz, Germany).

2.2.2 Cell Biological and Immunological Methods

2.2.2.1 Cultivation of cells

All cells were cultured under aseptic conditions in specified medium including 1 % penicillin/streptomycin. All cells were cultured in an incubator at 37°C with 95 % humidity and 5 % CO_2.

2.2.2.2 Determination of cell numbers

Use of Neubauer chamber in combination with Trypan Blue is a practical method to determine the cell density and vitality in a cell suspension. Trypan Blue is a polyanionic azo dye that is not able to penetrate cells with an intact membrane potential. For this reason vital cells remain unstained (refractive), while due to the leaky cell membrane dead cells are stained with Trypan Blue. Aliquots taken from a cell suspension can be diluted with Trypan Blue solution and applied to Neubauer chamber. The cell density/number and percent vitality can be calculated by using the equations below:

Cell density = Cells counted in one quadrant x dilution factor x 10^4/mL

Total cell number = Cell density x volume of cell suspension (mL)

% vitality = 100 x (# of all cells - # of blue cells) / # of all cells

2.2.2.3 Generation of peripheral blood mononuclear cells (PBMCs) from human blood samples

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation from buffy coats obtained from healthy blood bank donors (Transfusion Center Mainz). The term buffy coat describes the interface between plasma and red blood cells resulting from centrifugation of coagulation-inhibited blood samples. Buffy coat mainly consists of leucocytes and platelets and represents a by-product from converting blood donations to erythrocyte concentrates and plasma products. For isolation of PBMCs, blood samples were diluted with PBS and gently pipetted on a ficoll layer, a sucrose containing solution with a defined density of 1.077 g/mL. Separation of mononuclear cells from polynuclear cells and erythrocytes was achieved by centrifugation (678 x g, 21°C, 25 min). The cells localize according to their density, which influences the sedimentation speed under centrifugal force. Based on

a higher density erythrocytes and granulocytes migrate through the ficoll and are pelleted at the bottom. A milky ring composed of lymphocytes and monocytes is located directly on the top of the ficoll cushion overlaid by plasma and platelets. The ring containing the PBMCs was aspirated and washed twice with PBS/2 mM EDTA prior to further use.

2.2.2.4 Isolation of different cell populations from PBMCs

Different cell populations were isolated from PBMCs using magnetic activating cell sorting (MACS). This method is based on the specific labeling of cells with magnetic particles (diameter app. 50 nm). These microbeads are directly or indirectly linked to the cells by antibodies highly specific for cell surface molecules. The magnetically labeled cells are separated with the help of columns containing a steel wool matrix. When such columns are placed in a magnet, the column matrix provides a magnetic field strong enough to retain magnetically labeled cells, while unlabeled cells are washed out. Aliquots of cell preparations with a final concentration of 10^7 cells/80 μL were labeled with 15 μL microbeads for 15 min at 4°C. After removing unbound beads by a washing step, cells were diluted in an appropriate amount of MACS buffer. The column was placed in a magnet and equilibrated with MACS buffer before the cell suspension was poured into the column reservoir. The column was rinsed three times with MACS buffer to remove unbound cells, then removed from the magnet and cells were flushed out with media with the aid of a plunger, cell density was calculated and cells were cultured $2 * 10^6$ cells/mL.

2.2.2.5 Generation of monocytes and monocytes derived dendritic cells (DCs) from PBMCs

Monocytes were enriched from PBMCs with anti-CD14 microbeads according to the manufacturer's instructions. To obtain immature dendritic cells (iDC), monocytes were differentiated for 5 days in human medium supplemented with 1000 U/mL GM-CSF and 1000 U/mL IL-4.

2.2.2.6 Production of viral supernatant

Viral particles for transduction of human T cells were generated by co-transfection of plated HEK 293T cells with lentiviral destination vectors encoding genes of interest together with pCMVΔR8.91 (encoding gag-pol) and M620 (encoding the GALV envelop) using Fugene6. Viral particles for transduction of murine T cells were generated by transfection of a potent retrovirus packaging cell line (e.g. Platinum-E) with retroviral destination vector encoding

genes of interest using Fugene6. Supernatant from transfected cells was collected after 48 h and 72 h and titrated. Viral supernatant was produced and titrated by the viral service unit (TRON, Mainz, Germany). Supernatants with titer of $1*10^7$ were used.

2.2.2.7 Viral Transduction of T cells

Human T cells were stimulated with 2 μg/mL plate-bond αCD3, 2 μg/mL soluble αCD28, 50 U/mL IL-2, 5 μg/mL IL-7 and 10 μg/mL IL-15 for 24 h prior viral transduction. Murine T cells were stimulated with 5 μg/mL specific Antigen (see table 2.5) 24 h prior viral transduction. Retronectin (20 μg/mL) coated plates were loaded three times with viral supernatant by centrifugation (1.500 x g, 15 min, 15°C). After washing with PBS to remove unbound virus particles, cells were loaded on virus coated plates. After cells were incubated for 4 h at 37°C, viral supernatant with 8 μg/mL polybrene was reloaded onto the cells (790 x g, 1 h, 37°C). After co-incubation of cells with viral supernatant for 1 h at 37°C, viral supernatant was removed and medium was placed back to the cells. This procedure was repeated 48 h after stimulation of T cells. Six days after stimulation, T cells were cleaned from cell debris by ficoll centrifugation. Therefore cells were collected and loaded on a ficoll layer. Separation of living cells and cell debris was achieved by centrifugation (678 x g, 21°C, 25 min). All living cells were located directly on the top of the ficoll, collected and used for further analysis.

2.2.2.8 Cytokine Expression Analysis

$5*10^5$ transduced human T cells were stimulated with $2*10^5$ autologous iDCs (see section 2.2.2.5) and different amounts of αCD3. For co-stimulation independent analysis, $5 * 10^5$ transduced human T cells were stimulated with different amounts of plate-bound αCD3 in the presence or absence of soluble αCD28. $5*10^5$ transduced murine T cells were stimulated with $2*10^5$ CD90.2 depleted splenocytes from Balb/c mice as APCs with different amounts of specific antigen (see table 2.5). After 2 h of stimulation, cytokine export was blocked by adding 5 μg/mL Brefeldin A to the cells. After additional 4 h incubation at 37°C, cells were fixed with 2 % PFA. Cytokine expression was analyzed using intracellular staining (see section 2.2.3.2) and measurement on FACS CantoII.

2.2.2.9 Analysis of Cytokine Release

Transduced human T cells were sorted due to their GFP expression (see section 2.2.3.1). $5 * 10^5$ sorted T cells were then stimulated with $2 * 10^5$ γ-irradiated autologous iDCs (see section 2.2.2.5) and different amounts of αCD3 up to 5 days. After different time points, supernatant was collected and the cytokine release was analyzed using a multiplex suspension array (see section 2.2.3.4).

2.2.2.10 Gene Expression Analysis

For gene expression analysis, cells were collected and total RNA was extracted (see section 2.2.1.6), reverse transcribed into cDNA (see section 2.2.1.7) and gene expression was analyzed using Fluidigm (see section 2.2.1.11).

2.2.2.11 Proliferation Analysis with Cell Proliferation Dye eFluor 670 (CPD-eFluor670)

The Cell Proliferation Dye eFluor 670 (CPD-eFluor670) was used to analyze the proliferation of cells. CPD-eFluor670 binds to cellular proteins containing primary amines. When cells divide, the dye is distributed to the daughter cells leading to a reduction of fluorescence which can be detected by FACS analysis. To label cells with CPD-eFluor670, $1 * 10^6$ cells were stained in 500 μL PBS with 50 μL prediluted CPD-eFluor670 solution (final 5 μM). After adding CPD-eFluor670 solution, the cells were mixed and incubated for 10 min at 37°C. To remove unbound CPD-eFluor670, the cells were washed with complete medium and subsequently adjusted to the required concentration.

2.2.2.12 Analyzing apoptosis using Annexin V and Red Active Caspase Staining Kit

The loss of plasma membrane asymmetry is one of the earliest features of apoptosis. In this process the membrane phospholopid phosphatidylserine (PS) is translocated from the inner to the outer face of the plasma membrane leading to exposition of PS to the external cellular environment. Annexin V is a calcium dependent phopholipid-binding protein with high affinity for PS. Annexin V can bind to cells with exposed PS only. Since externalization of PS occurs in the early stages of apoptosis, Annexin V can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. The loss of membrane integrity accompanies also the last stages of cell death. Therefore Annexin V staining is used

in conjunction with a vital dye such as propidium iodide (PI) to distinguish between early apoptotic cells (Annexin V^+/PI^-) and late apoptotic or already dead cells (Annexin V^+/PI^+). Another interesting feature of apoptosis is the activation of caspases. The Red Active Caspase Staining Kit enables the detection of activated caspase-3 in living cells. The assay utilizes the caspase-3 inhibitor VAD-FMK conjugated to sulfo-rhodamine (Red-VAD-FMK) as a fluorescent *in situ* marker. Red-VAD-FMK is cell permeable, non-toxic and irreversibly binds to activated caspase-3 in apoptotic cells.

To detect active capase-3, 0,3 μL of Red-VAD-FMK were added to the cells and incubated at 37°C for 1 h. To remove unbound Red-VAD-FMK, the cells were washed with 200 μL BD Wash Buffer. After surface staining (see section 2.2.3.2) cells were washed twice with cold PBS. After the second wash step, cells were splitted for Annexin V staining. Therefore cells were resuspended either in 100 μL Annexin Binding Buffer or in 100 μL EGTA Buffer. 5 μL labeled Annexin V were added to cells and incubated for 15 min at RT in the dark. For flow cytometry analysis, cells were resuspended 300 μL of the required buffer with 1 μg/mL PI and analyzed within 1 h on FACS CantoII.

2.2.2.13 Cytotoxicity Assay

For evaluation of cell-mediated cytotoxicity the luciferase assay was used. This assay measures lytic activity of effector cells by calculating the number of viable luciferase expressing target cells following co-incubation. For transduced murine T cells, A20-Luc Cl.9 cells provided by Jan Diekmann were used as target cells. The target cells were stably transfected with the luciferase gene coding for the firefly luciferase from the firefly *Photinus pyralis*. Luciferase is an enzyme catalyzing the oxidation of luciferin. The reaction is ATP-dependent and takes place in two steps:

$$(luciferin + ATP \rightarrow luciferyladenylate + PPi)$$

$$(luciferyladenylate + O_2 \rightarrow oxyluciferin + AMP + light)$$

$1 * 10^4$ target cells per well were plated in white 96-well plates and co-cultivated with varying numbers of T cells with different stimulation conditions (different αCD3 or antigen concentration) in a final volume of 100 μL. 3 h later, 50 μL of a D-Luciferin containing reaction mix (see

table 2.6) was added to the cells. By addition of ATPase to the reaction mix, luminescence resulting from luciferase released from dead cells was diminished. The bioluminescence emitted by viable cells was measured using the Tecan Infinite 200 reader after different incubation times. Cytotoxic activity was calculated in regard to luminescence values obtained after complete cell lysis induced by the addition of 10 μL Triton-X 100 and in relationship to luminescence emitted by target cells alone. Data output was in counts per second (CPS) and percent specific lysis was calculated as follows:

$$(1\text{-}(CPS_{exp} - CPS_{min})/(CPS_{max} - CPS_{min})) * 100$$

2.2.3 Cellular Anlysis Methods

2.2.3.1 Flow Cytometry

Flow cytometry is a useful technique for phenotyping cells on the single cell level. It simultaneously analyzes and measures multiple physical parameters of cells as they move in a fluid stream through a beam of light. Protein expression can be analyzed by fluorescence labeled antibodies, which bind to their respective targets. Being excited by the laser, cells scatter the laser light and fluoresencent molecules bound to the cells emit fluorescent light at certain wavelengths. Both are detected by sensitive photomultiplier tubes (PMT). To discriminate between viable and dead cells, the DNA intercalating dye propidium iodid (PI) was used. Flow cytometry studies were performed on a FACSCantoII or a Fortessa machine and analyzed using FlowJo software. Flow cytometry sorting of cells was performed on a FACSAria cell sorter using the FACSDiva software.

2.2.3.2 Staining of cell surface marker

For staining of cells with fluorochrome labeled antibodies, cells were harvested by centrifugation (300 x g, 8 min, 4°C) and resuspended in 100 μL FACS buffer to wash the cells. Determined amounts of fluorochrome labeled antibodies were mixed in FACS buffer, 50 μL were added to the cells and incubated for 20 min at 4°C in the dark. The cells were washed with FACS buffer and resuspended in 200 μL FACS buffer for analysis.

Staining of murine blood involved an erythrocyte lysis step by incubation of cells in 300 μL BD Blood Lysis Buffer (8 min, RT, in dark) after direct staining of 50 μL blood. Stained cells were then washed with 3 mL PBS, pelleted by centrifugation (450 x g, 6 min, RT) and resuspended in 200 μL FACS buffer.

2.2.3.3 Intracellular staining

For an intracellular staining, the cells were fixed for 20 min at RT using 2 % PFA and washed with FACS buffer. After surface staining (see section 2.2.3.2), cells were permeablized by washing with 500 μL 1x BD permeabilization solution 2. Determined amounts of fluorochrome labeled antibodies were diluted in 1x BD perm/wash solution, 50 μL of the staining mix was added to the cells and incubated for 20 min at 4°C in the dark. The cells were washed with 1x BD perm/wash solution and resuspended in 200 μL FACS buffer for analysis.

2.2.3.4 Cytokine Release Analysis using a Multiplex Suspension Array

Multiplex Suspension Array permits multiplexing of different Enzyme Linked Immunosorbent Assays (ELISA) within a single sample. Each assay is performed on the surface of a 5.5 μm polysterene bead. The beads are filled with different ratios of two fluorescent dyes, resulting in an array of 100 distinct spectral addresses. Each set of beads can be conjugated with a capture molecule (e.g.receptors, antibodies, antigens, DNA-molecules, enzyme substrates). These conjugated beads can be mixed and incubated with the sample of interest. To detect and quantify each engaged analyte, a fluorescent labeled reporter molecule, that binds to the analyte, is added.

To analyze the cytokines released after co-incubation with γ-raddiated iDCs (see section 2.2.2.9) an individual Bio-Plex Pro cytokine Assay (Bio-Rad, Munich, Germany) was used. The collected supernatant was centrifuged (10.000 x g, 5 min, 4°C) to completely remove precipitates before adding samples to the bead-coated wells and incubated for 30 min at RT. After three wash steps to remove unbound molecules, a biotinylated detection antibody was added to create a sandwich complex. The final detection complex was formed with the addition of streptavidin-phycoerythrin (SA-PE) conjugate. Phycoerythrin serves as a fluorescent reporter. A standard for each used assay was provided by the manufacturer to perform a standard curve

on each plate, medium was used as blank. The readout was performed on Bio-Plex 200 machine (Bio-Rad, Munich, Germany). Here the beads were analyzed by two lasers individually. The red classification laser excites the dyes in each bead to identify the specific beads address. The green reporter laser excites the reporter molecule associated with the beads to quantify the captured analyte. The concentration of cytokines in each sample was calculated in relation to the standard curve and blank samples directly by the Bio-Plex Manager 6.0 Software (Bio-Rad, Munich, Germany).

2.2.3.5 Tissue fixation and Haematoxylin/Eosin staining

Tumors were isolated, fixed and paraffinated until further analysis. For analysis paraffinated samples were sectioned (3 μm) and paraffin was removed by incubation with xylol. Samples were then rehydrated using decreasing ethanol concentrations (100 - 70 %) and stained with haematoxylin solution for 5 min at RT. After washing with water for 10 min, samples were stained with eosin solution for 2 min at RT and dehydrated using increasing ethanol concentrations (70 -100 %) and fixated with xylol.

2.2.3.6 Immunohistochemistry (IHC)

Immunohistochemistry enables the identification of the localization of proteins in the cells of a tissue section by labeling them with specific antibodies. The labeling can be performed either directly, by the use of the labeled antigen-specific antibodies or indirectly, by detecting unlabeled primary antibody with labeled species-specific secondary antibodies. The latter allows the amplification of the absolute numbers of labels per coupled antigen. After rehydration of the sectioned samples (see section 2.2.3.5) an epitope retrieval was followed 10 min in citratebuffer at 120°C. For quenching endogenous peroxidases, samples were incubated 15 min in PBS + 0.3 % H_2O_2 and incubated 30 min with PBS + 10% goat serum to block unspecific antibody binding. For immunohistochemical staining, the samples were incubated over night at 4°C with the primary unlabeled antibody of interest. After washing the samples three times with PBS, the species defined secondary antibody coupled with the HRP was incubated for 5 min at RT. The substrate incubation times was depending on each antibody and is indicated in the results part. The samples were stained with haematoxylin for counterstaining before

dehydration and fixation was done (see section 2.2.3.5). Pictures were made and analyzed with with the help of the functional unit IF/IHC (TRON, Mainz, Germany).

2.2.4 Animal Experiments

2.2.4.1 Blood retrieval

Mice were anesthetized using isofluran, 100-150 μL blood was retrieved from the retro-orbital vein via a micro haematocrit capillary and transferred to a clean reaction tube containing Heparin (1000 U/mL) or EDTA as an anticoagulant.

2.2.4.2 Preparation of single cell suspension from murine lymph nodes and spleens

Preparation of spleen

BALB/c, BALB/c-TCR-HA or BALB/c-CL4 mouse was sacrificed by cervical dislocation or CO_2 exposure, spleen was removed and placed in a 1.5 mL eppendorf tube with 500 μL PBS. The plunger of a 5 mL syringe served as a grinder to homogenize spleen while the homogenate was being filtered through cell strainer (70 μm) to a 50 mL centrifugation tube. After centrifugation of spleen cell suspension (300 x g, 8 min, RT), the pellet was resuspended in 1 mL erythrocyte lysis buffer for 3-5 min at RT. The lysis reaction was stopped with addition of 5-10 mL PBS and cells were pelleted by centrifugation (300 x g, 8 min, RT). After resuspension of cells in murine medium, cell density was calculated and cells were cultured $2 * 10^6$ cells/mL.

Preparation of lymph nodes (LN)

After sacrification of BALB/c, BALB/c-TCR-HA or BALB/c-CL4 mouse using cervical dislocation or CO_2 exposure, a short cut within the region of the inguinal LN was made with scissors and the inguinal LN was removed due to its opaque appearance compared to the surrounding adipose tissue. The LN was minced into small fragments by forceps and biochemical disruption of the connective and adipose tissue was carried out by incubation in 500 μL PBS with 1 mg/mL Collagenase D for 30 min at RT. The resulting homogenate was filtered through cell strainer (70 μm) to a 50 mL centrifugation tube. The LN cell suspension was then washed with PBS, centrifugated (300 x g, 8 min, RT). After resuspension of cells in murine medium, cell density was calculated and cells were cultured $2 * 10^6$ cells/mL.

2.2.4.3 Tumorgeneration

For tumorgeneration a B cell lymphoma cell line (A20) expressing hemagglutine (HA) was used. $2*10^5$ A20-HA tumor cells were injected *subcutanously* (s.c.) into the flank of the mice using insulin syringes.

2.2.4.4 Adoptive transfer of cells

Intravenous (i.v.) injection route was employed to adoptively transfer cells. For this purpose, an indicated number of cells, resuspended in 100 μL PBS, was injected i.v into the retro-orbital vein using insulin syringes.

2.2.5 Statistical Analysis

Statistical analysis was performed in GraphPad Prism software employing two-way ANOVA corrected with Bonferroni´s multiple-comparison test factor. Values of $p < 0.05$ were considered statistically significant.

3 Results

3.1 miR181a overexpression in human T cells

3.1.1 Lentiviral transduction of primary human T cells with miR181a

To study miR181a in human T cells, the wildtype sequence of human miR181a was obtained from Geneart (Regensburg, Germany) and cloned into the 3' UTR of EmGFP reporter gene (subsequently termed GFP) in the Gateway pENTR vector. As a control, *lacZ* targeting miRNA (subsequently termed ctrl miRNA) provided by the manufacturer, was used and cloned in a similar manner. The pENTR vectors were recombined with a pENTR vector carrying the human EF1α promotor and a lentiviral destination vector. This allows the simultaneous expression of miR181a, or ctrl miRNA, with GFP. Analyzing the GFP expression by flow cytometry enables the identification of miR181a or ctrl miRNA transduced cells.

For transduction of human T cells, PBMCs were isolated from Buffy coats and $CD4^+$ or $CD8^+$ T cells were enriched using magnetic activated cell sorting (MACS). T cells were then activated with 2 μg/mL plate bound αCD3 and 2 μg/mL soluble αCD28 in the presence of IL-2, IL-7, and IL-15. After 24 h and 48 h of activation, T cells were transferred onto virus coated plates, either coated with lentiviral vectors encoding miR181a or ctrl miRNA in the 3' UTR of the reporter gene GFP. The expression of the reporter gene GFP was analyzed by flow cytometry, four days after the last transduction (see Figure 3.1).

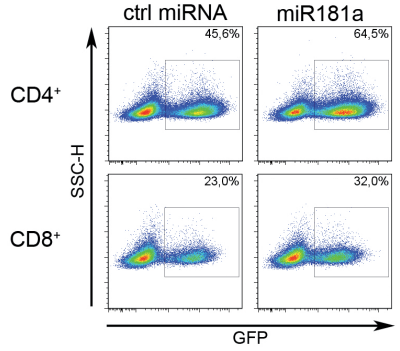

Figure 3.1: Lentiviral transduction of primary human T cells.
MACS sorted primary human $CD4^+$ and $CD8^+$ T cells were activated with 2 μg/mL plate bound αCD3 and 2 μg/mL soluble αCD28 in the presence of IL-2, IL-7, and IL-15. After 24 h of activation, T cells were transferred onto virus coated plates either coated with lentiviral vectors containing miR181a or ctrl miRNA in the 3' UTR of the reporter gene GFP. 48 h after activation, T cells were placed on freshly coated plates. Expression of the reporter gene GFP was analyzed by flow cytometry, four days after the last transduction. Shown is one representative example out of three independent experiments.

In the upper panel of 3.1, the analysis of primary human $CD4^+$ T cells transduced with lentiviral vectors containing ctrl miRNA (left) or miR181a (right) in the 3' UTR of the reporter gene GFP, is shown. Here, higher numbers of T cells expressing GFP could be detected when transduced with lentiviral vectors encoding for miR181a (64.5 %) compared to GFP expressing T cells transduced with ctrl miRNA (45.6 %). In the lower panel, the analysis of primary human $CD8^+$ T cells transduced with lentiviral vectors coding for ctrl miRNA (left) or miR181a (right) in the 3' UTR of the reporter gene GFP, is shown. Again, T cells transduced with miR181a showed higher numbers of GFP positive T cells (32.0 %) than T cells transduced with ctrl miRNA (23.0 %). Interestingly, $CD4^+$ T cells (upper panels) showed higher transduction efficiencies as indicated by the higher GFP expression compared $CD8^+$ T cells (lower panels). Nearly twice as many $CD4^+$ T cells were transduced compared to $CD8^+$ T cells, as shown by GFP expression as well as transduced with miR181a or ctrl miRNA.

In conclusion, human T cells could efficiently be transduced with lentiviral vectors either encoding miR181a or ctrl miRNA.

3.1.2 Overexpression of miR181a in primary human T cells downregulates the expression of multiple phophatases

For validating the overexpression of miR181a using lentiviral transduction of T cells, the amount of miR181a in transduced cells was analyzed using qRT-PCR. For that, T cells transduced with miR181a or ctrl miRNA were sorted based on their GFP expression. GFP positive cell populations (see Figure 3.2 A) were used for RNA extraction and qRT-PCR analysis. The amount of miR181a in human T cells transduced with miR181a was determined using an miR181a specific primer and normalized to the amount of miR181a obtained by human T cells transduced with ctrl miRNA (see Figure 3.2 B). The expression was calculated relative to the expression of three house-keeping genes (HPRT, GAPDH and β-Actin). In $CD4^+$ T cells, miR181a is overexpressed approximately three-fold in cells transduced with miR181a compared to cells transduced with ctrl miRNA. In $CD8^+$ T cells, transduction with miR181a leads to a roughly 12-fold overexpression compared to cells transduced with ctrl miRNA.

Li et. al have shown that miR181a affects the expression of multiple phosphatases during thymocyte development, enabling the recognition of weak antigens [Li et al., 2007]. Therefore,

the expression of known target genes was analyzed in T cells transduced with miR181a and compared to T cells transduced with ctrl miRNA. Again, the expression of the analyzed genes was calculated relative to the expression of three house keeping genes (HPRT, GAPDH, and β-Actin). The overexpression of miR181a in CD4$^+$ T cells led to significant downregulation of SHP2, DUSP5, and DUSP6 (see Figure 3.2 C, left panel). In CD8$^+$ T cells, the downregulation of the analyzed phosphates was stronger than in CD4$^+$ T cells. PTPN22 was also significantly reduced, in addition to SHP2, DUSP5, and DUSP6 when miR181a is overexpressed (see Figure 3.2 C, right panel).

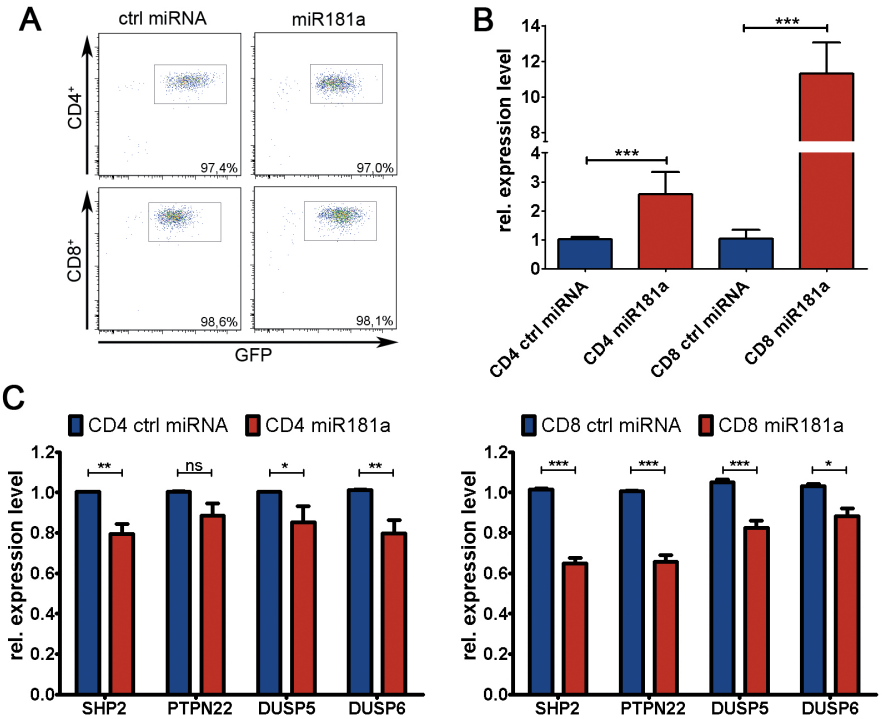

Figure 3.2: Overexpression of miR181a in primary human T cells downregulates the expression of multiple phophatases.
Primary human CD4$^+$ and CD8$^+$ T cells were transduced with lentiviral vectors containing miR181a or ctrl miRNA in the 3' UTR of the reporter gene GFP. T cells were then sorted based on their GFP expression.
(A) Analysis of cells after sorting using flow cytometry.
(B) Relative expression levels of miR181a in CD4$^+$ and CD8$^+$ T cells were determined by qRT-PCR.
(C) Relative expression levels of different target genes in CD4$^+$ (left panel) and CD8$^+$ (right panel) T cells were determined by qRT-PCR. Equal numbers of cells were used for RNA extraction. Equal amounts of RNA were used for qRT-PCR. HPRT, GAPDH and β-Actin were used for quantification. Expression values were normalized to T cells transduced with crtl miRNA. n=3; Error bars are SD; Two-way ANOVA (ns – not significant; * indicates significant differences; * $p<0.05$; ** $p<0.01$; *** $p<0.001$) Representative results of one out of three experiments were shown (A).

Importantly, the extent of downregulation of target genes correlates with the amount of miR181a overexpression. In $CD4^+$ T cells, a three-fold overexpression of miR181a causes a downregulation of target genes between ~10 to ~20 %. In $CD8^+$ T cells, the nearly 12-fold overexpression of miR181a results in ~10 to ~35 % downregulation of the known phosphatases. In conclusion, the overexpression of miR181a in primary human T cells leads to downregulation of multiple phosphatases that counteract activation inducing kinases.

3.1.3 miR181a enhances cytokine production in primary human T cells

Primary human $CD4^+$ and $CD8^+$ T cells were transduced with miR181a or ctrl miRNA and restimulated with autologous iDCs and different amounts of αCD3 for 6h to analyze the effects of miR181a overexpression in human T cells on cytokine production. The expression of the cytokines IFNγ and TNFα in $CD4^+$ and $CD8^+$ T cells transduced with miR181a or ctrl miRNA was analyzed by intracellular cytokine staining (see Figure 3.3 A and 3.3 C). The percentages of cytokine producing cells were determined relating to GFP positive $CD4^+$ and $CD8^+$ T cells (see Figure 3.3 B and 3.3 D). In $CD4^+$ T cells, miR181a overexpression resulted in higher numbers of IFNγ (Figure 3.3 B, left panel) and TNFα (Figure 3.3 B, right panel and Figure 3.3 A) producing cells upon restimulation with iDCs and higher concentrations of αCD3. In $CD8^+$ T cells, the overexpression of miR181a has no influence on IFNγ (Figure 3.3 D, left panel) and TNFα (Figure 3.3 D, right panel and Figure 3.3 C) production after short-term restimulation.

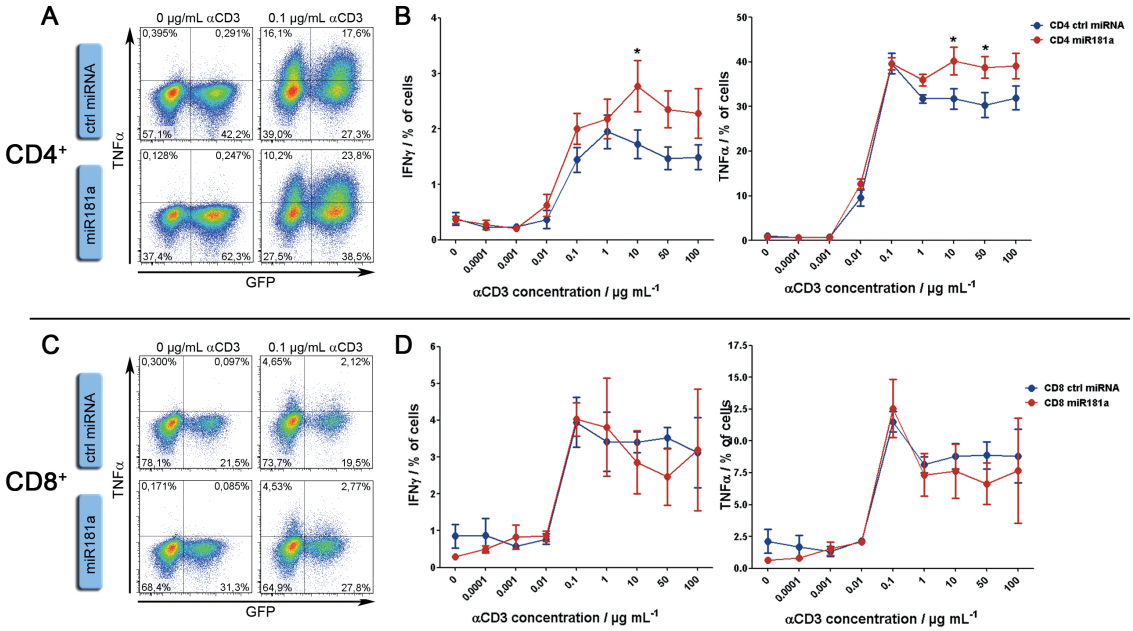

Figure 3.3: miR181a overexpression in primary human T cells leads to higher numbers of reacting CD4$^+$ T cells after short-term restimulation.
Primary human CD4$^+$ and CD8$^+$ T cells transduced with miR181a or ctrl miRNA were restimulated with autologous iDCs and different amounts of αCD3 six days after primary stimulation. Cells were restimulated for 6h and the expression of cytokines was analyzed by intracellular staining using flow cytometry.
(A and C) Analysis of TNFα expression in CD4$^+$ (A) and CD8$^+$ (C) T cells transduced with miR181a or ctrl miRNA after restimulation with autologous iDCs and indicated amounts of αCD3.
(B and D) Cytokine expression was analyzed in GFP positive CD4$^+$ (B) and CD8$^+$ (D) T cells. n=4; Error bars are ±SD; Two-way ANOVA (* indicates significant differences; * $p<0.05$) Representative results of one out of three experiments were shown (A and C).

Only a weak influence of miR181a on cytokine production in CD4$^+$ T cells could be detected after restimulation for 6h. Therefore, the long-term effects of miR181a overexpression in primary human T cells were analyzed. For that, T cells transduced with miR181a or ctrl miRNA were sorted based on their GFP expression. GFP positive cell populations were then restimulated with γ-irradiated autologous iDCs and different amounts of αCD3 for five days. After time points from 12 h to 108 h, the concentrations of the IFNγ, TNFα, IL-4, IL-10, IL-17 were analyzed using a multiplex suspension array. The concentrations of cytokines in the supernatant of CD4$^+$ T cells (see Figure 3.4) and CD8$^+$ T cells (see Figure 3.5) restimulated with γ-irradiated autologous iDCs and 0.1 μg/mL αCD3 are depicted.

For CD4$^+$ T cells, there was no difference detectable between miR181a and ctrl miRNA transduced cells in IFNγ and TNFα secretion after long-term restimulation (see Figure 3.4 upper panels). Remarkably, the concentration of IFNγ continuously increased over time (see Figure 3.4 upper left panel), while, the concentration of TNFα reached a peak after 24h before decreasing back to starting concentrations after 72h (see Figure 3.4 upper right panel).

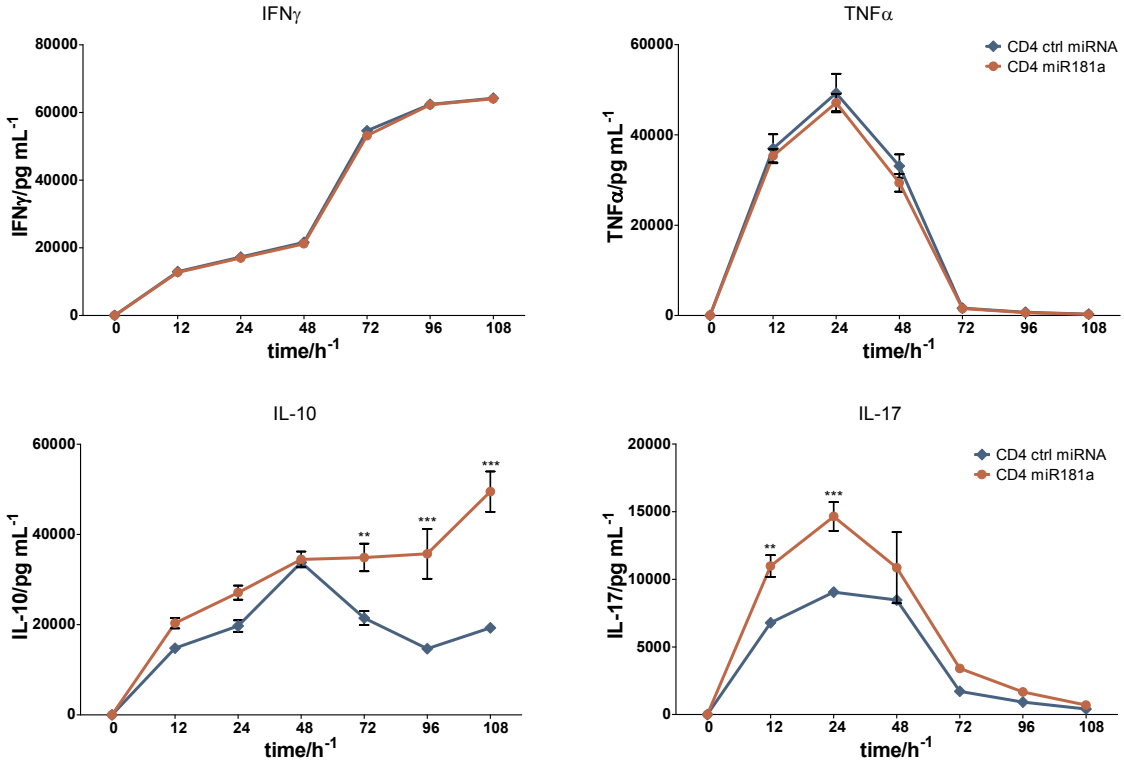

Figure 3.4: miR181a increases and prolongs cytokine expression in primary human CD4$^+$ T cells.
MACS sorted human CD4$^+$ T cells were transduced with lentiviral vectors encoding miR181a or ctrl miRNA in the 3'UTR of the reporter gene GFP. Four days after the last transduction, CD4$^+$ T cells were sorted based on their GFP expression. Sorted cells were then co-incubated with γ-irradiated autologous iDCs and 0.1 μg/mL αCD3 for five days. The secretion of cytokines was analyzed using a Multiplex Suspension Array after different time points of stimulation. n=3; Error bars are ±SD; Two-way ANOVA (* indicates significant differences; ** $p<0.01$; *** $p<0.001$)

In contrast, the secretion of IL-10 was prolonged in CD4$^+$ T cells overexpressing miR181a (miR181a CD4$^+$ T cells) compared to CD4$^+$ T cells transduced with ctrl miRNA (ctrl miRNA CD4$^+$ T cells) (see Figure 3.4 lower left panel). While ctrl miRNA CD4$^+$ T cells reach a maximal secretion level of IL-10 after 48h, miR181a overexpression enables stable and even enhanced secretion of this cytokine. In addition, the expression of IL-17 was increased in miR181a CD4$^+$

T cells without affecting the kinetic of cytokine release (see Figure 3.4 lower right panel). After 24 h the secretion of IL-17 reached a peak in both samples. Interestingly, miR181a $CD4^+$ T cells produced nearly twice as much of IL-17, compared to ctrl miRNA $CD4^+$ T cells. After 24 h, the secretion of IL-17 decreased and after 48 h there was no difference in IL-17 secretion between $CD4^+$ T cells transduced either with miR181a or ctrl-miRNA.

Despite the fact that there was no influence on cytokine production in $CD8^+$ T cells overexpressing miR181a (miR181a $CD8^+$ T cells) after restimulation for 6 h, restimulation with γ-irradiated autologous iDCs and different amounts of αCD3 for five days showed significant differences in IFNγ secretion without changing the kinetic (see Figure 3.5 upper left panel). This was also observed for the secretion of IL-10 (see Figure 3.5 lower left panel) and IL-4 (see Figure 3.5 lower right panel). The concentration of all three cytokines increased over time and reached a maximum level after 48 h. After 48 h, the cytokine release decreased very quickly for IL-4 and IL-10, whereas the decline of IFNγ secretion was slower. Interestingly, the production of IFNγ was significantly higher in miR181a $CD8^+$ T cells compared to $CD8^+$ T cells transduced with ctrl miRNA (ctrl miRNA $CD8^+$ T cells) for all time points analyzed between 12 h and 96 h. Whereas miR181a $CD8^+$ T cells showed no differences in IL-4 and IL-10 secretion compared to ctrl miRNA $CD8^+$ T cells after 72 h.

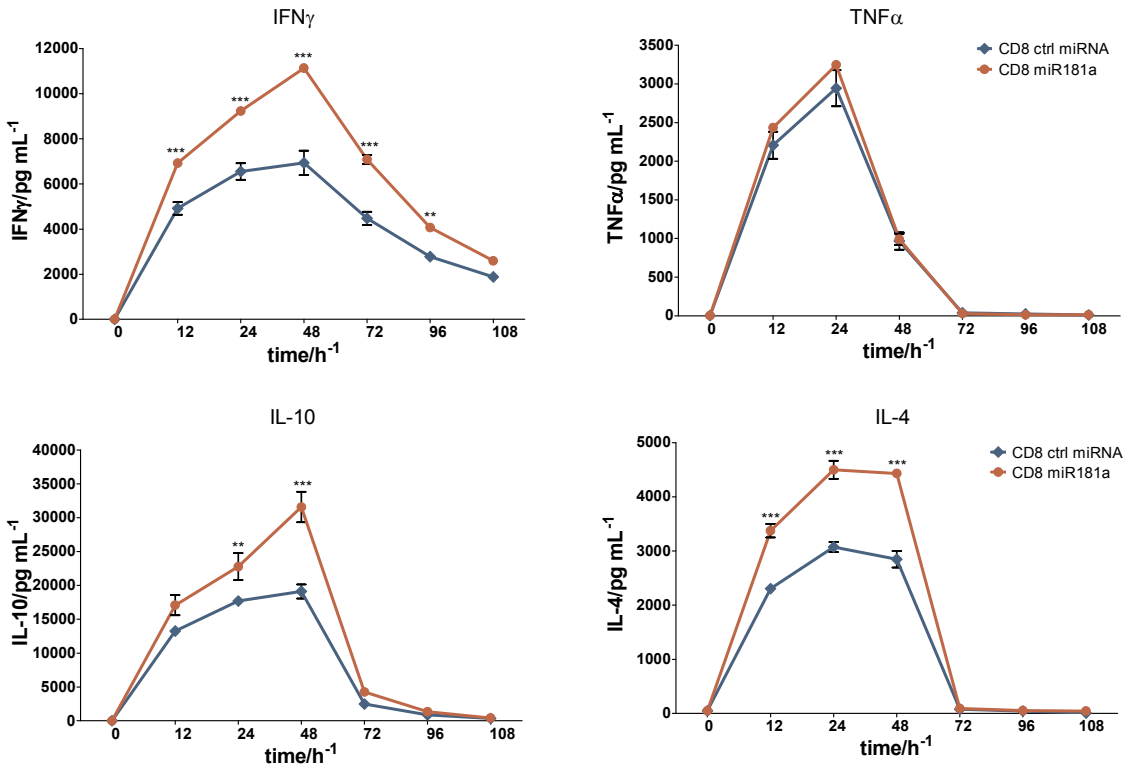

Figure 3.5: miR181a increases cytokine expression in primary human CD8$^+$ T cells. MACS sorted human CD8$^+$ T cells were transduced with lentiviral vectors encoding miR181a or ctrl miRNA in the 3'UTR of the reporter gene GFP. Four days after the last transduction, CD8$^+$ T cells were sorted based on their GFP expression. Sorted cells were then co-incubated with γ-irradiated autologous iDCs and 0.1 μg/mL αCD3 for five days. The secretion of cytokines was analyzed using a Multiplex Suspension Array after different time points of stimulation. n=3; Error bars are ±SD; Two-way ANOVA (* indicates significant differences; ** p<0.01; *** p<0.001)

Remarkably, there was no difference detectable for TNFα secretion after long-term restimulation of CD8$^+$ T cells (see Figure 3.5 upper right panel) either transduced with miR181a or ctrl miRNA. Similar to the release of IL-4 and IL-10, the amount of cytokine release increased over time and reached a maximum after 24 h. After 48 h, the secretion of TNFα decreased rapidly. In summary, miR181a overexpression did not drastically change cytokine production in human T cells after short-term restimulation. However, after long-term restimulation, an increased and prolonged cytokine expression in human T cells overexpressing miR181a could be detected. These results are specific effects on certain cytokines. In conclusion, miR181a is able to enhance the cytokine production of primary human T cells.

3.1.4 Activation of miR181a overexpressing primary human T cells is independent of αCD28 mediated co-stimulation

Effective activation of T cells requires two signals. The first signal is delivered through the antigen-specific T cell receptor, which recognizes the peptide bound into the MHC-complex. The second signal is generated by cognate interactions through adhesion molecules of T cells and target cells. Many tumor cells do not express any co-stimulatory molecules preventing full activation of T cells. Therefore, it would be beneficial if miR181a overexpressing primary human T cells would be independent of co-stimulatory signals for full activation. To this end, the effect of CD28 signaling in miR181a overexpressing T cells was analyzed.

Primary human $CD4^+$ and $CD8^+$ T cells were transduced with miR181a or ctrl miRNA and restimulated with different amounts of plate bound αCD3 in the presence or absence of the co-stimulatory molecule αCD28 for 6 h to analyze the effects of miR181a overexpression in primary human T cells on cytokine production. The expression of different cytokines in $CD4^+$ and $CD8^+$ T cells transduced with miR181a or ctrl miRNA was analyzed by flow cytometry using intracellular staining (see Figure 3.6).

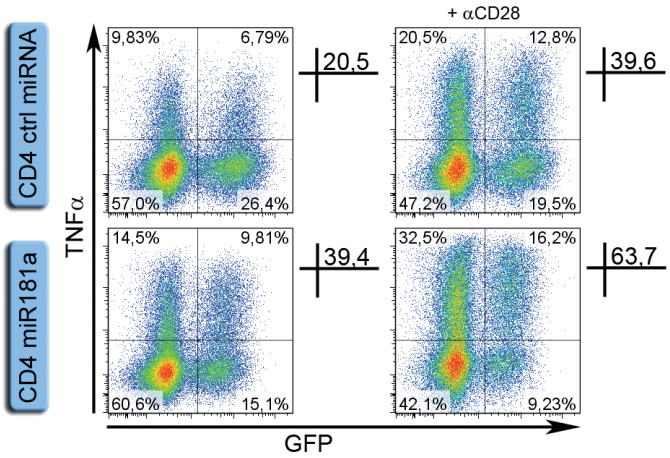

Figure 3.6: Activation of primary human T cells with and without the co-stimulatory molecule αCD28.
Primary human T cells transduced with miR181a or ctrl miRNA were restimulated with 10 μg/mL plate bound αCD3 in the presence or absence of 2μg/mL soluble αCD28. Cells were restimulated for 6h and the expression of TNFα was analyzed by intracellular staining using flow cytometry. Numbers beside the plots indicate percentages of cytokine expressing cells within the GFP positive population. Representative results of one out of three experiments were shown.

In the upper panels, the analysis of TNFα producing ctrl miRNA CD4$^+$ T cells after restimulation with αCD3 in the abscence (left panel) or presence (right panel) of αCD28 is shown. In the lower panels the analysis of TNFα producing miR181a CD4$^+$ T cells after restimulation (as described before) is depicted. Overexpression of miR181a in primary human CD4$^+$ T cells (see Figure 3.6, lower panels) leads to a two-fold increase of TNFα producing cells compared to ctrl miRNA CD4$^+$ T cells under the same conditions (see Figure 3.6, upper panels). Interestingly, the same number of TNFα producing CD4$^+$ T cells overexpressing miR181a could be detected in the absence of the co-stimulatory molecule αCD28 (see Figure 3.6, lower left panel) compared to ctrl miRNA CD4$^+$ T cells in the presence of αCD28 (see Figure 3.6, upper right panel). The addition of αCD28 to miR181a CD4$^+$ T cells leads to further enhancement of responding cells (see Figure 3.6, lower right panel).

The percentages of cytokine producing cells were determined relating to GFP positive CD4$^+$ T cells (see Figure 3.7). The numbers of cytokine producing cells increased in an αCD3 concentration dependent manner. Remarkably, the percentage of cytokine expressing cells after restimulation with αCD3 only in miR181a CD4$^+$ T cells (shown in Figure 3.7 with brighter red lines or bars) was similar to the numbers of cytokine expressing cells in ctrl miRNA CD4$^+$ T cells after restimulation with αCD3 and αCD28 (shown in Figure 3.7 with darker blue lines or bars). The co-stimulation of miR181a overexpressing primary human CD4$^+$ T cells (shown in Figure 3.7 with darker red lines or bars) leads to a further enhancement of reacting cells. This could be detected for TNFα (see Figure 3.7 left panels) and IFNγ (see Figure 3.7 right panels) in cytokine producing CD4$^+$ T cells.

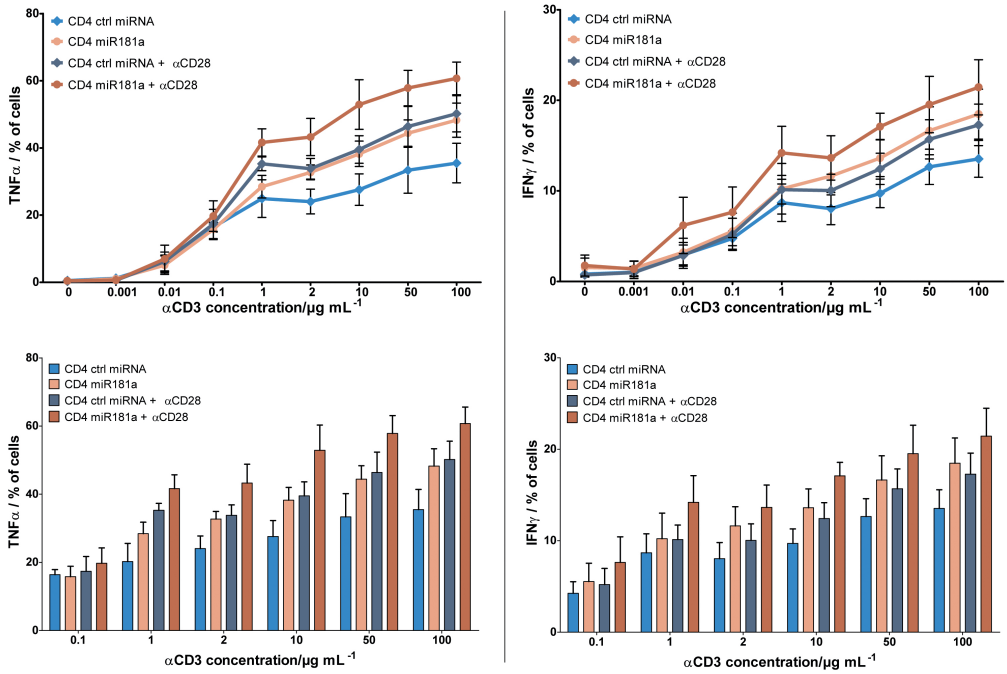

Figure 3.7: Activation of miR181a overexpressing primary human CD4$^+$ T cells is independent of αCD28 mediated co-stimulation.
Primary human CD4$^+$ T cells transduced with miR181a or ctrl miRNA were restimulated with different amounts of plate bound αCD3 in the presence or absence of 2 μg/mL soluble αCD28. Cells were restimulated for 6h and the expression of cytokines was analyzed by intracellular staining using flow cytometry. Analysis of TNFα and IFNγ expression in transduced CD4$^+$ T cells with indicated amounts of plate bound αCD3 in the presence or absence of 2 μg/mL soluble αCD28. n=3; Error bars are ±SD.

Additionally, the percentages of cytokine producing cells were determined in relation to GFP positive CD8$^+$ T cells (see Figure 3.8). Comparable to CD4$^+$ T cells, the numbers of cytokine producing cells increased in an αCD3 concentration dependent manner. In contrast to CD4$^+$ T cells, no differences in the numbers of cytokine producing cells could be detected after restimulation, either with or without the co-stimulatory molecule αCD28 (see Figure 3.8). Furthermore, there were no distinctions between miR181a overexpressing human CD8$^+$ T cells and CD8$^+$ T cells transduced with ctrl miRNA. This was true of TNFα (see Figure 3.8 left panels) and IFNγ (see Figure 3.8 right panels) producing cells in CD8$^+$ T cells.

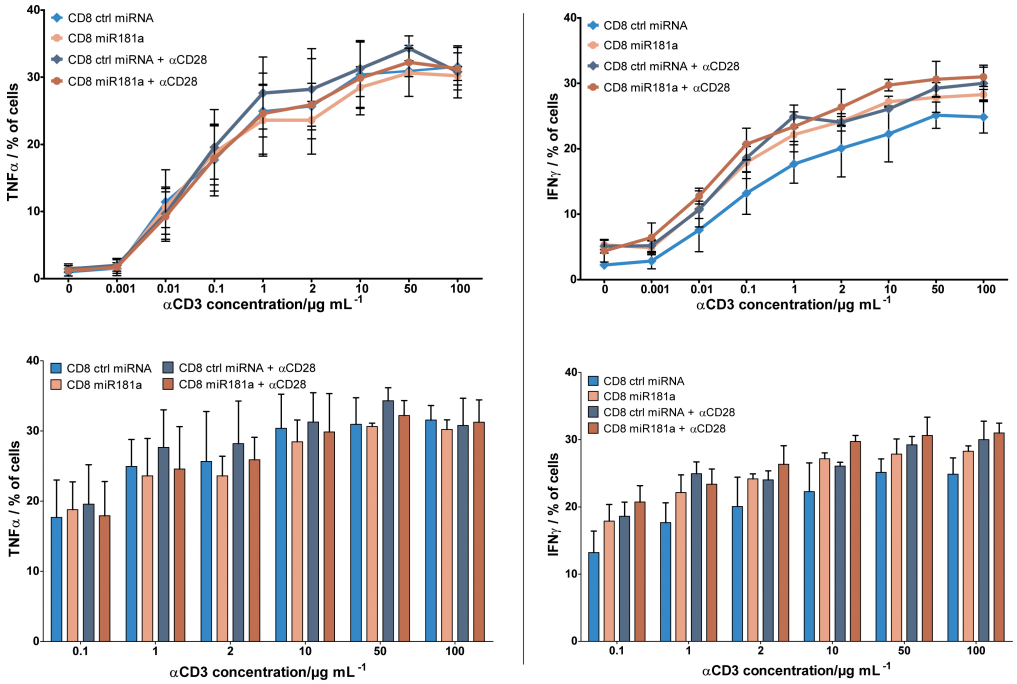

Figure 3.8: miR181a overexpression primary human CD8$^+$ T cells did not affect αCD28 mediated co-stimulation.
Primary human CD8$^+$ T cells transduced with miR181a or ctrl miRNA were restimulated with different amounts of plate bound αCD3 in the presence or absence of 2 μg/mL soluble αCD28. Cells were restimulated for 6h and the expression of cytokines was analyzed by intracellular staining using flow cytometry. Analysis of TNFα and IFNγ expression in transduced CD8$^+$ T cells with indicated amounts of plate bound αCD3 in the presence or absence of 2 μg/mL soluble αCD28. n=3; Error bars are ±SD.

In summary, there was no effect of miR181a overexpression in primary human CD8$^+$ T cells on cytokine production detectable after restimulation, in the presence or absence of soluble αCD28. In contrast, miR181a overexpression in CD4$^+$ T cells results in higher numbers of TNFα and IFNγ producing cells upon restimulation, even in the absence of co-stimulation.

To analyze the long-term effects of miR181a in primary human T cells after restimulation with and without co-stimulatory molecules, transduced T cells were sorted due to their GFP expression and restimulated with αCD3 in the presence or absence of αCD28. After indicated time points, the relative expression levels of different mRNA-transcripts were analyzed using a high-throughput, microfluidic chip-based, multi-analyte system. Expression levels of all genes were normalized to the amount of the house keeping gene HPRT and the first time point (12h). The results for transduced $CD4^+$ T cells are depicted as a heatmap in figure 3.9.

Here, many transcription factors such as GATA-binding protein 3 (GATA3), T box domain 21 (TBx21 or T-bet), forkhead box P3 (FOXP3) and eomesodermin (EOMES) involved in T cell differentiation into Th1 and Th2 states or for transcription of cytotoxic effector molecules are overexpressed in $CD4^+$ miR181a T cells with and without αCD28 co-stimiluation. Additionally, activation induced molecules and interleukin receptors such as perforin 1 (PRF1), CD137, IL-7-receptor (IL-7R), IL-12-receptor (IL-12R) and IL-13 are highly expressed in miR181a overexpressing $CD4^+$ T cells compared to $CD4^+$ ctrl miRNA T cells. Furthermore, the expression of the homing receptors CD62L and CD44 and also the chemokine receptors CCR3 and CCR7 are higher presented in $CD4^+$ miR181a T cells. Interestingly, the expression of the programmed cell death protein 1 (PDCD1) is also overexpressed in these cells compared to $CD4^+$ ctrl miRNA T cells. These effects are increasing by longer incubation times and with additionally co-stimulation by αCD28.

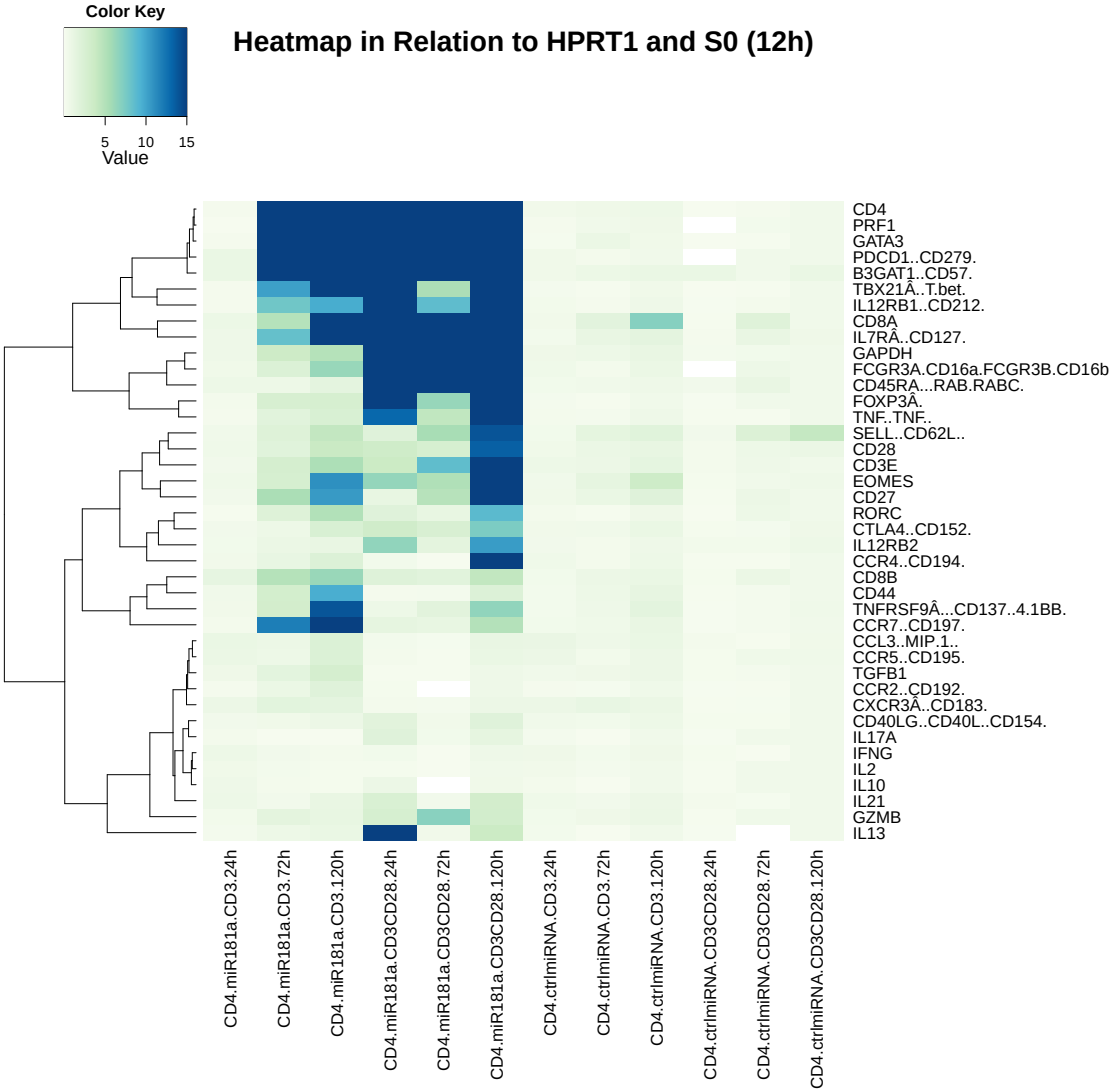

Figure 3.9: Heatmap of selected genes in transduced CD4$^+$ T cells after stimulation with αCD3 in the presence or absence of αCD28.
MACS sorted human CD4$^+$ T cells were transduced with lentiviral vectors encoding miR181a or ctrl miRNA in the 3'UTR of the reporter gene GFP. Four days after last transduction CD4$^+$ T cells were sorted due to their GFP expression. Sorted cells were then incubated with 0.1 μg/mL plate-bound αCD3 in the presence or absence of 2 μg/mL αCD28 for five days. Relative expression levels of different mRNA-transcipts were determined by fluidigm technology after different time points of stimulation. n=2; Data are represented as a heatmap of selected genes (rows) and analyzed samples (columns).

In figure 3.10 the results for transduced CD8$^+$ T cells are summarized. Here, only with co-stimulation the pro-inflammatory cytokine IL-17 and the cytotoxicity associated molecules perforin 1 (PRF1), beta-1,3-glucuronyltransferase-1 (B3GAT) and the chemokine CCL3 are higher expressed in CD8$^+$ miR181a T cells compared to CD8$^+$ ctrl miRNA T cells. Remarkably, the expression of IL-10 shows increased levels in CD8$^+$ T cells overexpressing miR181a. This is

observable in CD8$^+$ miR181a T cells restimulated either with and without αCD28. Interestingly, molecules associated with the homing process of T cells such as CD44 and CD62L and also some chemokine receptors (CCR2, CCR5, and CCR7) are downregulated in CD8$^+$ miR181a T cells compared to CD8$^+$ ctrl miRNA T cells after 72h of restimulation without αCD28.

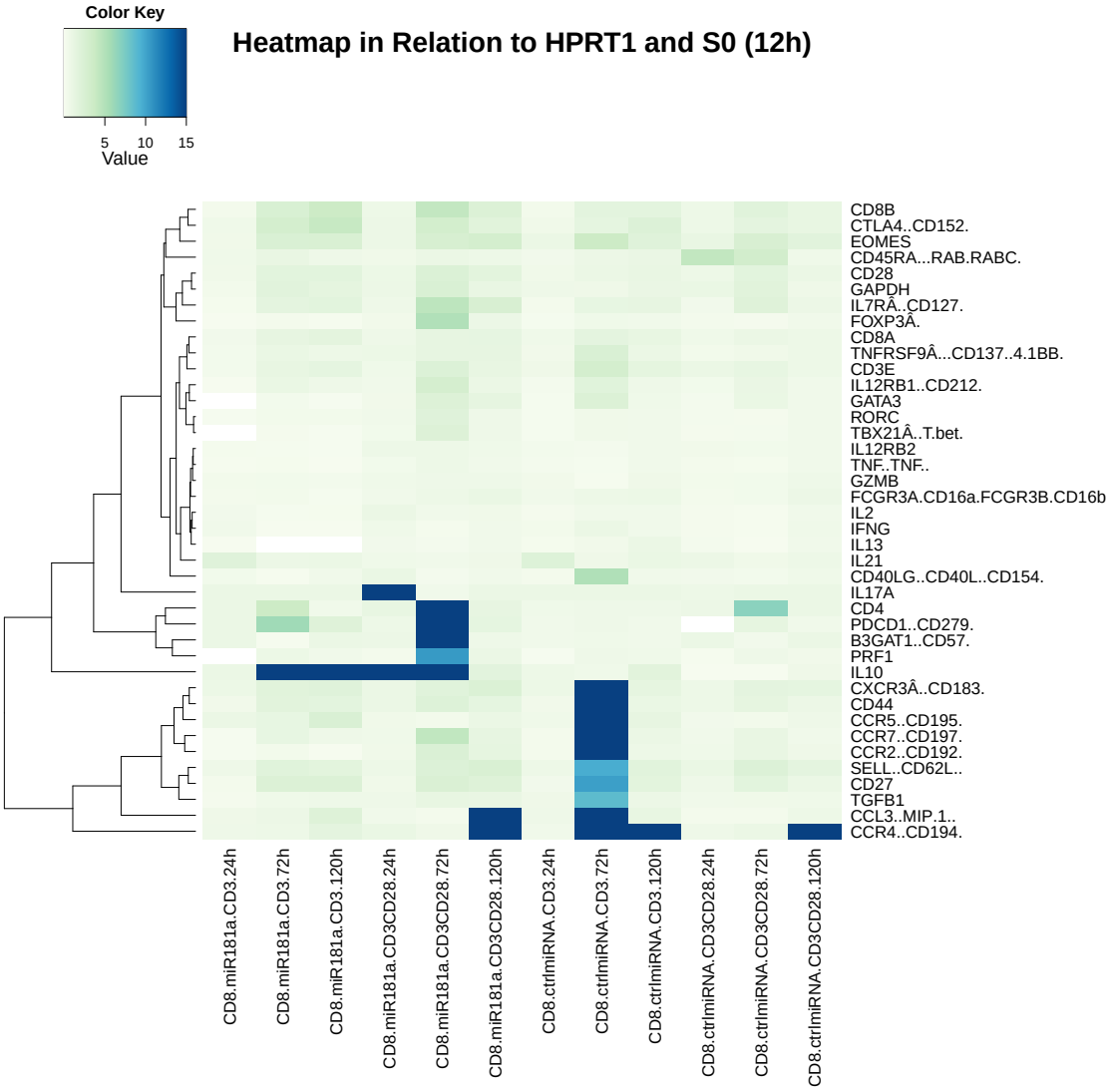

Figure 3.10: Heatmap of selected genes in transduced CD8$^+$ T cells after stimulation with αCD3 in the presence or absence of αCD28.
MACS sorted human CD8$^+$ T cells were transduced with lentiviral vectors encoding miR181a or ctrl miRNA in the 3'UTR of the reporter gene GFP. Four days after last transduction CD8$^+$ T cells were sorted due to their GFP expression. Sorted cells were then incubated with 0.1 μg/mL plate-bound αCD3 in the presence or absence of 2 μg/mL αCD28 for five days. Relative expression levels of different mRNA-transcipts were determined by fluidigm technology after different time points of stimulation. n=2; Data are represented as a heatmap of selected genes (rows) and analyzed samples (columns).

In conclusion, miR181a overexpression affects the expression pattern of different genes in both $CD4^+$ and $CD8^+$ T cells. These include genes for T cell differentiation, activation induced molecules, cytotoxic effector molecules, interleukins and interleukin receptors, chemokine receptors, and molecules involved in the homing process of T cells. The influcence of miR181a on gene expression is stronger in primary human $CD4^+$ T cells than in $CD8^+$ T cells.

3.1.5 Overexpression of miR181a in human $CD4^+$ T cells results in reduced proliferation

The Cell Proliferation Dye eFluor 670 (CPD-eFluor670) was used to analyze the proliferation of T cells transduced either with miR181a or ctrl miRNA. CPD-eFluor670 binds to cellular proteins containing primary amines. When cells divide, the dye is distributed to the daughter cells leading to a reduction of fluorescence, which can be detected by FACS analysis. Labeled cells were restimulated with autologous iDCs and different amounts of αCD3. Four days after restimuation, the reduction of the fluorescence intensity of CPD-eFluor670 was analyzed in GFP positive T cells by flow cytometry (see Figure 3.11). In figure 3.11 A the fluorescent intensity of CPD-eFluor670 in $CD4^+$ T cells transduced with ctrl miRNA (upper panel) or miR181a (lower panel) restimulated with autologous iDCs and indicated amounts of αCD3 are depicted as overlaying histograms. For both populations a similar reduction of CPD-eFluor670 fluorescence intensity could be detected in an αCD3 concentration dependent manner.

For a better understanding, the GeoMean of CPD-eFluor670 was calculated for GFP positive $CD4^+$ T cells (see Figure 3.11 B, left panel). Here the reduction of the fluorescence intensity was very strong after restimulation with iDCs and additional αCD3, compared to restimulation with iDCs only. The decline of fluorescence intensity of CPD-eFluor670 was weaker in miR181a $CD4^+$ T cells compared to ctrl miRNA $CD4^+$ T cells.

Additionally, the percentages of proliferating cells in GFP positive $CD4^+$ T cells were determined (see Figure 3.11 B, right panel). The numbers of proliferating cells were significantly lower in primary human $CD4^+$ T cells overexpressing miR181a than in $CD4^+$ T cells transduced with ctrl miRNA. For $CD8^+$ T cells, there were no differences detectable in the GeoMean of CPD-eFluor670 (see Figure 3.11C, left panel) or the quantity of proliferating cells (see Figure 3.11 C, right panel) either transduced with miR181a or ctrl miRNA.

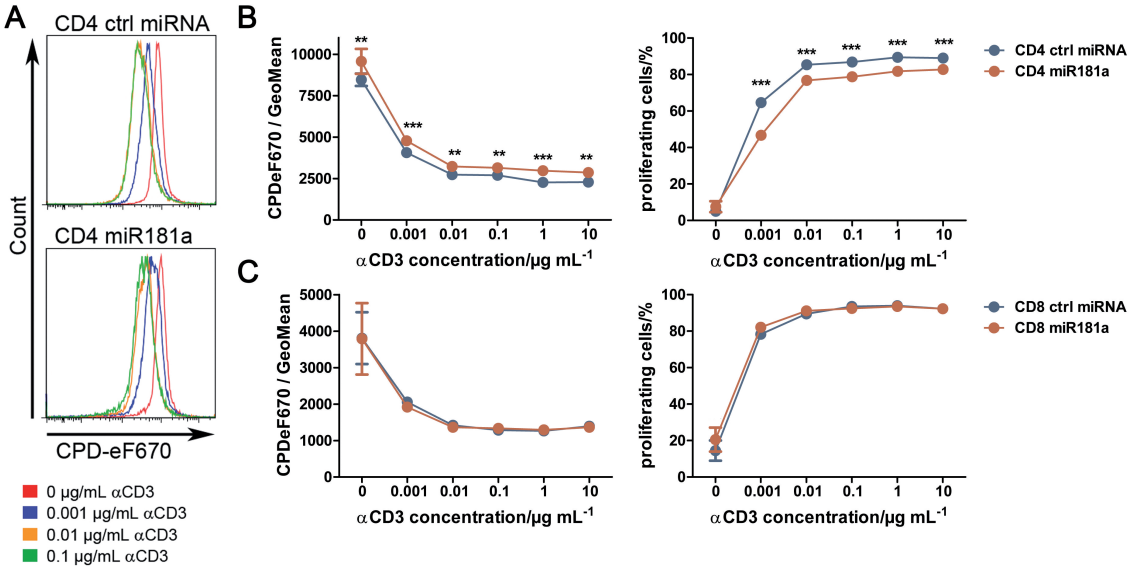

Figure 3.11: Proliferation of miR181a overexpressing primary human T cells.
Primary human T cells transduced with miR181a or ctrl miRNA were labeled with CPD-eFluor670 and restimulated with autologous iDCs and different amounts of αCD3.
(A) The fluorescence intensity of CPD-eFluor670 in GFP positive $CD4^+$ T cells was analyzed by flow cytometry four days after restimulation. Representative results of one out of three experiments are shown.
(B and C) The decrease of CPD-eFluor670 fluorescence intensity (left panel) and the number of proliferating cells (right panel) in GFP positive $CD4^+$ (B) and $CD8^+$ (C) T cells was calculated four days after restimulation. n=3; Error bars are ±SD; Two-way ANOVA (* indicates significant differences; ** $p<0.01$; *** $p<0.001$)

In conclusion, the overexpression of miR181a in primary human T cells does not change the proliferation of $CD8^+$ T cells, but leads to less proliferation of $CD4^+$ T cells.

3.1.6 miR181a has an anti-apoptotic effect on T helper cells

To analyze the viability of miR181a overexpressing T cells, MACS-sorted $CD4^+$ and $CD8^+$ T cells either transduced with miR181a or ctrl miRNA were restimulated with autologous iDCs and different amounts of αCD3. Apoptosis of GFP positive cells was analyzed after several time points by flow cytometry using AnnexinV staining. AnnexinV staining was used in conjunction with the vitality dye propidium iodide (PI) to distinguish between early apoptotic cells ($AnnexinV^+/PI^-$) and late apoptotic or already dead cells ($AnnexinV^+/PI^+$).

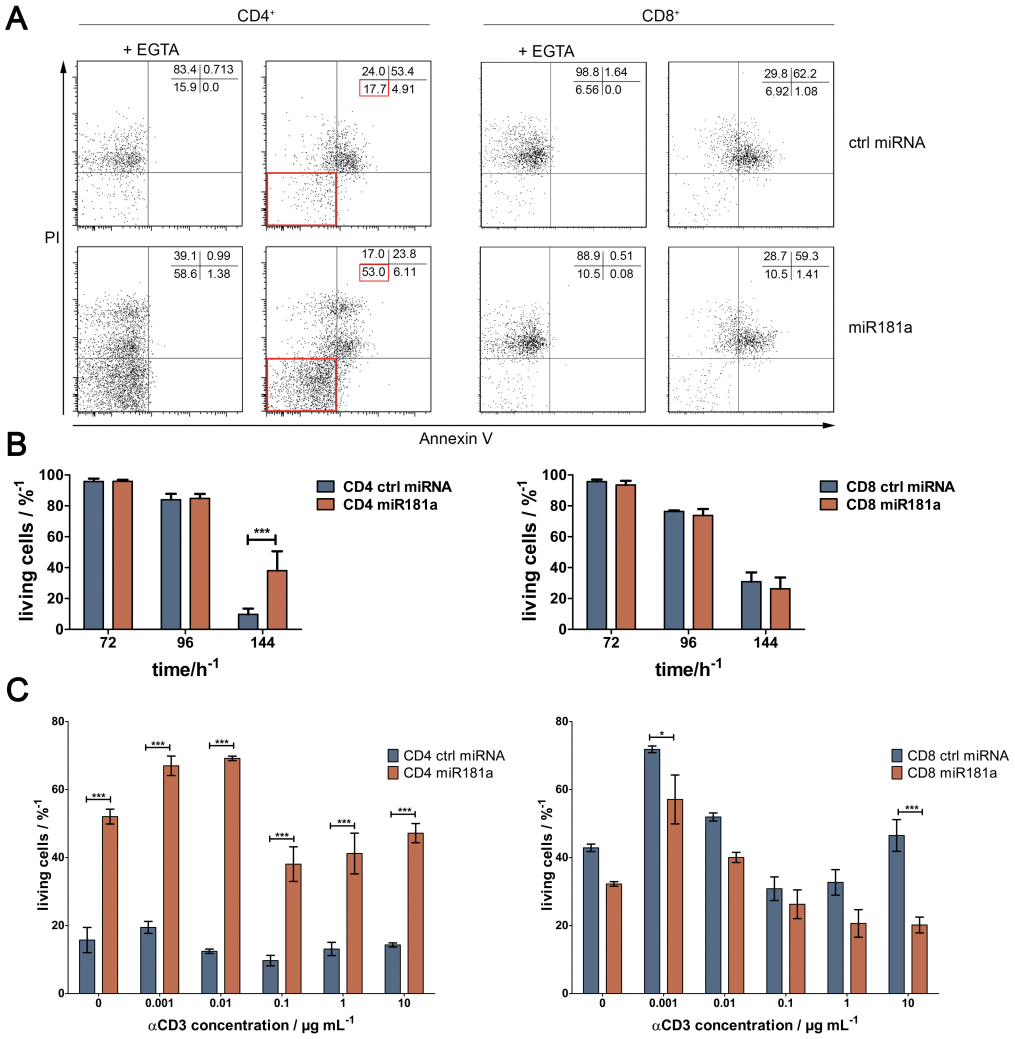

Figure 3.12: miR181a renders T helper cells resistant to apoptosis.
Primary human T cells transduced with miR181a or ctrl miRNA were restimulated with autologous iDCs and different amounts of αCD3 four days after the last transduction. Apoptosis of GFP positive cells was analyzed after several time points using flow cytometry.
(A) Representative FACS-Plots of Analysis on day six after restimulation with autologous iDCs and 0.1 μg/mL αCD3. Representative results of one out of three experiments are shown.
(B) Living cells (AnnexinV$^-$/PI$^-$) after different time points of restimulation with autologous iDCs and 0.1 μg/mL αCD3 in GFP positive CD4$^+$ (left panel) and CD8$^+$ (right panel) T cells were determined.
(C) Living cells (AnnexinV$^-$/PI$^-$) 144 h after restimulation with autologous iDCs and different amounts of αCD3 in GFP positive CD4$^+$ (left panel) and CD8$^+$ (right panel) T cells. n=3; Error bars are ±SD; Two-way ANOVA (* indicates significant differences; * p<0.05; ** p<0.01; *** p<0.001)

In figure 3.12, the analysis of living cells in primary human T cells either transduced with miR181a or ctrl miRNA after restimulation with autologous iDCs and αCD3 is shown. Living cells are characterized by negative staining for both AnnexinV and PI (see Figure 3.12 A). For

the first time points (72 h and 96 h) of analysis there were no differences in viability observed between miR181a $CD4^+$ T cells and ctrl miRNA $CD4^+$ T cells (see figure 3.12 B, left panel). After 144 h there was a significant higher number of living $CD4^+$ T cells detectable that overexpress miR181a than for ctrl miRNA T cells. This could also be observed for other restimulation conditions using different concentrations of αCD3 144 h after starting the restimulation (see figure 3.12 C, left panel).

For $CD8^+$ T cells transduced with miR181a or ctrl miRNA, there were no differences in viability after restimulation for all time points analyzed (see Figure 3.12 B, right panel). 144 h after starting restimualtion with iDCs and different amounts of αCD3, miR181a overexpressing $CD8^+$ T cells showed a reduced viability than ctrl miRNA $CD8^+$ T cells (see Figure 3.12 C, right panel). For the most concentrations tested, these differences are not significant. Only for 0.001 μg/mL αCD3 and 10 μg/mL αCD3, were there significant differences detectable.

In conclusion, apoptosis of $CD8^+$ T cells is more or less unaffected by ectopic expression of miR181a, but miR181a overexpression renders $CD4^+$ helper cells resistant to apoptosis after long term restimulation.

3.2 miR181a overexpression in murine Infl.-HA TCR^{tg} T cells

3.2.1 Retroviral transduction of murine Infl.-HA TCR^{tg} T cells

To study the effect of miR181a overexpression in murine Infl.-HA TCR^{tg} T cells, wildtype sequence of murine miR181a was obtained from Geneart (Regensburg, Germany) and cloned into the 3' UTR of EmGFP reporter gene (subsequently termed GFP) in the Gateway pENTR vector. As a control, *lacZ* targeting miRNA (subsequently termed ctrl miRNA) provided by the manufacturer, was used and cloned in a similar manner. The pENTRvectors were then recombined with a retroviral destination vector. The simultaneous expression of miR181a or ctrl miRNA and GFP in murine T cells is driven by the long terminal repeat (LTR). Analyzing the GFP expression by flow cytometry enables the identification of miR181a or ctrl miRNA transduced cells.

For transduction of murine T cells, spleen and LN were isolated from transgenic mice bearing an influenza virus hemagglutinin (HA) peptide specific T cell receptor that recognizes HA either in the context of the MHC class I or MHC class II molecule. The isolated splenocytes were then stimulated with 5 μg/mL antigenic peptide in the presence of IL-7 and IL-15. After 24 h and 48 h of activation, cells were transferred onto virus coated plates either coated with retroviral vectors encoding miR181a or ctrl miRNA in the 3' UTR of the reporter gene GFP. The expression of the reporter gene GFP was analyzed by flow cytometry, four days after the last transduction (see 3.13).

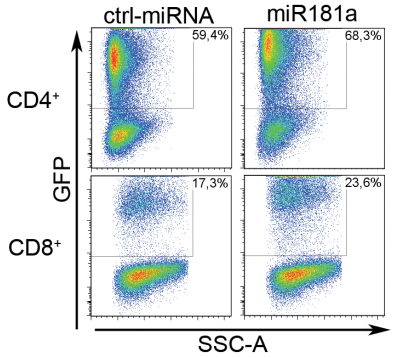

Figure 3.13: Retroviral transduction murine Infl.-HA TCR^{tg} T cells.
Murine Infl.-HA TCR^{tg} T cells were isolated from mice transgenic for an influenza virus hemagglutinin (HA) peptide specific T cell receptor, either in the context of MHC class I or in the context of MHC class II molecule. Isolated Infl.-HA TCR^{tg} T cells were stimulated with 5 μg/mL antigen specific peptide. After 24 h of stimulation, activated cells were transferred onto virus coated plates either coated with retroviral vectors containing miR181a or ctrl miRNA in the 3' UTR of the reporter gene GFP. 48 h after activation, T cells were placed on freshly coated plates. Expression of the reporter gene GFP was analyzed by flow cytometry, four days after the last transduction. Shown is one representative example out of three independent experiments.

In the upper panel, the analysis of Infl.-HA TCR^{tg} CD4$^+$ T cells transduced with retroviral vectors containing ctrl miRNA (left) or miR181a (right) in the 3' UTR of the reporter gene GFP is shown. Here, higher numbers of T cells expressing GFP could be detected when the cells were transduced with retroviral vectors encoding for miR181a (68.3%) compared to murine T cells transduced with ctrl miRNA (59.4%). In the lower panel, the analysis of Infl.-HA TCR^{tg} CD8$^+$ T cells transduced with retroviral vectors coding for ctrl miRNA (left) or miR181a (right) in the 3' UTR of the reporter gene GFP is shown. Again, T cells transduced with miR181a showed higher numbers of GFP positive T cells (23.6%) than T cells transduced with ctrl miRNA (17.3%).

Strikingly, Infl.-HA TCR^{tg} CD4^{+} T cells (upper panels) shows higher transduction effciencies as indicated by the GFP expression than Infl.-HA TCR^{tg} CD8^{+} T cells (lower panels). This was also observed in human T cells (compare chapter 3.1.1). In conclusion, murine Infl.-HA TCR^{tg} T cells could effciently transduced with retroviral vectors either encoding for miR181a or ctrl miRNA.

3.2.2 Overexpression of miR181a in murine Infl.-HA TCR^{tg} T cells downregulates the expression of multiple phophatases

To prove the overexpression of miR18a using retroviral transduction of murine Infl.-HA TCR^{tg} T cells, the amount of mature miR181a in transduced cells was analyzed using qRT-PCR. Therefore, murine Infl.-HA TCR^{tg} T cells transduced with miR181a or ctrl miRNA were used for RNA extraction and qRT-PCR analysis. The amount of miR181a in transduced Infl.-HA TCR^{tg} T cells was determined using an miR181a specific primer and normalized to the amount of miR181a obtained by Infl.-HA TCR^{tg} T cells transduced with ctrl miRNA (see Figure 3.14 A). The expression was calculated as relative to the expression of three house keeping genes (HPRT, GAPDH and β-Actin). In Infl.-HA TCR^{tg} CD4^{+} T cells, miR181a is overexpressed approximately two-fold in miR181a transduced cells compared to cells transduced with ctrl miRNA. In Infl.-HA TCR^{tg} CD8^{+} T cells, transduction with miR181a leads to a roughly six-fold overexpression compared to cells transduced with ctrl miRNA. These percentages were calculated from a mixture of transduced and untransduced cells, because there was no sorting before RNA extraction. Thus, the amount of overexpression in transduced murine Infl.-HA TCR^{tg} T cells might be higher than depicted here.

Similar to human T cells, the expression of known target genes was analyzed in murine Infl.-HA TCR^{tg} miR181a T cells and compared to T cells transduced with ctrl miRNA. Again, the expression of the analyzed genes was calculated relative to the expression of three house keeping genes (HPRT, GAPDH, and β-Actin). The overexpression of miR181a in Infl.-HA TCR^{tg} CD4^{+} T cells leads to significant downregulation of SHP2 and PTPN22. (see Figure 3.14 B, left panel). In Infl.-HA TCR^{tg} miR181a CD8^{+} T cells, the downregulation of all analyzed phosphates (SHP2, PTPN22, DUSP5 and DUSP6) was stronger than in CD4^{+} T cells. Here, all phosphatases showed a significant reduction when miR181a was overexpressed (see Figure

3.14 B, right panel).

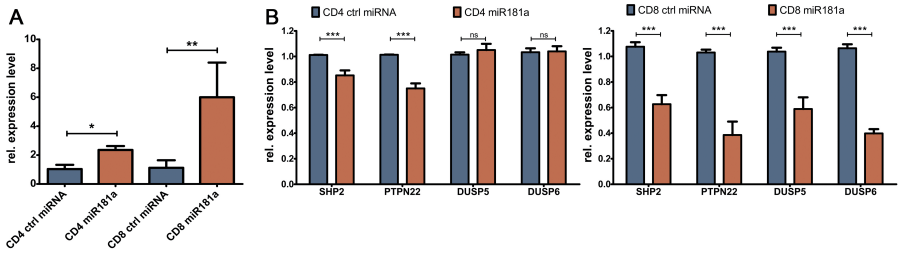

Figure 3.14: Overexpression of miR181a in murine Infl.-HA TCR^{tg} T cells downregulates the expression of multiple phophatases.
Isolated murine Infl.-HA TCR^{tg} CD4$^+$ and CD8$^+$ T cells were transduced with retroviral vectors containing miR181a or ctrl miRNA.
(A) Relative expression levels of miR181a in CD4$^+$ and CD8$^+$ T cells were determined by qRT-PCR.
(B) Relative expression levels of different target genes in CD4$^+$ (left panel) and CD8$^+$ (right panel) T cells were determined by qRT-PCR. Equal amounts of cells were used for RNA extraction. Equal numbers of RNA were used for qRT-PCR. HPRT, GAPDH and β-Actin were used for quantification. Expression values were normalized to T cells transduced with crtl miRNA. n=3; Error bars are SD; Two-way ANOVA (ns – not significant; * indicates significant differences; * p<0.05; ** p<0.01; *** p<0.001)

Importantly, the downregulation of target genes correlates to the amount of miR181a overexpression. In murine Infl.-HA TCR^{tg} CD4$^+$ T cells only a two-fold overexpression of miR181a could be detected after transduction with miR181a containing retroviral vectors. Here, the increase of miR181a leads to a reduction of SHP2 and PTPN22 between ~10 to ~20%. In CD8$^+$ T cells, the nearly six-fold overexpression of miR181a results in ~40 to ~60% downregulation of all analyzed phosphatases (SHP2, PTPN22, DUSP5 and DUSP6). Again, these percentages were calculated from a mixture of transduced and untransduced cells. Thus, the amount of downregulation in transduced murine Infl.-HA TCR^{tg} T cells might be stronger than depicted here. In conclusion, the overexpression of miR181a in Infl.-HA TCR^{tg} T cells leads to downregulation of multiple phosphatases that counteract activation inducing kinases.

3.2.3 miR181a enhances cytokine expression in murine Infl.-HA TCR^{tg} T cells

Murine Infl.-HA TCR^{tg} T cells were transduced with miR181a or ctrl miRNA and restimulated with T cell depleted splenocytes from Balb/c mice as APCs and different amounts of antigen for 6 h to analyze the effects of miR181a overexpression in murine T cells on activation and cytokine production. The expression of activation induced molecules such as CD40L and CD137 and the cytokines IFNγ and TNFα in CD4$^+$ and CD8$^+$ Infl.-HA TCR^{tg} T cells transduced with miR181a

or ctrl miRNA was analyzed (see Figure 3.15 A and 3.15 C). The percentages of responding cells was determined related to GFP positive CD4$^+$ and CD8$^+$ Infl.-HA TCR^{tg} T cells (see Figure 3.15 B and 3.15 D).

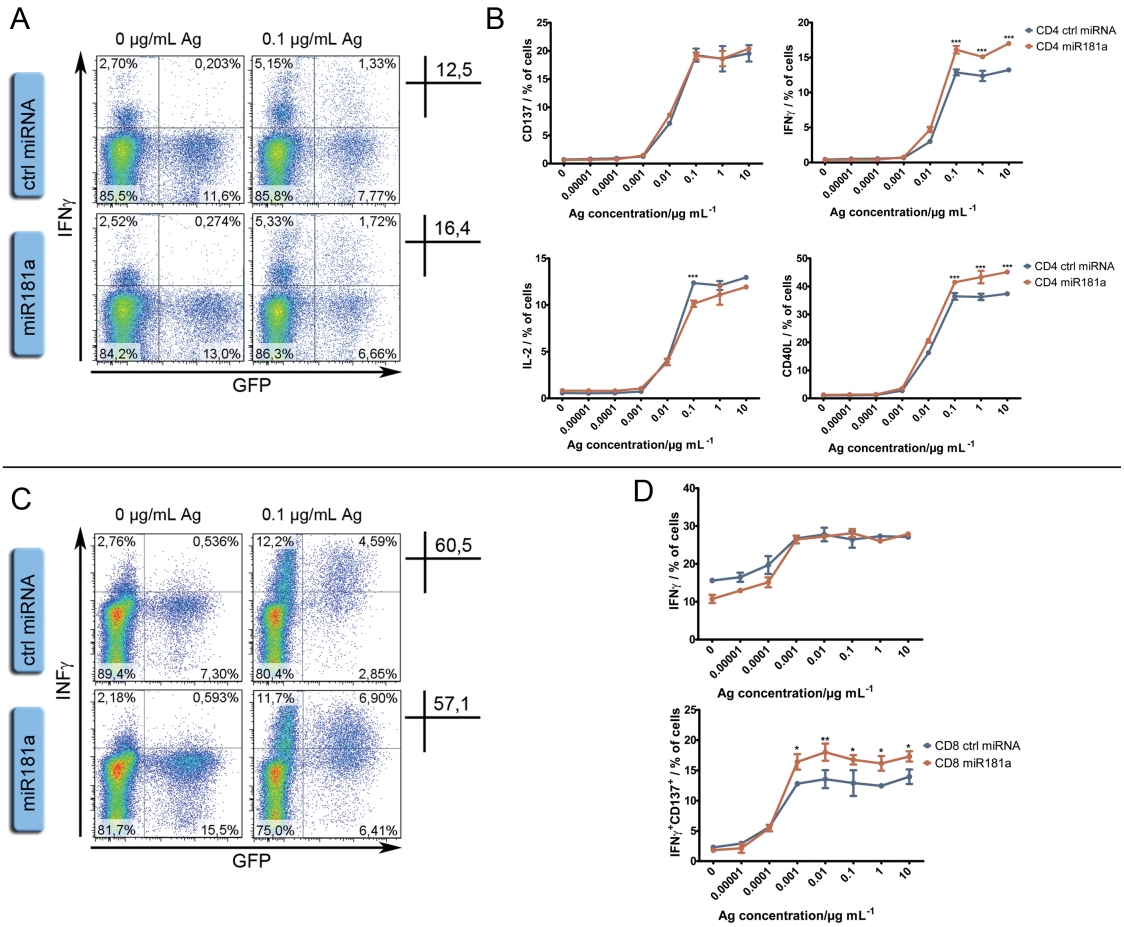

Figure 3.15: miR181a overexpression in murine Infl.-HA TCR^{tg} T cells leads to higher numbers of reacting T cells after short-term restimulation.
Murine Infl.-HA TCR^{tg} T cells transduced with miR181a or ctrl miRNA were restimulated with CD90.2 depleted splenocytes from Balb/c mice as APCs and different amounts of antigen 6 days after primary stimulation. Cells were restimulated for 6 h and cytokine expression was analyzed by intracellular staining using flow cytometry.
(A and C) Analysis of IFNγ expression in CD4$^+$ (A) and CD8$^+$ (C) Infl.-HA TCR^{tg} T cells transduced with miR181a or ctrl miRNA after restimulation with APCs and indicated amounts of antigen.
(B and D) Cytokine expression was analyzed in GFP positive CD4$^+$ (B) and CD8$^+$ (D) Infl.-HA TCR^{tg} T cells. n=3; Error bars are ±SD; Two-way ANOVA (* indicates significant differences; * $p<0.05$; ** $p<0.01$; *** $p<0.001$) Representative results of one out of three experiments were shown (A and C).

In murine Infl.-HA TCR^{tg} CD4$^+$ T cells, miR181a overexpression results in significant higher numbers of IFNγ (Figure 3.15 A and 3.15 B, upper right panel) and CD40L (Figure3.15 B, lower right panel) producing cells upon restimulation at higher concentrations of antigen (0.1 - 10 μg/mL). Meanwhile, the numbers of IL-2 (3.15 B, lower left panel) producing cells were not strongly enhanced by miR181a overexpression in murine Infl.-HA TCR^{tg} CD4$^+$ T cells. In contrast, the numbers of CD137 (3.15 B, upper left panel) producing cells were unaffected by miR181a overexpression in murine Infl.-HA TCR^{tg} CD4$^+$ T cells.

In murine Infl.-HA TCRtg CD8$^+$ T cells, the overexpression of miR181a has no influence on the number of IFNγ (Figure 3.15 C and 3.15 D, uper panel) producing cells after short-term restimulation. Regarding CD137 and IFNγ double positive cells, the numbers of these reacting cells increased significantly in miR181a overexpressing cells compared to ctrl miRNA transduced cells (see Figure 3.15 D, lower panel). In conclusion, miR181a overexpression in murine Infl.-HA TCR^{tg} T cells is able to enhance the numbers of reacting cells.

3.2.4 miR181a changes the expression pattern of different genes in murine Infl.-HA TCR^{tg} T cells

To analyze the influence of miR181a overexpression in murine Infl.-HA TCR^{tg} T cells on the expression of different genes, transduced T cells were sorted due to their GFP expression and the relative expression levels of different mRNA-transcripts were analyzed using a high-throughput, microfluidic chip-based, multi-analyte system. Expression levels of all analyzed genes were normalized to the amount of the house keeping gene HPRT. The results are depicted as fold change (FC) in miR181a T cells compared to ctrl miRNA T cells.

In figure 3.16 the results for transduced Infl.-HA TCR^{tg} CD4$^+$ T cells are shown. Here the mRNA for certain interleukines (IL-2, IL-18, and IL-4) and chemokines (CXCL1, CXCL11, and CCL4) are downregulated in miR181a transduced T cells compared to Infl.-HA TCR^{tg} ctrl miRNA CD4$^+$ T cells (blue area in figure 3.16). The mRNA coding for the immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO) and also the intercellular adhesion molecule-1 (ICAM1) are downregulated in Infl.-HA TCR^{tg} miR181a CD4$^+$ T cells. Importantly, also IFNγ shows a gentle downregulation for miR181a transduced Infl.-HA TCR^{tg} CD4$^+$ T cells. A few genes are not influenced by miR181a (black area in figure 3.16). These include chemokines (e.g. CCL12,

CCL19, CXCL9, and CXCL12), some interleukines (e.g. IL-21, IL-12, and IL-33), and also INFα, IFNβ, and TNF receptor.

There are also many genes overexpressed in Infl.-HA TCR^{tg} miR181a CD4^{+} T cells compared to ctrl miRNA transduced T cells (dark green to yellow area in figure 3.16). A gentle overexpression could be observed for the transcription factors T box domain 21 (TBX21), forkhead box O1 (FOXO1), and GATA-binding protein 3 (GATA3) in Infl.-HA TCR^{tg} miR181a CD4^{+} T cells. The transcription factor Eomesodermin (EOMES) shows a significant higher overexpression in Infl.-HA TCR^{tg} miR181a CD4^{+} T cells compared to ctrl miRNA transduced T cells (lighter green bar in figure 3.16). All of these transcription factors are involved in T cell differentiation. Other overexpressed factors include chemokine receptors (CCR7, CCR4, CXCR3, CXCR4, CCR5, and CCR2), chemokines (CCL3, CCL5, CXCL5, CXCL10, CCL22, CCL2, and CCL17), interleukines (IL-6, IL-1, and IL-10) and interleukin receptors (IL-12R, IL-2RA, IL-12RB1, and IL-12RB2). Importantly, also the surface molecules CTLA4, ICOS, and CD28 with contrary function during T cell activation are overexpressed in Infl.-HA TCR^{tg} miR181a CD4^{+} T cells. Additionally, molecules involved in T cell killing and death (Fas, Fas-Ligand, and PDCD1) are upregulated in Infl.-HA TCR^{tg} miR181a CD4^{+} T cells compared to ctrl miRNA transduced T cells.

The overexpression of miR181a in murine Infl.-HA TCR^{tg} CD4^{+} T cells changes the expression of different genes. Remarkable, the expression of molecules involved in T cell activation and regulation are overexpressed in these cells.

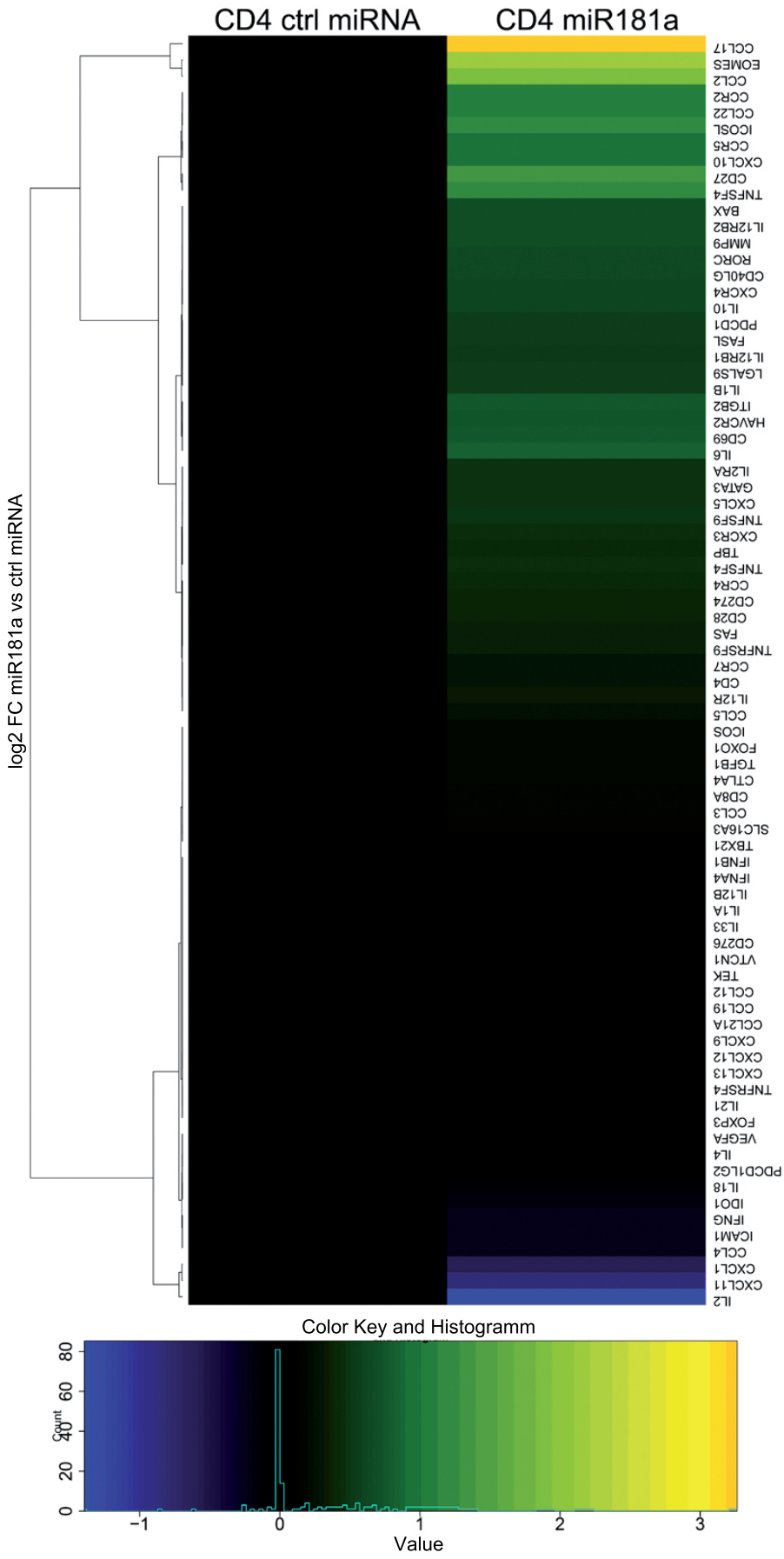

Figure 3.16: Gene expression analysis of miR181a overexpressing murine CD4$^+$ Infl.-HA TCR^{tg} T cells.
Murine CD4$^+$ Infl.-HA TCR^{tg} T cells were transduced with retroviral vectors encoding miR181a or ctrl miRNA in the 3′UTR of the reporter gene GFP. Four days after last transduction Infl.-HA TCR^{tg} T cells were sorted due to their GFP expression. Relative expression levels of different mRNA-transcipts were determined by fluidigm technology. Data are represented as a heatmap of selected genes in relation to ctrl miRNA transduced cells.

In figure 3.17 the results for transduced Infl.-HA TCR^{tg} CD8$^+$ T cells are shown. For Infl.-HA TCR^{tg} miR181a CD8$^+$ T cells only few genes are downregulated compared to ctrl miRNA transduced cells (blue area in figure 3.17). This include the chemokine 11 (CXCL11), the chemokine receptor 7 (CCR7), RORC, and IL-4. No changes in expression could be observed for programmed cell death ligand 1 also known as CD274 and indoleamine 2,3-dioxygenase (IDO) (black area in figure 3.17).

Many genes analyzed in transduced Infl.-HA TCR^{tg} miR181a CD8$^+$ T cells are overexpressed (dark green to red area in figure 3.17). A gentle overexpression could be observed for the transcription factors T box domain 21 (TBX21), forkhead box O1 (FOXO1), and GATA-binding protein 3 (GATA3). Again, the transcription factor Eomesodermin (EOMES) shows a higher overexpression in Infl.-HA TCR^{tg} miR181a CD8$^+$ T cells compared to ctrl miRNA transduced T cells (lighter green bar in figure 3.17). The surface molecules CTLA4, ICOS, and CD28 with contrary function during T cell activation are gently overexpressed in Infl.-HA miR181a CD8$^+$ TCR^{tg} T cells. Also for INFα, IFNβ, and TNF receptor a moderate overexpression in Infl.-HA TCR^{tg} miR181a CD8$^+$ T cells could be detected. Other overexpressed genes include chemokines (e.g. CXCL13, CXCL12, CXCL9, CXCL5, CCL21, CCL10, CCL5, CCL2, and CCL17), the chemokine receptor CCR4, interleukines (e.g. IL-6, IL-18, IL-1, IL-12, IL-10, and IL-2), and interleukin receptors (IL-12RB2, IL-12R, IL-2RA, and IL-12RB1). Again, molecules involved in T cell killing and death (Fas, Fas-Ligand, and PDCD1) are upregulated in Infl.-HA TCR^{tg} miR181a CD8$^+$ T cells compared to ctrl miRNA transduced T cells. The highest overexpression could be observed for IFNγ and different chemokine receptors (CXCR3, CCR2, CXCR4, and CCR5) (yellow to red area in figure 3.17).

The overexpression of miR181a in murine Infl.-HA TCR^{tg} CD8$^+$ T cells leads more to overexpression than to downregulation of the analyzed genes. Importantly, the expression of molecules involved in T cell activation and regulation and also the effector molecule IFNγ are upregulated in miR181a overexpressing cells.

In conclusion, miR181a overexpression in murine Infl.-HA TCR^{tg} T cells affects the expression of different genes in both CD4$^+$ and CD8$^+$ T cells. This include genes for T cell differentiation, cytotoxic effector molecules, interleukins and interleukin receptors, chemokines and chemokine receptors, and molecules involved in T cell killing. Importantly, transcription fac-

tors involved in T cell differentiation and surface molecules important for regulation of T cell activation are overexpressed in both Infl.-HA TCR^{tg} miR181a $CD4^+$ and $CD8^+$ T cells. Whereas, IFNγ is downregulated in Infl.-HA TCR^{tg} miR181a $CD4^+$ T cells and upregulated in Infl.-HA TCR^{tg} miR181a $CD8^+$ T cells. The influcence of miR181a on gene expression is stronger in Infl.-HA TCR^{tg} $CD8^+$ T cells than in $CD4^+$ T cells. In contrast to the human expression analysis (see section 3.1.3), murine Infl.-HA TCR^{tg} T cells were analyzed directly after sorting without restimulation, showing the status of transdued T cells without target contact.

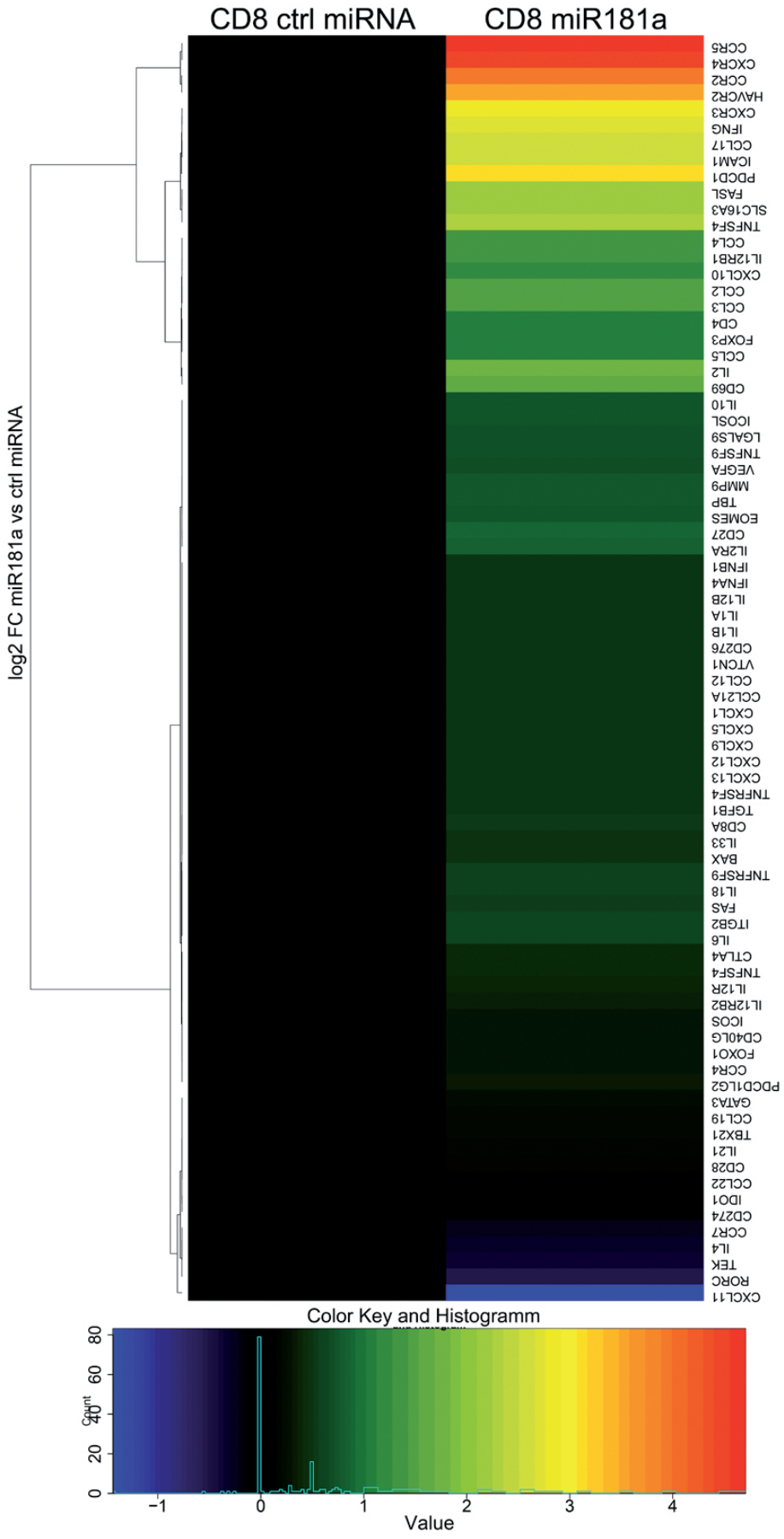

Figure 3.17: Gene expression analysis of miR181a overexpressing murine CD8$^+$ Infl.-HA TCR^{tg} T cells.
Murine CD8$^+$ Infl.-HA TCR^{tg} T cells were transduced with retroviral vectors encoding miR181a or ctrl miRNA in the 3′UTR of the reporter gene GFP. Four days after last transduction Infl.-HA TCR^{tg} T cells were sorted due to their GFP expression. Relative expression levels of different mRNA-transcipts were determined by fluidigm technology. Data are represented as a heatmap of selected genes in relation to ctrl miRNA transduced cells.

3.2.5 miR181a does not influence the proliferation of murine Infl.-HA TCR^{tg} T cells

Similarly as described for human T cells, murine Infl.-HA TCR^{tg} T cells were labeled with CPD-eFluor670 to analyze the proliferation of T cells transduced either with miR181a or ctrl miRNA. Labeled cells were restimulated in the presence of different amounts of antigen. Four days after restimulation, the reduction of the fluorescence intensity of CPD-eFluor670, as indication of proliferation, was analyzed in GFP positive T cells by flow cytometry (see Figure 3.18). In figure 3.18 A the fluorescence intensity of CPD-eFluor670 in murine CD4$^+$ Infl.-HA TCR^{tg} T cells transduced with ctrl miRNA (upper panel) or miR181a (lower panel) restimulated with indicated amounts of antigen are depicted as overlaying histograms. For both transduced populations a similar reduction of CPD-eFluor670 fluorescence intensity could be detected in an antigen concentration dependent manner.

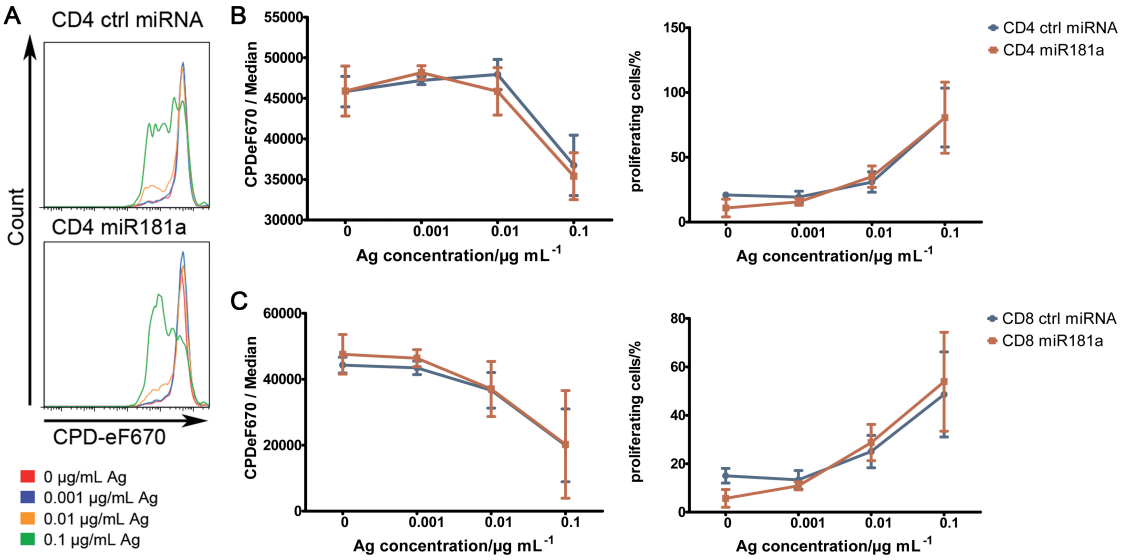

Figure 3.18: Proliferation of murine Infl.-HA TCR^{tg} T cells.
Murine Infl.-HA TCR^{tg} T cells transduced with miR181a or ctrl miRNA were labeled with CPD-eFluor670 6 days after primary stimulation and restimulated with CD90.2 depleted splenocytes from Balb/c mice as APCs and different amounts of antigen.
(A) The fluorescence intensity of CPD-eFluor670 in GFP positive CD4$^+$ Infl.-HA TCR^{tg} T cells was analyzed by flow cytometry four days after restimulation with APCs and indicated amounts of antigen.
(B and C) The decrease of CPD-eFluor670 fluorescent intensity (left panel) and the percentage of proliferating cells (right panel) in GFP positive CD4$^+$ (B) and CD8$^+$ (C) Infl.-HA TCR^{tg} T cells was calculated four days after restimulation. n=3; Error bars are ±SD; Representative results of one out of three experiments were shown (A).

For a better understanding, the median of CPD-eFluor670 was calculated for GFP positive murine CD4$^+$ Infl.-HA TCR^{tg} T cells (see Figure 3.18 B, left panel). Here the reduction of the fluorescent intensity was very strong after restimulation with 0.1 μg/mL antigen. Lower antigen concentrations did not influence the fluorescent intensity. There were no differences in fluorescent intensity detectable between murine CD4$^+$ Infl.-HA TCR^{tg} T cells either transduced with miR181a or ctrl miRNA. Additionally, the percentages of proliferating cells within GFP positive murine CD4$^+$ Infl.-HA TCR^{tg} T cells was determined (see Figure 3.18 B, right panel). The numbers of proliferating cells were similar in murine CD4$^+$ Infl.-HA TCR^{tg} T cells with and without miR181a overexpression. For murine CD8$^+$ Infl.-HA TCR^{tg} T cells, there were also no differences detectable in the median of CPD-eFluor670 (see Figure 3.18 C, left panel) or the quantity of proliferating cells (see Figure 3.18 C, right panel) either transduced with miR181a or ctrl miRNA.
In conclusion, the overexpression of miR181a in murine Infl.-HA TCR^{tg} T cells has no influence on proliferation.

3.2.6 miR181a has an anti-apoptotic effect on murine Infl.-HA TCR^{tg} T cells

To analyze the viability of miR181a overexpressing murine T cells, transduced CD4$^+$ and CD8$^+$ Infl.-HA TCR^{tg} T cells were restimulated with different amounts of antigen. Apoptosis in GFP positive cells was analyzed after several time points by flow cytometry using AnnexinV staining. The viability dye PI was used in conjuction with AnnexinV to distinguish between early apoptotic cells (AnnexinV$^+$/PI$^-$) and late apoptotic or already dead cells (AnnexinV$^+$/PI$^+$).
In figure 3.19 the analysis of living cells in transduced murine Infl.-HA TCR^{tg} T cells is summarized. After 72 h, there were no differences in viability observed for CD4$^+$ Infl.-HA TCR^{tg} T cells transduced with either miR181a or ctrl miRNA after restimulation with APCs and 0.01 μg/mL antigen (see figure 3.19 A). After 96 h there was a significantly higher number of living cells detectable for CD4$^+$ Infl.-HA TCR^{tg} T cells overexpressing miR181a than for ctrl miRNA CD4$^+$ T cells. This could also be observed for other restimulation conditions using different concentrations of antigen 96 h after starting restimulation (see figure 3.19 B). After 144 h there were no significant differences in viability detectable for CD4$^+$ murine Infl.-HA TCR^{tg} T cells either transduced with miR181a or ctrl miRNA.

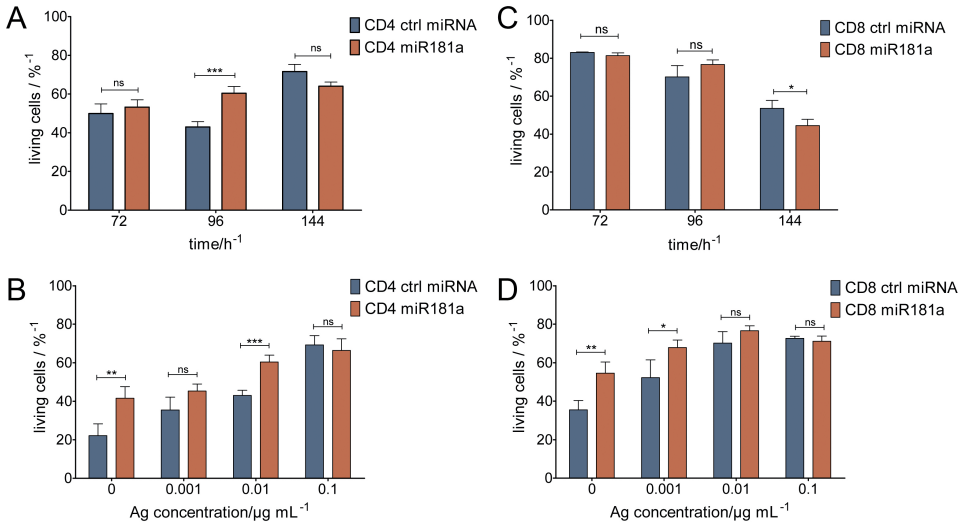

Figure 3.19: miR181a has an anti-apoptotic effect on murine Infl.-HA TCR^{tg} T cells. Murine Infl.-HA TCR^{tg} T cells transduced with miR181a or ctrl miRNA were restimulated with CD90.2 depleted splenocytes from Balb/c mice as APCs and different amounts of antigen six days after stimulation. Apoptosis of GFP positive cells was analyzed after several time points using flow cytometry.
(A and C) The proportion of living cells (AnnexinV$^-$/PI$^-$) after different time points of restimulation with APCs and 0.01 μg/mL antigen in GFP positive CD4$^+$ (A) and CD8$^+$ (C) Infl.-HA TCR^{tg} T cells was determined.
(B and D) The proportion of living cells (AnnexinV$^-$/PI$^-$) on day four after restimulation with APCs and different amounts of antigen in GFP positive CD4$^+$ (B) and CD8$^+$ (D) Infl.-HA TCR^{tg} T cells was determined. n=3; Error bars are ±SD; Two-way ANOVA (ns – not significant; * indicates significant differences; * $p<0.05$; ** $p<0.01$; *** $p<0.001$)

For CD8$^+$ Infl.-HA TCR^{tg} T cells, there was no significant difference in viability after 72 h and 96 h of restimulation with 0.01 μg/mL antigen (see figure 3.19 C). 144 h after starting restimulation with 0.01 μg/mL antigen, miR181a overexpressing murine CD8$^+$ Infl.-HA TCR^{tg} T cells showed a reduced viability compared with ctrl miRNA CD8$^+$ T cells. However, using lower concentrations of antigen, a significantly higher number of viable CD8$^+$ Infl.-HA TCR^{tg} T cells could be observed when miR181a was overexpressed (see figure 3.19 D).

In conclusion, miR181a overexpression in murine Infl.-HA TCR^{tg} T cells is able to enhance their viability upon antigen specific restimulation.

3.2.7 miR181a enhances the cytolytic activity of murine Infl.-HA TCR^{tg} cytotoxic CD8^{+} T cells

Using TCR^{tg} T cells allows the analysis of antigen-specific lysis of target cells and are therefore a good model system to analyze the effects of miR181a on cytotoxic activity. Murine Infl.-HA TCR^{tg} CD8^{+} T cells transduced with either miR181a or ctrl miRNA were co-incubated with a B cell lymphoma cell line (A20) stably expressing luciferase (A20-Luc Cl.9) as target cells and different amounts of antigen six days after primary stimulation. Additionally, different effector to target cell ratios (E:T ratio) were tested. The bioluminescence emitted by viable cells was measured after different incubation times. Cytotoxic activity was calculated in regard to luminescence values obtained after complete cell lysis of target cells induced by the addition of Triton-X 100 and in relationship to luminescence emitted by target cells alone.

The calculated percentage of cell lysis is depicted in figure 3.20. Here the cell lysis using Infl.-HA TCR^{tg} CD8^{+} T cells overexpressing miR181a as effector cells is shown in shades of red (brighter red means lower concentration of antigen). Infl.-HA TCR^{tg} CD8^{+} T cells transduced with ctrl miRNA are represented in shades of blue (brighter blue means lower concentration of antigen). In table 3.1 the statistical analysis of the cell lysis obtained from Infl.-HA TCR^{tg} CD8^{+} T cells transduced with ctrl miRNA versus miR181a overexpressing Infl.-HA TCR^{tg} CD8^{+} T cells is summarized.

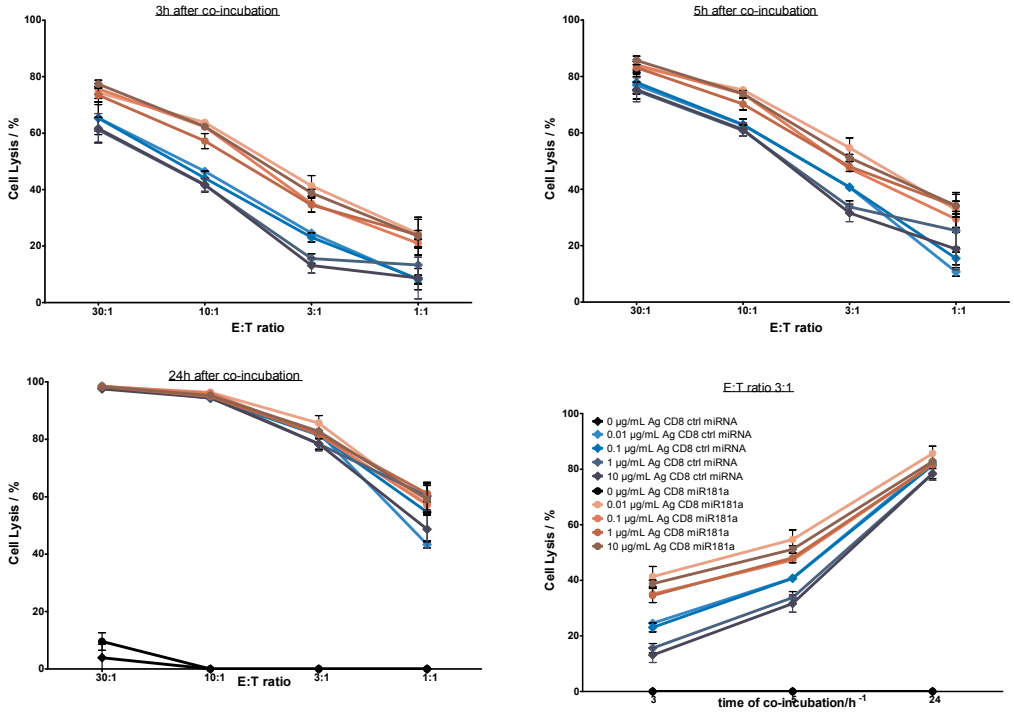

Figure 3.20: miR181a enhances cytotoxic activity of murine Infl.-HA TCR^{tg} T cells. Murine Infl.-HA TCR^{tg} CD8^{+} T cells were transduced with retroviral vectors containing miR181a or ctrl miRNA. Transduced T cells were co-incubated with a B cell lymphoma cell line (A20) stably expressing luciferase (A20-Luc Cl.9) as target cells and different amounts of antigen six days after stimulation. After different time points, luciferase activity of viable cells was measured and the cytotoxic activity was calculated in regard to luminescence values obtained after complete cell lysis induced by the addition of Triton-X 100 and in relationship to luminescence emitted by target cells alone. n=3; Error bars are ±SD.

After 3 h of co-incubation with A20-Luc Cl.9 target cells, there was a significant higher cell lysis detectable using miR181a overexpressing Infl.-HA TCR^{tg} CD8^{+} T cells than could be observed for Infl.-HA TCR^{tg} ctrl miRNA CD8^{+} T cells (see figure 3.20, upper left panel and table 3.1). This difference could be detected at different antigen concentrations (depicted in different color shades) and E:T ratios used. This advantage of miR181a overexpressing Infl.-HA TCR^{tg} CD8^{+} T cells was still observable after 5 h of co-incubation (see figure 3.20, upper right panel and table 3.1). After 24 h there were no differences in cytotoxic activity detectable for E:T ratio between 3:1 and 30:1 (see figure 3.20, lower left panel and table 3.1). At a E:T ratio of 1:1 there was still a significant higher target cell lysis detectable when miR181a overexpressing Infl.-HA TCR^{tg} CD8^{+} T cells were used as effector cells (see figure 3.20, lower left panel and table 3.1).

Table 3.1: Statistical Analysis of Cytotoxic Activity.
T cells transduced with ctrl miRNA vs. T cells transduced with miR181a. Two-way ANOVA (ns – not significant; * indicates significant differences; * p<0.05; ** p<0.01; *** p<0.001)

Ag concentration	E:T ratio	After 3 h	After 5 h	After 24 h
0.01 μg/mL	30:1	**	*	ns
	10:1	***	***	ns
	3:1	***	***	ns
	1:1	***	***	***
0.1 μg/mL	30:1	**	ns	ns
	10:1	***	***	ns
	3:1	***	*	ns
	1:1	***	***	ns
1 μg/mL	30:1	***	**	ns
	10:1	***	**	ns
	3:1	***	***	ns
	1:1	**	**	ns
10 μg/mL	30:1	***	***	ns
	10:1	***	***	ns
	3:1	***	***	ns
	1:1	***	***	***

In conclusion, these results demonstrate that miR181a is able to enhance the cytotoxic activity of murine Infl.-HA TCR^{tg} $CD8^+$ T cells.

3.3 *In vivo* models to study the effects of miR181a on anti-tumoral activity

3.3.1 Experimental setup to study the effects of miR181a overexpression *in vivo*

The expression of model antigens in tumor cell lines enables the targeted elimination of tumors using TCR^{tg} T cells and provides the opportunity to study the influence of intrinsic modulations of T cells. To study the effect of miR181a on anti-tumoral activity of murine Infl.-HA TCR^{tg} T cells, a tumor model with tumor cells expressing the antigen hemagglutinine (HA) was established. Therefore, a B cell lymphoma cell line expressing HA (A20-HA) was used for tumor generation. Of those, $2*10^5$ A20-HA tumor cells were injected subcutaneously into the flank of Balb/c mice. The growing tumors can easily be monitored by measuring the size using

a caliper square. During tumor development, Infl.-HA TCR^{tg} T cells were transduced with retroviral vectors encoding for miR181a or ctrl miRNA. Ten days after tumor inoculation and six day after T cell activation, tumor bearing mice were irradiated with 5 Gy and an indicated number of transduced cells was injected intravenously (i.v.) 4 h after irradiation. The tumor growth, survival, and T cell phenotype in blood samples were monitored. The experimental setup is depicted in figure 3.21.

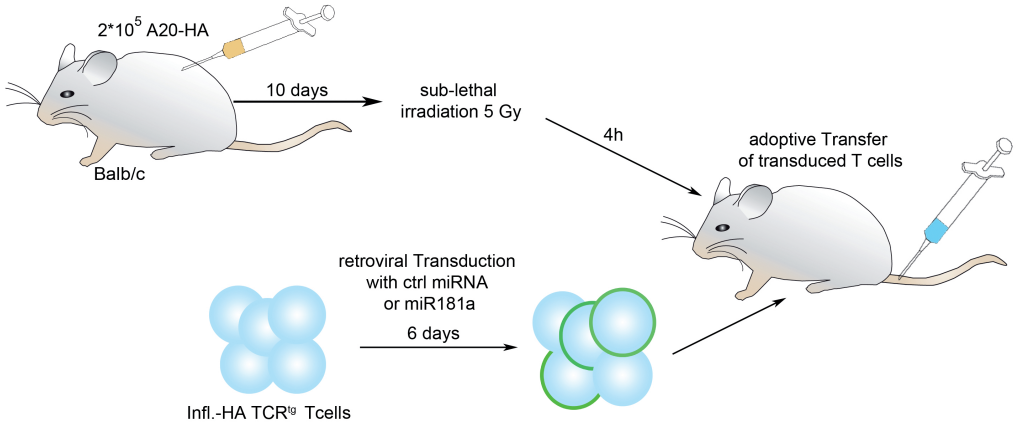

Figure 3.21: Experimental setup to study the effects of miR181a overexpression *in vivo*. For tumor generation a B cell lymphoma cell line (A20) expressing hemagglutinine (HA) was used. 2*10^5 A20-HA tumor cells were injected subcutaneously into the flank of Balb/c mice. In the meantime, Infl.-HA TCR^{tg} T cells were transduced with retroviral vectors encoding for miR181a or ctrl miRNA. Ten days after tumor inoculation, tumor bearing mice were irradiated with 5 Gy and an indicated number of transduced cells was injected intravenously (i.v.) 4 h after irradiation. The tumor growth, survival, and T cell phenotype in blood samples were monitored.

3.3.2 miR181a overexpression in murine Infl.-HA TCR^{tg} CD8$^+$ T cells does not affect tumor control

To study the effect of miR181a on anti-tumoral activity of murine Infl.-HA TCR^{tg} CD8$^+$ T cells, transduced Infl.-HA TCR^{tg} CD8$^+$ T cells were injected i.v. into A20-HA tumor bearing mice. Before transfer of cells, the transduction efficiency was analyzed using flow cytometry (see figure 3.22). In the upper panel, the analysis of Infl.-HA TCR^{tg} CD8$^+$ T cells transduced with ctrl miRNA is shown. Here, approximately 35 % of the cells express the reporter gene GFP (see 3.22, upper left panel). In the lower panel, the analysis of Infl.-HA TCR^{tg} CD8$^+$ T cells transduced with miR181a is shown. Similar as reported before (see chapter 3.2.1), the transduction using miR181a coding retroviral vectors leads to higher numbers of GFP expressing

cells. Here, approximately 49 % of the cells express the reporter gene GFP (see 3.22, lower left panel). Additionally, the expression of Vβ8.2 as a marker for the transgenic TCR was analyzed. For both, Infl.-HA TCR^{tg} CD8^{+} T cells either transduced with miR181a or ctrl miRNA, nearly all cells carry the transgenic TCR, enabling the recognition of A20-HA tumor cells. These cells were injected i.v. into A20-HA tumor bearing mice. As a control, splenocytes from Balb/c wildtype mice, unable to recognize the tumor cells, were injected into A20-HA tumor bearing mice.

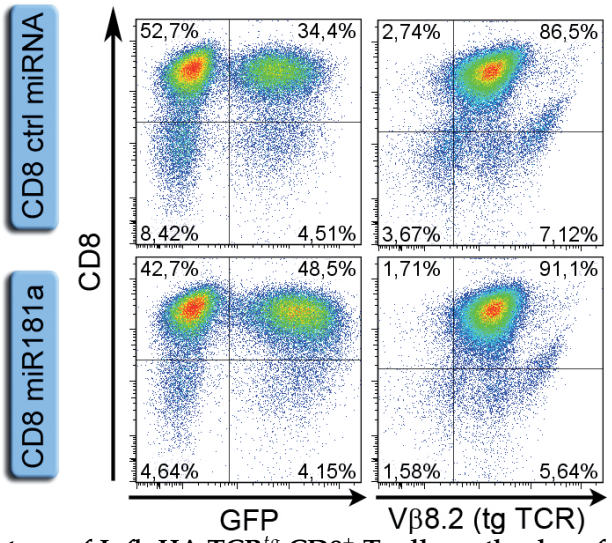

Figure 3.22: Phenotype of Infl.-HA TCR^{tg} CD8^{+} T cells on the day of transfer into A20-HA tumor bearing mice.
Murine Infl.-HA TCR^{tg} T cells were isolated from mice transgenic for an influenza virus hemagglutinin (HA) peptide specific T cell receptor in the context of MHC class I molecule. Isolated Infl.-HA TCR^{tg} CD8^{+} T cells were stimulated with 5 μg/mL antigen specific peptide. After 24 h of stimulation, activated cells were transferred onto virus coated plates either coated with retroviral vectors containing miR181a or ctrl miRNA in the 3' UTR of the reporter gene GFP. 48 h after activation, T cells were placed on freshly coated plates. Six days after stimulation, expression of the reporter gene GFP was analyzed by flow cytometry before cells were transferred into A20-HA tumor bearing mice.

After T cell transfer, tumor growth and the phenotype of T cells in blood samples were monitored. Seven days after transfer, approximately the same numbers of GFP positive cells could be detected in A20-HA tumor bearing mice either treated with Infl.-HA TCR^{tg} CD8^{+} miR181a or CD8^{+} ctrl miRNA T cells (see figure 3.23 A). Further characterization within GFP positive cells showed the same phenotype of these cells regarding their central memory (T$_{cm}$) and effector (T$_{eff}$) potential (see figure 3.23 B). After 17 days, there was a significant higher number of GFP positive cells detectable in A20-HA tumor bearing mice treated with Infl.-HA TCR^{tg} CD8^{+}

ctrl miRNA T cells than treated with Infl.-HA TCR^{tg} CD8^{+} T cells overexpressing miR181a (see figure 3.23 C). Regarding the phenotype within GFP positive cells, there were no differences detectable (see figure 3.23 D).

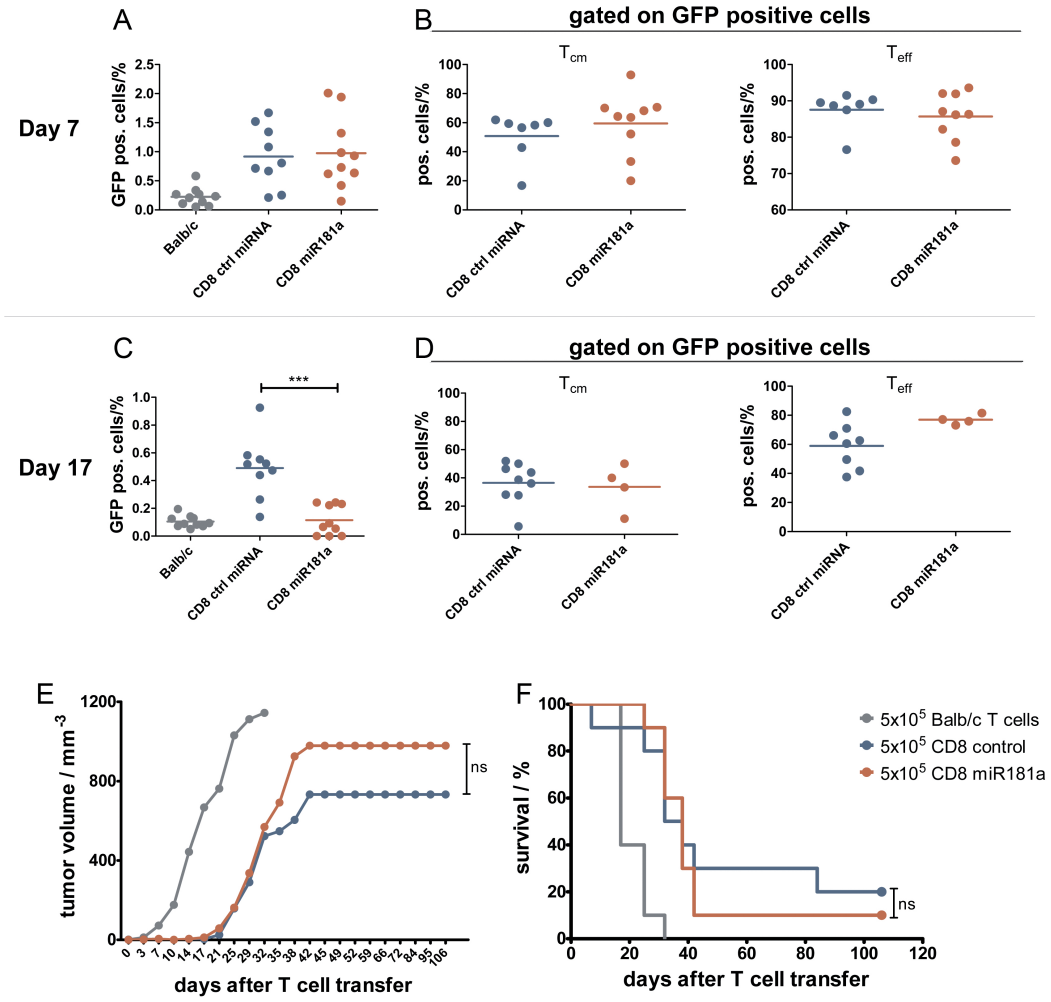

Figure 3.23: miR181a overexpression in murine Infl.-HA TCR^{tg} CD8^{+} T cells does not affect tumor control.
Murine Infl.-HA TCR^{tg} CD8^{+} T cells transduced with miR181a or ctrl miRNA were injected *i.v.* into A20-HA tumor bearing mice. The phenotype of cells in blood samples was analyzed by flow cytometry.
(A) Percentages of GFP positive cells in blood samples on day 7 after transfer.
(B) Percentages of central memory (CD44^{+}/CD62L^{+}/CD127^{+}) (left panel) and effector (CD44^{+}/CD62L^{-}/CD127^{-}) (right panel) T cells within GFP^{+}/Vß8.2^{+} cells in blood samples on day 7 after transfer.
(C) As in (A) on day 17 after transfer.
(D) As in (B) on day 17 after transfer.
(E and F) Tumor growth (E) and survival (F) are depicted (n = 10 mice per group) displayed as mean. Symbols represent individual mice and the line is the mean (A-D), One-way ANOVA (*** p<0.001).

In figure 3.23 E the tumor growth is depicted. The control group treated with Balb/c T cells showed a fast tumor growth detectable 7 days after T cell transfer. For the groups treated with Infl.-HA TCR^{tg} $CD8^+$ T cells either transduced with miR181a or ctrl miRNA, a delayed tumor growth could be detected. There was no prove for advantage of miR181a overexpressing Infl.-HA TCR^{tg} $CD8^+$ T cells. This was also observable in the survival curve (see figure 3.23 F).
To characterize the A20-HA tumor model, tumors were isolated when the criteria of termination were reached and analyzed by Immunohistochemistry (IHC). The results are summarized in figure 3.24. Haematoxylin and Eosin staining (HE staining) enables the analysis of the structure of the isolated tumors. Here, all A20-HA tumors showed capsules, necrotic areas of different size, hemorrhages in the necrotic areas and some muscle cells at the border areas of the tumor. These observations were independently from the treatment of A20-HA tumor bearing mice. Additionally the A20-HA tumor cells were stained with an α-paired box gene 5 (αPAX5) antibody. PAX5 is a B cell lineage specific transcription factor. All tumors showed a distributed strong staining all over the tissue, demonstrating that the tumor consists mainly of the A20-HA B cell lymphoma tumor cells and not of different tumor cells. Despite the fact, that no specific antibody for HA was available, no direct conclusions of target identification and specific recognition by Infl.-HA TCR^{tg} $CD8^+$ T cells could be made. To analyze the infiltration of T cells and also the infiltration of transduced T cells, tumors were stained with αCD3 and αGFP antibody. In figure 3.24 just a low amount of CD3 positive cells in the tumors could be found. These cells were mainly identified in the edge and in the connective tissue and not in the middle of the tumors. Also very low amount of GFP positive cells were found in the treated tumors. In the Balb/c control group no GFP cells were identified, showing that the staining for GFP in ctrl miRNA or miR181a Infl.-HA TCR^{tg} T cells treated mice the GFP staining was specific for transduced T cells. Here, no differences between $CD8^+$ miR181a T cells treated A20-HA tumor bearing mice compared to $CD8^+$ ctrl miRNA T cells could be detected.

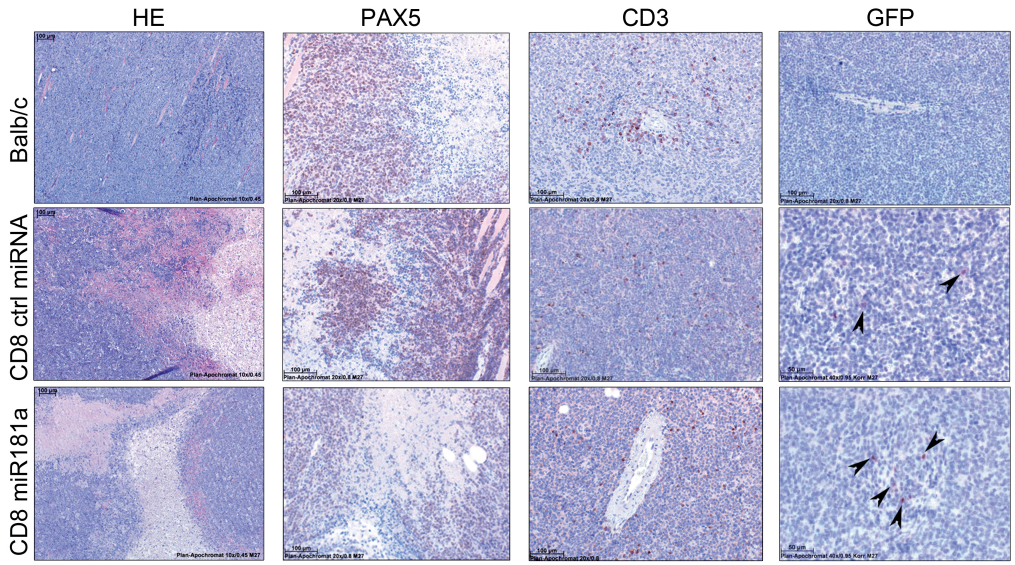

Figure 3.24: Immunohistochemistry Analysis of isolated A20-HA tumors after treatment with transduced Infl.-HA TCR^{tg} CD8^{+} T cells.
Murine Infl.-HA TCR^{tg} CD8^{+} T cells transduced with miR181a or ctrl miRNA were injected *i.v.* into A20-HA tumor bearing mice. After reaching the criteria of termination, mice were scarified and tumors were isolated. For analysis, paraffinated tumor samples were sectioned (3 μm) and analyzed by HE staining and immunohistochemistry using αPAX5, αCD3 and αGFP antibodies. The magnification and size of scale bar is indicated for each picture separately. Shown are represented figures out of ten isolated tumors for each group. Arrows indicate positive signals for GFP staining.

In summary, the transfer of miR181a overexpressing Infl.-HA TCR^{tg} CD8^{+} T cells into A20-HA tumor bearing mice has no positive effect on tumor control or the survival of tumor bearing mice.

3.3.3 Co-transfer of miR181a overexpressing Infl.-HA TCR^{tg} CD4^{+} and CD8^{+} T cells leads to improved tumor control and prolonged survival.

To study the effect of miR181a on anti-tumoral activity of murine Infl.-HA TCR^{tg} CD4^{+} T cells, miR181a or ctrl miRNA transduced T cells were injected i.v. into A20-HA tumor bearing mice. Before transfer of cells, the transduction efficiency was analyzed using flow cytometry (see figure 3.25).

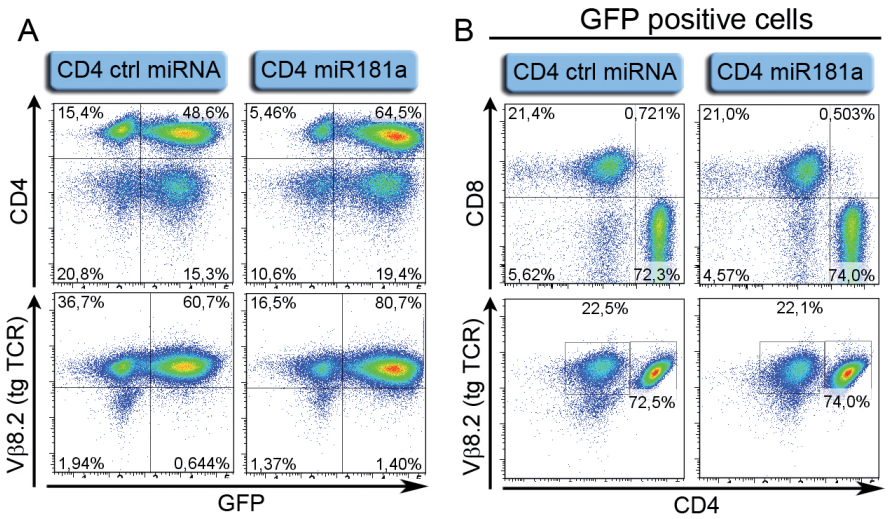

Figure 3.25: Phenotype of Infl.-HA TCR^{tg} CD4^{+} T cells on the day of transfer into A20-HA tumor bearing mice.
Murine Infl.-HA TCR^{tg} T cells were isolated from mice transgenic for an influenza virus hemagglutinin (HA) peptide specific T cell receptor in the context of MHC class II molecule. Isolated Infl.-HA TCR^{tg} CD4^{+} T cells were stimulated with 5 μg/mL antigen specific peptide. After 24 h of stimulation, activated cells were transferred onto virus coated plates either coated with retroviral vectors containing miR181a or ctrl miRNA in the 3′ UTR of the reporter gene GFP. 48 h after activation, T cells were placed on freshly coated plates. Six days after stimulation, the cells were analyzed by flow cytometry before adoptive transfer into A20-HA bearing mice.
(A) Infl.-HA TCR^{tg} T cells transduced with miR181a or ctrl miRNA were stained with αCD4 (upper panel) and αVß8.2 (lower panel) antibodies and GFP expression was analyzed on the day of transfer.
(B) Analysis of CD4 and CD8 (upper panel) and CD4 and Vß8.2 (lower panel) T cells within GFP positive cells on the day of transfer.

In figure 3.25 A the expression of the reporter gene GFP was analyzed in comparison to the expression of CD4 (upper panels) and the transgenic TCR (lower panels) of murine Infl.-HA TCR^{tg} T cells either transduced with ctrl miRNA (left panels) or miR181a (right panels). For murine Infl.-HA TCR^{tg} ctrl miRNA T cells approximately 76 % of CD4^{+} T cells express the reporter gene GFP. Interestingly nearly 43 % of CD4^{-} cells also express GFP. Similar results could be observed for murine Infl.-HA TCR^{tg} miR181a T cells. Here, approximately 92 % of CD4^{+} T cells express the reporter gene GFP and roughly 65 % of CD4^{-} cells also express GFP. In addition, all cells express Vβ8.2 as a marker for the transgenic TCR and are therefore able to recognize the tumor cells. Looking closer at the phenotype within GFP positive cells (see figure 3.25 B), about 21 % of the cells are CD8^{+} in both Infl.-HA TCR^{tg} T cells, transduced with ctrl miRNA (see figure 3.25 B, upper left panel) or miR181a (see figure 3.25 B, upper right panel). Again the expression of Vβ8.2 was verified within GFP positive cells for Infl.-HA TCR^{tg} ctrl

miRNA T cells (see figure 3.25 B, lower left panel) and miR181a overexpressing Infl.-HA TCR^{tg} T cells (see figure 3.25 B, lower right panel). These results show, that there was a co-transfer of Infl.-HA TCR^{tg} CD4^{+} T cells with roughly 20 % Infl.-HA TCR^{tg} CD8^{+} T cells. Two different cell numbers (1*10^{4} and 5*10^{4} cells per mice) were injected into the retro-orbital vein of A20-HA tumor bearing mice. Again, splenocytes from Balb/c wildtype mice (5*10^{4} cells per mouse) were injected into A20-HA tumor bearing mice as a control.

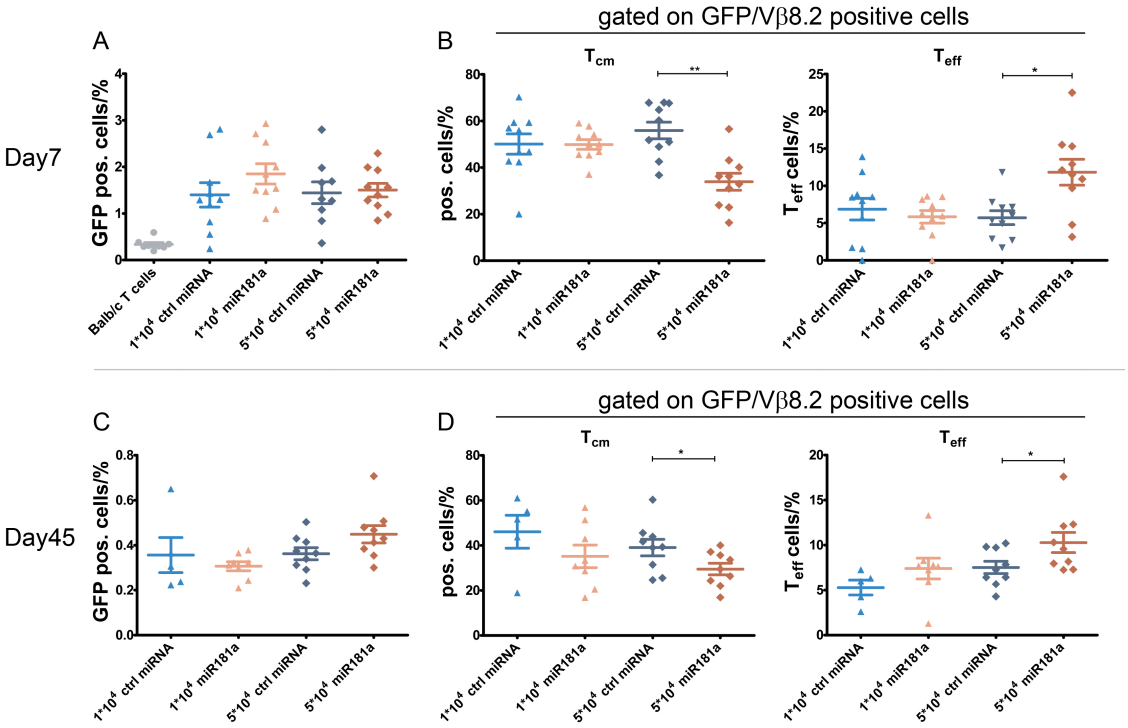

Figure 3.26: miR181a permits the expansion of effector T cells *in vivo*.
Murine Infl.-HA TCR^{tg} T cells transduced with miR181a or ctrl miRNA were injected *i.v.* into A20-HA bearing mice. The phenotype of cells in blood samples was analyzed by flow cytometry .
(A) Percentages of GFP positive cells in blood samples on day 7 after transfer.
(B) Percentages of central memory (CD44^{+}/CD62L^{+}/CD127^{+}) (left panel) and effector (CD44^{+}/CD62L^{-}/CD127^{-}) (right panel) T cells within GFP^{+}/Vß8.2^{+} cells in blood samples on day 7 after transfer.
(C) As in (A) on day 45 after transfer.
(D) As in (B) on day 45 after transfer. Symbols represent individual mice and the line is the mean ±SEM (A-D), One-way ANOVA (* p<0.05, ** p<0.01).

After T cell transfer, tumor growth and the phenotype of T cells in blood samples were monitored. Seven days after transfer, approximately the same numbers of GFP positive cells could be detected in A20-HA tumor bearing mice either treated with Infl.-HA TCR^{tg} T cells transduced with miR181a or ctrl miRNA (see figure 3.26 A). Further characterization within GFP

positive cells showed the same cell phenotype with regard to their central memory (T_{cm}) and effector (T_{eff}) potential (see figure 3.26 B) when transferring $1*10^4$ Infl.-HA TCR^{tg} T cells. The transfer of higher numbers of cells ($5*10^4$ cells per mice) overexpressing miR181a leads to changes in phenotype. Here, a significantly lower number of cells showed a central memory phenotype when overexpressing miR181a, which results in a significantly higher number of effector T cells. The transfer of higher numbers of cells ($5*10^4$ cells per mice) transduced with ctrl miRNA does not change the phenotype. These results could also be observed on day 45 after transfer (see figure 3.26 C and 3.26 D).

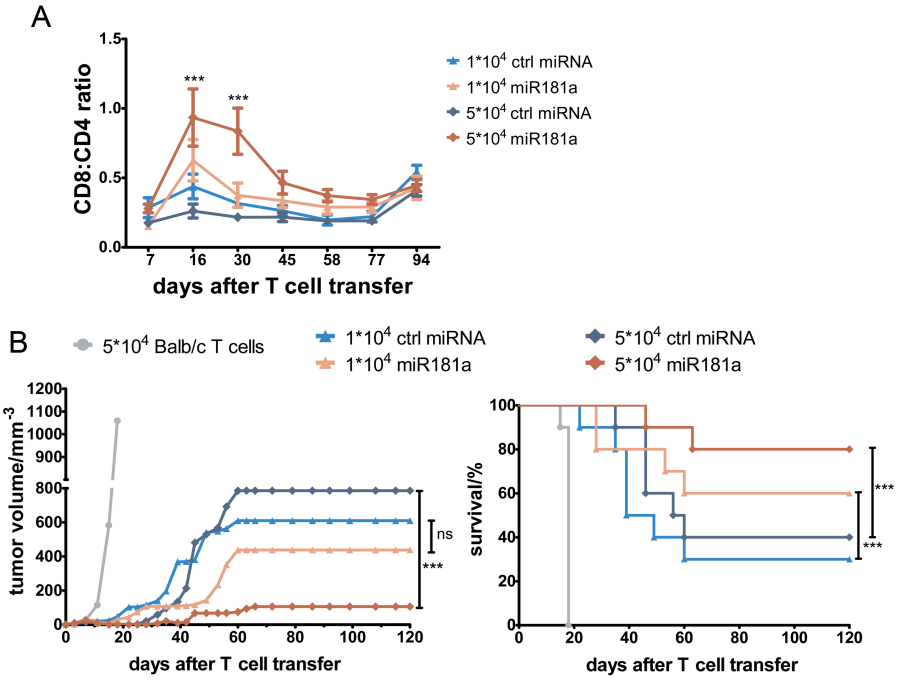

Figure 3.27: Expansion of Infl.-HA TCR^{tg} $CD8^+$ T cells by miR181a overexpression improves tumor control.
Murine Infl.-HA TCR^{tg} T cells transduced with miR181a or ctrl miRNA were injected *i.v.* into A20-HA bearing mice.
(A) The ratio of CD8:CD4 T cells within $GFP^+/V\beta8.2^+$ cells was analyzed in blood samples at indicated time points after T cell transfer using flow cytometry displayed as mean ±SEM.
(B) Tumor growth (left panel) and survival (right panel) are depicted (n = 10 mice per group) displayed as mean values. Two-way ANOVA (ns – not significant; * indicates significant differences; *** $p<0.001$).

In figure 3.27 A, the ratio of CD8:CD4 T cells within $GFP^+/V\beta8.2^+$ cells analyzed after indicated time points is summarized. Here, higher ratios of CD8:CD4 T cells could be detected in blood samples of A20-HA tumor bearing mice when treated with Infl.-HA TCR^{tg} T cells transduced with miR181a compared to ctrl miRNA transduced cells. This could be observed for both

transferred cell numbers. However, only high numbers of transferred cells leads to a significant increase of $CD8^+$ T cells within $GFP^+/V\beta8.2^+$ on day 16 and on day 30 after transfer into A20-HA tumor bearing mice.

In figure 3.27 B the tumor growth is depicted. The control group treated with Balb/c T cells showed a fast tumor growth after T cell transfer. For the groups treated with Infl.-HA TCR^{tg} T cells either transduced with miR181a or ctrl miRNA, a delayed tumor growth could be detected. Here the retardation of tumor growth was stronger after transfer of Infl.-HA TCR^{tg} T cells overexpressing miR181a than after transfer of Infl.-HA TCR^{tg} ctrl miRNA T cells (see figure 3.27 B, left panel). This was observed for both cell numbers transferred. After transfer of $5*10^4$ Infl.-HA miR181a TCR^{tg} T cells, only in two mice a tumor developed. Additionally, the transfer of miR181a overexpressing Infl.-HA TCR^{tg} T cells leads to a significant higher survival rate than transfer of Infl.-HA TCR^{tg} T cells transduced with ctrl-miRNA. After transfer of $1*10^4$ Infl.-HA TCR^{tg} miR181a T cells six out of ten mice survived. Eight out of ten mice survived after transfer of $5*10^4$ Infl.-HA TCR^{tg} miR181a T cells.

Again, tumors were isolated when the criteria of termination were reached and analyzed by IHC (see figure 3.28). As described before, the HE staining showed capsules, necrosis of different sizes, hemorrhages in the necrotic areas and some muscle cells at the border of the tumor. These observations were independently of the treatment of A20-HA tumor bearing mice. PAX5 staining showed a similar distributed strong staining all over the tissue as described before, demonstrating that the tumor consists mainly of the A20-HA tumor cells. The infiltration of T cells and also the infiltration of transduced T cells was analyzed by CD3 and GFP staining. Here, only low amounts of CD3 positive cells could be found in the tumor. These cells were mainly identified at the border and in the connective tissue of the tumors, not in the center of the tumors. Moreover, only low amount of GFP positive cells were found in the treated tumors. As expected, in the Balb/c control group no GFP cells could be identified. Interestingly, tumors treated with miR181a overexpressing Infl.-HA TCR^{tg} showed higher amounts of GFP positive cells then tumors treated with ctrl miRNA T cells. This could be observed for both cell numbers transfered into A20-HA tumor bearing mice (data not shown), showing that more miR181a overexpressing T cells were able to infiltrate into to established tumors.

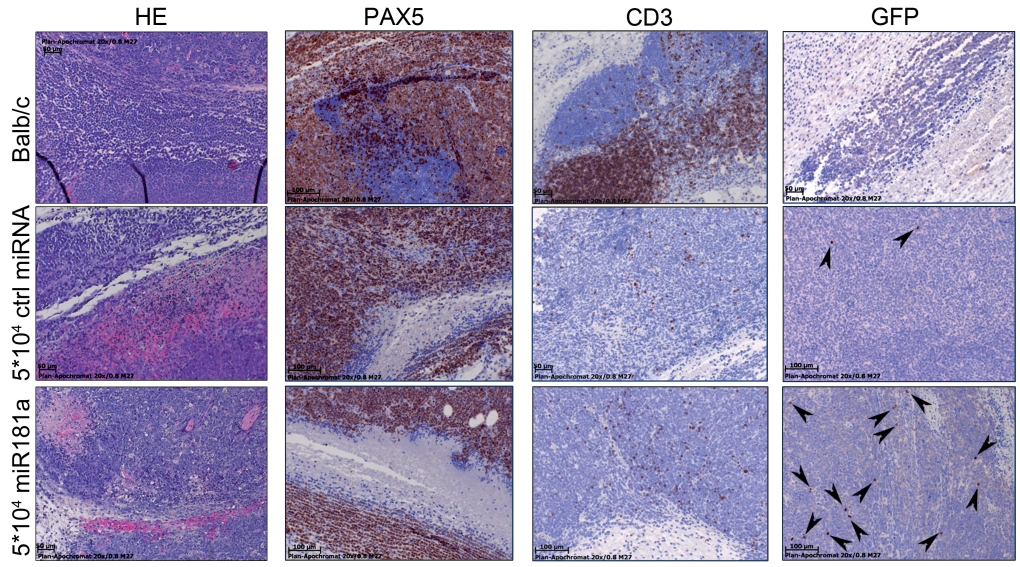

Figure 3.28: Immunohistochemistry Analysis of isolated A20-HA tumors after treatment with transduced Infl.-HA TCR^{tg} CD4^{+} and CD8^{+} T cells.
Murine Infl.-HA TCR^{tg} CD4^{+} T cells transduced with miR181a or ctrl miRNA were injected *i.v.* into A20-HA tumor bearing mice. After reaching the criteria of termination, mice were scarified and tumors were isolated. For analysis, paraffinated tumor samples were sectioned (3 μm) and analyzed by HE staining and immunohistochemistry using αPAX5, αCD3 and αGFP antibodies. The magnification and size of scale bar is indicated for each picture separately. Shown are representative figures of isolated tumors for each group. Arrows indicate positive signals for GFP staining.

In summary, the co-transfer of miR181a overexpressing Infl.-HA TCR^{tg} CD4^{+} and CD8^{+} T cells leads to improved tumor control and prolonged survival of A20-HA tumor bearing mice. This effect is characterized by higher amounts of effector T cells and the expansion of Infl.-HA TCR^{tg} CD8^{+} T cells.

4 Discussion

Immunotherapy of cancer by adoptive transfer of tumor-specific T cells is a promising treatment for selective tumor destruction. However, in patients many T cells against auto-antigens show only weak anti-tumoral response. To overcome this barrier it is necessary to improve the efficacy of these T cells. Activation and activity of T cells are tightly controlled to inhibit unwanted T cell responses and to reduce the risk of autoimmunity. Both are regulated by extrinsic signals and intrinsic mechanisms which suppress T cell activation. The intrinsic mechanisms include the expression of phosphatases that counteract the activation-inducing kinases. Modifying the expression of these phosphatases allows the targeted modulation of T cell reactivity that could be used to enhance the potency of T cells for adoptive cell therapy of cancer. The microRNA miR181a has been shown to be highly expressed in immature T cells that recognize low-affinity antigens [Li et al., 2007]. Therefore, this study analyzed the potency of miR181a overexpression in primary T cells to enhance their potential for adoptive cell therapy.

4.1 Ectopic expression of miR181a in primary human T cells

4.1.1 Lentiviral transduction of primary human T cells with miR181a leads to efficient downregulation of target genes

To my knowledge this is the first study of ectopic expression of miR181a in primary human T cells. The introduction by viral gene transfer was used to enable stable expression of miR181a over the life span of the cells. After primary activation of MACS-sorted $CD4^+$ and $CD8^+$ human T cells, cells were transduced with lentiviral vectors either encoding miR181a or ctrl miRNA. Both miRNAs were cloned into the 3' UTR of GFP reporter gene, enabling the analysis of transduction efficiency by measuring the GFP expression using flow cytometry. The gene expression in the present study was driven by the elongation factor 1 *alpha* (EF1α) promoter. Salmon et al. could show that lentiviral vectors containing EF1α promoter could efficiently transduce approximately 25% of activated human T cells [Salmon et al., 2000]. Additionally, Zhou et al. showed in 2003 that lentiviral transduction of isolated human $CD4^+$ and $CD8^+$ T cells achieves 45% up to 50% of transduced cells when vectors encoding EF1α promoter were used [Zhou et al., 2003]. In the present study the transduction efficiency was between 23% (ctrl miRNA $CD8^+$ T cells) and 65% (miR181a $CD4^+$ T cells) and is thereby consistent with previous pub-

lished data. Interestingly, $CD4^+$ T cells show higher transduction efficiencies than $CD8^+$ T cells. In $CD4^+$ T cells, nearly twice as many $CD8^+$ T cells express GFP as well as transduced with miR181a or ctrl miRNA. This demonstrates that primary human $CD4^+$ T cells are more susceptible for viral gene transfer and further manipulation than primary human $CD8^+$ T cells. This is consistent with previous published observations regarding transduction of human T cells [Jones et al., 2009].

Human T cells transduced with miR181a showed higher numbers of GFP positive T cells than T cells transduced with ctrl miRNA. This was observed for both $CD4^+$ and $CD8^+$ T cells and was not a consequence of different titers, because viral supernatants with nearly identical titers were used for transduction. This could indicate that miR181a influences not only the expression of intrinsic genes, but also can drive the expression of incorporated genes, such as GFP in this setting. Consistent with these findings, Li et al. identified 2006 several dsRNAs that activate gene expression by targeting noncoding regulatory regions in gene promotors [Li et al., 2006]. Additionally, they showed that gene induction by promoter targeting dsRNAs is sequence-specific. One year later, Janowski et al. reported about the induction of progesteron receptor and major vault protein by promotor-targeting dsRNAs [Janowski et al., 2007]. These reports indicate the ability of small dsRNAs to induce gene expression. In 2008, Place et al. showed that miR-373 targets the promotor sequences of E-cadherin and cold-shock domain-containing protein C2 and induces their gene expression [Place et al., 2008]. Their findings indicate a new function by which miRNAs could regulate gene expression. Probably, there could be a better translation of the mRNA conditioned by the secondary structure resulting from the mRNA-miRNA duplex. Thus, coupling of miR181a with antigen specific receptors (TCRs or CARs) could probably increase the expression of these receptors and could therefore enhance the number of antigen specific T cells for adoptive T cell therapy. Analyzing the amount of miR181a using qRT-PCR showed a three-fold overexpression of miR181a in human $CD4^+$ T cells after transduction with miR181a compared to ctrl miRNA transduced $CD4^+$ T cells. In primary human $CD8^+$ T cells, transduction with miR181a leads to a roughly 12-fold overexpression compared to T cells transduced with ctrl miRNA. Murine immature DP thymocytes show approximately six- to ten-fold higher expression of miR181a than mature SP thymocytes [Li et al., 2007]. Thereby, the lentiviral transduction of primary human T cells achieves a miR181a level

compared to those find in the more sensitive immature thymocytes. It was previously shown that miR181a is expressed in murine SP T cells at different levels [Li et al., 2007]. In murine $CD4^+$ T cells nearly twice as much miR181a could be detected compared to miR181a amount in murine $CD8^+$ T cells. The present analysis showed the same relationship in primary human T cells (data not shown). Thereby, the basal level of miR181a in $CD4^+$ T cells is higher than in $CD8^+$ T cells. After viral transduction with lentiviral vectors encoding miR181a, there can be a stronger upregulation of miR181a in $CD8^+$ T cells, because ctrl miRNA transduced cells have a lower amount of natural miR181a compared to $CD4^+$ T cells.

miRNAs and short interfering RNAs (siRNAs) are involved in gene regulation. Both were found to associate with RNA-induced silencing complexes (RISCs) [Hammond et al., 2000] where they recognize their target RNA by Watson-Crick base pairing. There are some distinctions to notice. First, miRNAs were processed from stem-loop precursors (for further explanation see chapter 1.5),whereas siRNAs were found to be excised from long, fully complementary double-stranded RNAs [Tomari and Zamore, 2005]. Second, and also the key difference between miRNAs and siRNAs is in the precision of their ends. In contrast to siRNAs, which have more heterogeneous ends, miRNAs have highly exact ends. This probably allowed them to interact with greater specificity on substrate mRNAs without a need for stringent complementarity or large overlap [Dogini et al., 2014]. One unique aspect of gene regulation by miRNA is its complexity. It could be shown that a single miRNA can regulate the expression of different mRNAs [Yanaihara et al., 2006], [Selbach et al., 2008]. Additionally, one mRNA can be regulated by multiple miRNAs [Yanaihara et al., 2006]. Usually, miRNA-binding sites in animal mRNAs lie in the 3'UTR and are present in multiple copies [Pillai et al., 2004]. It also thought that classic RNA interference by siRNA transfection is relatively short-lived lasting only 5–7 days [Dykxhoorn et al., 2003], whereas miRNAs generates a more stable gene regulation. The overexpression of miR181a in primary human T cells leads to significant downregualtion of activation inhibiting phosphatases. Here, the downregulation of SHP2, DUSP5, DUSP6, and PTPN22 was stronger in $CD8^+$ T cells than in $CD4^+$ T cells. The downregulation of target genes correlates with the amount of miR181a overexpression and is comparable to published data concerning miR181a overexpression in murine T cells [Li et al., 2007].

4.1.2 miR181a enhances cytokine production in primary human T cells

Regarding the effects of ectopic expression of miR181a in primary human T cells on cytokine production, it was apparent that miR181a is able to enhance the reactivity of human T cells in two different ways. For short-term restimulation with autologous iDCs and αCD3, overexpression of miR181a in $CD4^+$ T cells leads to higher numbers of pro-inflammatory cytokine (TNFα and IFNγ) producing cells. These observation indicate that miR181a overexpression could lead to enhanced numbers of T_H1 cells which directs cell-mediated immunity by activation of macrophages and $CD8^+$ T cells after short-term restimulation, which could result in enhanced anti-tumoral immune responses. It is known that TNFα induces hemorrhagic necrosis in a certain set of tumor types. Manusama et al. reported that hemorrhagic necrosis was much greater in tumor bearing rats with TNFα in addition to chemotherapeutic drugs than without TNFα treatment [Manusama et al., 1996b]. Additionally, they could show a synergistic anti-tumor effect by the combination treatment with TNFα and chemotherapeutic drugs [Manusama et al., 1996a]. But TNFα alone induced only a mild central necrosis, and there could no objective tumor response be observed [Manusama et al., 1996a]. Importantly, the cell lines they used were not sensitive to TNFα *in vitro*, which was also be shown by other reports describing a lack of effect of TNFα in cell lines [Ruggiero et al., 1987], [Watanabe et al., 1988]. Further studies in mice and rats showed a systemic low dose of TNFα augments the anti-tumor activity of pegylated liposomal doxorubicin [Hagen et al., 2000], [Brouckaert et al., 2004]. Van Horssen et al. suggest that cells of the tumor stroma may be responsible for the observed anti-tumor effect of TNFα. They propose that the TNF-Receptor-1 was upregulated in tumor vessels and could bind TNFα leading to hyperpermeability and enhanced extravasation of immune cells [van Horssen et al., 2006]. This could explain the massive hemorrhagic necrosis of tumors. Several studies have shown that a low dose of TNFα results in comparable responses, suggesting that a lower dose still may induce anti-vascular effects [Hill and Thomas, 1994], [Bonvalot et al., 2005]. The healthy vessels were not addressed: no apoptosis and no extravasation could be observed. The local administration by isolated limb perfusion of TNFα in combination with the alkylating agent melphalan could be shown to be an effective treatment for metastatic melanoma and unresectable soft tissue sarcomas [Grunhagen et al., 2006], [Grunhagen et al.,

2009], [Deroose et al., 2011]. Additionally, TNFα has also been reported to promote regression of unresectable metastases from colorectal cancer when delivered in combination with melphalan [Alexander et al., 2009]. The increased amounts of TNFα producing CD4$^+$ miR181a T cells could thereby improve cancer treatment and tumor destruction. But TNFα can also promote differentiation of tumour-associated myeloid cells into cells that express endothelial cell markers, and could thereby promote angiogenesis and tumour growth [Li et al., 2009], [Li et al., 2011]. Similar to TNFα, IFNγ also has two faces: it can have cytostatic/cytotoxic antitumor functions [Brown et al., 1987] as well as cytoproliferative effects depending on the context. The first demonstration of anti-proliferative effects of IFNγ in melanoma cells was reported by Fisher et al. [Fisher et al., 1986]. Weber and Rosenberg showed that IFNγ induces upregulation of MHC class I genes, which increase tumor immunogenicity [Weber and Rosenberg, 1988]. These enables the recognition and elimination of tumor cells by cytotoxic T lymphocytes, which were recruited to the tumor site via IFNγ-induced chemokines [Kunz et al., 1999], [Mukai et al., 1999]. Kortylewski et al. reported that IFNγ had significant growth inhibitory activity on four different human melanoma cell lines [Kortylewski et al., 2004]. Therefore, the increased numbers of IFNγ producing CD4$^+$ T cells overexpressing miR181a could enhance the recognition and elimination of tumor cells by the recruitment cytotoxic T lymphocytes and could inhibit the growth of tumor cells. In contrast, Taniguchi et al. showed that IFNγ was a potent enhancer of lung colonization of *i.v.* inoculated B16 melanoma cells [Taniguchi et al., 1987]. Additionally, intra-tumoral expression of IFNγ was shown to be associated with a more aggressive phenotype in human melanomas [Brocker et al., 1988]. In contrast, IFNγ and TNFα were also shown to be necessary for tumor eradication as therapeutic effectiveness of adoptively transferred TILs [Barth et al., 1991]. Antibodies against IFNγ and TNFα abrogated the ability of TIL cultures to mediate tumor regression [Barth et al., 1991]. Additionally, anti-IFNγ antibodies were shown to inhibit the therapeutic effect of adoptively transferred T cells [Tuttle et al., 1993]. These observations indicate that TNFα and IFNγ producing CD4$^+$ miR181a T cells could improve tumor control. There are additional experiments needed to point out the role of TNFα and IFNγ producing CD4$^+$ miR181a T cells in tumor progression.

After long-term restimulation higher production of the pro-inflammatory cytokine IL-17 could be observed. IL-17 is highly secreted by T$_H$17 cells [Park et al., 2005]. T$_H$17 cells are a kind of

effector T helper cells which regulates tissue inflammatory reactions [Park et al., 2005]. Kryczek et al. find high proportions of T_H17 cells in tumors of patients with ovarian cancer, whereas the frequency of T_H17 cells in tumor-draining lymph nodes and peripheral blood is similar to that found in healthy donors [Kryczek et al., 2007], [Kryczek et al., 2009a]. Their findings indicate that T_H17 cells may be induced by and/or recruited to the tumor microenvironment. Furthermore, it could be shown in many tumor models that in IL-17-deficient mice tumor growth and lung metastasis is accelerated compared to IL-17 competent mice [Hirahara et al., 2001] [Martin-Orozco et al., 2009], [Kryczek et al., 2009b]. Moreover, the forced expression of IL-17 in tumor cells could suppress tumor progression [Hirahara et al., 2001] [Martin-Orozco et al., 2009], [Kryczek et al., 2009b]. In patients with prostate cancer, an inverse correlation between T_H17 cell differentiation and tumor progression could be shown [Sfanos et al., 2008]. Von Euw et al. showed that the blockage of CTLA4 induces T_H17 cells in patients with melanoma and that the levels of IL-17 detected in tumor-associated water-retention positively predicts patient survival [Euw et al., 2009]. Correspondingly, IL-17 was positively associated with tumor-infiltrating IFNγ^+ effector T cells [Kryczek et al., 2009a]. Martin-Orozco et al. showed that T_H17 cell-mediated anti-tumor activity was also linked to dendritic cell recruitment into the tumor microenvironment or into tumor-draining lymph nodes [Martin-Orozco et al., 2009]. These data suggest that T_H17 cells can have protective roles in tumour immunity. The presented results give evidence that the overexpression of miR181a in $CD4^+$ T cells could postitively affect T_H17 differentiation after long-term stimulation. Additionally, miR181a overexpression in $CD4^+$ T cells enables prolonged and higher secretion of IL-10 after long-term restimulation. IL-10 is expressed by T_H2 cells and T_H9 [Dardalhon et al., 2008]. There are several studies indicating that T_H9 cells are derivatives of T_H2 cells as a consequence of induction of additional transcription factors such as PU.1 (purine-rich box 1) and IRF4 (interferon regulatory factor 4) [Chang et al., 2009], [Chang et al., 2010], [Staudt et al., 2010]. Two research groups could independently show that T_H9 cells exhibit remarkable therapeutic efficacy in cancer models [Lu et al., 2012], [Purwar et al., 2012]. In a highly aggressive B16 melanoma model, they could demomstrate that induction of T_H9 cells is associated with potent anti-cancer effects and favorable outcomes of cancer-bearing mice [Lu et al., 2012], [Purwar et al., 2012]. Therefore, the enhanced expression of IL-10 indicates that miR181a overexpressing $CD4^+$ T cells shows a more

T_H2/T_H9 character, which could enhance anti-cancer effects as could be exhibit by prior studies. Consequently, miR181a overexpression changes the phenotype of $CD4^+$ T cells depending on the length of activation which could lead to more potent immune responses. In future experiments it would be important to analyze the expression of IL-9 to further characterize miR181a overexpressing $CD4^+$ T cells.

For $CD8^+$ T cells miR181a overexpression has no effect on the number of reacting cells after short-term restimulation. For longer and even more stable interactions, $CD8^+$ T cells overexpressing miR181a induce prolonged and higher production of IFNγ. Interestingly, also the production of the cytokines IL-4 and IL-10 was increased in $CD8^+$ T cells overexpressing miR181a. In 2000, Vukmanovic-Stejic and colleagues described three different $CD8^+$ T cell subsets, according to their cytokine production: Tc1 cells produces IFNγ no IL-4, Tc0 cells producing IL-4 in addition to IFNγ, and Tc2 cells producing IL-4 but no IFNγ [Vukmanovic-Stejic et al., 2000]. The production of other cytokines such as IL-2, granulocyte macrophage colony-stimulating factor, IL-6, IL-10, and TNFα was not limited to a particular subset and was common to the three classes. Regarding this classification, miR181a overexpression in $CD8^+$ T cells leads to higher amounts of Tc0 cells. Vukmanovic-Stejic et al. could demonstrate that Tc1 and Tc2/0 cells showed comparable cytotoxicity and produced similar levels of perforin and Fas L [Vukmanovic-Stejic et al., 2000]. Delfs et al. additionally demonstrates, that IL-4 producing $CD8^+$ T cells promote the recruitment of secondary effectors like eosinophils [Delfs et al., 2001]. Berg et al. could show a tumor suppressive function for IL-10 in a mouse model for colorectal cancer [Berg et al., 1996]. Another study also showed that IL-10 overexpressing animals appear to be resistant to tumor induction compared to $IL10^{-/-}$ mice [Mumm et al., 2011]. Further studies using a pegylated form of IL-10 showed increased $CD8^+$ cytotoxic T cells in tumor tissues and enhanced INFγ production [Mumm et al., 2012], which emphasize the tumor suppressive function of IL-10. In 2013, Visekruna et al. reported IL-9 producing $CD8^+$ T cells which were characterized by specific IFNγ and IL-10 expression patterns and called them Tc9 cells [Visekruna et al., 2013]. They showed that IL-9 producing Tc9 cells display diminished cytotoxicity. According to this findings, Lu et al. recently showed that Tc9 cells were less cytolytic *in vitro* but surprisingly exhibit greater antitumor responses against advanced tumors in OT-I/B16-OVA and Pmel-1/B16 melanoma models [Lu et al., 2014]. They

could demonstrate that after adoptive transfer, Tc9 cells persist longer and differentiated into IFNγ and granzyme-B (GrzB)-producing cytolytic Tc1-like effector cells [Lu et al., 2014]. In the present study, miR181a overexpresion in primary human CD8$^+$ T cells increases the amount of IFNγ, IL-4 and IL-10 after long-term restimulation and thereby changes their phenotype into more potent subsets which could enhance their cytotoxic effect.

4.1.3 αCD28 independent activation of miR181a overexpressing primary human T cells

As previously mentioned, tumors present different barriers for effective anti-tumor immunity, e.g. most tumor cells do not express any ligands for co-stimulatory molecules. The most important co-stimulatory molecule is CD28 which mediates T cell activation. To analyze miR181a function for T cell activation, primary human T cells overexpressing miR181a in the absence of co-stimulatory molecules were tested. In CD8$^+$ T cells there were no differences detectable between restimulation with and without the co-stimulatory molecule αCD28 after short-term restimulation. There were also no distinctions detectable between CD8$^+$ T cells either transduced with miR181a or ctrl miRNA. Fluidigm technology enables the simultaneous analysis of gene expression on mRNA level of different genes by using a high-throughput, microfluidic chip-based, multi-analyte system. Fluidigm analysis after long-term restimulation conditions showed that miR181a CD8$^+$ T cells express higher amounts of pro-inflammatory cytokines (IL-17) and cytotoxicity associated molecules (PRF1, B3GAT) indicating that miR181a CD8$^+$ T cells exhibit stronger cytotoxcicity potential than ctrl miRNA CD8$^+$ T cells.

For CD4$^+$ T cells, a CD28-independent activation of human T cells overexpressing miR181a could be shown. This effect was very strong in primary human CD4$^+$ T cells after short-term restimulation. Here, miR181a overexpressing T cells showed the same amounts of reacting cells in the abscence of αCD28 as ctrl miRNA transduced cells showed in combination with the co-stimulatory molecule. The numbers of reacting cells increased further by addition of αCD28. Nearly twice as many as ctrl miRNA transduced cells showed IFNγ and TNFα production. Analyzing transduced cells by Fluidigm-Technology after long-term restimulation conditions showed that in miR181a overexpressing CD4$^+$ T cells many transcription factors (T-bet, GATA3, FOXP3, EOMES) involved in T cell differentiation were upregulated. Similar results regarding the transcriptional regulators CDX2 and GATA6 regulated by miR181 were shown by Ji et al.

[Ji et al., 2009]. T-bet was identified as "master" transcription factor that regulates T_H1 cell differentiation [Szabo et al., 2000], whereas GATA3 drives T_H2 cell differentiation [Zheng and Flavell, 1997]. Szabo et al. showed that T-bet expression correlates with IFNγ expression in T_H1 and NK cells [Szabo et al., 2000]. In 2003, Hori et al. reported that FOXP3 is specifically expressed in naturally arising $CD4^+$ regulatory T cells and that the transfer of *Foxp3* converts naïve T cells toward a regulatory T cell phenotype similar to that of naturally occurring $CD4^+$ regulatory T cells [Hori et al., 2003]. They conclude from these results that *Foxp3* is the key regulatory gene for the development of regulatory T cells. Importantly, EOMES which is expressed in only very low levels in $CD4^+$ T cells [Pearce et al., 2003] was also upregulated in $CD4^+$ miR181a T cells. EOMES mainly regulates IFNγ production in NK cells and $CD8^+$ T cells. Previous studies showed that EOMES plays a direct role in IFNγ production and T_H1 development in $CD4^+$ T cells [Suto et al., 2006], [Yang et al., 2008]. Endo et al. showed in 2011 that EOMES is highly expressed in memory T_H2 cells [Endo et al., 2011]. They reported that EOMES interact with GATA3 and prevent its binding to the *Il-5* promotor. Thereby upregulation of EOMES downregulates IL-5 expression in memory T_H2 cells. Additionally, they could show that EOMES could be detected also in memory CD4+ T cells induced by the immunization of antigen *in vivo* and that the expression of EOMES was downregulated in memory T_H2 cells after secondary challenge [Endo et al., 2011]. Therefore, the upregulation of EOMES in $CD4^+$ miR181a T cells could explain the higher IFNγ production. Additionally, miR181a overexpressing $CD4^+$ T cells could exhibit better memory T_H2 cell character by the higher expression of EOMES. The results in the present study were generated by a pool of transduced cells and indicates that miR181a can target transcriptional factors of differentiation and could thereby influence the type of reacting $CD4^+$ T cells. Single cell analysis are required to specifically point out the exact coherences.

Additionally, the expression of interleukin receptors (IL-2R, IL-7R), receptors involved in the homing process of T cells (CD62L, CD44) and activation induced molecules was increased in miR181a $CD4^+$ T cells even without co-stimulatory molecules. These results give evidence that miR181a is not only able to enhance the sensitivity of T cells as has been shown by Li et al. [Li et al., 2007] but can also direct the homing process of T cells. Henao-Meja et al. recently showed that miR181 target *SELL* encoding for CD62L [Henao-Mejia et al., 2013] and could

therefore influence the surface expression of CD62L in T cells. Importantly, the expression of PDCD1 was also increased in miR181a $CD4^+$ T cells compared to ctrl miRNA $CD4^+$ T cells. This upregulated expression could be a consequence of the higher concentration of cytotoxic effector molecules released from these cells. The surface expression of all of these molecules was not addressed in this study and have to be validated in further experiments.

The present results can not distinguish between different T helper subsets but clearly point out that miR181a overexpression enhances the activation of $CD4^+$ T cells even without αCD28. Further experiments are needed to specifically characterize the function of miR181a in the different subsets of $CD4^+$ T cells.

4.1.4 miR181a preserve T helper cells from apoptosis and renew T cells for adoptive cell therapy

For adoptive cell therapy, T cells are isolated from the patient and enriched *ex vivo* by stimulation of these cells. During this procedure, the cells can undergo activation-induced cell death (AICD), in which activation through the T cell receptor results in apoptosis. This phenomenon was first described in 1991 when Kawabe et al. reported that T cells responding to an antigenic stimulus first expand in number and then decrease [Kawabe and Ochi, 1991]. The decrease they observed was via apoptotic death. *In vitro*, AICD can be induced by stimulating T cells to expand by receptor ligation and the addition of IL-2 and then activating the T cells again [Lenardo, 1991], [Lenardo et al., 1999]. To study if miR181a overexpression could prevent human T cells from AICD the viability of transduced cells was analyzed. The present results showed that miR181a overexpressing $CD4^+$ T cells are less apoptotic after 144 hours of restimulation with APCs and different amount of αCD3 than $CD4^+$ T cells transduced with ctrl miRNA. Additionally, miR181a overexpressing $CD4^+$ T cells showed fewer proliferation than ctrl miRNA transduced $CD4^+$ T cells. For $CD8^+$ T cells there was no difference detectable between miR181a overexpressing cells or ctrl miRNA transduced cells either regarding on proliferation or viability of these cells. This demonstrates that miR181a enables $CD4^+$ T cells to live longer by lower proliferation rate. The mechanism of reduced induction of apoptosis has to be analyzed in more detail by future experiments, e.g. the expression of anti-apoptotic molecules (Bcl-2, Bcl-xl).

A second challenge of *ex vivo* T cell enrichment by stimulation is a process known as replicative senescence. This occurs when cells reach an irreversible stage of cell cycle arrest following multiple rounds of replication [Effros, 2007]. A critical change as T cells progress to senescence is the loss of gene and surface expression of CD28. For *in vitro* T cell cultures Effros et al. observed approximately 95% CD28-negative T cells within the $CD8^+$ T cell subset [Effros et al., 1994]. Additionally, they could show that older persons have higher proportions of CD28-negative, late-differentiated cells within the $CD8^+$ T cell subset (>60%) compared to <10% in younger adults [Effros et al., 1994]. Another study showed an increased frequency of CD28-negative $CD4^+$ T cells in older persons [Koetz et al., 2000]. Additionally, Li et al. reported that naïve $CD4^+$ T cells from elderly individuals have an increased protein expression of DUSP6 due to a decline in repression by miR181a [Li et al., 2012]. This leads to a reduced signaling capacity of the ERK pathway. Here, transduction of T cells from elderly patients with miR181a could regenerate them to enable full T cell activation by impairing the initial ERK signal and also submit CD28-independent activation of these cells. The renewing effect of miR181a on T cells would be also beneficial for *in vitro* expansion of antigen specific T cells for adoptive cell therapy.

4.2 Ectopic expression of miR181a in murine Infl.-HA TCR^{tg} T cells

4.2.1 Retroviral transduction of murine Infl.-HA TCR^{tg} T cells with miR181a leads to efficient downregulation of target genes

To study the effects of miR181a after antigen specific activation of T cells, murine Infl.-HA TCR^{tg} T cells were used. Similar to human T cells, murine Infl.-HA TCR^{tg} $CD4^+$ T cells showed higher transduction efficiencies either transduced with miR181a or ctrl miRNA than murine Infl.-HA TCR^{tg} $CD8^+$ T cells. For both cell types, transduction with retroviral vectors encoding for miR181a leads to higher numbers of GFP positive T cells than using retroviral vectors encoding ctrl miRNA. This was also observed for human T cells, showing that both murine and human T cells have similar properties for genetic manipulation using viral gene therapy.

Similar to human T cells, the amount of miR181a was more increased in murine Infl.-HA TCR^{tg} CD8^{+} T cells than in murine Infl.-HA TCR^{tg} CD4^{+} T cells after retroviral transduction with miR181a encoding vectors compared to ctrl miRNA transduced cells. Here, miR181a was overexpressed nearly two-fold in murine Infl.-HA TCR^{tg} CD4^{+} T cells and roughly six-fold in murine Infl.-HA TCR^{tg} CD8^{+} T cells. Consistent with these findings, Li et al. achieved a three- to five-fold upregulation of miR181a amount after retroviral transduction of T cells from 5C.C7 TCR transgenic mice [Li et al., 2007]. As previously mentioned, miR181a is six- to ten-fold higher present in murine immature DP thymocytes than in mature SP thymocytes [Li et al., 2007]. Accordingly, the retroviral transduction of mature murine Infl.-HA TCR^{tg} CD8^{+} T cells leads to similar miR181a amounts as could be found in more sensitive immature DP thymocytes. Li et al. showed in 2007 that murine SP CD4^{+} T cells express nearly twice as much miR181a than murine SP CD8^{+} T cells. The upregulation of miR181a in murine Infl.-HA TCR^{tg} CD8^{+} T cells after retroviral transduction is stronger than in Infl.-HA TCR^{tg} CD4^{+} T cells, because crtl miRNA transduced cells express a lower basal level of miR181a. Again, the higher amount of miR181a leads to concentration depended downregulation of the activation inhibiting phosphatases DUSP5, DUSP6, PTPN22 and SHP2. These results are consistent with published data [Li et al., 2007].

4.2.2 miR181a overexpression in murine Infl.-HA TCR^{tg} T cells changes the expression of many genes

Murine Infl.-HA TCR^{tg} T cells can be specifically activated using antigenic peptides resulting in a more physiologic activation compared to human T cells with unspecific activation using αCD3. Here, miR181a overexpression in Infl.-HA TCR^{tg} CD4^{+} T cells leads to higher numbers of INFγ and CD40L producing cells after short-term restimulation. In contrast, the numbers of INFγ producing cells were unaffected in Infl.-HA TCR^{tg} CD8^{+} T cells overexpressing miR181a. Regarding CD137^{+}/IFNγ^{+} double positive cells, miR181a overexpression is able to enhance the number of reacting Infl.-HA TCR^{tg} CD8^{+} T cells. Accordingly, miR181a overexpression in murine Infl.-HA TCR^{tg} T cells could increase their reactivity, this is consistent with previously published data [Li et al., 2012], [Ebert et al., 2009].

Not only cytokines and activation induced molecules are changing under miR181a influence. Analyzing the gene expression in miR181a overexpressing cells using fluidigm technology shows that miR181a overexpression in murine Infl.-HA TCR^{tg} T cells results in various changes in gene expression. The cells were analyzed without restimulation with antigenic peptides, representing the status quo of the T cells before specific activation by target cells. The analyzed genes include genes for interleukins, interleukin receptors, cytokines, cytokine receptors, molecules involved in T cell differentiation, activation, and regulation, and cytotoxic effector molecules. Transcription factors participating in T cell differentiation (TBX21, FOXO1, GATA3, EOMES) and surface molecules important for regulation of T cell activation (CTLA4, ICOS, CD28) are overexpressed in both Infl.-HA TCR^{tg} miR181a CD4^{+} and CD8^{+} T cells. These changes demonstrate that miR181a could change the expression transcriptional factors in murine cells in a similar way as has been previously described for human T cells and thereby also influence the differentiation of murine T cells. Importantly, the overexpression of miR181a leads to upregulation of many chemokines, chemokine receptors, interleukins, and interleukin receptors in both Infl.-HA TCR^{tg} CD4^{+} and CD8^{+} T cells, demonstrating that these cells could have a higher activation potential. Similar to human T cells, IL-10 was upregulated in both Infl.-HA TCR^{tg} miR181a CD4^{+} and CD8^{+} T cells. A tumor suppressive function of IL-10 was previuosly shown [Berg et al., 1996], [Lin and Karin, 2007], [Mumm et al., 2011] and [Mumm et al., 2012]. Instrestingly, also IL-12 was upregulated in murine Infl.-HA TCR^{tg} miR181a CD8^{+} T cells. It could be shown that T cells which express IL-12 can have enhanced anti-tumor function [Kerkar et al., 2010] and are able to resist immunosupression by regulatory T cells [Pegram et al., 2012]. Hence, miR181a overexpressing murine Infl.-HA TCR^{tg} T cells showed increased expression of IL-10 and IL-12, they could have an improved tumor suppressive function and furthermore could be resistant to immunosuppression by T$_{reg}$ cells. Additionally, CCR2 was upregulated in both Infl.-HA TCR^{tg} CD4^{+} and CD8^{+} T cells overexpressing T cells. Craddock et al. could show that the coexpression of the chemokine receptor CCR2b together with tumor specific CARs increased migration of T cells to tumors leading to significantly increased anti-tumor activity [Craddock et al., 2010]. Additionally, Moon et al. reported that a single intravenous injection of 20 million mesoCAR + CCR2b T cells into immunodeficient mice bearing established tumors resulted in a 12.5-fold increase in T cell tumor infiltration [Moon

et al., 2011]. These observations indicate that CAR T cells bearing a functional chemokine receptor can overcome the inadequate tumor localization and can improve anti-tumor efficacy *in vivo*. The upregulation of CCR2 in miR181a overexpressing Infl.-HA TCR^{tg} CD4$^+$ and CD8$^+$ T cells could improve migration of T cells to tumors and thereby enhance anti-tumor efficacy. Surprisingly, IFNγ is downregulated in Infl.-HA TCR^{tg} miR181a CD4$^+$ T cells and upregulated in Infl.-HA TCR^{tg} miR181a CD8$^+$ T cells. These results are contrary to the results from flow cytometry analysis of murine Infl.-HA TCR^{tg} miR181a T cells after restimulation with antigenic peptides. These contrasts could be due to the different experimental designs and further Fluidigm analysis after activation of miR181a overexpressing murine Infl.-HA TCR^{tg} T cells is needed. Remarkably, the changes in expression pattern are stronger in Infl.-HA TCR^{tg} CD8$^+$ T cells, especially the upregulation of molecules involved in T cell activation and regulation.

4.2.3 miR181a enhances the cytolytic activity of murine Infl.-HA TCR^{tg} cytotoxic CD8$^+$ T cells and prevents apoptosis in murine Infl.-HA TCR^{tg} T cells

As previously mentioned, IFNγ is upregulated in Infl.-HA TCR^{tg} miR181a CD8$^+$ T cells. IFNγ is one of the most important cytokine regarding T cell cytotoxicity. The upregulation of IFNγ in Infl.-HA TCR^{tg} miR181a CD8$^+$ T cells could increase their cytotoxicity. Therefore, the cytotoxic effect of Infl.-HA TCR^{tg} miR181a CD8$^+$ T cells was analyzed in a target specific manner. Here, miR181a is able to enhance the cytotoxic activity of Infl.-HA TCR^{tg} miR181a CD8$^+$ T cells even in very low effector to target ratios. Kezuka et al. reported that OT-I TCR^{tg} T cells *in vitro* activated with OVA-pulsed APCs maintain their cytotoxic capability [Kezuka and Streilein, 2000]. Here the lysis of target cells was between 30 % and 35 % for a E:T ratio of 2:1 [Kezuka and Streilein, 2000], which is consistent with the lysis of Infl.-HA TCR^{tg} ctrl miRNA CD8$^+$ T cells for a E:T ratio of 3:1 from the present study. Whereas miR181a overexpressing Infl.-HA TCR^{tg} CD8$^+$ T cells leads to lysis of approximately 40 % of target cells at the same E:T ratio. Additionally, in all E:T ratios tested approximately 20 % higher cytolytic activity could be observed using Infl.-HA TCR^{tg} miR181a CD8$^+$ T cells compared to ctrl miRNA transduced T cells. These results were generated with only roughly 25 % of miR181a overexpressing CD8$^+$ T cells in the used cell mixture. The real effect of miR181a overexpression in Infl.-HA TCR^{tg} CD8$^+$ T cells on their cytolytic activity might be stronger. Hence, miR181a overexpression in murine Infl.-HA

TCR^{tg} CD8$^+$ T cells enhance their cytotoxic activity. Interestingly, Infl.-HA TCR^{tg} CD8$^+$ T cells reached a plateau using the lowest concentration of antigen tested (0.01 μg/mL). Additional experiments with a pure population of Infl.-HA TCR^{tg} miR181a CD8$^+$ T cells and additional lower concentrations of antigen are needed to reveal their true cytotoxicity.

Similar to human CD4$^+$ T cells, miR181a overexpression in murine Infl.-HA TCR^{tg} T cells could increase their lifespan. Thereby, miR181a has an anti-apoptotic effect on both Infl.-HA TCR^{tg} miR181a CD4$^+$ and CD8$^+$ T cells. Previous findings demonstrated that thymocytes from miR181-deficient mice have a decreased lifespan *in vivo* and showed increased apoptosis in culture [Henao-Mejia et al., 2013]. The present results give evidence that overexpression of miR181a in murine T cells could enhance their survival in culture and *in vivo*.

4.3 The co-transfer of miR181a overexpressing Infl.-HA TCR^{tg} CD4$^+$ and CD8$^+$ T cells enhances effective tumor control

The effect of miR181a overexpression on anti-tumoral activity of transgenic T cells were tested in two different mouse models. In the first model a pure population of murine Infl.-HA TCR^{tg} CD8$^+$ T cells either transduced with miR181a or ctrl miRNA were injected in A20-HA tumor bearing mice. The results from *in vitro* experiments give evidence for higher cytotoxic effects of Infl.-HA TCR^{tg} CD8$^+$ T cells when miR181a is overexpressed. Against the expectation, the overexpression of miR181a in Infl.-HA TCR^{tg} CD8$^+$ T cells did not lead to better tumor control or enhanced survival of A20-HA tumor bearing mice. The analysis of isolated tumors shows that Infl.-HA TCR^{tg} CD8$^+$ T cells are not efficiently able to infiltrate into the tumor either transduced with miR181a or ctrl miRNA. The persistence of transferred GFP positive cells in peripheral blood was lower in Infl.-HA TCR^{tg} miR181a CD8$^+$ T cells than in ctrl miRNA transduced cells. Consistent with these findings, Schietinger et al. showed that miR181a was highly expressed in tolerant CD8$^+$ T cells, which are unable to proliferate in response to antigen stimulation [Schietinger et al., 2012]. The overexpression of miR181a in Infl.-HA TCR^{tg} CD8$^+$ T cells alone did not enhance tumor control and could perhaps induce tolerance.

Dudley et al. demonstrated in a study that using highly selected tumor reactive CD8$^+$ T cells did not result in tumor regression [Dudley et al., 2005]. They suggested that the presence of CD4$^+$ T cells were necessary to mediate tumor rejection. It was shown that CD4$^+$ T cells could recruit

and sustain antigen-specific cytotoxic $CD8^+$ T cells [Pardoll and Topalian, 1998]. Previous studies reported that $CD4^+$ T cells are able to promote tumor rejection by their ability to secrete IL-2 [Antony et al., 2005]. Consistent with this, studies which tested the coadministration of $CD4^+$ and $CD8^+$ CAR T cells showed that T cell persistence could not be increased by additionally exogenous IL-2 administration [Mitsuyasu et al., 2000]. Thus, in the second model a co-transfer of Infl.-HA TCR^{tg} $CD4^+$ and $CD8^+$ T cells transduced with miR181a or ctrl miRNA were analyzed. Here, the transfer of Infl.-HA TCR^{tg} miR181a T cells leads to improved tumor control and prolonged survival of A20-HA tumor bearing mice. This was achieved by higher amounts of effector T cells and the expansion of Infl.-HA TCR^{tg} $CD8^+$ T cells. The persistence and predominant expansion of adoptively transferred T cells remains a limiting factor of adoptive cell therapy. miR181a overexpressing cells were shown to upregulate the expression of IL-10 and IL-12 *in vitro*. For both an enhanced anti-tumor function could be reported [Berg et al., 1996], [Lin and Karin, 2007], [Mumm et al., 2011],[Kerkar et al., 2010]. Additionally, the enhanced expression of pro-inflammatory cytokines e.g. IFNγ was observed. A positive effect of IFNγ on adoptively transferred T cells were shown previously by several studies [Fisher et al., 1986], [Brown et al., 1987], [Barth et al., 1991], [Tuttle et al., 1993], [Kortylewski et al., 2004].

Only two out of ten ($5*10^4$ T cells transferred) mice and four out of ten ($1*10^4$ T cells transferred) mice in the miR181a groups developed growing tumors. The analysis of the isolated tumors from these mice showed a higher infiltration of GFP positive T cells compared to isolated tumors from mice from the ctrl miRNA groups. These results indicate that miR181a overexpression in Infl.-HA TCR^{tg} $CD4^+$ and $CD8^+$ T cells enables these cells to infiltrate the tumors efficiently. This could be due to the higher expression of chemokin receptors as e.g. CCR2 is upregulated in both Infl.-HA TCR^{tg} miR181a $CD4^+$ and $CD8^+$ T cells. CCR2 was previously shown to increase the migration of tumor-specific T cells to established tumors leading to significantly increased anti-tumor activity [Craddock et al., 2010], [Moon et al., 2011]. Interestingly, only $5*10^4$ Infl.-HA TCR^{tg} miR181a T cells are needed to reach a survival rate of 80 % in the present study. These data suggest that the overexpression of miR181a enables the usage of lower amounts of antigen specific T cells for effective adoptive cell therapy.

The present results show that the complex effects of miR181a overexpression and the co-transfer of Infl.-HA TCR^{tg} miR181a $CD4^+$ and $CD8^+$ T cells leads to improved anti-tumor activity. Additional *in vivo* experiments using a pure population of transduced murine Infl.-HA TCR^{tg} $CD4^+$ T cells are needed to clarify their part in tumor control.

4.4 Conclusion

There are several studies ongoing to improve adoptive cell therapy. Many groups are working on major issues, e.g. identification of potent targets, searching for highly specific receptors (TCRs and CARs), methods to efficently engineer T cells, strategies to improve the persistence of transfered cells. The ability to redirect T cell specificity to target antigens of choice by specific receptors has been addressed intensely. Other aspects of T cell biology are further investigated by several researchers. These aspects include trafficking of effector cells and accumulation at the tumor site, long-term functional persistence, and engagement of co-stimulatory receptors to mediate a robust effector response [Kalos and June, 2013]. Zhang et al. showed that inducible IL-12 production of young tumor-infiltrating lymphocytes had enhanced effector function as demonstrated by increased IFNγ secretion [Zhang et al., 2012a]. Several studies demonstrated that TGFβ-signaling blockage could improve tumor treatment efficacy [Bollard et al., 2002], [Zhang et al., 2012b], and [Quatromoni et al., 2012]. Additionally, T cell intrinsic molecules are addressed. The downregulation of ubiquitin ligase Cbl-b in effector $CD8^+$ T cells improves the efficacy of adoptive therapy of leukemia in mice [Stromnes et al., 2010] and in a B16 melanoma model [Hinterleitner et al., 2012]. Also miRNAs were shown to regulate lymphocyte development and function. After T cell activation miR155 is upregulated [Haasch et al., 2002] to control cell proliferation and differentiation [O'Connell et al., 2008], [Turner and Vigorito, 2008]. Additionally, Dudda et al. could recently show that $miR155^{-/-}$ $CD8^+$ T cells were ineffective at controlling tumor growth [Dudda et al., 2013]. The overexpression of miR155 was able to enhance the anti-tumor response of tumor-specific $CD8^+$ T cells [Dudda et al., 2013]. The miRNA miR181a was shown to modulate T cell sensitivity in a way that immature T cells with higher amounts of miR181a are more sensitive to TCR stimuli [Li et al., 2007], [Ebert et al., 2009].

The present study demonstrates that ectopic expression of miR181a is able to enhance the sensitivity of both murine and human T cells. For primary human T cells the increased sensitivity by miR181a overexpression was proven in a simulating way by unspecific activation with αCD3. Using isolated murine T cells from transgenic mice allowed the analysis of miR181a in an antigen specific manner, representing physiologic T cell activation. In both cell types the increased amount of miR181a leads to downregulation of negative regulators of TCR signaling, such as DUSP5, DUSP6, PTPN22, and SHP2. These phosphatases are important to maintain a balance that permits activation of T cell responses against foreign peptides but not to self peptides. Overexpression of miR181a in human T cells leads to αCD28-independent activation. In addition, increasing the amount of miR181a enhance the cytolytic activity of murine TCR^{tg} T cells in an antigen-specific manner *in vitro*. The co-transfer of miR181a overexpressing murine Infl.-HA TCR^{tg} $CD4^+$ and $CD8^+$ T cells into A20-HA tumor bearing mice leads to improved tumor control and prolonged survival. This effect was characterized by higher amounts of effector T cells and a better infiltration of tumors. All of these effects were achieved by several changes in expression of different genes including molecules involved in T cell differentiation, activation and regulation, cytotoxic effector molecules, and chemokine and interleukin receptors in miR181a overexpressing T cells. The present results demonstrate that ectopic expression of miR181a in T cells is able to enhance effective tumor control and is therefore a promising candidate for improving adoptive cell therapy.

As previously mentioned, a majority of tumor-specific antigen targets are self or altered self. However, increasing the sensitivity of T cells by lowering the TCR threshold for activation by overepression of miR181a enhance the risk that modified T cells could respond not only to tumor tissues but also to healthy tissues and inducing autoimmunity. One innovative way to reduce the risk of autoimmunity is the use of chimeric antigen receptors (CARs) [Alvarez-Vallina, 2001], which recognize intracellular molecules. This approach may generate T cells with even greater specificities for tumor cells. The major challenge for both, TCRs and CARs, is the identification of a specific target (level and heterogeneity of expression, likelihood of antigen loss). This is a momentous field which is addressed by many groups. Finally, coupling miR181a in the 3′UTR of antigen specific receptors (TCRs or CARs) could further enhance the success of adoptive cell therapy by their pleiotropic effects.

A Appendix

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Danksagung

Curriculum vitae

Statutory Declaration

I hereby declare that I wrote the dissertation submitted without an unauthorized external assistance and used only sources acknowledged in the work. All textual passages which are appropriated verbatim or paraphrased from published and unpublished texts as well as all information obtained from oral sources are duly indicated and listed in accordance with bibliographical rules. In carrying out this research, I complied with the rules of standard scientific practice as formulated in the statutes of Johannes Gutenberg-University Mainz to insure standard scientific practice.

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Kelkheim, den 03.03.2015 Kathleen Hobohm

Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig und ohne Verwendung anderer als der angegebenen Quellen und Hilfsmittel angefertigt habe. Die wörtlichen oder inhaltlich benutzen Quellen habe ich als solche kenntlich gemacht. Diese Arbeit hat in gleicher oder ähnlicher Form noch keiner Prüfungsbehörde vorgelegen.

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Kelkheim, den 03.03.2015 Kathleen Hobohm