

# Dissertation

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**Immunotherapy with tumor antigen-  
specific T cells in *ret* transgenic mouse  
melanoma model**

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# Summary

Metastatic melanoma is a severe disease with a high rate of lethality. It is known to be resistant to current therapies. Since melanoma is immunogenic the development of an immunotherapy can be a promising possibility to enhance an antitumor effect *in vivo*. Memory T cells (MTC) have abilities to respond quicker to antigens and to release a broader spectrum of cytokines than naïve T cells. The *ret* transgenic mouse melanoma model was used in this study since it resembles the pathological situation of human melanoma in contrast to transplantation models. It has been previously shown that the bone marrow (BM) is a major site for the persistence of tumor-specific MTC in cancer patients. In addition, melanoma-specific MTC were also found in the BM of *ret* transgenic mice without macroscopic tumors. Therefore, we isolated CD3<sup>+</sup> cells from the BM of *ret* transgenic mice with and without tumors.

Therefore, we isolated CD3<sup>+</sup> T cells from the BM of *ret* transgenic mice with and without tumors. After a 40 h *ex vivo* stimulation of bone marrow-derived T cells with melanoma antigen-loaded DC, which were treated with anti-PD-L1 antibody overnight, T cells revealed a higher IFN- $\gamma$  production and an increased T cell activation *in vitro*. Moreover, activated CD8<sup>+</sup> T cells displayed mainly a central memory phenotype and an increased level of CD69 expression after 40 h of co-culture with DC.

Labeled melanoma-specific, stimulated memory T cells from *ret* transgenic mice migrated to skin tumor lesions, metastatic lymph nodes (LN), BM and spleen after adoptive transfer into *ret* transgenic tumor-bearing mice. A similar migration pattern was detected using stimulated TRP-2 TCR transgenic effector T cells. Furthermore, migrated CD8<sup>+</sup> T cells showed an increase in effector memory (T<sub>EM</sub>) and effector phenotype at day 7 post injection and a decrease of central memory and naïve CD8<sup>+</sup> T cells within tumor

lesions, whereas at day 3, central memory, effector memory, naive and effector CD8<sup>+</sup> T cells were equally distributed.

To investigate the anti-tumor activity of melanoma-specific memory T cells *in vivo* we adoptively transferred MTC, which were prior activated with DC, into tumor-bearing mice by i.c. injections. Mice receiving memory T cells showed a significantly longer survival compared to the control group. Mice receiving the phosphodiesterase-5 inhibitor sildenafil and adoptive transfer of MTCs displayed a significantly higher survival rate than mice treated with sildenafil or PBS only.

We suggest that an adoptive transfer of melanoma-specific memory T cells activated with antigen-loaded DC, which were pre-treated with anti-PD-L1 antibodies, can enhance an anti-tumor response and therapeutic efficacy *in vivo*.

# Zusammenfassung

Das maligne Melanom ist gekennzeichnet durch ausgeprägtes Metastisierungspotenzial und es besteht Resistenz gegenüber gängige Behandlungsmethoden wie Chemotherapie und Bestrahlung. Deshalb ist es von großer Wichtigkeit neue Behandlungsstrategien zu entwickeln. Da das Melanom sehr immunogen ist, dient die Entwicklung einer Immuntherapie als eine vielversprechende Option. Ein Werkzeug hierfür ist die Verwendung des Spontanmelanom-Tiermodells, welches hinsichtlich genetischer, histopathologischer und klinischer Situation, dem humanen Melanom sehr ähnlich ist. Dieses Mausmodell wurde auch für die vorliegende Arbeit herangezogen. Gedächtnis-T-Zellen besitzen die Fähigkeit schnelleren auf ein Antigen zu antworten und sie sekretieren ein viel breiteres Cytokinspektrum als naive T-Zellen. Es wurde vor Kurzem gezeigt, dass das Knochenmark als der Hauptort für persistierende Tumor-spezifische Gedächtnis-T-Zellen in Krebspatienten ist. Außerdem wurden Melanom-spezifische Gedächtnis-T-Zellen im Knochenmark *ret* transgener Mäuse ohne makroskopische Tumore gefunden. Basierend darauf, haben wir für unsere Untersuchungen CD3<sup>+</sup> T-Zellen aus den Knochen *ret* transgener Mäuse mit und ohne Tumore isoliert.

Nach einer 40-stündigen Kokultur frisch isolierter T-Zellen mit Melanom-Antigen geladenen Dendritischen Zellen, welcher über Nacht mit einem anti-PD-L1 Antikörper behandelt wurden, zeigten T-Zellen eine erhöhte IFN- $\gamma$ -Produktion und somit eine verstärkte T-Zell-Aktivierung, wenn der anti-PD-L1 Antikörper eingesetzt wurde. Desweiteren zeigten stimulierte T-Zellen eine erhöhte CD69 Expression und die Kultur stellte hauptsächlich den „*central memory*“ CD8<sup>+</sup> T-Zell Phänotyp dar.

Markierte Melanom-spezifische Gedächtnis-T-Zellen aus *ret* transgenen Mäusen migrierten in den Tumor, die metastasierten Lymphknoten, das Knochenmark und die



Milz nach einem Adoptiven Transfer. Ein ähnliches Migrationsverhalten zeigten TRP-2 TCR transgene „effector“ T-Zellen. Zudem zeigten die migrierten Zellen an Tag 7 ein Ansteigen des „effector memory“ und „effector“ CD8<sup>+</sup> T-Zell-Phänotyps, während die Menge an naiven und „central memory“ T-Zellen abnahm. An Tag 3 nach dem Adoptiven Transfer war das Verhältnis von effector memory, central memory, naive und effector T-Zellen fast gleich.

Um die anti-Tumor Aktivität Melanom-spezifischer Gedächtnis-T-Zellen *in vivo* zu untersuchen, wurde 40 h nach Kokultur mit Tumor-Antigen beladenen DC, ein Adoptiver Transfer in Tumor-tragende *ret* transgene Mäuse durchgeführt. Mäuse, welche mit aktivierten Gedächtnis-T-Zellen behandelt wurden, zeigten eine signifikant höhere Überlebensrate im Vergleich zur Kontrollgruppe. In der Kombinationstherapie mit dem Phosphodiesterase-5 Inhibitor Sildenafil, zeigte die Kombinationsgruppe, Sildenafil und Adoptiver Transfer, ebenfalls eine höhere Überlebensrate als Gruppen, die nur mit Sildenafil oder PBS behandelt wurden. Im Überlebensversuch mit TRP-2 TCR transgenen T-Zellen konnte man nur die Tendenz einer höheren Überlebensrate sehen, verglichen mit der Kontrollgruppe.

Eine effektive Melanom-Immuntherapie könnte auf einem adaptiven Transfer *in vitro* restimulierter Gedächtnis-T-Zellen aus dem Knochenmark von Melanompatienten, zusammen mit Melanom-Antigen beladenen DC in Kombination mit der Neutralisierung des Tumormikromilieus basieren.

# I List of abbreviations

<b>A</b>		DTIC	dimethyl triazeno- imidazole carboxamide (dacarbazine)
AEC	3-amino-9- ethylcarbazole		
Ag	antigen		
APC	allophycocyanin	<b>E</b>	
APCs	antigen-presenting cells	EDTA	ethylenediaminetetra- acetic acid
<b>B</b>		e.g.	for example
BM	bone marrow	ELISPOT	enzyme-linked immunospot
BSA	bovine serum albumin	ERK	extracellular-signal regulated kinase
<b>C</b>		et al.	et alteri
CAR	chimeric antigen receptor	etc	et cetera
CCR	C-C chemokine receptor	<b>F</b>	
CD	cluster of differentiation	FACS	fluorescence activated cell sorting
ConA	concanavalin A	FCS	fetal calf serum
CPD	cell proliferation dye eFluor® 670	FDA	Food and Drug Administration
CTLs	cytotoxic lymphocytes	FITC	fluorescein- isothiocyanat
CTLA-4	cytotoxic T lymphocyte antigen 4	FMO	fluorescence minus one
Cy	cyanin	FoxP3	forkhead box P3
<b>D</b>		FSC	forward scatter
DC	dendritic cells	<b>G</b>	
DKFZ	Deutsches Krebsforschungszentru m	GM-CSF	granulocyte- macrophage colony- stimulating factor
DNA	desoxyribonucleic acid		
dH <sub>2</sub> O	distilled water		
DMSO	dimethylsulfoxide		

<b>H</b>		rm	recombinant murine
HRP	horseradish peroxidase	ROS	reactive oxygen species
		RPE	R-phycoerythrin
<b>I</b>		rpm	rounds per minute
i.e.	that is	RPMI	Roswell Park Memorial
IL	interleukin		Institute medium
IFN	interferon	RT	room temperature
IU	international unit		
		<b>S</b>	
<b>L</b>		SA	secretion assay
LN	lymph nodes	SEB	staphylococcal enterotoxin B
		SSC	side scatter
<b>M</b>		<b>T</b>	
MAPK	MAP kinase mitogen- activated protein kinase	TA	tumor-antigen
MHC	major histocompatibility complex	tb	tumor-bearing ret transgenic
MT	metallothionein	TCM	central memory T cell
MTCs	memory T cells	TCR	T cell receptor
		TEM	effector memory T cell
<b>P</b>		tg	<i>ret</i> transgenic without tumor
PBS	phosphate buffered saline	TILs	tumor infiltrating lymphocytes
PD-1	programmed death 1	Tim-3	T cell immunoglobulin mucin-3
PD-L1	programmed death 1 ligand 1	TL	tumor lysate
PE	phycoerythrin	TME	tumor microenvironment
PerCP	peridinin-chlorophyll- protein complex	TNF	tumor necrosis factor
pH	potentia hydrogenii	Tregs	regulatory T cells
PI	propidium iodide	TRP-2	tyrosinase related protein 2
PRRs	pattern recognition receptors	TRP-2 <sub>(180-188)</sub>	TRP-2 derived peptide (residues 180-188)
PTO	phosphorothioate		
<b>R</b>		<b>U</b>	
<i>ret</i>	rearranged during transfection	UV	ultraviolet

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# 1 Introduction

## 1.1 The immune system

The immune system is a very complex network of cells, tissues and molecules. The main task of the immune system is to defend the body against foreign invaders. To operate properly, an immune system must detect a large spectrum of agents, from viruses to parasitic worms, and distinguish them from the organism's own healthy tissue. As a respond to the immune system which detects and neutralizes intruders, pathogens can quickly evolve and adapt in order to escape the immune system. Therefore, the immune system has developed different defense mechanisms to identify and neutralize pathogens. All mechanisms are based on detecting structural features of the invaders that mark them as distinct from host cells (Chaplin, 2006; Hoffmann et al., 1999; Janeway, 2001a).

The immune system is made of two arms, one is the innate immune system, specific for infectious non-self, and another one is the adaptive immunity which is specific for all non-self-antigens (Janeway, 2001a).

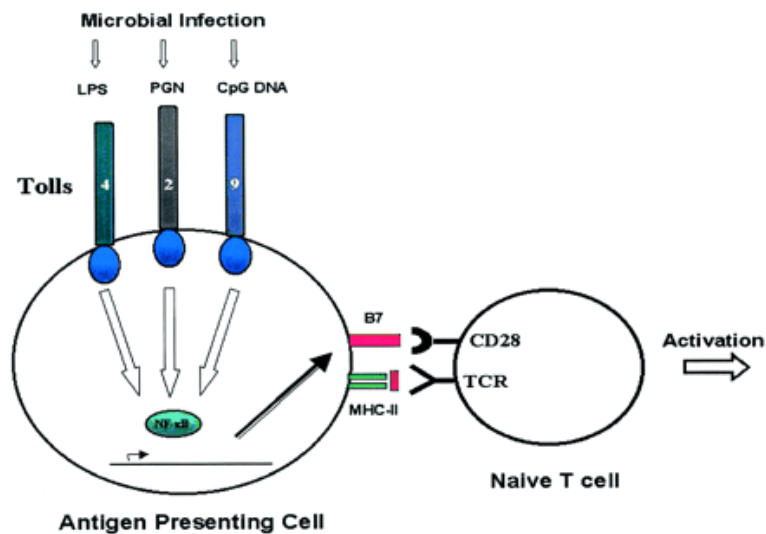
### 1.1.1 INNATE IMMUNITY

The first defense in the innate immunity occurs through the epithelial barrier and the mucociliary clearance. The recognition of the innate immune system is based on germline-encoded receptors. It includes the recognition of conserved repeating patterns from microorganisms, e.g. lipopolysaccharide (LPS) of Gram-negative bacteria, lipoteichoic acid (LTA) of Gram-positive bacteria, mannose and glycans, bacterial CpG DNA or double-stranded viral RNA. These molecules called pathogen-associated molecular patterns (PAMPs) can be found in the cell walls of Gram-negative bacteria, in

viruses, fungi and Gram-positive bacteria (Janeway, 1989). Receptors that can recognize them are known as pattern recognition receptors (PRRs) and they do not recognize self-structures of the host (Janeway, 2001b). There are different types of PRRs such as the macrophage mannose receptor, scavenger receptor or toll-like receptors (TLRs).

One very important event triggered by the activation of the innate immune system is the expression of co-stimulatory molecules, like CD80 (B7.1) and CD86 (B7.2) on the surface of antigen presenting cells (APCs), e.g. on dendritic cells (DC). Furthermore, the phagocytized molecules are either processed in the immunoproteasom of APCs and are presented on the major histocompatibility complex class I (MHC I) or they are degraded in endosomes and are presented on MHC class II (MHC II) which can be recognized as a complex by T lymphocytes (Hardardottir et al., 1995).

Hence, APCs link the innate immunity with the adaptive immune system by presenting antigens which, are recognized by the T cell receptor (TCR) of T lymphocytes.



**Fig.1: Signaling pathway triggered by Toll-like receptors.** Various PAMPs recognized by cognate PRRs expressed on APCs induce the expression of CD80 and CD86 molecules, which signal the presence of pathogens and allow activation of lymphocytes specific for antigens derived from the pathogens. Depicted are LPS recognition by TLR-4, proteoglycan recognition by TLR-2, and the recognition of CpG DNA by TLR-9 (Janeway, 2001b).



### 1.1.2 APCs

As mentioned above, APCs represent a link between the innate and adaptive immune system. T and B lymphocytes are known to be the major effector cells of the immune system. They can eliminate infected cells, neutralize and remove toxic substances. APCs are able to attain microbial and other antigens and display them on their surface in a sense that leads to the activation of T lymphocytes of the adaptive immune system.

It can be distinguished between professional and non-professional APCs. There are different types of professional APCs, e.g. DC, macrophages and B lymphocytes. These kinds of cells are very efficient in internalizing foreign proteins and processing them to display fragments of the antigen bound to MHC class I or II molecules. After facing a pathogen or cytokines, APCs get activated and migrate into the T cell areas of local lymph nodes. On their way there, they pass different stages of maturation which leads to the expression of co-stimulatory molecules and a stable expression of peptide:MHC complexes on their surface (Germain, 1994). The internalization of foreign antigens can occur through phagocytosis, receptor-mediated endocytosis or macropinocytosis. TCRs and the CD8 co-receptor of cytotoxic T cells (CTL) recognize peptides originating in the cytosol delivered to the cell surface and bound to the MHC class I whereas CD4<sup>+</sup> T cells recognize peptides arising in vesicular system and attached to MHC class II (Goldberg et al., 2002; Hiltbold and Roche, 2002).

Peptides that bind to MHC class I are usually derived from intracellular bacteria or viruses, which are then degraded by the immunoproteasome. They are then transported from the cytosol by proteins called transporters associated with antigen processing-1 and -2 (TAP-1 and TAP-2) to the endoplasmatic reticulum (ER) membrane. In the ER lumen, they bind to the MHC class I molecules, which then can be exported as a complex to the cell surface. These molecules are expressed on every nucleated cell of the body (Schubert et al., 2000; Uebel and Tampe, 1999; Vigneron et al., 2004).

MHC class II molecules are only found on APCs. They bind peptides for their presentation to CD4 T lymphocytes. Peptides binding to MHC class II molecules are

derived from extracellular pathogens or from pathogens, which have invaded the cell to replicate in intracellular vesicles. Vesicles containing MHC class II molecules fuse with endosomes containing processed peptides. Subsequently, peptides bind to the molecules and are transported to the cell surface (Hiltbold and Roche, 2002).

In addition to the peptide presentation on MHC class I and II, professional APCs express co-stimulatory molecules, like CD80 and CD86, which are important for proper activation of T cells.

### 1.1.3 DC

DC are by far the most important APCs. They were characterized in 1973 by Ralph Steinman and Zanvil Cohn (Steinman and Cohn, 1973) since then they have gained a solid interest from immunologists. DC arise from hematopoietic progenitors of the BM and circulate through the blood, non-lymphoid and lymphoid tissues (Inaba et al., 1992). A large part of the DC is constituted by myeloid derived DC, which evolve from CD34<sup>+</sup> myeloid progenitors compared to the lesser amount of CD14<sup>+</sup> lymphoid progenitors which differentiate into plasmacytoid DC (pDC) (Banchereau et al., 2000). pDC are a rare circulating population, which can produce large amounts of type I interferons in response to viruses or host-derived nucleic acid-containing complexes that bind to TLR7 and TLR9 in their compartment (Liu, 2005). Myeloid DC express TLR2 and TLR4 on their surface and bind to different bacteria derived products, producing high amounts of IL-12 (Leenen et al., 1998).

Immature DC have a high endocytic and phagocytic capacity important for antigen uptake, though, the expression of MHC class II and co-stimulatory molecules is very low which leads to a low stimulation of T cells. DC can recognize PAMPs of microbial products via PRRs, e.g. mannose-like receptors and the TLR family. (Iwasaki and Medzhitov, 2004; Reis e Sousa et al., 1993). During their maturation state, upon pathogen recognition, DC release high amounts of proinflammatory and anti-viral cytokines such as IFN- $\alpha$  and IL-12, which lead to the recruitment and activation of innate immune cells at the site of inflammation (Blander and Medzhitov, 2006; Reis e

Sousa, 2004). As soon as DC are mature, they lose their endocytic and phagocytic receptors, the expression of MHC II and co-stimulatory molecules like CD80, CD86 and CD40 on their surface increases to a high level. DC also change their morphology and activate the antigen-processing machinery (Allan et al., 2006). To activate T lymphocytes, DC express CCR7 receptors which respond to homeostatic chemokines CCL19 and CCL21 and guide DC through afferent lymphatics into the T cell areas of local draining lymph nodes. Here, DC select and activate antigen-specific T cells and induce their differentiation into effector cells, initiating thereby primary immune response (Banchereau et al., 2000; Rescigno, 2001).

Utilizing DC as a tool to treat cancer, various clinical trials have been conducted using DC transduced with viral vectors, DC loaded with proteins or peptides and DC loaded with tumor lysates (Engell-Noerregaard et al., 2009).

#### 1.1.4 ADAPTIVE IMMUNE SYSTEM

The adaptive immunity is also known as a specific or acquired immune system. It is classified in the cellular immunity and humoral immunity. The cellular immunity consists of T cells with their antigen-specific TCRs recognizing short antigen fragments bound to the MHC molecules and provides a basis of self-nonsel self discrimination (Blattman and Greenberg, 2004). The humoral immunity consists of B lymphocytes that express antigen-specific receptors and bind antigenic determinants present on soluble proteins, carbohydrates, or nucleic acids.

After the initiation of the primary immune response by pathogens, a couple of days are necessary for the clonal expansion and differentiation from naïve into effector T lymphocytes and antibody-secreting B lymphocytes. Afterwards, antigen-specific immune cells are able to eliminate the pathogens or infected cells and neutralize pathogen-derived products (Medzhitov and Janeway, 1999). After the infection removal, most effector cells undergo apoptosis but some cells build the immunological memory that ensures a rapid reinduction of Ag-specific antibody and effector T cells after subsequent encounter with

the same pathogen (Sallusto et al., 2004). Immunological memory provides a long lasting and often lifelong protection and in use as a tool for immunotherapy establishment against melanoma (Murali-Krishna et al., 1999; Umansky et al., 2008).

## 1.2 T cells

Cellular immunity is based on two major T cell subsets, CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which work together to mediate an effective immune response. These cells originate from the hematopoietic precursors in the bone marrow and mature in the thymus into naïve T cells (Borowski et al., 2002; Shortman and Wu, 1996). Each T cell expresses TCRs for a specific antigen which is formed during T cell maturation through somatic recombination. Thus, large amounts of T cells with a broad spectrum of receptors specific for a variety of peptide:MHC complexes emerges (Zerrahn et al., 1997). During positive selection in the thymus, T cells specific for endogenous MHC molecules are selected. The subsequent negative selection is important to eliminate T cells in the thymus stroma which bind with their TCR to self-antigens (Sprent and Webb, 1995).

CD4<sup>+</sup> T helper T cells are able to provide cytokine-mediated help to form the B cell-mediated humoral response as well as to improve the durability and quality of the CD8<sup>+</sup> T cell-mediated cytotoxic response. They can be further divided into type 1 and type 2 helper T cells (Mosmann et al., 1997). Type 1 helper T cells produce IL-2 and IFN- $\gamma$  after activation triggered by binding to the peptide:MHC II complex on APCs and facilitate in this way the CTL activity. This phenotype is considered to contribute to the anti-tumor immunity. Type 2 helper T cells promote humoral immune response by secreting IL-4, IL-5 and IL-6 (Trombetta and Mellman, 2005).

CTLs are able to induce death in infected cells. After activation, which occurs after binding to the peptide:MHC I complex on APCs, CTLs release cytokines and enzymes, e.g. perforin and granzymes, to lyse diseased cellular targets. Most of effector cells die after overcoming the infection (Barry and Bleackley, 2002). However, a few cells survive and remain as memory T cells. Here, the activation of memory T cells upon encounter

with the same antigen occurs after a very short time compared to the primary immune response, where the activation takes several days (Huang et al., 2004). Memory T cells are capable of quick differentiation into effector cells and secretion of a broad spectrum of cytokines.

The utilization of T cells for immune-mediated therapy of cancer has gained a deep interest of immunologist. Opportunities of therapeutic manipulation of T cells to maximize anti-tumor immunity exist in different stages of T cell maturation and activation, e.g. monoclonal antibodies (mAb) blocking CTLA-4 on APCs to inhibit the interaction with CD28 on T cells that has been recently approved for the treatment of metastatic melanoma (Lipson and Drake, 2011). CD80 and CD86 interact with CD28 to promote T cell activation, and CD80/CD86 interact with the counter-regulatory molecule CTLA-4 to dampen it by feedback regulation (Ostrand-Rosenberg et al., 2002).

### 1.2.1 MEMORY T CELLS

Developing an immune memory, which enables to react quickly to antigens upon the re-exposure with a higher potency and efficacy, is a hallmark of the adoptive immunity. This is characterized by 1) the response of memory T cells in the presence of lower antigen concentration, 2) the rapid clonal proliferation upon antigen stimulation (Barber et al., 2003; Wherry et al., 2003), 3) the potential to release a broader spectrum of cytokines, 4) the expression of peripheral tissue homing receptors and 5) a rapid display of effector functions (Dutton et al., 1998; Tanel et al., 2009). Moreover, memory T cells undergo antigen-independent self-renewal stimulated by cytokines such as IL-2, IL-7, IL-15 and IL-21 to maintain the memory T cell pool (Kieper et al., 2002; Luckey et al., 2006; Schluns et al., 2000). Memory T cells maintain in the body many years after antigen exposure, even though the pathogen was eliminated (Combadiere et al., 2004). Memory T cells differ from naïve T cells in their expression of surface molecules like CD45RB (mouse), CD45RA (human), CD45RO (human), CD44 (mouse), CD62L and CCR7 (human/mouse) (Sallusto et al., 1999).

### 1.2.2 MEMORY T CELLS SUBSETS

Mouse as well as human memory T cells can be subdivided into different subgroups based on the expression of various cell surface molecules, e.g. homing markers, chemokines and cytokines (Masopust et al., 2001; Sallusto et al., 2004; Sallusto et al., 1999). As shown in Table.1, both CD4<sup>+</sup> and CD8<sup>+</sup> T cells can be divided into naïve (T<sub>N</sub>), central memory (T<sub>CM</sub>) and effector memory T cells (T<sub>EM</sub>). Naïve T cells express high levels of homing markers L-selectin (CD62L) and CCR7, binding the chemokines CCL19 and CCL21, which are required for cell extravasation through high endothelial venules (HEV) into the secondary lymphoid organs (Forster et al., 1999).

Surface marker (mouse)	Naïve T cells	Central memory T cells	Effector memory T cells
<b>CD45RB</b> (CD4 <sup>+</sup> )	high	low	low
<b>CD44</b> (CD4 <sup>+</sup> /CD8 <sup>+</sup> )	low	high	high
<b>CD62L</b> (CD4 <sup>+</sup> /CD8 <sup>+</sup> )	high	high	low
<b>CCR7</b> (CD4 <sup>+</sup> /CD8 <sup>+</sup> )	high	high	low

Table.1: Murine memory T cell surface markers.

Murine central memory T cells do not differ much from naïve T cells regarding their phenotype except the expression of CD45RB. T<sub>CM</sub> reside in the lymph nodes, tonsils and the bone marrow (Campbell et al., 2001; Feuerer et al., 2001b; Sallusto et al., 1999). Upon exposure to antigens, long-lived T<sub>CM</sub> can rapidly differentiate into cells equipped with effector functions; they are more sensitive to antigenic stimulation and less dependent on costimulation (Sallusto et al., 2004; Sallusto et al., 1999). Furthermore, T<sub>CM</sub> have the ability of self-renewal and secrete high amounts of IL-2, which is necessary for the proliferation and differentiation of T<sub>N</sub> to become effector T cells. Compared to short-lived T<sub>EM</sub>, T<sub>CM</sub> have been shown to mediate a protective immunity because of their long-term persistence. This feature designates them as “stem cells” of the adoptive immune response (Bouneaud et al., 2005; Klebanoff et al., 2005; Zaph et al., 2004).

Circulating CD4<sup>+</sup> and CD8<sup>+</sup> effector T cells are found in the peripheral blood, spleen and non-lymphoid tissue where they can respond quickly to foreign antigens by the production of effector molecules such as IFN- $\gamma$ , IL-4 and IL-5 (Sallusto et al., 1999). In

addition,  $CD8^+$   $T_{EM}$  produce large amounts of perforin. In contrast to  $T_{CM}$ , effector memory T cells do not express CCR7 and CD62L (Sallusto et al., 1999). However, they display characteristic sets of chemokines and adhesion molecules for homing into inflamed tissue. In addition,  $T_{EM}$  respond with a higher potency to antigens than  $T_{CM}$  (Sallusto et al., 2004).

### 1.2.3 MODELS OF MEMORY T CELL DIFFERENTIATION

Currently, there are three different models of memory T cell differentiation described in the literature (Fearon et al., 2006; Ganusov, 2007; Kalia et al., 2006; Lefrancois and Marzo, 2006).

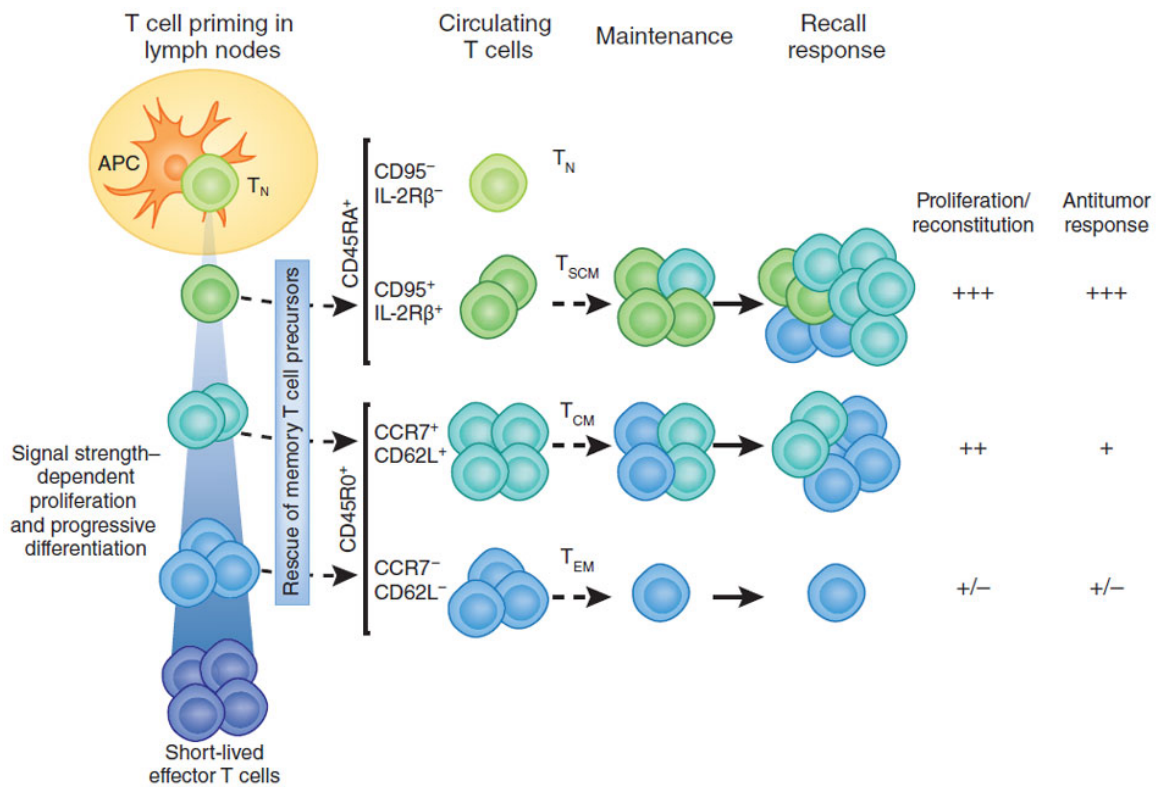
The first model is called stem cell-associated differentiation (SCAD). During the expansion phase of the immune response, terminally differentiated effector T cells are produced by sustained self-renewal of memory T lymphocytes. After antigen clearance, which is also known as the contraction phase, effector T cells undergo apoptosis and only a small amount of memory T cells remains in the system (Ahmed and Gray, 1996; Fearon et al., 2006; Wodarz et al., 2000)

In the linear differentiation (LD) model, during the expansion phase activated T lymphocytes with an effector phenotype proliferate and die in the contraction phase after the antigen elimination and a few cells differentiate into long-lived memory T cells (Ahmed and Gray, 1996; Antia et al., 2005; Kaech et al., 2002; Lefrancois and Marzo, 2006).

The last model is called progressive differentiation (PD). Here, proliferating T cells progress through different stages of differentiation during the expansion phase. Cells receiving a weak signal will differentiate into memory T cells, those receiving a strong signal will transform into effector T lymphocytes (Fearon et al., 2006; Fearon et al., 2001; Lefrancois and Marzo, 2006).

However, there are some variations of these models. In the PD model three distinct stimulation patterns according to signal strength through antigens are described (Lanzavecchia and Sallusto, 2005). Thus, the circulation of a low number of antigens in

the system leads to an incomplete activation of T lymphocytes through low stimulation. These T cells undergo apoptosis and the differentiation into memory T cells does not occur. In contrast, a circulation of high amounts of foreign antigen leads to an enhanced stimulation of T cells by the recruitment of additional APCs with upregulated MHC molecules on their surface and removal of the antigens. During this activation, some naïve T cells stop their transformation into effector cells and undergo differentiation into either  $T_{CM}$  or  $T_{EM}$  depending on the intensity of the antigen signal.  $T_{CM}$  maintain in the body for a long time whereas  $T_{EM}$  undergo apoptosis if they are not exposed to the same antigen. In case of constitutive strong antigen stimulation, naive T cells differentiate only into effector T cells Fig.2.



**Fig.2: Memory T cell differentiation.** A model based on strength and quality of stimulatory signals. After T cell activation and during differentiation into memory T cells lose naïve properties and acquire effector properties. After elimination of the antigen, a few T cells remain as memory T cells (Sallusto and Lanzavecchia, 2011).



#### 1.2.4 CD8<sup>+</sup> MEMORY STEM CELLS

Conventionally, memory T cells are divided into two subtypes based on their homing molecules CD62L and CCR7. However, a new subset of mouse memory T cells with stem cell like properties has been recently described (Gattinoni et al., 2009; Zhang et al., 2005). These self-renewal T cells, so called memory stem T cells (T<sub>SCM</sub>), have a phenotype similar to naive T cells (CD44<sup>low</sup> and CD62L<sup>high</sup>). Moreover, T<sub>SCM</sub> were found to express the stem cell antigen (Sca)-1 and high levels of the antiapoptotic molecule B cell lymphoma- 2 (Bcl-2), the  $\beta$ -chain of IL-2 and IL-15 receptor (IL-2R $\beta$ ), and the chemokine (C-X- C motif) receptor CXCR3 (Gattinoni et al., 2009). This memory subtype displayed an enhanced self-renewal capacity and the multipotency to generate all memory and effector T cell subsets *in vitro* (Gattinoni et al., 2009; Zhang et al., 2005). Recently, T<sub>SCM</sub> were also found within a CD45RO<sup>-</sup>, CCR7<sup>+</sup>, CD45RA<sup>+</sup>, CD62L<sup>+</sup>, CD27<sup>+</sup>, CD28<sup>+</sup> and IL-7Ra<sup>+</sup> T cell compartment characteristic for naive T cells. Furthermore, they expressed high levels of CD95, IL-2R $\beta$ , CXCR3 and LFA-1 and displayed various characteristics of memory T cells (Gattinoni et al., 2011).

These recently identified memory T cells with stem cell properties have major implications for the design of new vaccination strategies and adoptive immunotherapy.

#### 1.2.5 BONE MARROW AS A NICHE FOR MEMORY T CELLS

It has been demonstrated that the persistence of memory plasma cells occurs in the bone marrow in so-called survival niches. Therefore, the question was addressed whether T cells can also reside in the BM (Manz et al., 1997; Radbruch et al., 2006; Tokoyoda et al., 2009a). Recent studies have shown that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells persist in the bone marrow stroma niches which serve as a reservoir for memory T cells (Beckhove et al., 2004; Duffy et al., 2012; Feuerer et al., 2001a; Feuerer et al., 2004; Feuerer et al., 2001b; Mazo et al., 2005; Tokoyoda et al., 2009b). An essential role of IL-7 for memory CD4<sup>+</sup> T cells and IL-15 for memory CD8<sup>+</sup> T cells has been demonstrated (Snell et al., 2012; Surh and Sprent, 2008). In the BM, CD4<sup>+</sup> T helper memory cells persist in close

contact with VCAM-1<sup>+</sup> stroma cells, which produce IL-7 to maintain these T lymphocytes (Tokoyoda et al., 2009b).

The recruitment of memory T cells from the peripheral blood to the bone marrow follows by different mechanisms including the interaction of very late activation antigen (VLA)-4 expressed on memory T cells and the vascular cell adhesion molecule (VCAM)-1, which is constitutively expressed in the bone marrow (Mazo et al., 2005). The binding of mucosal addressin cell adhesion molecule (MAdCAM)-1 to  $\alpha_4\beta_7$  integrin molecules, which is upregulated on memory T cells, is another mechanism of T cell recruitment into the bone marrow (Berlin et al., 1993; Erle et al., 1994; Mazo et al., 2005).

### 1.2.6 TUMOR-SPECIFIC MEMORY T CELLS

Tumor antigen specific memory T cells have been found close to dormant tumor cells in the bone marrow of mice previously vaccinated with tumor cells. It has been described that these memory T cells provide protection against further tumor challenge (Khazaie et al., 1994; Muller et al., 1998).

Patients with breast cancer harbored tumor antigen-specific memory T cells in their bone marrow, which could be reactivated *ex vivo* with previously generated DC pulsed with the tumor lysate. After stimulation, they produced IFN- $\gamma$  and attained cytotoxic activity against tumor antigens *in vitro* and *in vivo* (Beckhove et al., 2004; Feuerer et al., 2001b). The BM is considered to be the site of persistence for tumor specific memory T cell and low amounts of dormant tumor cells which are kept under control by the host immune control (Feuerer et al., 2001a). Tumor specific memory T cells were not only found in the BM of breast cancer patients but also in patients with pancreatic cancer and melanoma (Beckhove et al., 2004; Feuerer et al., 2001a; Muller-Berghaus et al., 2006; Schmitz-Winnenthal et al., 2005).

Utilizing the *ret* transgenic mouse melanoma model, it has been demonstrated that the bone marrow of mice with primary skin tumors contained high numbers of melanoma antigen-specific CD8<sup>+</sup> T cells, with mainly effector memory phenotype (Umansky et al., 2008). Furthermore, melanoma specific CD8<sup>+</sup> effector memory T cells were detected in mice with disseminated melanoma cells in the LN and the BM but without macroscopic

tumors and no further tumor progression (Umansky et al., 2008). Since tumor specific memory T cells from the BM of *ret* transgenic mice could be reactivated *ex vivo*, they were used for adoptive transfer in tumor-bearing mice (Umansky et al., 2008). Reactivated memory T cells from breast cancer patients were reported to recognize and reject xenotransplanted autologous tumors (Beckhove et al., 2004).

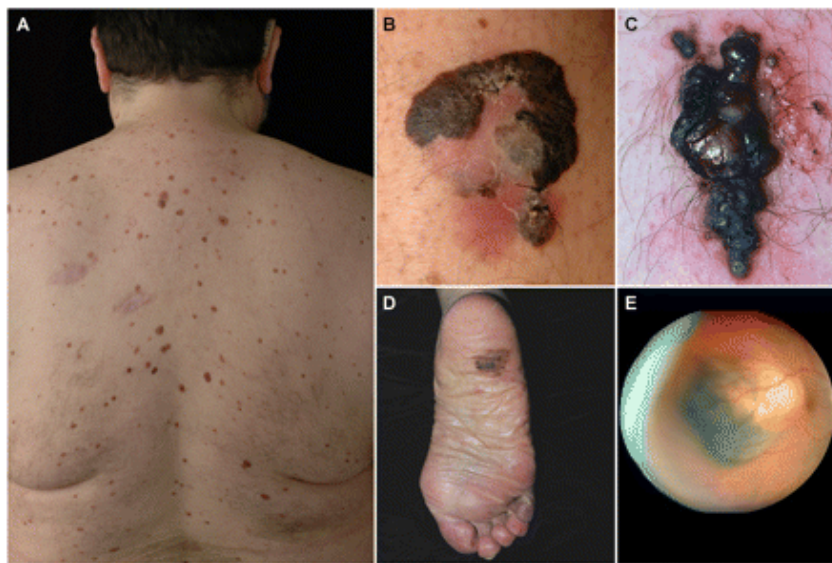
### 1.3 Melanoma

Malignant melanoma is an aggressive malignancy of transformed melanocytes. It is known to be resistant to standard therapy, i.e. chemo- and radiotherapy (Finn et al., 2012). It is the most rapidly increasing malignancy in Western population in terms of incidence and is directly related to improving life standards such as travelling to sea resorts and getting tanned (Sapoznik et al., 2012). Although only approximately 5% of skin cancers account for melanoma, it is responsible for most skin cancer related deaths (Kanavy and Gerstenblith, 2011). Risk factors for melanoma development are solar UV radiation, fair skin, dysplastic nevi syndrome, and a family history of melanoma (Garibyan and Fisher, 2010; Ilkovitch and Lopez, 2008; Whiteman et al., 2006). Melanoma is more common in Caucasians than in African or Asian people. It is affecting generally young and middle-aged people, unlike other solid tumors and it is the most common malignancy in women aged 25-29 years (Brewer et al., 2011).

Most commonly melanoma arises from the malignant transformation of melanin-producing melanocytes in the skin, rarely in noncutaneous melanocytes such as the retina and uvea of the eyes, respiratory, gastrointestinal, and genitourinary mucosal surfaces, or the meninges (Tsao et al., 2012). Clinicians have traditionally divided the disease into different subgroups based on the TNM classification of malignant tumors. Stage I melanoma is described as an invasive melanoma where the tumor cell spread *in situ*. If the tumor reaches the size of 1.5 mm and more, it can be assigned to stage II, which is high risk melanoma with a 5-year survival rate of 45-79%. In stage III, the tumor begins to metastasize into regional lymph nodes and the skin. Stage IV has a 5-

year survival rate of 7-19% and the tumor cells form distant metastases by spreading through the peripheral blood and lymphatic system into the brain, lung, liver skin and the bone marrow (Balch, 2002). In general, patients with thin lesions ( $>0.75$  mm) have a 5-year survival rate of greater than 99%, whereas patients with lesions  $>4$  mm showed a 5-year survival rate of less than 50% (Balch et al., 2003).

According to the progression and invasion of melanoma, four subtypes can be described: superficial spreading melanoma, lentigo maligna melanoma, acral lentiginous melanoma, and nodular types (Reed and Martin, 1997).



**Fig.3: Different melanoma types.** (A) Patient with various melanoma lesions. (B) Superficial spreading and (C). nodular melanoma. (D) Acral lentiginous melanoma and (E) ocular melanoma. *Tsao, H et al., Genes & Dev. 2012*

### 1.3.1 STANDARD THERAPIES OF MALIGNANT MELANOMA

Surgical excision of the tumor with adequate margins and assessment for the presence of detectable metastatic disease along with short- and long-term follow-up belongs to the common standard procedure. However, surgery can be beneficial only for localized (primary) melanoma (Landthaler et al., 1989).

Diverse chemotherapeutic agents have been used for treatment against melanoma but without a significant increase in the overall survival of patients (Bajetta et al., 2002). For instance, dacarbazine and temozolamide are used as a single therapy or in

combination as a polychemotherapy. Systemic therapy as a single-agent chemotherapy has not been successful until now. In contrast, polychemotherapy has increased the response rate but not the survival rate (Serrone et al., 2000).

Patients with non-resectable distant metastases or with regionally and advanced melanoma are treated with radiotherapy. Although it is proven to be ineffective it is still commonly used after surgical resection to treat left tumor tissue (Mendenhall et al., 2008).

As adjuvant therapy interferon- $\alpha$  (IFN- $\alpha$ ) is used after surgical removal since it has been shown an improvement in disease-free survival when applied in a high-dose manner (Kirkwood et al., 1996). IFN- $\alpha$  has an anti-proliferative effect on tumor cells by the downregulation of oncogenes and the induction of tumor suppressor genes. Therefore, it is associated with an enhancement of tumor immunogenicity through the upregulation of MHC class I on tumor cells (Bracci et al., 2007; Fang et al., 2008). Therapy with IFN- $\alpha$  can increase the overall survival up to 15% (Mohr et al., 2003).

### 1.3.2 MELANOMA IMMUNOTHERAPY

Melanoma that is known as an immunogenic cancer has been considered as a prime target for immunotherapeutic approaches. Therefore, various immunological therapy strategies have been used in clinical trials as a promising treatment possibility compared to conventional therapies (Ortenberg et al., 2012). Ongoing efforts are made to find effective treatments of metastatic melanoma based on the immune system modulation (Sapoznik et al., 2012). Melanoma cells induce both innate and adaptive immune responses. Furthermore, immune cells are migrating to and infiltrate melanoma lesions (Thompson et al., 2010). Nevertheless, clinical studies are not very successful probably due insufficient immune cell number, their low cytotoxic potential and the inhibitory tumor microenvironment (Anichini et al., 2004; Harlin et al., 2006; Rosenberg et al., 2005; Yuan et al., 2010). In the past 25 years, immunological research has led to the improvement of our knowledge about molecular mechanisms of the immune response.

Moreover, this better understanding has allowed the development of new methods to induce and manipulate the anti-tumor immune response *ex vivo* and *in vivo* (Lizee et al., 2013). Recent studies have demonstrated that the regression of metastases after immunotherapy was linked to the activation of genes responsible for antigen presentation and interferon-mediated tumor rejection (Carretero et al., 2012). Several systemic treatment approaches include the administration of immune-stimulating cytokines, immunization with tumor cells or molecules, adoptive transfer of T cells, blocking antibodies against inhibitory molecules and inhibitors of mutated kinases (Klein et al., 2011; Rosenberg et al., 2008; Shepherd et al., 2010; Weber, 2010).

### 1.3.3 CYTOKINES

Cytokines are small signaling proteins that are secreted by various immune cells. These signaling molecules function as regulators and immune-modulating agents. IL-2 is produced by T cells and enables the growth and expansion of T cells, expressing IL-2 receptors on their surface (Morgan et al., 1976). In 1992, IL-2 was approved as the first immunotherapy for the treatment of metastatic renal cancer and in 1998 for the treatment of advanced melanoma (Rosenberg, 2012). Treatment with high-dose IL-2 can result in a complete regression, around 5-10% of patients, and additionally 10% in partial regression (Atkins et al., 1999; Rosenberg et al., 1998; Schwartzentruber et al., 2011; Smith et al., 2008). Furthermore, around 70% of complete responders to IL-2 therapy do not relapse which means that they are probably cured (Rosenberg et al., 1988).

Other cytokines such as IL-21 were also studied in early-phase clinical trials. This cytokine can activate cytotoxic T cells promoting potent anti-tumor response in pre-clinical studies and patients (Frederiksen et al., 2008; Hashmi and Van Veldhuizen, 2010; Rasmussen et al., 2010).

Other cytokines involved in the activation (such as IL-7 and IL-15) have been investigated for a possible clinical application (Klebanoff et al., 2011b). Some immunotherapies aim the differentiation of CD8<sup>+</sup> T cells at the tumor site toward effector and memory phenotypes since it is believed that these cells can elicit the most efficient anti-tumor response. In this respect, IL-7 and IL-15 used alone or in

combination may promote the maturation of anti-tumor CD8<sup>+</sup> T cells and induce the regression of melanoma metastases (Le et al., 2009).

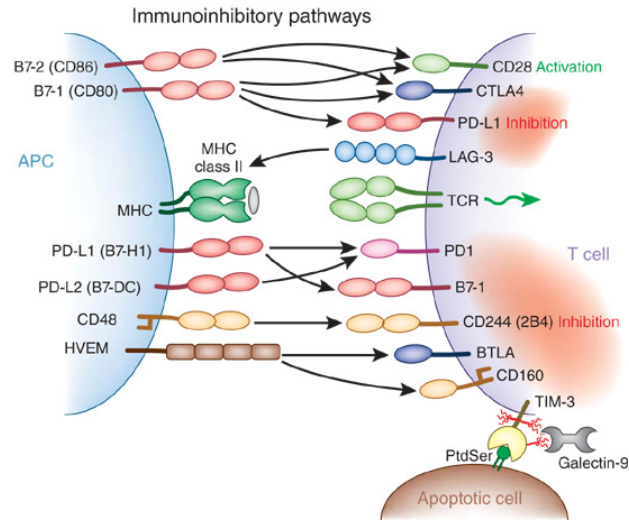
#### 1.3.4 CANCER VACCINES

Another approach to elicit potent anti-tumor immune responses without side effects is the immunization with whole tumor cells, tumor specific peptides, recombinant viruses, DC, and naked DNA combined with different adjuvants (Allison, 1999; Banchereau and Palucka, 2005; Gorelik and Flavell, 2001; McGilvray et al., 2009; Nestle et al., 1998; Zaks and Rosenberg, 1998). The aim is to stimulate innate and adaptive immunity to recognize and eliminate tumor cells. Although therapeutic cancer vaccines showed an impressive anti-tumor activity in numerous animal models, their clinical benefit in cancer patients leaves much to be desired. Multiple clinical trials have achieved only low overall survival with rare complete responders (Klebanoff et al., 2011a; Rosenberg et al., 2004).

#### 1.3.5 ANTIBODIES

Therapeutic antibodies have an advantage to circulate in the peripheral blood and lymph system where they can bind to targets. Monoclonal antibodies can be subdivided into either inhibitory or activating antibodies against co-inhibitory or co-stimulatory molecules respectively. Various approaches have been used in recent clinical trials (Ascierto et al., 2010; Robert et al., 2011; Sznol, 2010; Vonderheide et al., 2007).

The lack of co-stimulation leads to peripheral T cell tolerance with no immune response or cell death through apoptosis (Croft, 2009). Pre-clinical studies in animal models have shown that sustaining co-stimulatory signaling will enhance T cell reactivity against tumor antigens (Croft, 2009). This facilitates an induction of cytotoxic CD8<sup>+</sup> T cell response, leading to the tumor elimination by cell death (Croft, 2009). Some monoclonal antibodies utilized in the clinical trials are mentioned below.



**Fig.4: Immunoinhibitory pathways.** Modified from Freeman et al. 2012 (Freeman and Sharpe, 2012).

*Ipilimumab*, a monoclonal antibody, has been approved in March 2011 as an antibody-based immune therapy for late-stage metastatic melanoma. This antibody increases the anti-tumor immunity by inhibiting CTLA-4, which acts as a negative regulator in the immune response (Korman et al., 2006). CTLA-4 is a homologue of T cell costimulatory CD28 but with higher binding affinity to its ligand (e.g. CD86). Once CTLA-4 is upregulated on the surface of activated cytotoxic T lymphocytes, it leads to cell cycle arrest and inhibition of proliferation of these cells, and as a consequence causes immune evasion (Korman et al., 2006; Peggs et al., 2006). In clinical trials, the treatment with ipilimumab of patients with inoperable stage III and IV melanoma achieved response rates range from 5-15%, with durable responses (Thumar and Kluger, 2010). Moreover, combination studies, including ipilimumab, to attain higher response rates in melanoma patients are in progress (Sondak et al., 2011).

Another antagonistic antibody, blocking the negative T cell checkpoint, is directed against programmed death cell death (PD)-1 (MDX-1106; BMS-936558). PD-1 is expressed mainly on activated T cells and APCs (Weber, 2010). Its ligands are PD-L1 (B7-H1) and PD-L2 (B7-H2). PD-L1 is expressed on macrophages, DC, B cells and T cells (Keir et al., 2008; Weber, 2010). High level of PD-L1 expression was also found on multiple tumor cells including melanoma (Dong et al., 2002; Kronig et al., 2012). It is believed that melanoma cells can induce durable PD-1 signaling which leads to T cell dysfunction and T cell exhaustion (Kronig et al., 2012). Therefore, since PD-1 is



expressed on tumors and stroma cells, PD-1 blockade might restore immune cell functions and induce anti-tumor response. Pre-clinical studies on PD-1 deficient mice showed an increased T cell response and cytokine production, leading thereby to the inhibition of the hematogenous spread of B16 melanoma cells (Iwai et al., 2002). The blockade of PD-L1 could enhance therapeutic efficiency of the combined immunotherapy in mice with B16 melanoma (Pilon-Thomas et al., 2010). Based on these findings, early phase clinical trials were initiated with anti-PD-1 antibodies as a single agent. Objective tumor response could be observed in 37.5% of patients (Brahmer et al., 2010). Furthermore, preliminary data suggest a relationship between PD-L1 expression on tumor cells and clinical response since patients with PD-L1-negative tumors had no objective response (Topalian et al., 2012).

The combination of MDX-1106 with ipilimumab in patients with stage III or IV melanoma has been initiated in a phase I clinical trial (Davar et al., 2013).

### 1.3.6 ADOPTIVE CELL THERAPY

Based on the fact that host's immune system is capable of producing anti-tumor response, various attempts have been performed to evoke an effective immune-mediated elimination of the tumor (Wu et al., 2012). As a highly promising approach, adoptive transfer of tumor-reactive autologous T cells into cancer patients is currently under intense investigation (Shapira-Frommer and Schachter, 2012). This strategy utilizes *ex vivo* cultured autologous T lymphocytes with specificity for tumor antigens. For an efficient treatment, these lymphocytes need to have the ability for the homing to tumor sites and the potential to destroy tumor cells *in vivo* (Shapira-Frommer and Schachter, 2012; Zito and Kluger, 2012).

#### Adoptive therapy of tumor infiltrating lymphocytes

Since Steven Rosenberg first demonstrated that T lymphocytes infiltrated in melanoma metastasis could be grown in presence of IL-2 and are capable to recognize melanoma cells, adoptive cell therapy (ACT) protocols have developed (Dudley et al., 2008). In

1998, it was demonstrated that these autologous tumor infiltrating lymphocytes (TILs) have the ability to mediate tumor regression (Rosenberg et al., 1988). However, the durability rates were very low or absent. Therefore, lymphodepletion regimens were introduced prior to adoptive transfer. Lymphodepletion was performed to eliminate immune regulatory cells, to reduce the competition for growth factors and also to achieve an increase in serum concentrations of IL-7 and IL-15, which promote T cell growth (Dudley et al., 2008).

After using cyclophosphamide to deplete immune suppressor cells and adding a high dose IL-2 administration to the adoptive transfer, an objective response of 34% among 86 melanoma patients was detected (Rosenberg et al., 1994). Among different lymphodepletion protocols, such as fludarabine, cyclophosphamide and combination of both, the total body irradiation (TBI) was the most effective in maintaining transferred polyclonal TIL populations and expansion of cytotoxic CD8<sup>+</sup> T cells in the blood circulation *in vivo* (Bernatchez et al., 2012; Dudley et al., 2008). Lymphodepletion prior to adoptive transfer of TILs in combination with high dose bolus IL-2 has been shown to lead to around 50% of clinical response rates in patients with metastatic melanoma in nonrandomized phase II clinical trials (Dudley et al., 2005; Laurent et al., 2010). Those results remain to be validated in phase III clinical trials, although they are promising.

### 1.3.7 TARGETED THERAPY

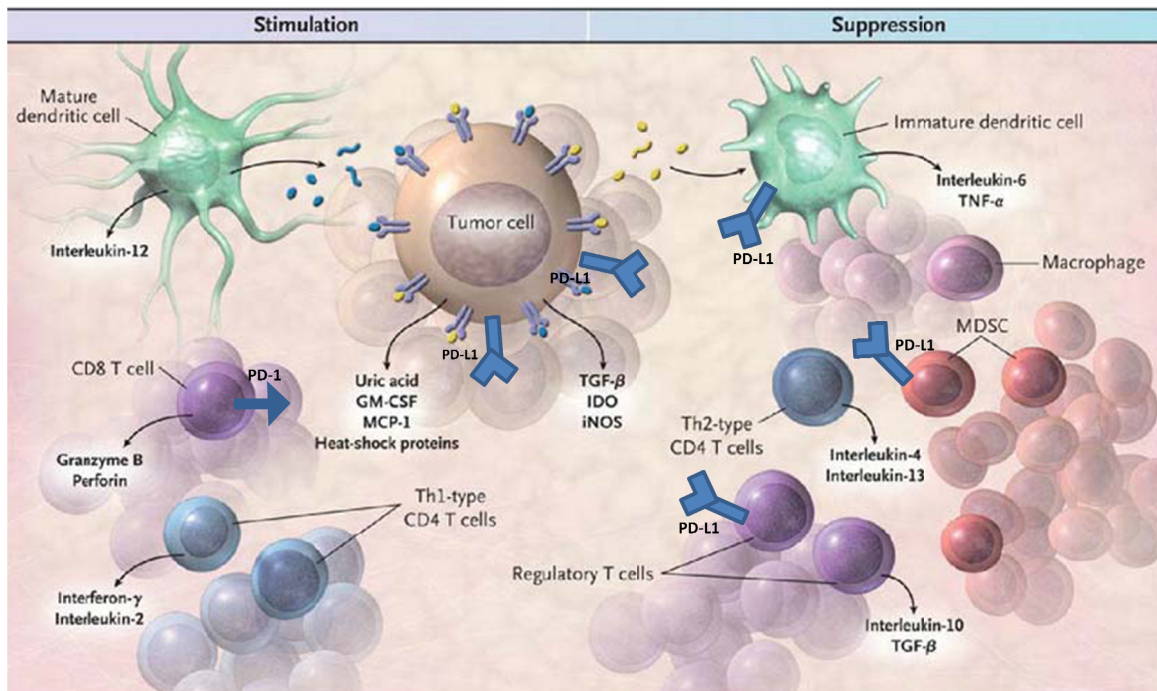
Another therapy approach is targeting signaling pathway leading to the tumor progression (Inamdar et al., 2010). Several such drugs including BRAF and MEK inhibitors are now under evaluation (Spagnolo and Queirolo, 2012). Promising results have been recently achieved in phase I and II trials. A BRAF-inhibitor *Vemurafenib* has been approved in August 2011 for the treatment of patients with unresectable or metastatic melanoma with BRAF V600E mutation (Bernatchez et al., 2012; Ribas et al., 2012). Phase I and II trial that combined a selective BRAF inhibitor and a selective MAPK kinase (MEK) inhibitor, showed a significant improvement of progressive-free survival in patients with metastatic melanoma (Flaherty et al., 2012). In addition, the

treatment with BRAF inhibitors has been shown to increase T cell infiltration of melanoma lesions (Boni et al., 2010; Comin-Anduix et al., 2010; Wilmott et al., 2012).

In summary, tremendous efforts have been made for the development of new therapeutic strategies of metastatic melanoma. Multiple treatment options show promising results. However, to attain an efficient immune response against the tumor, a combination of different approaches, including adoptive immunotherapy seem to be the most auspicious way to go.

### 1.3.8 TUMOR-INDUCED IMMUNOSUPPRESSION

Although multiple therapeutic approaches show promising effects on the anti-tumor reactivity of T cells; such T cells activation does not always correlate in clinical trials with the therapeutic benefit. The immunosuppressive tumor microenvironment (TME) might play a role in the failure of these treatment approaches (Wu et al., 2012). Tumors have developed mechanisms to create an immunosuppressive network which can negatively influence immunotherapy. Although T cells are capable of infiltrating tumors, they fail to eliminate tumor cells due to their defected lytic machinery or the lack of active attack (Hanahan and Weinberg, 2011). This might be due to the presence of co-inhibitory molecules or the downregulation of tumor specific antigens or MHC molecules on tumor cells (Vesely et al., 2011). Since the downregulation of MHC class I favors tumor recognition by natural killer (NK) cells, tumor cells adjust their MHC I expression to minimize the recognition by either NK or T cells (Vivier et al., 2012). Furthermore, effector T cells are not capable to function properly due to the inhibition by CD4<sup>+</sup>CD25<sup>high</sup>, FoxP3<sup>+</sup> regulatory T cells (Tregs), CD19<sup>+</sup> CD25<sup>high</sup> regulatory B cells, IL-13 producing NKT cells in the TME (Jacobs et al., 2009; Jacobs et al., 2012; Kuniwa et al., 2007; Olkhanud et al., 2011). Additionally, local myeloid cells are trimmed by TME to convert into myeloid-derived suppressor cells (MDSCs).



**Fig.5: Immunostimulatory and immunosuppressive factors in the tumor microenvironment.** The TME consists of different immunosuppressive cells like MDSCs, Tregs and immature DC. The immunosuppression can occur directly, e.g. through the interaction of PD-1 on activated T cells with its ligand PD-L1 on suppressive cells in the TME or indirectly, e.g. through the secretion of TGF- $\beta$ , IL-13 and IL-10. Modified from (Finn, 2008).

MDSCs have different phenotypes. Two main MDSC subtypes have been found in tumor-bearing mice, i.e. granulocytic MDSCs (G-MDSC, CD11b<sup>+</sup> Gr1<sup>high</sup> Ly6G<sup>+</sup>) and monocytic MDSCs (M-MDSC, CD11b<sup>+</sup> Gr1<sup>high</sup> Ly6C<sup>high</sup>) (Kusmartsev and Gabrilovich, 2006; Ostrand-Rosenberg and Sinha, 2009; Serafini et al., 2006). Both subtypes are capable to inhibit effector T cells by antigen-specific and non-specific mechanisms (Gabrilovich and Nagaraj, 2009). G-MDSCs produce reactive oxygen species (ROS) mediated through the increased activity of NADPH oxidase (NOX). In the absence of NOX2 activity, MDSCs lose their ability of T cell inhibition and differentiate into mature DC (Corzo et al., 2009). The activation of inducible nitric oxide synthase (iNOS) and arginase (ARG)-1 leads to L-arginine depletion and increased production of nitric oxide (NO) (Dolcetti et al., 2010; Movahedi et al., 2008; Youn et al., 2008). MDSCs can block antigen recognition on tumor cells by nitration of tyrosines in the T-cell receptor TCR-CD8 complex and their inhibition of the interaction with MHC molecules (Nagaraj et al., 2007). The suppressive activity of ARG-1 is reflected by the downregulation of TCR  $\zeta$ -chain expression (Rodriguez et al., 2002). Recently, it has been reported that the manipulation of the TME with phosphodiesterase-5 inhibitor sildenafil could partially

restore  $\zeta$ -chain expression in T cells and significantly prolong survival of tumor-bearing mice (Meyer et al., 2011).

Other approaches of T cell inhibition is the interaction of PD-1 expressed on T lymphocytes with PD-L1, which is expressed on tumor cells, MDSCs and Tregs (Jacobs et al., 2009; Ozao-Choy et al., 2009), Fig.5. PD-1 negatively regulates T cell responses through interaction of PD-1 with PD-L1, which leads to reduced proliferation and IFN- $\gamma$  secretion (Freeman et al., 2000).

## 1.4 Melanoma mouse model

To develop new strategies of melanoma immunotherapy, it is essential to use a reliable animal melanoma model. Conventional transplantation mouse melanoma models (e.g., B16) are based on the transplantation of tumor cells, in which the natural history of the disease is not comparable with the clinical situation. Therefore, the *ret* transgenic mouse melanoma model, which closely resembles human melanoma with respect to tumor genetics, histopathology and clinical development is the better choice (Kato et al., 1998; Umansky et al., 2008). In this spontaneous transgenic melanoma model, the human *ret* receptor tyrosine kinase is overexpressed in melanocytes under the control of the mouse metallothionein-I (MT) promoter-enhancer. Activation of *ret* kinase overexpression during tumor progression is associated with the activation of other downstream signaling molecules such as mitogen-activated protein kinase (MAPK), Erk2 and c-Jun. During the malignant stage, tumors showed as well a high activity of matrix metalloproteinases (MMP)-2 and MMP-9 (Kato et al., 1998; Kato et al., 2001).

The mechanism of spontaneous melanoma development in *ret* transgenic mice is still under investigation. So far is known that *ret* kinase overexpression during tumor progression is associated with the activation of other downstream signaling molecules such as mitogen-activated protein kinase (MAPK), Erk2 and c-Jun (Kato et al., 1999). During the malignant stage, tumors showed also a high activity of matrix metalloproteinases (MMP)-2 and MMP-9 (Kato et al., 1999; Kato et al., 2001; Kato et al., 2006; Phay and Shah, 2010).

Mice develop spontaneously malignant cutaneous melanoma, metastasizing to lymph nodes, lungs spleen, kidney, liver and brain (Kato et al., 1998; Kato et al., 2004). This metastatic profile resembled that of human malignant (Polsky et al., 2002).

## 1.5 Aim of the study

Melanoma is known to be very aggressive and associated with high lethality rates once it metastasized. It shows poor response to common treatments. Therefore, the investigation of novel therapeutic strategies is very important. Immunotherapeutic approaches are particularly promising because of the immunogenic properties of melanoma.

The objective of this work is to determine whether melanoma-specific memory T cells, which have been previously shown to persist in the bone marrow of patients with different cancer types, can be utilized for an adoptive immunotherapy against melanoma in *ret* transgenic mice. Therefore, the phenotype and effector functions of *in vitro* activated melanoma-specific memory T cells from the bone marrow of *ret* transgenic mice were characterized *in vitro*. Furthermore, migration properties of these cells and their anti-tumor effect were examined *in vivo*. In addition, an adoptive transfer of melanoma-specific T cells with was performed after modulation of the tumor microenvironment with PDE-5 inhibitor sildenafil.

# 2 Materials

## 2.1 Mice

All mice were crossed and kept under specific pathogen-free conditions in the animal facility of the German Cancer Research Center (Heidelberg). Experiments were performed in accordance with government and institute guidelines and regulations.

### 2.1.1 *RET* TRANSGENIC SPONTANEOUS MELANOMA MOUSE MODEL

*Ret* transgenic mice (C57BL/6 background) express the human *Ret* proto-oncogene in melanocytes under the control of mouse metallothionein-I promoter-enhancer. These mice were kindly provided by Dr. I. Nakashima (Japan) (Kato et al., 1998) and were kept under the guidelines of the animal facility of the German Cancer Research Center.

### 2.1.2 TRP-2 TCR TRANSGENIC MOUSE MODEL

Mice with T cell receptor transgene (TCR Tg) specific for the TRP-2<sub>(180-188)</sub> epitope were kindly provided by Dr. A. Hurwitz (USA), crossed and kept under the guidelines of the animal facility of the German Cancer Research Center.

### 2.1.3 C57BL/6

The C57BL/6 (BL/6) wild type mice were provided by Elevage Janvier, crossed and kept in the mouse facility of the German Cancer Research Center.

## 2.2 Reagents

### 2.2.1 CHEMICALS

3-Amino-9-ethyl-carbazol (AEC) tablets	Sigma, Cat.#A6926
Ammonium chloride (NH <sub>4</sub> Cl)	Merck,Cat.#101141
Bovine serum albumin (BSA)	Sigma, Cat.#7030-50G
Concanavalin A	Amersham Biosciences, Cat. 170450-01
Dimethylsulfoxid (DMSO)	Merck, Cat.#109678
0,5M EDTA (pH 8.0)	GIBCO, Cat. #15575-098
100% Acetic acid (CH <sub>3</sub> COOH)	Merck, Cat.#100063
Fatal bovine serum (FBS)	PAN Biotech GmbH, Cat.#3702- P260718
Isofluran	DeltaSelect
Potassium hydrogen carbonate (KHCO <sub>3</sub> )	Roth, Cat. #P748
Sodium acetate (CH <sub>3</sub> COONa)	neoLab, Cat.#4720
Sodium azide (NaN <sub>3</sub> )	Roth, Cat.#K305
Sodium carbonate (Na <sub>2</sub> CO <sub>3</sub> )	AppliChem, Cat.#A3900
N,N – Dimethylformamid	Sigma, Cat. D-4551
0.4% Trypan blue solution	Sigma, Cat.#T8154
Tween20	Sigma, Cat. #P-2287



## 2.2.2 BUFFERS AND MEDIA

### Enzyme-linked immunosorbent spot (ELISPOT)

<b>AEC buffer</b>	1 AEC tablet (20mg)
	Dimethylformamide 2,5 ml
	0,2M Sodium acetate 8,4 ml
	0,2M acetic acid 3,5 ml
	H <sub>2</sub> O bidest. 35,6 ml
	Hydrogen peroxide 25 µl

An AEC tablet (20 mg) was dissolved in 2.5 ml of dimethylformamide in a 50 ml Falcon tube. Then, 8.4 ml of 0.2 M sodium acetate, 3.5 ml of 0.2 M acetate acid and 35.6 ml H<sub>2</sub>O were added. After mixing, the solution was filtered through 0.45 µm filter and 25 µl of H<sub>2</sub>O<sub>2</sub> was added. The prepared AEC buffer should be kept in dark and used within one month after preparation.

<b>Working solution</b>	1x PBS
	BSA, 0,5% (w/v)

#### Coating buffer

Solution A: 1,59g Na<sub>2</sub>CO<sub>3</sub> in 100 ml H<sub>2</sub>O

Solution B: 2,93g NaH<sub>2</sub>CO<sub>3</sub> in 100 ml H<sub>2</sub>O

**Working coating buffer** 1 ml A + 1 ml B + 8 ml H<sub>2</sub>O  
pH 9.6

**Washing buffer** 1x PBS, Tween 20, 0,25% (v/v)

### Flow cytometry (FACS)

**FACS buffer** 1 x PBS  
2% FBS,  
0.2% NaN<sub>3</sub>  
2 mM EDTA



	APC-H7	rat IgG2a	<b>BD 560182</b>
	Alexa Flour	rat IgG2a	<b>BioLegend</b>
<b>anti-mouse CD3</b>	PerCP-Cy5.5	rat IgG2a	<b>BD 555276</b>
	APC	rat IgG2a	<b>BD 553066</b>
	FITC	rat IgG2a	<b>BD 553062</b>
	V500	syrian hamster IgG2	<b>BD Horizon</b>
	pacific blue	syrian hamster IgG2	<b>BD 558214</b>
<b>anti-mouse CD45RB</b>	FITC	rat IgG2a	<b>BD 553100</b>
<b>anti-mouse CD62L</b>	FITC	rat IgG2a	<b>BD 553150</b>
	APC	rat IgG2a	<b>BD 553152</b>
<b>anti-mouse I-A/I-E</b>	FITC	rat IgG2a	<b>BD 553623</b>
<b>anti-mouse CD4</b>	PE	rat IgG2b	<b>BD 553730</b>
	pacific blue	rat IgG2b	<b>BioLegend</b>
<b>anti-mouse CD40</b>	PE	rat IgG2a	<b>BD 553791</b>
<b>anti-mouse CD44</b>	PE	rat IgG2b	<b>BD 553134</b>
	FITC	rat IgG2b	<b>BD 561859</b>
	PE-Cy7	rat IgG2b	<b>eBioscience</b>
<b>Anti-mouse CD69</b>	Alexa Flour	armenian hamster IgG	<b>BioLegend</b>
	APC-Cy7	armenian hamster	<b>BD 561240</b>
	PE-Cy7	armenian hamster IgG	<b>BioLegend</b>
<b>anti-mouse CD80 (B7.1)</b>	PE	hamster IgG2 k	<b>BD 553769</b>
<b>anti-mouse CD86 (B7.2)</b>	PE	rat IgG2a	<b>BD 553692</b>
<b>Anti-mouse CD45.2</b>	V500	rat IgG2a	<b>BD Horizon</b>
	PerCP-Cy5.5	IgG2a, κ	<b>BD 552950</b>
<b>CD279 (PD-1)</b>	FITC	armenian hamster IgG	<b>eBioscience</b>

	PE	armenian hamster IgG	eBioscience
CD274 (B7-H1, PD-L1)	PE	rat IgG2a $\lambda$	BD 558091

Table 2: Antibodies for flow cytometry

**ELISPOT**

	Name	Reactivity	Clone	Company/cat. #
Primary antibody	IFN $\gamma$	rat anti-mouse	RMMG-1	Biosource Europe AMC 4834
Secondary antibody	IFN $\gamma$ , biotinylated	rat IgG <sub>1</sub> , $\kappa$	XMG1.2	BD 554410
Streptavidin-HRP	-	-	-	BD 557630

Table 3: Antibodies used for ELISPOT

**Other antibodies/ Dextramer/ peptides**

Antibody	Format	Reactivity	Company/ cat.#
anti-mouse CD274 (B7-H1)	purified	rat IgG2a, $\lambda$	eBioscience 14-5982-82
		rat IgG <sub>2b</sub> , $\kappa$	BD 553142

Table 4: Other antibodies

	Format	Allele	Peptide	Company/ cat.#
MHC -Dextramer	PE	H-2 Kb	SVYDFFVWL	Immudex JD2199
TRP-2 <sub>(180-188)</sub> peptide	purified		SVYDFFVWL	DKFZ Heidelberg

Table 5: TRP-2 Dextramer and peptide

## 2.3 Kits

<b>T cell enrichment</b>	Dynal® Mouse T Cell Negative Isolation Kit, Invitrogen, Cat. #114.13
<b>IFN-<math>\gamma</math> secretion assay</b>	Mouse IFN- $\gamma$ Secretion Assay – Cell Enrichment and Detection Kit, Miltenyi, Cat. # 130-090-517

## 2.4 Routine laboratory materials

### 2.4.1 DEVICES

<b>ELISPOT Reader</b>	Bioreader 3000, Biosys
<b>FACS machine</b>	FACS Canto II, 8 colors, BD
<b>Refrigerator (-80 °C)</b>	HeraFreeze, Heraeus
<b>Incubator</b>	HeraCell, Heraeus
<b>Refrigerator (-20 °C)</b>	Premium, Liebherr
<b>Microscopes</b>	DMIL, Leica
<b>pH meter</b>	766, Calimatic
<b>Laminar flow</b>	Hera Safe, Thermo Electron Cooperation
<b>Vortex</b>	REAX top, Heidolph Vortex Genie 2, Scientific Industries
<b>Balance</b>	BP 3100P, Sartorius
<b>Water bath</b>	DC3, HAAKE / GFL

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<b>Centrifuges</b>	Labofuge 400R, Heraeu Biofuge primo R, Heraeus Varifuge K, Heraeus
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#### 2.4.2 ROUTINE LABORATORY MATERIALS

<b>ELIPOT plates</b>	Silent Screen Plate 96-well clear w/o Lid Biodyne B Membrane N/Str PS, Nunc, Cat. # 256154
<b>Needles</b>	0,4x19 mm Mikrolance, BD 0,3x13 mm Mikrolance, BD
<b>Pipets</b>	2-20 µl, 20-200 µl, 200-1000µl, Rainin
<b>Object carrier</b>	76x26 mm SuperFrostPlus, Menzel-Gläser, Cat. J1800AMNZ
<b>Cover glass</b>	24 x 24 mm , Roth
<b>Tubes 15 ml / 50 ml</b>	Polypropylen, BD Falcon 0.5 ml tubes, Eppendorf 1.5 ml tubes, Eppendorf 2 ml tubes, Eppendorf
<b>Syringes</b>	1 ml Plastipak, BD
<b>Cell culture plates</b>	96-well-Platte, Greiner 24-well plate, TPP 6-well plate, Greiner

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**Cell strainer** 40  $\mu\text{m}$ ; 100  $\mu\text{m}$ , BD Falcon

**Sterile filter** 0,45  $\mu\text{m}$ , sterile, Rotilabo, Roth

### 2.4.3 SOFTWARE FOR DATA ANALYSIS

**FlowJo** (Version 7.6.1) Tree Star, Inc., Ashland, USA

**GraphPad PRISM** (Version 5) GraphPad Software, Inc., San Diego, USA

## 3 Methods

### 3.1 Preparation of single cell suspensions from mouse organs

Mice were sacrificed by cervical dislocation.

#### 3.1.1 SPLEEN

Mouse spleens were collected in 15 ml Falcon tube with 2 ml of ice-cold PBS. Single cell suspensions were prepared by smashing the spleen with the plunger of a 5 ml syringe through a 40  $\mu$ m cell strainer. Then the cells were washed with cold PBS once at 1200 rpm for 10 min. Red blood cells were lysed with 1 ml of lysis buffer on ice for 3 min followed by the addition of 9 ml PBS to stop the reaction. Cells were centrifuged (1200 rpm, 10 min) and the cell pellets were resuspended in appropriate buffers for different assays.

#### 3.1.2 LYMPH NODES

Isolated mouse lymph nodes were smashed through a 40  $\mu$ m cell strainer using plunger of a 5 ml syringe. The cell strainer was washed with cold PBS to remove the remaining cells. The cells were washed with PBS once at 1200 rpm, 10 min and resuspended in FACS buffer for flow cytometry.



### 3.1.3 BONE MARROW

Freshly isolated femurs and tibiae from the mouse were shortly treated with 70% ethanol to avoid contamination. Then the bones were cut at both ends and the BM was flushed with ice cold PBS using a 10 ml syringe with a 23G needle. The cells were collected in a 10 cm Petri dish and dissociated by pipetting with a 1 ml pipette. Then, cells were washed once with PBS and red blood cells were lysed with 1 ml of lysis buffer for 3 min on ice. After washing with cold PBS Cold PBS (1200 rpm, 10 min), the pellet was resuspended in an appropriate buffer depending on the following assay.

### 3.1.4 TUMOR

The freshly isolated tumor mass was smashed through a 40  $\mu$ m cell strainer by a 5 ml syringe plunger into a 50 ml Falcon tube. Cells were washed with cold PBS at 1200 rpm for 10 min. The cell pellet was resuspended in an appropriate buffer.

### 3.1.5 ASSESSMENT OF CELL CONCENTRATION

The cell number was determined using a Neubauer counting chamber. A sample of 10  $\mu$ l was diluted with trypan blue in a ratio 1:100 and two quadrates were counted and divided by two to attain a precise number of cells. The following formula was used:

$$n \times 10^4 \times \text{dilution factor (10)} = \text{cell number / ml}$$

## 3.2 Generation of bone marrow derived dendritic cells

### 3.2.1 DC CULTURE

DC were generated from BM precursors of C57BL/6 mice. A single cell suspension from the BM was prepared as described and resuspended in RPMI-1640 medium supplemented with 10% FCS and 1% P/S (complete medium). To deplete all adherent

non-DC precursors, cells were incubated in a 75 cm<sup>2</sup> flask overnight at 37°C, 5% CO<sub>2</sub> and 95% humidity. Then the supernatant containing precursor cells was removed, washed with RPMI-1640 + 10% FCS and 1% P/S. After centrifugation the pellet was washed with RPMI-1640 + 10% FCS and 1% P/S and resuspended in complete RPMI-1640 medium (1x10<sup>6</sup>/ml). 1000 U/ml of recombinant murine GM-CSF (rmGM-CSF) and 1000 U/ml of recombinant murine IL-4 (rmIL-4) were added. Three ml of the cell suspension was plated in each well of a 6-well plate followed by a 7 days culture at 37°C, 5% CO<sub>2</sub> and 95% humidity. On day 3 and 6, 1.5 ml of medium from each well were carefully removed, collected in a 50 ml Falcon tube and centrifuged (1300 rpm, 5 min). Next the pellet was resuspended in the same volume of complete RPMI-1640 medium. GM-CSF and IL-4 were added (each 1000 U/ml), and the wells were filled with freshly prepared medium. The day before DC harvesting, DC were activated with 3 µg/ml of CpG1668 phosphorothioate (PTO)-modified. In addition, the PD-L1 on DC was blocked with 10 µg/ml of purified anti-PD-L1 antibody. DC were pulsed with 500 µg tumor lysate per well overnight. The pulsing of stimulated DC with TRP-2 derived peptide (TRP-2<sub>180-188</sub>) was performed for 2 hours. Then, DC were harvested by carefully pipetting and collected in a 50 ml Falcon flask for further procedures.

### 3.2.2 PHENOTYPE ANALYSIS OF DC

DC phenotype was detected by flow cytometry. In order to avoid unspecific binding of the antibodies to the Fc receptor, cells were incubated for 15 min with Fc block solution. After washing with FACS buffer samples were incubated with mAbs against MHC class II-PE, CD11c-APC, CD40-FITC, CD80-PerCP/Cy5.5 and CD86-PE-Cy7 for 30 min at 4°C in the dark followed by two washing steps and resuspension in 200 µl of FACS buffer.

### 3.2.3 PREPARATION OF TUMOR LYSATES

In order to make intracellular proteins accessible for DC pulsing with tumor antigens, tumor cells were lysed by mutual freezing in liquid nitrogen and thawing at 37°C (Feurerer et al., 2001a).

The tumors were squeezed through 45 µm cell strainers and collected in a petri dish with 2 ml of ice cold PBS. The suspension was collected in a preferably small amount of PBS (1-2 ml/tumor) so that in the end a high concentration of the protein was attained. The suspension was transferred into a 15 ml flask and flash-frozen for 10 min in liquid nitrogen. Then the cells were thawed at 37°C in water bath for another 10 min. This procedure was repeated four times. Afterwards the cell debris was centrifuged (2000 rpm, 7 min, 4°C), the supernatant containing protein was transferred into 2 ml Eppendorf tubes and the tumor lysates (TL) were frozen at -80°C until use. The protein concentration was analyzed by Bradford Assay according to manufacturers' instruction.

## 3.3 Negative selection of T cells from the bone marrow of *ret* transgenic mice

The negative T cell isolation kit is based on a monoclonal antibody cocktail which binds all non-T cells like B-and NK cells, monocytes, macrophages, granulocytes and DC. Magnetic beads are bound to these antibodies and the bead-bound cells can be subsequently separated on a magnet and discarded. The benefit of this method is that the untouched T cells in the remaining eluate are not stimulated and can be used for further experiments.

The negative selection of T cells from the BM of at least 10 mice was performed using the Dynal® Mouse T Cell Negative Isolation kit according to manufacturer's protocol. Then the cells were counted, washed with PBS and the pellet was resuspended in complete medium. Then, cells were adjusted to an appropriate concentration for the co-culture with DC.

## 3.4 Co-culture of T cells with dendritic cells

### 3.4.1 CO-CULTURE

In order to stimulate melanoma antigen-specific T cells from the bone marrow of *ret* transgenic mice, they were co-cultured with tumor antigen loaded DC as described in 6.2.1. Within 40 hours of co-culture, only T cells, which already encountered tumor-specific antigens (memory T cells), can be stimulated. The co-culture was performed in 96-well plates (round-bottom).

For the stimulation of memory T cells  $5 \times 10^5$  freshly isolated T cells (6.3) were cultured with  $1 \times 10^5$  *in vitro* generated tumor antigen-loaded DC (6.2.1) in 150  $\mu$ l of complete medium per well (T cell: DC ratio= 5:1) at 37°C, 5% CO<sub>2</sub> and 95% humidity. DC were derived from the BM progenitors of C57BL/6 wild type mice. After 40 h of co-culture cells were harvested by carefully pipetting and used for flow cytometry or adoptive transfer experiments.

### 3.4.2 T CELL EXPANSION

To detect optimal conditions for *in vitro* activation and expansion of memory T cells, a cytokine cocktail containing IL-21 (25 ng/ml), IL-15 (50 ng/ml) and IL-7 (10 ng/ml) was added to the co-culture on day 2 and the medium was exchanged every 2-3 days with fresh cytokines.

## 3.5 Generation of TRP-2-specific CD8<sup>+</sup> effectors

For generation of TRP-2-specific CD8<sup>+</sup> T cells, pooled lymph node cells and splenocytes from TCR tg mice were cultured in T cell medium with 1  $\mu$ M of TRP-2<sub>(180-188)</sub> peptide and human recombinant IL-2 (20 IU/ml, PeproTech) for 3 days. Then, cells were expanded with media containing 20 IU/ml IL-2 for another 2 days. On day 5, cells were centrifuged, adjusted to a number of  $1 \times 10^7$  cells in 100  $\mu$ l and injected into tumor-bearing *ret* transgenic mice. In case of migration experiments, cells were stained with 5-

(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) before the adoptive transfer as described by Parish CR, *et al.* (2009). Therefore,  $1 \times 10^7$  cells were resuspended in 0.5 ml PBS supplemented with 10% FCS (PBS-10%FCS) and 0.5 ml CFSE solution (1  $\mu$ M solution: 2  $\mu$ l from 5 mM CFSE stock solution mixed with 10 ml PBS). Then, cells were incubated at 37°C for 10 min, gently shaken and 4 ml of pre-warmed PBS-10% FCS was added to stop the reaction. Cells were washed in PBS (1400 rpm, 8 min, 4°C) and resuspended in pre-warmed sterile PBS and counted.

## 3.6 Flow cytometry

Flow cytometry allows the simultaneous multiparametric analysis of single cells based on the emission of electronic light when a cell passes the electronic detection apparatus. The fluid containing cells which are labeled with fluorescent markers are sucked through a capillary and are focused in the center. Thereby, the cell aggregates are dissolved by the strong acceleration which allows the passage through a laser beam of a single cell. The cell that passes the laser beam emits scattered light, which is dependent on the size, structure of the cell membrane and the intracellular content of the cell. Two different scatterings exist, the forward scatter (FSC) which contains information about the size and the side scatter (SSC) that is a measure for granularity of the cell (Baumgarth, N. 2000, Radbruch, 2000). Since the emitted fluorescence is proportional to the amount of antibody-bound epitopes, with the help of fluorescent markers phenotype of the cell can be determined. The more antigens are expressed on the cell bound to the antibody the stronger is their emission of the light. Different fluorescent dyes have different absorption and emission spectra which allows staining of different epitopes of a cell.

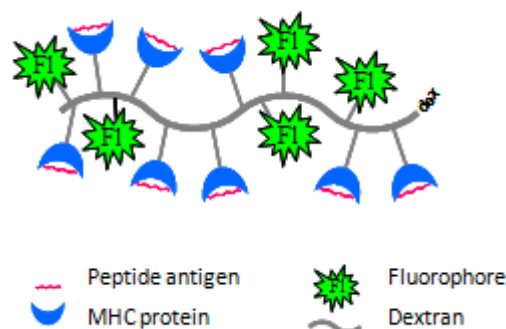
### 3.6.1 STAINING OF CELL SURFACE MARKERS

$1 \times 10^6$  cells/well cells were transferred into a 96-well plate (round bottom). After centrifugation (1900 rpm, 1 min) the supernatant was discarded and the pellet was resuspended in 50  $\mu$ l of FACS buffer containing Fc-block. After 15 min of incubation at

4°C cells were washed with 150 µl/well FACS buffer and centrifuged (1900 rpm, 1 min). Afterwards, the cell pellet was resuspended in 50 µl FACS buffer containing fluorescent-conjugated antibodies (list of used antibodies see 2.2.4). The cells were incubated for at least 20 min at 4°C in the dark. Before measurement the cells were washed twice with FACS buffer and the pellet was resuspended in 10-200 µl FACS buffer. The measurement was performed with the FACSCanto II (BD) using the BD Diva Software V.6.1.1. FlowJo software 7.6.1 (Tree Star) was used to analyze at least 100,000 events. Data were expressed as dot plots.

### 3.6.2 MHC DEXTRAMER STAINING OF TRP-2-SPECIFIC CD8 T CELLS

The MHC Dextramer staining is based on the interaction of the TCR on T cells and MHC complex on antigen-presenting cell (APC). The multimer staining technique was developed by Altman et al. (1996). It enables the detection of antigen-specific T cells *ex vivo*. MHC multimers are reagents that carry multiple MHC-peptide complexes. They have the ability to bind simultaneously to multiple TCRs on a single T cell. The antigen which the MHC complexes contain is the TRP-2 derived peptide<sub>180-188</sub> (SVYDFVWL).



**Fig.6: Schematic drawing of the MHC Dextramer. Source: Immudex, Denmark**

After the Fc-block cells were incubated with 10 µl of PE-labeled MHC Dextramer for 10 min at 4°C in the dark. Then antibodies for CD8 T cells were added followed by incubation for another 20 min. Then the cells were washed with FACS buffer twice and

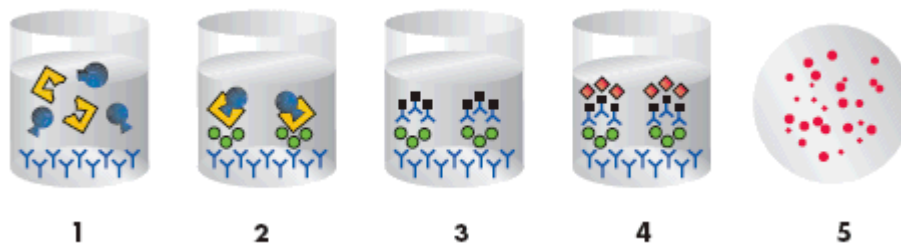
the pellet was resuspended in 100-200  $\mu$ l FACS buffer for the measurement using FACSCanto II.

## 3.7 IFN- $\gamma$ ELISPOT

### 3.7.1 PRINCIPLE OF THE ASSAY

ELISPOT assays have an unsurpassed sensitivity in detecting low frequency antigen-specific T cells that secrete effector molecules, including granzyme and perforin (Lehmann *et al.* 2012). This assay provides an effective method of measuring antibody or cytokine production of immune cells on a single cell level (Janeway *et al.* 2002).

Fig.7 shows the principle of ELISPOT. For the detection of cytokines released by stimulated T cells micro titer plates with a nitrocellulose bottom are coated with an antibody against the cytokine of interest. Then, cells are incubated in the coated wells. During incubation, the cells secrete cytokines which bind to the coating antibodies in the area of secretion. After incubation, the cells are removed and biotinylated secondary antibodies and streptavidin-bound enzyme conjugates are added. After incubation, a substrate is given. The spots, which appear during the development of the assay, represent a single cell secreting the cytokine of interest (Schmittl et al., 2000).



**Fig.7: Principle of ELISPOT.**1. Coat membrane with antibodies. Add immune cells and incubate. 2.Responding cells produce cytokines. The cytokine of interest is then bound by the antibody.3 Wash to remove cells. Add biotinylated antibodies which bind to the cytokine-antibody complex. 4 Add avidin-enzyme conjugate. 5 Add enzyme substrate and each responding cell will result in one spot.  
<http://www.millipore.com/immunodetection/id3/elispot>

### 3.7.2 IFN- $\gamma$ PRODUCTION BY STIMULATING BM T CELLS

In order to demonstrate the functionality of BM-derived memory T cells, a co-culture with antigen pulsed DC was performed (see 3.4). The co-culture was made in an ELISPOT plates that were equilibrated with 15  $\mu$ l 70% ethanol for 1 min, washed twice with 200  $\mu$ l/well PBS and coated with rat anti-mouse IFN- $\gamma$  primary antibody (10  $\mu$ g/ml in coating buffer; 100  $\mu$ l/well). The plates were sealed with parafilm and incubated at 4°C over night.

At the same day, DC were stimulated, treated with anti-PD-L1 and pulsed with antigen (see 3.2.1).

One day later, the unbound antibody was washed off twice with 200  $\mu$ l/well of sterile water. The membrane was blocked with 150  $\mu$ l/well RPMI-1640 supplemented with 10% heat inactivated FCS for at least 2 h at 37°C. The blocking medium was decanted and 5x10<sup>5</sup> freshly separated T cells were cultured with 1x10<sup>5</sup> generated DC per well (T cell:DC ratio of 5:1) in 150  $\mu$ l complete medium. For positive controls, BM-derived T cells and splenocytes were treated with concavalin A (ConA, 10 mg/ml). The plates were incubated for 40 h at 37°C, 5% CO<sub>2</sub> and 95% humidity.

Then cells were washed 6 times with PBS/0,05% Tween<sup>®</sup>20 (PBS-T), incubated with 50  $\mu$ l/well biotinylated IFN- $\gamma$  secondary antibody (rat anti-mouse, 1  $\mu$ g/ml in sterile BSA buffer) for 2 h at 37°C, 5% CO<sub>2</sub> and 95% humidity. Plates were washed again 6 times with PBS-T and incubated with diluted streptavidin-HRP (1:500 in PBS, 100  $\mu$ l/well) for 2 h at 37°C followed by washing three times with PBS-T and three times with PBS. For the spot development, AEC substrate was freshly prepared (see 2.2.2). Spot development was started after the addition of 50  $\mu$ l/well AEC substrate and incubation for 3-10 min at RT in the dark. The reaction was stopped under running water and extensive washing. Plates were dried between paper towels overnight. The quantification of the spots was analyzed in the ELISPOT reader Bioreader<sup>®</sup>-3000 (BioSys).



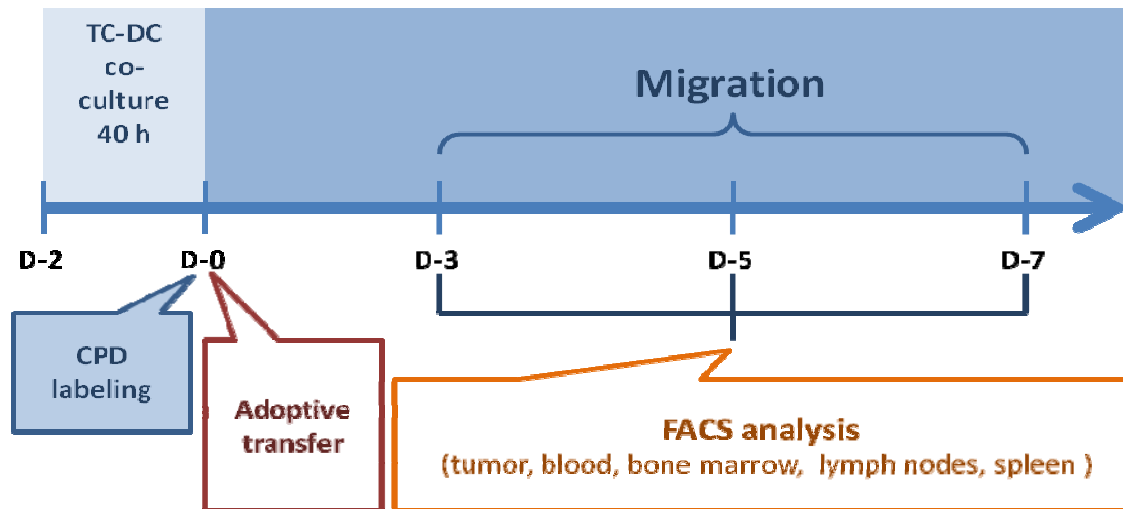
## 3.8 IFN- $\gamma$ secretion assay

### 3.8.1 PRINCIPLE OF THE METHOD

In addition to ELISPOT, antigen-specific IFN- $\gamma$  secretion by T cells can be detected with the Mouse IFN- $\gamma$  Secretion Assay Detection Kit (Miltenyi Biotec). The cells are re-stimulated either with APCs or with a peptide followed by attachment of the IFN- $\gamma$ -specific Catch Reagent to the cell surface. During the IFN- $\gamma$  secretion phase, IFN- $\gamma$  binds immediately to the Catch Reagent on the secreting lymphocytes. Then these cells are labeled with the IFN- $\gamma$  Detection Antibody conjugated to a fluorescent dye. The IFN- $\gamma$  secretion can be detected in different T cell subsets. Analysis of IFN- $\gamma$  secreting T cells is performed by flow cytometry.

For the detection of IFN- $\gamma$  secreting CD8 T cells in the T cell-DC co-culture the IFN-Secretion Assay Detection Kit (APC\*) was used according to manufacturer's instructions. T cells were co-culture with DC for 40 h as described in 3.4 followed by a staining with anti-CD3 and anti-CD8 fluorescent labeled antibodies. T cells cultured without DC were taken as a negative control. Con A or staphylococcal enterotoxin B (SEB) was added to splenocytes for unspecific stimulation and IFN- $\gamma$  secretion as a positive control. Propidium iodide (PI) was added directly before measurement to exclude dead cells.

### 3.9 Analysis of T cell migration



**Fig.8: Experimental setup of T cell migration.**

Upon the 40 h co-culture of T cells and antigen-loaded cells were labeled with cell proliferation dye eFlour<sup>®</sup>670 (CPD) by mixing of  $1 \times 10^7$  cells with 5  $\mu\text{M}$  of CPD in 1 ml PBS to a final CPD concentration of 2,5  $\mu\text{M}$ . The mixture was incubated at 37°C for 10 min with continuous shaking. To stop the reaction 10 ml RPMI-1640 containing 10% FCS were added. After incubation of 1 min at 37°C the cells were placed on ice for 4 min followed by two washing steps with PBS (1200 rpm, 10 min, 4°C). The cells were counted and adjusted to a number of  $3 \times 10^6$  cells in 100  $\mu\text{l}$  of sterile PBS. Then, intracardiac (i.c.) injections into ret transgenic tumor-bearing mice were performed with the help of Dr. med. vet. Michaela Socher. Mice treated with PBS were used as a control group. On day 3, 5 and 7 after adoptive transfer mice were sacrificed by cervical dislocation. Tumors, lymph nodes (axillar, brachial, inguinal and mandibular), bone marrow (femur and tibia) and the spleen were resected and single cell suspensions were prepared as shown in 3.1. The cells were stained with respective fluorescence-labeled antibodies. The migration of CPD-labeled CD8<sup>+</sup> T cells into the secondary lymphoid organs and tumor lesions was detected by flow cytometry (Feuerer et al., 2001a).

### 3.10 Statistical analyses

Statistical analyses were performed using Excel 2007 (Microsoft) and GraphPad Prism 5 (GraphPad Software, Inc.). Results were expressed as mean $\pm$ SD. To determine significant differences between two groups, P values were calculated by unpaired t-test.

# 4 Results

## 4.1 Analysis of maturation status of generated DC

The status of DC generated from the BM of C57BL/6 mice was determined using staining with antibodies against CD11c and MHC class II as well as costimulatory molecules (CD40, CD80 and CD86) followed by flow cytometry (Fig.9).

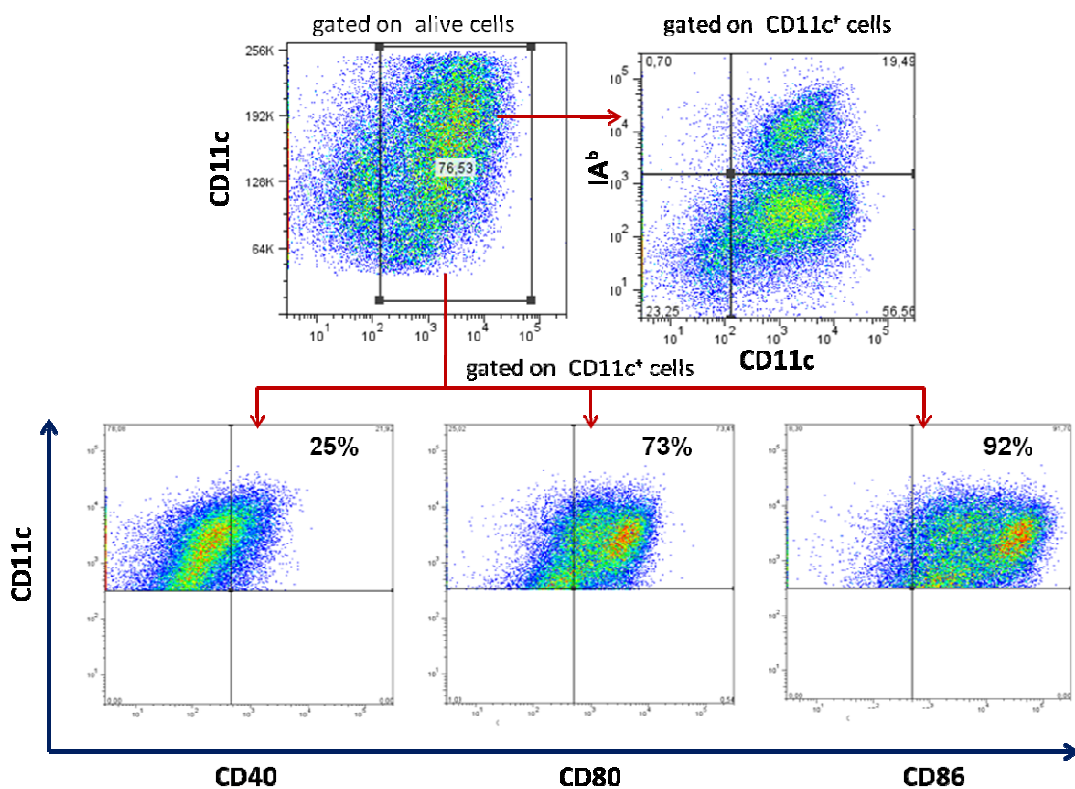


Fig.9: Expression of DC markers at day 8. DC were generated *in vitro* from BM progenitors of non-transgenic C57BL/6 mice in complete RPMI-1640 medium supplemented with rmGM-CSF (PeproTech) and rmIL-4 (eBioscience) (1000 U/ml each).

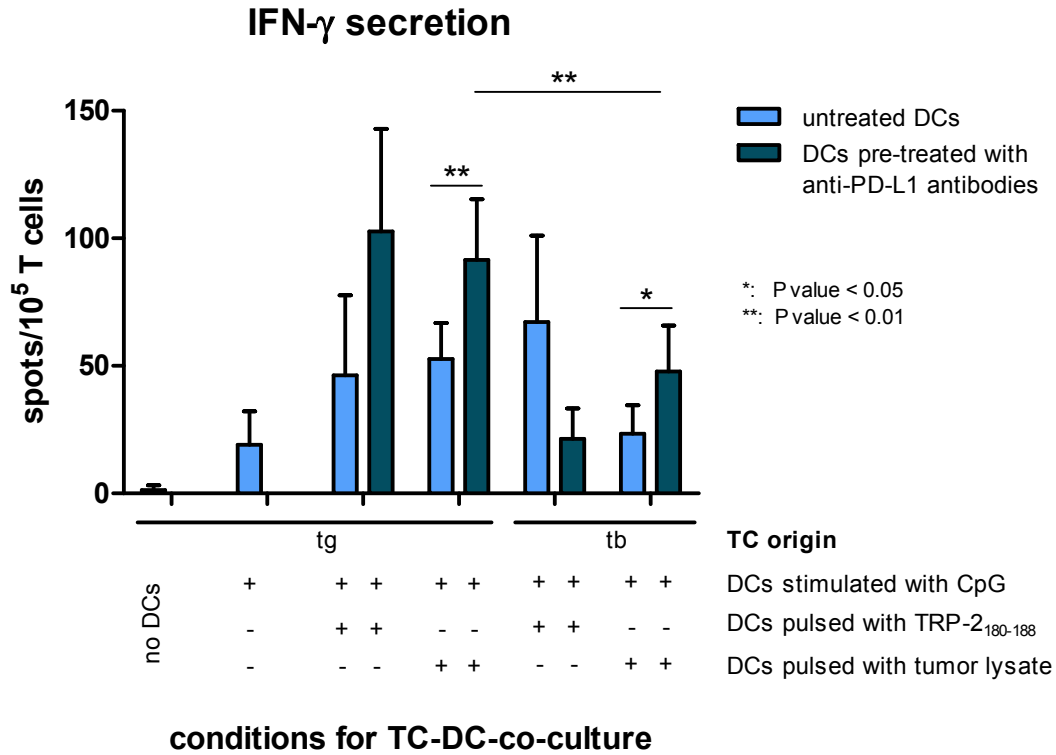
Cells, which express CD11c, were considered as mature DC. Around 76% of the cells in culture expressed CD11c and 19% CD11c and MHC-II (double positive). Out of CD11c<sup>+</sup> cells, 25% expressed CD40, 73% CD80 and 92% CD86. Hence, the majority of CD11c<sup>+</sup> cells expressed co-stimulatory molecules, which is important for T cell activation.

## 4.2 T cells from the bone marrow of *ret* transgenic mice

### 4.2.1 T CELL FUNCTION OF MEMORY T CELLS *EX VIVO*

Next, we determined the anti-tumor reactivity of memory T cells, which were isolated from the BM of *ret* transgenic mice with macroscopic tumors or without visible tumors. Bone marrow-derived T cells were co-cultured for 40 h with DC loaded with tumor lysate or TRP2<sub>(180-188)</sub> peptide (see 3.4). Such short-term co-culture is enough to activate tumor-specific memory T cells but is not sufficient to stimulate their naive counterparts.

It is known that the interaction between PD-1 on T cells and PD-L1 on DC can negatively regulate the T cell immune response (Fourcade et al., 2010; Freeman et al., 2000). Moreover, the blockade of PD-L1 leads to an enhanced therapeutic efficacy of the immunotherapy against melanoma (Pilon-Thomas et al., 2010). In order to block PD-1/PD-L1 interaction during the co-culture of T cells with DC, the latter were pretreated with anti-PD-L1 antibodies prior to co-culture (6.4.1). IFN- $\gamma$  secretion was measured using the ELISPOT assay.

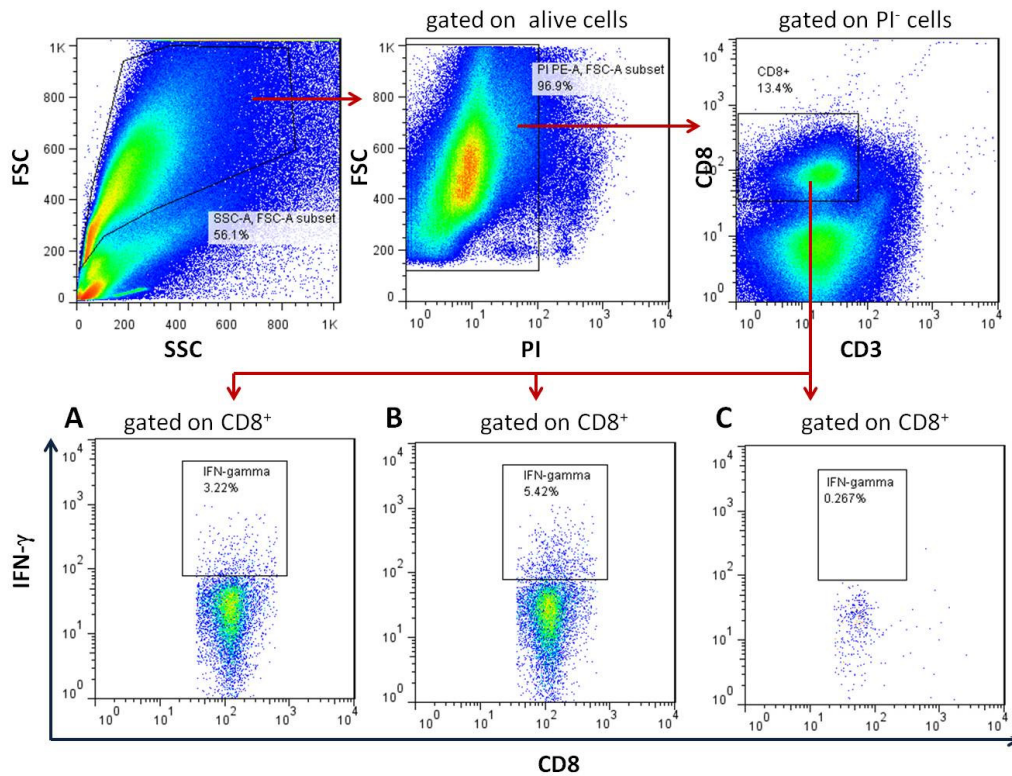


**Fig.10: ELISPOT.** DC were generated *in vitro* from BM progenitors of non-transgenic C57BL/6 mice in complete RPMI-1640 medium supplemented with rmGM-CSF (PeproTech) and rmIL-4 (eBioscience) (1000 U/ml each). Immature DC were stimulated with CpG and treated with anti-PD-L1 antibodies (eBioscience), where indicated, followed by pulsing with whole tumor lysate for 20 h or with TRP-2 derived peptide (TRP-2<sub>180-188</sub>) for 2 h. DC were co-cultured with freshly isolated CD3<sup>+</sup> T cells from the BM of *ret* transgenic tumor free (tg) or tumor-bearing (tb) mice (DC:T cell ratio, 1:5) for 40 h. The secretion of IFN- $\gamma$  was measured by ELISPOT assay. The graph shows the results of two independent performed assays (each in triplicates).

After co-culture with DC pulsed with tumor lysate or TRP-2<sub>(180-188)</sub> peptide, T cells were able to secrete IFN- $\gamma$  (Fig.10). The amount of IFN- $\gamma$  secreting T cells from mice without visible tumors (tg) was similar after DC pulsing with tumor lysate or TRP-2<sub>(180-188)</sub> peptide. In contrast, DC pulsed with TRP-2<sub>(180-188)</sub> peptide induced higher amounts of IFN-g producing T cells isolated from tumor-bearing (tb) mice than DC pulsed with tumor lysate. After PD-L1 blockade on DC, the number of IFN- $\gamma$  secreting T cells from tg mice was significantly increased both upon the co-culture with DC loaded with the peptide or tumor lysate. On the other hand, the pretreatment of DC with anti-PD-L1 antibodies did not lead to an enhancement of IFN- $\gamma$  secreting T cells in tb mice if DC were pulsed with the TRP-2<sub>(180-188)</sub> peptide. Therefore, the pretreatment of DC with anti-

PD-L1 antibodies leads to an increase of IFN- $\gamma$  secretion in T cells upon stimulation with melanoma antigen-pulsed DC.

To confirm our results with ELISPOT assay, we used the IFN- $\gamma$  secretion assay. In this setup, IFN- $\gamma$  secretion of T cells from mice without visible tumors was detected after *in vitro* stimulation with DC pretreated with anti-PD-L1 antibodies and pulsed with TRP2<sub>(180-188)</sub> peptide before co-culture (see 6.4.1).



**Fig.11: IFN- $\gamma$  secretion assay.** DC were generated *in vitro* from BM progenitors of non-transgenic C57BL/6 mice in complete medium supplemented with rmGM-CSF and rmlL-4 (1,000 U/ml each). Immature DC were stimulated with CpG and treated with anti-PD-L1 antibodies, followed by pulsing with TRP-2 derived peptide (TRP-2180-188) for 2 h. DC were co-cultured with freshly isolated CD3+ T cells from the BM of ret transgenic tumor free mice (DC:T cell, 1:5). After 40 h, the secretion of IFN- $\gamma$  was measured using the IFN- $\gamma$  secretion assay. As a negative control (C), T cells were cultured for 40 h in the absence of DC confirming that the secretion depended on the presence of DC.

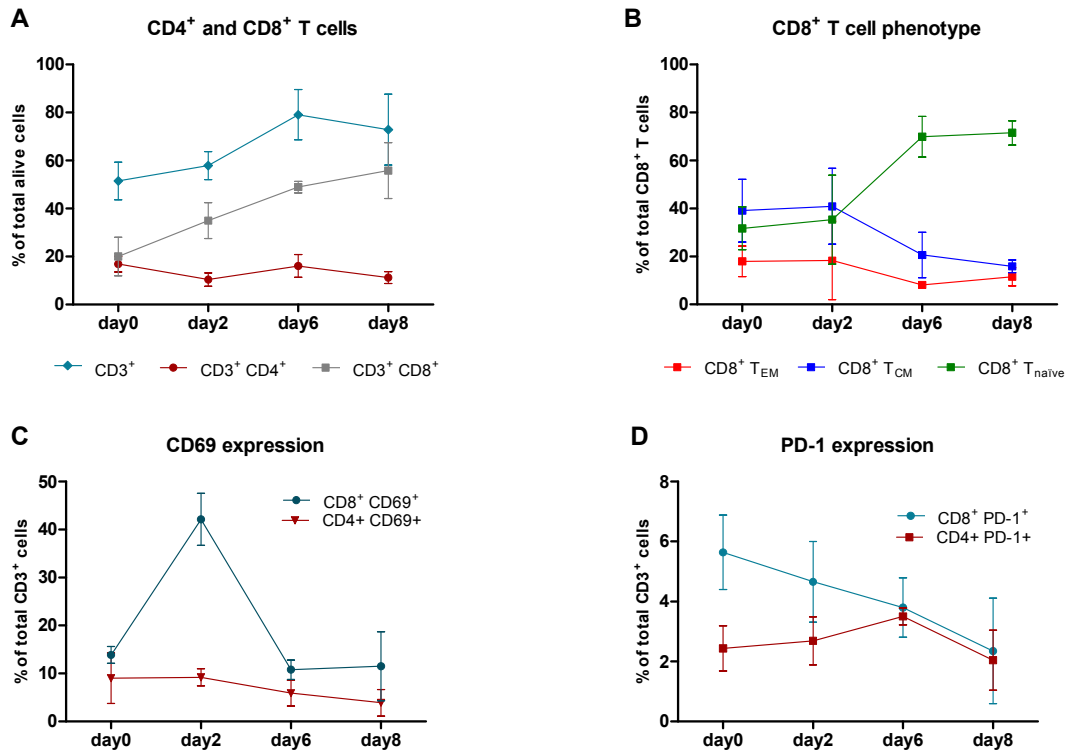
Fig.11 shows the proportion of IFN- $\gamma$  secreting T cells after co-culture with TRP-2<sub>(180-188)</sub> peptide pulsed DC either pretreated with anti-PD-L1 antibodies or without the pretreatment. After PD-L1 blockade, we detected more IFN- $\gamma$  secreting T cells as compared with the co-culture without anti-PD-L1 pretreatment (5,42% vs. 3,22%). These data confirms that the PD-L1 blockade on DC prior to co-culture leads to an increased stimulation of T cells.

#### 4.2.2 T CELL EXPANSION

Since for an adoptive transfer an efficient amount of melanoma-antigen-specific T cells is required, the T cell expansion was initiated ( $5 \times 10^5$  T cells and  $1 \times 10^5$  DC). The absolute amount of cells decreased during the culture (data not shown). However, a relative enrichment of CD3<sup>+</sup> and CD8<sup>+</sup> T cells could be observed (Fig. 12A). The number of CD4<sup>+</sup> T cells remained stable during the co-culture. At day 2, we observed no changes in the frequency of central and effector memory as well as naive subsets within total CD8<sup>+</sup> T cells (Fig. 12B). Afterwards, the relative proportion of naive T cells within CD8<sup>+</sup> T cells increased considerably, whereas the proportion of memory T cells diminished. CD4<sup>+</sup> T cells showed a decrease in the memory phenotype and an increase of naive T cells already from the beginning of the co-culture (data not shown).

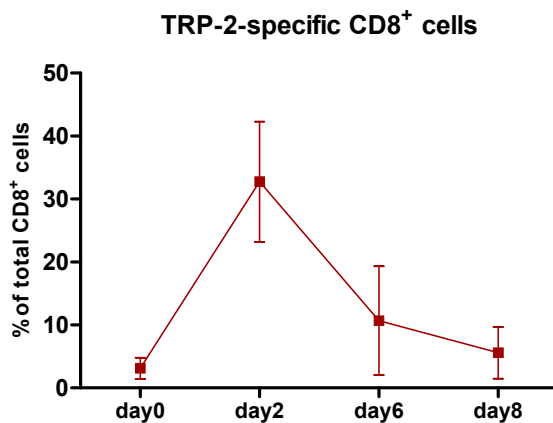
The proportion of the early activation marker CD69 expressed on CD8<sup>+</sup> T cells in the co-culture increased from  $13,9\% \pm 1,8\%$  at day 0 to  $42,1\% \pm 5,4\%$  at day 2 (Fig. 12C). Then, the percentage of CD69 expressing CD8<sup>+</sup> T cells increased to  $11,5\% \pm 7,2\%$  at day 8. In contrast, the proportion of CD4<sup>+</sup> T cells with an activated phenotype was decreased.





**Fig. 12: Phenotypical analysis of co-cultured T cells.** CD3<sup>+</sup> T cells were separated from the BM of *ret* transgenic mice with or without visible tumors, co-cultured with BM derived *in vitro* generated DC (T cell:DC-ratio, 5:1) and analyzed at the depicted time points. A) Relative amounts of CD3<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> cells during the co-culture. B) Proportions of MTC subpopulations within CD8<sup>+</sup> T cells. C) Relative amounts of CD69 expressing CD4<sup>+</sup> and CD8<sup>+</sup> T cells during co-culture. D) Frequencies of exhausted (PD-1<sup>+</sup>) CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

Furthermore, no accumulation of exhausted (PD-1<sup>+</sup>) T cells occurred during the co-culture (Fig. 12D). The amounts of PD1<sup>+</sup> T cells was approximately the same for both T cell subsets at all measured time points remaining below 10% of total T cells.



**Fig.13: TRP-2-specificity of CD8<sup>+</sup> T cells.** Proportion of TRP-2 specific within total CD8<sup>+</sup> T cells is shown.

Fig.13 depicts the TRP-2-specificity of CD8<sup>+</sup> T cells during the co-culture. The proportion of melanoma-specific T cells measured by Dextramer staining varied at different time points. The tumor-specificity of T cells increased notably from 3,1%  $\pm$  1,7% on day 0 up to 32,8%  $\pm$  9,6% after 40 h of co-culture. During the co-culture, after day 2, the TRP-2-specificity decreased to 10% on day 8.

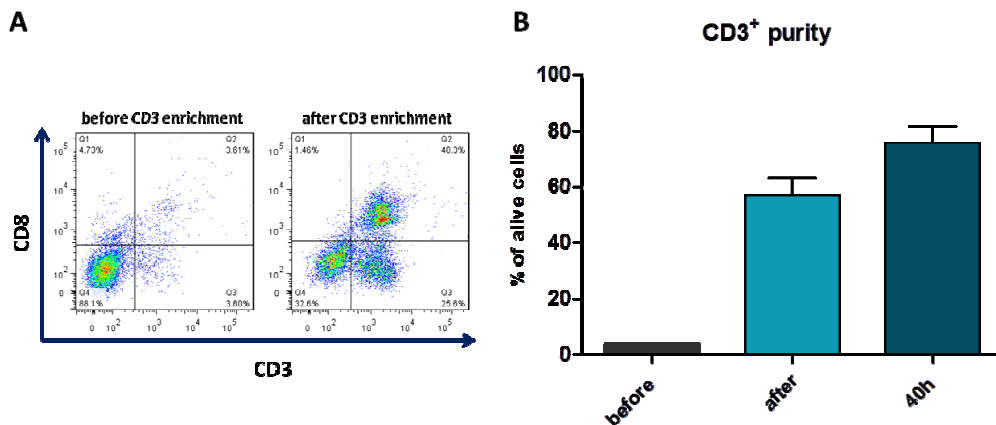
Based on these data, T cells restimulated by melanoma antigen-loaded DC were used for adoptive transfer experiments.

#### 4.2.3 PHENOTYPE ANALYSIS OF T CELLS FROM THE BONE MARROW OF *RET* TRANSGENIC MICE AFTER *IN VITRO* RESTIMULATION

##### T cell purity after enrichment and 40 h of co-culture

In total,  $2,2 \times 10^9 \pm 0,8 \times 10^9$  bone marrow cells could be isolated from femurs and tibiae of 40 mice (n=6). Using negative T cell selection kit, we were able to isolate untouched T cells, which were not activated by antibody binding and could be used for T cell-DC co-culture.

The average of the T cell amount in the BM of *ret* transgenic mice was around 5-7%. Therefore, the total amount isolated of T cells was  $3 \times 10^7$  cells that corresponds to 56.9%  $\pm$  6.5% of freshly separated alive cells (Fig.14B).



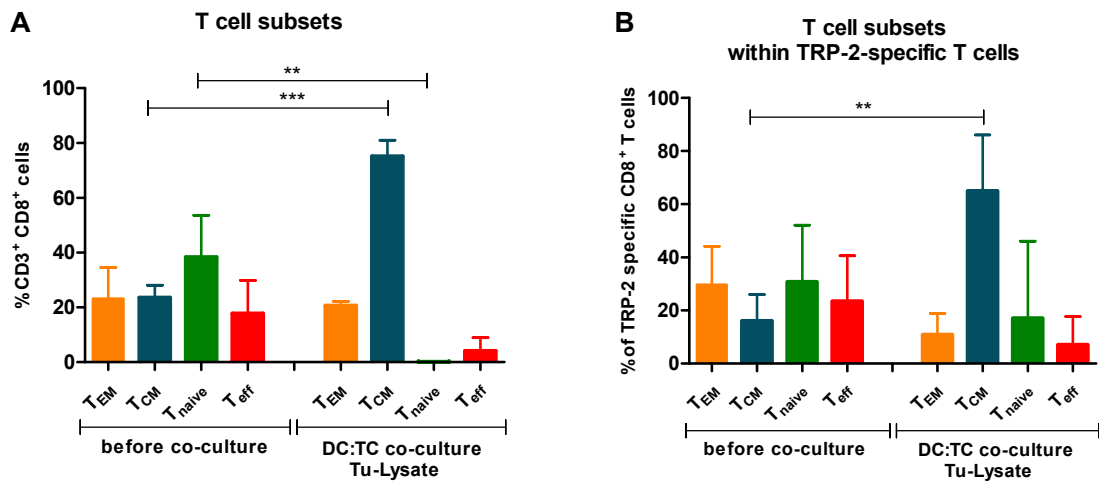
**Fig.14: CD3<sup>+</sup> T cell purification.** CD3<sup>+</sup> T cells from the bone marrow of *ret* transgenic mice were separated using Dynal<sup>®</sup> Mouse T Cell Negative Isolation Kit (Invitrogen). A) shows a dot plot of CD3<sup>+</sup> and

CD8<sup>+</sup> T cells before and after separation. B) represents the frequency of CD3<sup>+</sup> T cells before and after separation as well as upon 40 h co-culture with melanoma-antigen pulsed DC.

After co-culture with TL-loaded DC loaded with tumor lysate, we achieved around 75,7%  $\pm$  6,1% CD3<sup>+</sup> T cells within the total alive cells. Therefore, we could isolate from *ret* transgenic mice a sufficient amount of bone marrow CD3<sup>+</sup> T cells that was increased after the stimulation with DC.

### Phenotype analysis of CD8<sup>+</sup> T cells after coculture

Fig.15A demonstrates different phenotype subsets within CD8<sup>+</sup> T cells before and after co-culture with DC pulsed with tumor lysate.



**Fig.15: Phenotype analysis after co-culture.** CD3<sup>+</sup> T cells were separated from the BM of *ret* transgenic mice with or without visible tumors, co-cultured with bone marrow-derived *in vitro* generated, tumor lysate-pulsed DC (T cell:DC-ratio, 5:1) followed by flow cytometry. A) T cell subsets within CD3<sup>+</sup>CD8<sup>+</sup> T cells before and after co-culture. B) TRP-2 Dextramer staining. Different T cell subsets within TRP-2-specific CD8<sup>+</sup> T cells are shown.

Among freshly isolated T cells, CD8<sup>+</sup> T cell subset shows a prevalence for naive phenotype. After stimulation with tumor lysate-loaded DC the relative amount of naive T cells was decreased (from 38.4%  $\pm$  15.3% before co-culture to 0.2%  $\pm$  0.1% after 40 h of co-culture, Fig.15A). Furthermore, the relative amount of the central memory T cell subtype increased after stimulation (23.6%  $\pm$  4.6% vs. 75.2%  $\pm$  5.9% after co-culture). Comparing effector and effector memory phenotype before and after co-culture, we could not observe any significant difference within CD8<sup>+</sup> T cells. However, we could detect an

increase in TRP-2-specific CM T cells (from  $16.0\% \pm 10.0\%$  to  $64.9\% \pm 21.1\%$ ) after the stimulation with DC. Moreover, these cells dominated within the CD3<sup>+</sup> CD8<sup>+</sup> T cell subset.

Summarizing, after the co-culture, mainly central memory T cells specific for the melanoma antigen TRP-2 were found.

#### 4.2.4 T CELL MIGRATION AFTER INTRAPERITONEAL INJECTIONS

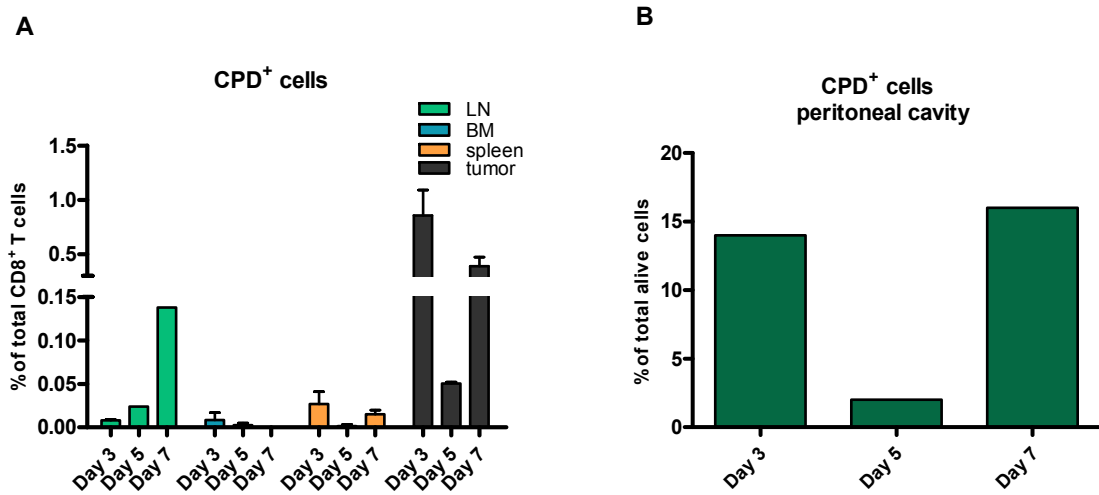
An adoptive immunotherapy against cancer can only be effective when the transferred T cells are able to migrate into tumor lesions, where they can display their cytotoxic activity. It is also important that T cells migrate into the secondary lymphoid organs where they can encounter a tumor-antigens presented by DC.

We analyzed the migration properties of *in vitro* activated memory T cells in *ret* transgenic tumor-bearing mice after intraperitoneal (i.p.) injections. Before the transfer, one part of the cell culture, including DC, was analyzed by flow cytometry and the other part was labeled with CPD (see 3.9). At day 3, 5 and 7 after adoptive transfer of CPD-labeled DC and T cells, the migration to tumor lesions, metastatic lymph nodes, BM and spleen was measured. One representative of 10 experiments is shown in Fig.1.  $2 \times 10^6$  cells per mouse were injected i.p. (Fig.16) followed by the migration assessment. Some CPD-labeled cells were found in tumor lesions at day 3 and 7 after injections (Fig.16A). However, at day 5, considerably less CPD<sup>+</sup> cells could be detected in the tumor. This is one of two experiments, where CPD<sup>+</sup> cells could be detected. In all other experiments, it was not possible to track any labeled cells (data not shown). In the lymph nodes (LN), some cells could be tracked only at day 7.

The analysis of the cells in the peritoneal cavity (Fig.16B) revealed that most of the CPD-labeled cells were remaining at the injection site. About 95% of the CPD<sup>+</sup> cells expressed CD3, out of which approximately 2.5% were CD8<sup>+</sup>. At day 5 after the injection, the relative amount of labeled cells in the peritoneal cavity was considerably

less. At day 7 after the inoculation, the proportion of CPD<sup>+</sup> cells increased to 16.1% of alive cells in the peritoneum.

Therefore, adoptively transferred cells showed migration properties but the main part of injected cells remained in the peritoneal cavity.



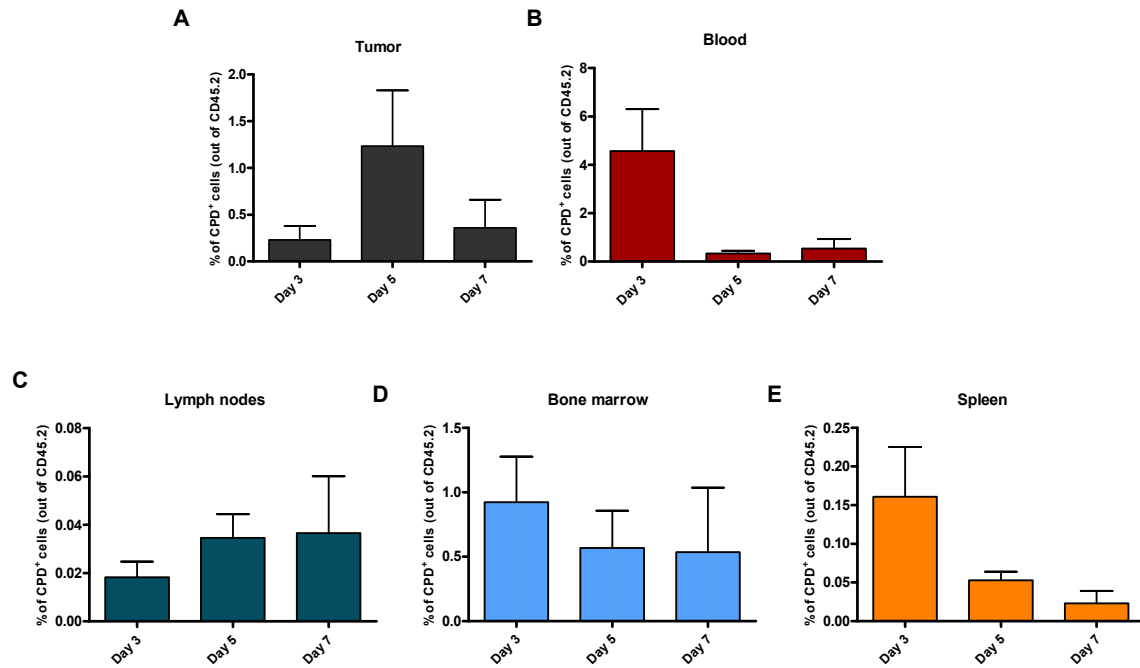
**Fig.16: T cell migration after i.p. injections.** CD3<sup>+</sup> T cells were separated from the BM of *ret* transgenic mice with or without macroscopic tumors and co-cultured with BM derived *in vitro* generated DC (T cell:DC ratio, 5:1) for 40 h. The cells were labeled with CPD and injected i.p. into tumor-bearing mice. A) At day 3, day 5 and day 7 after the injection, the migration of the cells to primary skin tumors and to lymphoid organs (LN: lymph nodes, BM, spleen) was determined. The injection was performed with  $2.0 \times 10^6$  CPD-labeled cells/mouse (n=2). In B) non-migrating cells in the peritoneal cavity were assessed (graph right panel).

#### 4.2.5 T CELL MIGRATION AFTER INTRACARDIAC INJECTIONS

Since the migration of adoptively transferred T cells was found to be non-efficient, we injected the cells intracardially (i.c.). Freshly isolated T cells from *ret* transgenic mice were co-cultured with tumor lysate-loaded DC for 40 h (see 3.4). Then,  $3 \times 10^6$  CPD-labeled cells in 100  $\mu$ l of sterile PBS (sPBS) were prepared. I.c. injections were performed under the supervision of Dr. med. vet. Michaela Socher. At day 3, 5 and 7 the migration of CPD-labeled cells in tumor lesions, blood, lymph nodes, BM and the spleen was assessed (Fig.17).

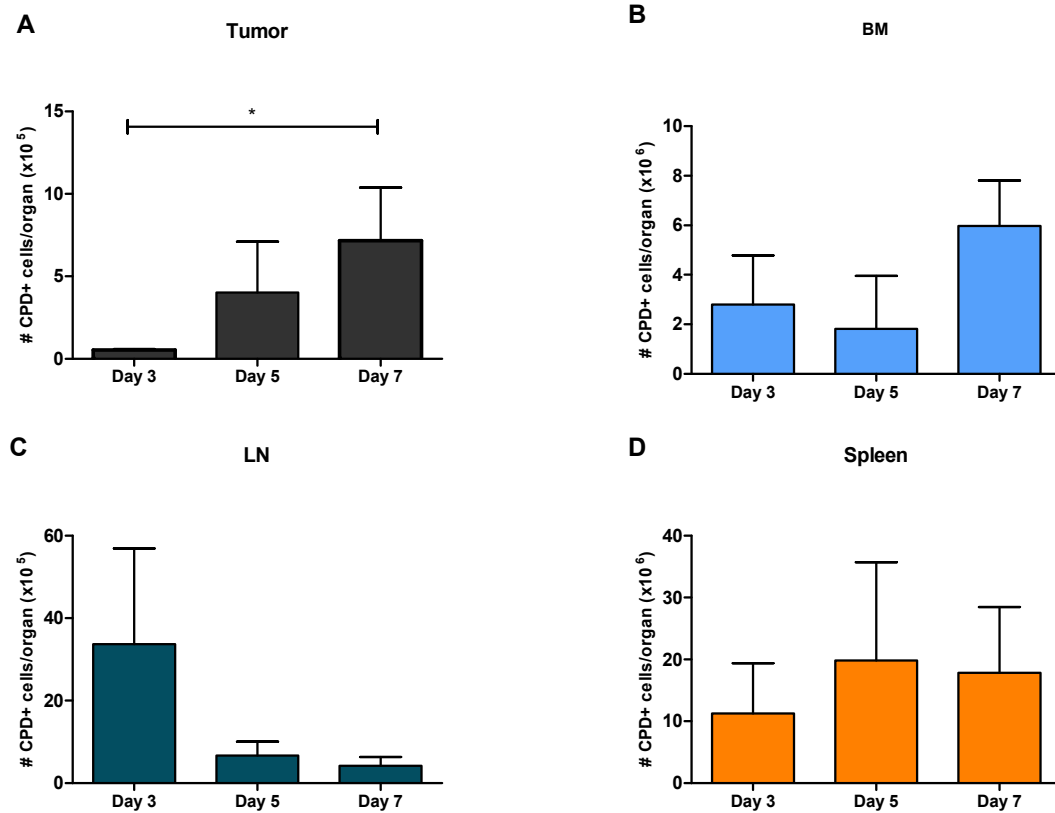
Fig.17A shows that the maximal migration of CPD<sup>+</sup> cells into tumor lesions was achieved at day 5. As expected, we could detect a decrease of CPD<sup>+</sup> cells in the peripheral blood (Fig.17B). Also in metastatic lymph nodes, labeled cells were present

(Fig.17C). In addition, we found migrated cells in lymphoid organs like the bone marrow and the spleen (Fig.17D-E).



**Fig.17: T cell migration after i.c. injections.** CD3<sup>+</sup> T cells were separated from the BM of *ret* transgenic mice with or without macroscopic tumors and co-cultured with BM derived *in vitro* generated DC (T cell:DC ratio, 5:1) for 40 h. The cells were labeled with CPD and injected i.c. into tumor-bearing mice. At day 3, day 5 and day 7 after the injection, the cell accumulation in the primary skin tumors (A), blood (B), lymph nodes (C), bone marrow (D) and the spleen (E)) was determined. The injections were performed with  $3.0 \times 10^6$  CPD-labeled cells in 100  $\mu$ l/mouse (n=5).

The absolute amount of migrated T cells in the tumor, LN, BM and the spleen was presented in Fig.18. We detected a significant increase of CPD-labeled cells, out of all leukocytes (CD45.2<sup>+</sup>), from day 3 ( $0.5 \times 10^5$  cells  $\pm$   $0.04 \times 10^5$  cells) to day 7 ( $7.2 \times 10^5$  cells  $\pm$   $3.2 \times 10^5$  cells, Fig.18A) in the tumors of *ret* transgenic mice. In the lymphoid organs, the absolute amount of T cells remained more or less the same, except for the lymph nodes, where the absolute number decreased over time ( $33.7 \times 10^5$  cells  $\pm$   $23.2 \times 10^5$  cells at day 3 vs.  $4.2 \times 10^5$  cells  $\pm$   $2.1 \times 10^5$  cells at day 7, Fig.18C).

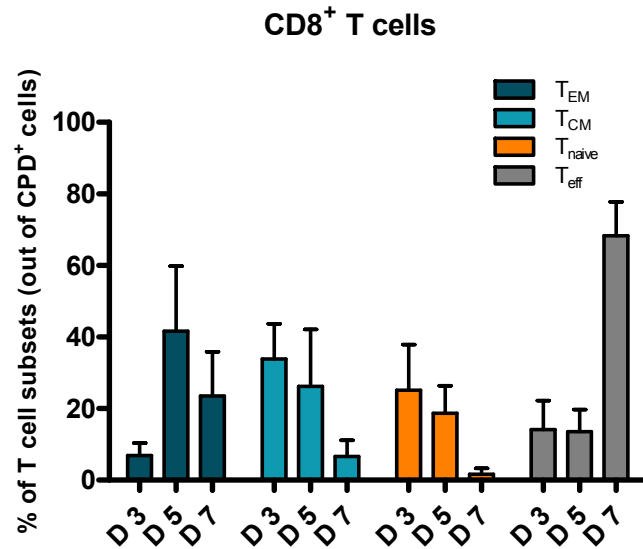


**Fig.18: Absolute amount of CPD<sup>+</sup> migrated cells.** (n=5 for each time point). Five mice per measured time point. CD3<sup>+</sup> T cells were separated from the BM of *ret* transgenic mice with or without macroscopic tumors and co-cultured with BM derived *in vitro* generated DC (T cell:DC ratio, 5:1) for 40 h. The cells were labeled with CPD and injected i.c. into tumor-bearing mice. At day 3, day 5 and day 7 after the injection, the absolute amount of migrated cells into primary skin tumors (A) bone marrow (B), lymph nodes (C) and spleen (D) was determined. The injections were performed with  $3.0 \times 10^6$  CPD-labeled cells in 100  $\mu$ l/mouse.

These data indicate that the *in vitro* activated T cells can efficiently migrate into tumor lesions.

#### 4.2.6 PHENOTYPE ANALYSIS OF MIGRATED CELLS IN THE TUMOR

To assess the phenotype of CPD-labeled CD8<sup>+</sup> T cells in infiltrated tumors, cells were stained with antibodies against CD62L, CD44 and CD45RB.



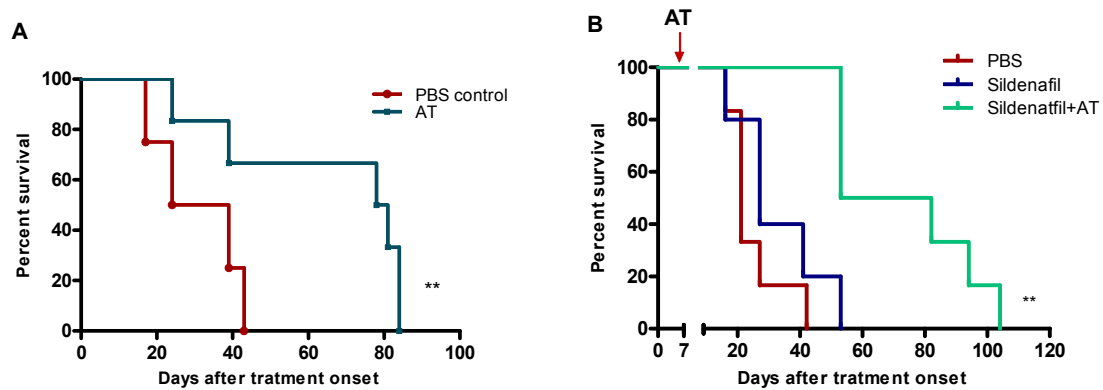
**Fig.19: T cell subsets within CD8<sup>+</sup> T cells.** CD3<sup>+</sup> T cells were separated from the BM of *ret* transgenic mice with or without macroscopic tumors and co-cultured with BM derived *in vitro* generated DC (T cell:DC ratio, 5:1) for 40 h. The cells were labeled with CPD and injected i.c. into tumor-bearing mice. On day 3, day 5 and day 7 after the injection the migration of the cells into to primary skin tumors was determined. The injections were performed with  $3.0 \times 10^6$  CPD-labeled cells in 100  $\mu$ l/mouse (n=5). After single cell suspension, tumor cells were stained with fluorescence-labeled antibodies against CD45.2, CD3, CD8, CD44 and CD62L. The measurement was performed by flow cytometry.

As displayed in Fig.19, at day 3 and 5 the distribution of different phenotypes within the CD8<sup>+</sup> T cell subsets was not significantly different. However, at day 7 the relative amount of effector CD8<sup>+</sup> T cells in the tumor increased from  $14.1\% \pm 18.0\%$  up to  $68.3\% \pm 13.5\%$ .

#### 4.2.7 IMMUNOTHERAPY WITH ADOPTIVELY TRANSFERRED ACTIVATED TUMOR-SPECIFIC MEMORY T CELLS

$3 \times 10^6$  cells per mouse were i.c. into tumor-bearing *ret* transgenic mice. The control group was treated with PBS. After the transfer, melanoma development in mice was monitored every second day to assure that they were not paralyzed or the tumor exceeded a marginal size.





**Fig.20: Survival of tumor-bearing mice after adoptive transfer.** A) CD3<sup>+</sup> T cells were separated from the BM of *ret* transgenic mice with or without macroscopic tumors and co-cultured with BM derived *in vitro* generated, tumor lysate-pulsed DC (T cell:DC ratio, 5:1) for 40 h. The cells were labeled with CPD and injected i.c. into tumor-bearing mice. The injections were performed i.c. with  $3.0 \times 10^6$  cells/mouse in 100  $\mu$ l. B) Seven days before AT mice were treated with sildenafil dissolved in drinking water (20 mg/d/kg). \*\*:  $P < 0.05$ .

Fig.20 shows the effect of an adoptive cell therapy with *in vitro* activated T cells on the survival of tumor-bearing mice. Already at day 43 after the transfer, the survival of mice in the therapy group was significantly higher than in the control group ( $P < 0.005$ ). In addition, the treated mice were more active than in the control group. Therefore, *ret* transgenic mice contained functionally active melanoma-specific memory T cells in their BM, which could be restimulated with melanoma antigen-loaded dendritic cells to exert anti-tumor effects *in vivo*.

MDSCs are known to inhibit tumor-reactive T cells. Previously, it has been shown by our group that MDSCs were accumulated in melanoma lesions and lymphatic organs of *ret* transgenic tumor-bearing mice. Phosphodiesterase-5 inhibitor (PDE-5) sildenafil was shown to restore the T cell reactivity and to prolong the survival of tumor-bearing mice upon *in vivo* application (Meyer et al., 2011). Therefore, we performed an adoptive transfer of *in vivo* activated BM derived T cells in combination with sildenafil Fig.20B. One week prior to the cell therapy, sildenafil was given to mice with the drinking water until the end of the experiment. One of two representative experiments is shown.

Fig.20 depicts the survival of tumor-bearing mice upon the adoptive transfer of activated T cells in combination with sildenafil. The overall survival was significantly increased in

the combinational therapy group compared to sildenafil treatment alone and mice treated with PBS ( $P < 0.05$ ).

Therefore, these results show an *in vivo* anti-tumor effect of bone marrow derived memory T cells from *ret* transgenic mice in combination with sildenafil treatment.

### 4.3 TRP-2-specific effector T cells from TRP-2 TCR transgenic mice

Tyrosinase-related protein 2 (TRP-2) is a melanogenic enzyme which is expressed by both melanocytes and melanomas (Wang et al., 1996). It is reported to be a candidate melanoma rejection antigen (Engelhard et al., 2002).

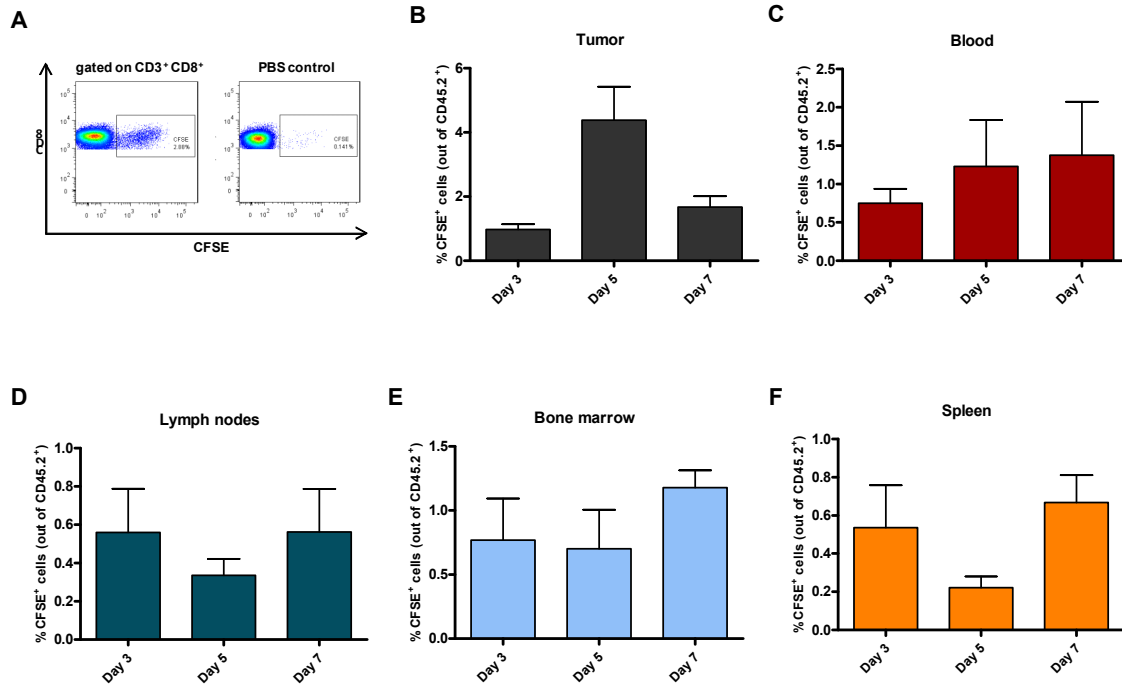
Recently, mice bearing T cell receptor transgene (TCR Tg) specific for TRP-2<sub>(180-188)</sub> epitope have been developed to study the role of self-reactive CD8<sup>+</sup> T cells in tumor immunity (Singh et al., 2009). Since the bone marrow of *ret* transgenic mice contains 5-7% T cells and therefore about 40 mice are needed to inject 6 mice for one survival experiment, we decided to use the TRP-2 TCR transgenic mouse model as a source of TRP-2-specific T cells for adoptive immunotherapy.

#### 4.3.1 MIGRATION OF TRP-2-SPECIFIC EFFECTOR T CELLS

We determined first the migration capability of *in vitro* activated TRP-2-specific effector T cells from lymph nodes and the spleen of TRP-2 TCR transgenic mice. Single cell suspensions from lymph nodes and spleens were prepared and cultured in presence of IL-2 and TRP-2<sub>(180-188)</sub> peptide for three days followed by the expansion for another two days in presence of IL-2 (see 3.5). T cells were stained with CFSE and  $1 \times 10^7$  cells were adoptively transferred into tumor-bearing *ret* transgenic mice i.c..

As shown in Fig.21B, the accumulation of adoptively transferred cells could be observed in the tumor at all three investigated time points with a maximum at day 5 ( $0.97\% \pm$

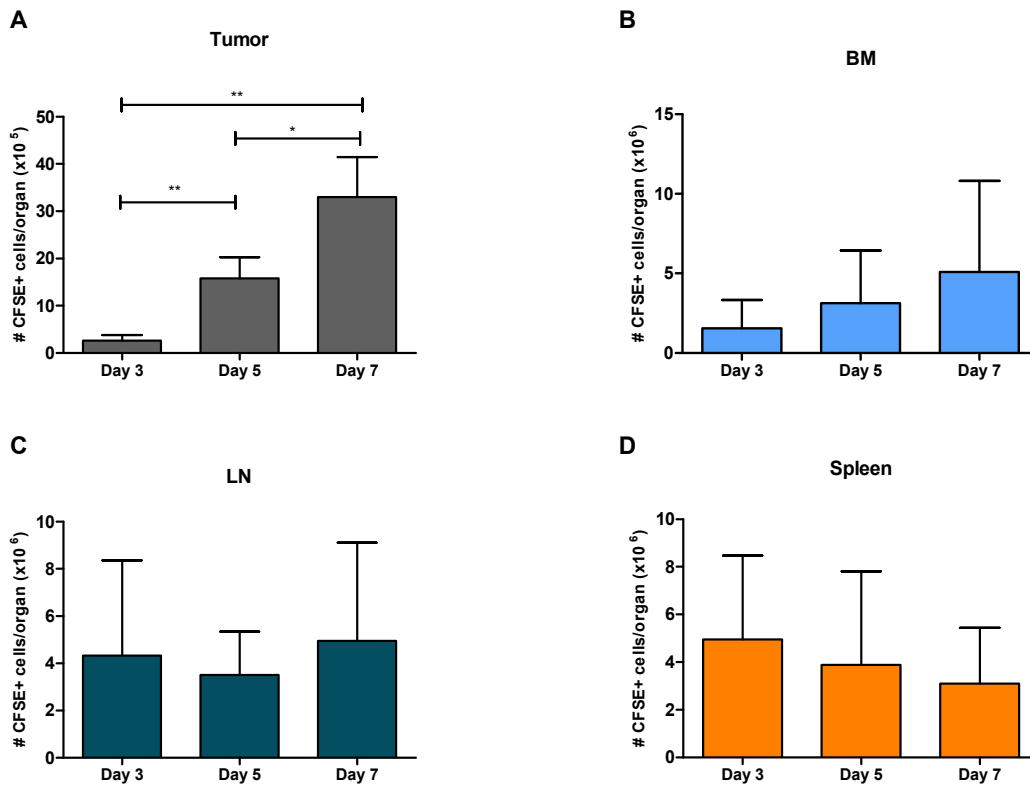
0.32% at day 3 vs.  $4.38\% \pm 2.08\%$  day 5 out of all CD45.2<sup>+</sup> cells). Also in all other analyzed organs, migrated CFSE<sup>+</sup> cells could be detected. However, there was no significant difference of the relative amount of tracked at different time points in all organs (Fig.21C-F).



**Fig.21: Migration of TRP-2-specific effector T cells.** Lymph node cells and splenocytes from TRP-2 TCR transgenic mice were cultured in T cell medium with 1  $\mu$ M of TRP-2<sub>(180-188)</sub> peptide and human recombinant IL-2 (20 IU/ml, PeproTech) for 3 days. Then, cells were expanded with media containing 20 IU/ml IL-2 for another 2 days. At day 5, T cells were stained with 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) before adoptive transfer. Then,  $1 \times 10^7$  CFSE-labeled T cells were injected into tumor-bearing *ret* transgenic mice. At the indicated time points mice were sacrificed and the migration was analyzed by flow cytometry. A) A representative dot plot of lymph node cells at day 5, gated on CD3<sup>+</sup> CD8<sup>+</sup> T cells. CD45.2<sup>+</sup> CFSE<sup>+</sup> cells in the tumor (B), peripheral blood (C), lymph nodes (D), bone marrow (E) and spleen (F) were determined by flow cytometry.

With respect to the absolute amount of migrated TRP-2-specific T cells, we could observe a significant increase of CFSE<sup>+</sup> cells in the tumor. Comparing day 3 with day 5, there was an 2.9-fold increase in the migration into tumor lesions ( $5.45 \times 10^5$  cells  $\pm$   $5.78 \times 10^5$  cells vs.  $15.75 \times 10^5$  cells  $\pm$   $4.5 \times 10^5$  cells; Fig.22A) and comparing day 3 with day 7 ( $33 \times 10^5$  cells  $\pm$   $8.49 \times 10^5$  cells), we could observe a 6-fold increase. In contrast, the absolute amount of CFSE<sup>+</sup> cells in lymph nodes, bone marrow and the spleen was consistent during the analyzed migration period (Fig.22B-C).

These data demonstrate the migration capability of *in vitro* generated TRP-2-specific effector CD8<sup>+</sup> T cells from of TRP-2 TCR transgenic mice into tumor lesions and lymphoid organs.

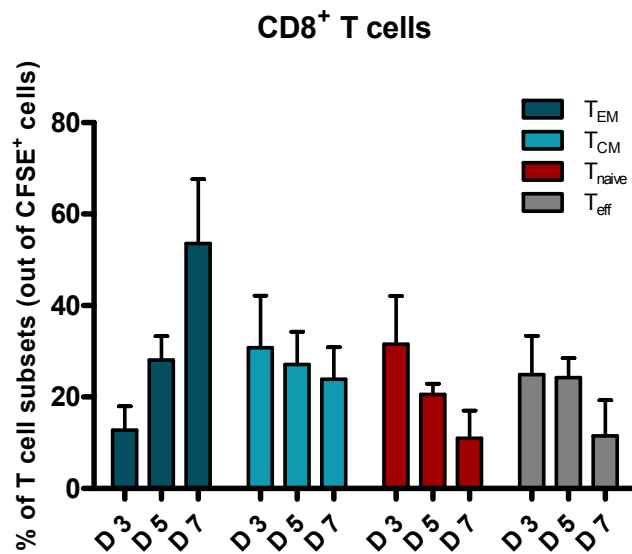


**Fig.22: Absolute amount of migrated TRP-2 TCR effector cells.** Lymph node cells and splenocytes from TCR tg mice were cultured in T cell medium with 1  $\mu$ M of TRP-2<sub>(180-188)</sub> peptide and human recombinant IL-2 (20 IU/ml, PeproTech) for 3 days. Then, cells were expanded with media containing 20 IU/ml IL-2 for another 2 days. At day 5, T cells were stained with 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) before AT. Then,  $1 \times 10^7$  CFSE-labeled T cells were injected into tumor-bearing *ret* transgenic mice. At the indicated time points mice were sacrificed and the migration was analyzed by flow cytometry in tumors (A), bone marrow (B), lymph nodes (C) and spleens (D). Three measured experiments with duplicates per measured time points are shown.

### 4.3.2 PHENOTYPE ANALYSIS OF CELLS MIGRATED INTO THE TUMOR

To define the phenotype of migrated  $CD8^+$  T cells, single cell suspensions were stained with antibodies against memory markers (CD44, CD62L).

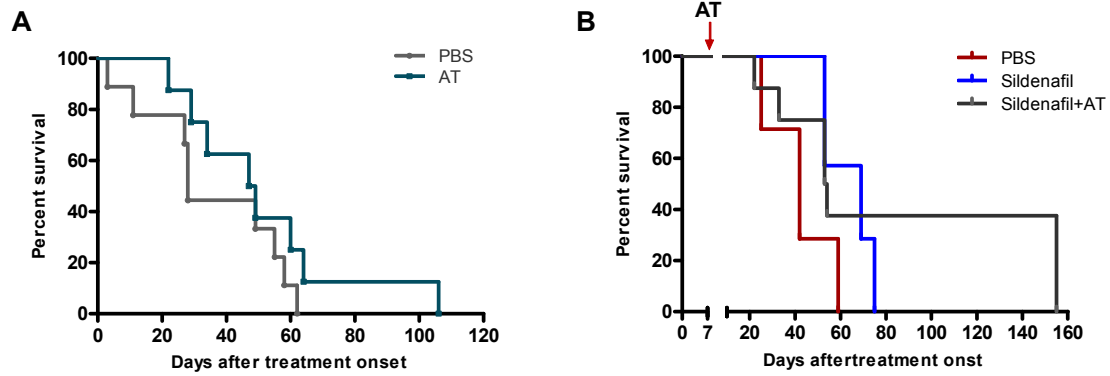
The frequency of the effector memory cells within migrated  $CD8^+$  T cells in the tumor increased from day 3 ( $12.7\% \pm 10.5\%$ ) to day 5 ( $53.6\% \pm 24.3\%$ ; Fig.18). In addition, we found a decrease in the relative amount of central memory, naive and the effector T cells at this period of time.



**Fig.23: CD8<sup>+</sup> T cell subsets in the tumor.** Splenocytes and lymph node cells from TRP-2 TCR transgenic mice were cultured in T cell medium with 1  $\mu$ M of TRP-2<sub>(180-188)</sub> peptide and human recombinant IL-2 (20 IU/ml, PeproTech) for 3 days. Then, cells were expanded with media containing 20 IU/ml IL-2 for another 2 days. At day 5, T cells were stained with 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) before adoptive transfer. Then,  $1 \times 10^7$  CFSE-labeled T cells were injected into tumor-bearing *ret* transgenic mice. At day 3, 5 and 7 after AT the phenotype of migrated CFSE<sup>+</sup> CD8<sup>+</sup> T cells was analyzed. Data from three experiments with duplicates are shown.

### 4.3.3 IMMUNOTHERAPY WITH ADOPTIVELY TRANSFERRED ACTIVATED TRP-2-SPECIFIC T CELLS FROM TRP-2 TCR TRANSGENIC MICE

In the first experiment (Fig.24A),  $1 \times 10^7$  *in vitro* activated TRP-2-specific effector  $CD8^+$  T cells were injected i.c. into tumor-bearing mice. We did not find statistically significant difference in survival as compared to the untreated group (PBS). However the median survival of the treated group was increased.



**Fig.24: Effect of immunotherapy with TRP-2 TCR transgenic T cells on melanoma development in *ret* transgenic mice.**  $1 \times 10^7$  *in vitro* activated T cells were injected alone (A) or in combination with (B). In the control group, mice were injected with 100  $\mu$ l of sterile PBS  $n=8$  per group. Both experiments were performed twice. One of each experiment is shown.

In the second experiment, tumor-bearing mice were additionally treated with the PDE-5 inhibitor sildenafil, starting on day seven before the adoptive immunotherapy (Fig.24B). In the group that included sildenafil therapy and adoptive transfer of TRP-2-specific CD8<sup>+</sup> T cells, we failed to observe statistically significant increase in mouse survival. However, we detected a tendency of increased survival in the group with combinational therapy.

In summary, we could observe a migration of BM derived reactivated melanoma antigen-specific memory T cells into tumor lesions. Moreover, a significantly prolonged survival of tumor-bearing mice upon the adoptive transfer of these cells could be detected.

# 5 Discussion

Malignant melanoma is one of the most aggressive forms of human cancer. In its metastatic stage, the lethality rate is very high, and its incidence is on the rise. Melanoma is resistant to common treatments such as chemotherapy and radiotherapy. Another therapeutic option, like the immunotherapy with high dose IL-2 comes with harmful side effects. Therefore, it is of importance to develop new strategies against malignant melanoma. Since melanoma is notorious to be immunogenic, multiple studies paid attention on developing therapies based on the modulation of hosts' immune system.

In the present study, we focused on adoptive immunotherapy in the *ret* transgenic melanoma mouse model. As described above, this model resembles clinical situation in metastatic melanoma patients.

## 5.1 Activated T cells secrete more IFN- $\gamma$ after PD-L1 blockade on DC prior to their coculture with T cells *in vitro*

It has been previously shown that memory T cells were located in the BM of breast cancer patients and patients with other cancer types (Feurerer et al., 2001b; Muller-Berghaus et al., 2006; Schirmacher et al., 2002; Schmitz-Winnenthal et al., 2005). Moreover, the reactivation of isolated memory T cells from the BM of breast cancer patients with DC has been demonstrated (Beckhove et al., 2004; Feuerer et al., 2001a). The reactivation of memory T cells from the BM of *ret* transgenic tumor-free mice and

mice with microscopic tumors with DC pulsed with tumor lysate has been also shown (Umansky et al., 2008). It has been reported that mature DC express PD-L1 on their surface, which can lead to T cell exhaustion upon binding to PD-1 (Gu et al., 2008; Pilon-Thomas et al., 2010; Schreiner et al., 2004). Therefore, we examined the T cell response after restimulation with DC upon PD-L1 blockade. T cells were isolated from the BM of *ret* transgenic mice and the response was measured using an IFN- $\gamma$  release via ELISPOT or IFN- $\gamma$  capture assay. As expected, there was a significantly increased secretion of IFN- $\gamma$  after PD-L1 blockade with anti-PD-L1 antibodies on DC prior to coculture as compared to the stimulation with untreated DC. An enhanced T cell activity after PD-L1 blockade on monocyte-derived DC has been previously observed (Brown et al., 2003).

In summary, the activity of T cells from the BM of *ret* transgenic tumor-free and tumor-bearing mice was enhanced after reactivation *in vitro* with DC treated with anti-PD-L1 antibodies prior to coculture with freshly isolated T cells.

## 5.2 T cell expansion

An effective expansion of melanoma specific T cells is required to obtain a sufficient number of cells for immunotherapy. After the isolation of T cells from the BM of *ret* transgenic and the 40 h coculture with DC we added IL-21, IL-15 and IL-7 to the T cell medium in order to provide stimuli for memory T cell maintenance and survival (Barker et al., 2010; Brincks and Woodland, 2010; Cha et al., 2010; Knutson and Disis, 2001; Schluns et al., 2000; Weng et al., 2002). It has been demonstrated that the transfer of memory T cells may mediate a strong anti-tumor response (Klebanoff et al., 2005). However, after 40 h of coculture the relative amount of CD8<sup>+</sup> T<sub>EM</sub> and CD8<sup>+</sup> T<sub>CM</sub> cells decreased. This phenomenon has also been observed with tumor infiltrating lymphocytes from melanoma patients using the rapid expansion protocol (REP) for T cell expansion (Dudley et al., 2003; Zhou et al., 2011). Furthermore, the relative amount of TRP-2-specific T cells did not increase but rather decrease during the culture. An *ex vivo*



expansion of tumor-specific T cells is not always feasible due to the low frequency of those cells (Fig.13;  $3.1\% \pm 1.7\%$  on day 0), resulting in loss of homing receptor, i.e. CD62L, and downregulation of CD44 (Zhou et al., 2011). An optimal memory T cell expansion could for instance be achieved with the recently published culture protocol for specific *in vitro* expansion of highly purified naïve CD8<sup>+</sup> T cells using peptide-pulsed DC in combination with sequential supplement of IL-21, IL-7 and IL-15. An expansion of approximately 200-fold of the initial antigen-specific population was achieved, and the anti-tumor reactive T cells showed a T<sub>CM</sub>-like phenotype expressing CD62L (Wolfl et al., 2011).

The early activation marker CD69 is one of the earliest cell surface antigens expressed by T cells following their activation (Ziegler et al., 1994). The CD69 expression on freshly isolated T cells was around 14.0%, and after the stimulation with tumor lysate pulsed DC, it increased up to 36.1%. We also measured the PD-1 expression on T cells which is linked to T cell exhaustion and is expressed on activated T cells (Freeman et al., 2000). However, the relative amount of PD-1 expressing cells did not increase after T cell stimulation with DC.

Since the TRP-2-specificity and the activation status of CD8<sup>+</sup> T cells was the highest after 40 h of coculture, we decided to use the cells for adoptive transfer at this time point.

### 5.3 Migration properties and phenotype of transferred T cells

Since the T cells after coculture were active and exhibited TRP-2 specificity we determined their migration after an adoptive transfer into *ret* transgenic tumor-bearing mice. To achieve an effective adoptive immunotherapy, T cells, which are transferred, should have the ability to migrate to the tumor site. As it has been previously shown, *ex vivo* expanded and adoptively transferred T cells from melanoma patients persist *in vivo*

and preferentially localize to tumor sites (Yee et al., 2002). They mediate an antigen-specific immune response characterized by, e.g. elimination of antigen-positive tumor cells and regression of metastases. In another study, transferred autologous, re-activated BM memory T cells were transferred together with DC into NOD/SCID mice harboring xenotransplanted human breast tumors (Beckhove et al., 2004). A co-localization CD4<sup>+</sup> and CD8<sup>+</sup> with DC was detected in the tumor transplants. Therefore, we transferred both T cells and DC, after 40 h of their co-culture into tumor-bearing mice. In the beginning of this study, an adoptive transfer was performed via i.p. injections since *ret* transgenic mice have hyperpigmented, dark tails, and the tail veins are difficult to reach. Very low numbers of injected T cells were able to migrate into tumor lesions at day 3, 5 and 7. At day 7 after the transfer, CPD-labeled T cells could be detected also in metastatic lymph nodes. We found a relatively high amount of CPD<sup>+</sup> cells in the peritoneal cavity. One reason could be that the concentration of chemokines responsible for T cell homing into the periphery was not enough, and the transferred T cells remained in the peritoneum. In addition, the amount of transferred cells was only 1.2-2.0x10<sup>6</sup> per mouse due to the low yield of T cells from the BM. In former publications, the amount of transferred T cells was at least 4x10<sup>6</sup> per mouse (Beckhove et al., 2004; Feuerer et al., 2001a; Umansky et al., 2008).

In further experiments we performed intracardiac injections of *in vitro* re-activated T cells and DC to avoid the persistence of transferred T cells in the peritoneal cavity. Adoptively transferred CPD-labeled cells were capable to migrate into tumor lesions. Furthermore, these cells could home to the BM and the secondary lymphoid organs such as spleen and metastatic lymph nodes. When we transferred *in vitro* activated CFSE-labeled TRP-2 TCR transgenic activated T cells, we observed a similar migration pattern of labeled cells. After the calculation of absolute numbers of migrated cells in respected organs and tumor lesions, we found that at day 3 after adoptive transfer, highest amounts of CPD<sup>+</sup> cells were detected in metastatic lymph nodes followed by spleen and the BM. In the tumor, the number of cells reached 1x10<sup>6</sup> cells, whereas in the BM it was 3x10<sup>6</sup>, in the spleen -1.5x10<sup>7</sup> and in metastatic lymph nodes -3.5x10<sup>7</sup>. Therefore, the number of detected cells was higher than initially injected (~3x10<sup>6</sup>

cells/mouse) indicating their proliferation *in vivo*. Later, the absolute amount of migrated cells significantly increased in the tumor, by contrast in spleen, the cell number did not change and in the lymph nodes they even decreased. In the BM, the absolute amount of tracked cells slightly increased at day 7. Since the BM was shown to be a preferential site for the migration and selective retention of memory T cells and DC (Feuerer et al., 2001a; Feuerer et al., 2001b), it is not surprising that adoptively transferred cells migrate into the BM.

It is feasible that migrated cells may proliferate in the BM, lymph nodes and the spleen and then migrate to the tumor site. It has been reported that in the BM, T cells are condensed in lymphoid follicles surrounding a blood vessel and the frequency of such follicles can be increased during infections, inflammation and autoimmunity (Bain, 2001; Custer, 1973). Moreover, BM can serve as a secondary lymphatic organ and it appears to be important for systemic T cell-mediated immune response as demonstrated earlier (Feuerer et al., 2003). Furthermore, T cells are homing to the BM and form multicellular clusters together with resident CD11c<sup>+</sup> DC, which can take up blood-borne antigen and serve as APCs for naïve or memory T cells (Schirmacher et al., 2003). As a result, interactions between BM derived-DC and antigen-specific T cells lead to the upregulation of CD69 on T cells and their subsequent clonal expansion (Schirmacher et al., 2003).

Next, we took a closer look at the phenotype of T cells migrated into the tumor. We found that at day 3 after adoptive transfer, there was no significant difference between effector, central memory and naïve CD8<sup>+</sup> T cells. The number of T<sub>EM</sub> cells was lower than that of the other CD8<sup>+</sup> T cell subsets. However, at day 5 and 7 post injection, the relative amount of effector memory CD8<sup>+</sup> T cells increased whereas the numbers of naïve and central memory T cells significantly decreased. A significant increase of effector CD8<sup>+</sup> T cells was found in the tumor at day 7 after i.c. injection. Our findings are similar to the data reported earlier (Pages et al., 2005). Presence of high levels of infiltrating memory and effector CD8<sup>+</sup> T cells was found in patients after examining local immune response within the tumor specimens of resected colorectal cancer (Pages

et al., 2005). Recently, Thompson *et al.* (2010) reported T cell infiltration into the tumor after an adoptive transfer of naïve T cells in B16 melanoma. OT-I T cells, which were found within the tumors, expressed effector functions.

When we used stimulated TRP-2 TCR transgenic effector T cells for an adoptive transfer, we could detect similar migration properties as in adoptive transfer of T cells from the BM of *ret* tg mice. T cells were trafficking into secondary lymph nodes as well as into BM of *ret* transgenic tumor-bearing mice. Furthermore, we have observed a continuous accumulation of CFSE<sup>+</sup> cells within the tumor. Here, we used  $1 \times 10^7$  T cells/mouse, it is 5 times more than we used in the experiment with BM derived T cells, but we observed a four times lower absolute amount of labeled T cells migrated into the spleen. On the contrary, more infiltrating T cells were found at day 7 in the tumor compared to the previous experimental setup with restimulated memory T cells ( $22.6 \pm 18.9 \times 10^5$  cells vs.  $7.2 \pm 3.2 \times 10^5$  cells). This can be due to the fact that these TRP-2 TCR transgenic T cells displayed CD8<sup>+</sup> effector T cell phenotype. It is known that once the antigen-specific effector T cells are activated they migrate through the endothelial cell wall to inflamed tissues or tumor sites where they exert their effector function by releasing cytotoxins such as perforin, granzymes and granulysin (Barry and Bleackley, 2002). Since effector T cells have a decreased expression of homing receptors like L-selectin (CD62L) and CC-chemokine receptor 7 (CCR7), they have a reduced potential for homing to secondary lymphoid organs (Finlay and Cantrell, 2011). However, with the increased expression of chemokine receptors CCR5 and CCR2 they have a greater capacity to migrate to inflamed tissues (Kaech et al., 2002). It is therefore reasonable that in our experiment with the adoptive transfer of TRP-2 TCR transgenic CD8<sup>+</sup> effector T cells we detected less CFSE<sup>+</sup> cells in the spleen and a greater absolute amount in the tumor compared to the adoptive transfer of restimulated memory T cells and DC. We found equal amounts of naive, effector, T<sub>CM</sub> and T<sub>EM</sub> CD8<sup>+</sup> TRP-2 TCR transgenic T cells at day 3 and 5. On day 7, we measured a slightly decreased relative amount of T<sub>eff</sub> and T<sub>naive</sub> CD8<sup>+</sup> cells but an increase in effector memory CD8<sup>+</sup> T cell numbers. In contrast, the relative amount of T<sub>eff</sub> CD8<sup>+</sup> cells increased when using activated BM-derived T cells from *ret* transgenic mice.

We hypothesize that transferred T cells migrate to secondary lymphoid organs, the BM and only a small number does migrate into tumor lesions. They are activated by APCs, clonally expanded and change their phenotype. Thereafter, effector T cells migrate into tumor lesions and exert their effector functions.

## 5.4 Melanoma-specific reactivity *in vivo* of *in vitro* activated T cells

We have shown that restimulated BM derived T cells from *ret* transgenic mice with and without visible tumors are capable to migrate into metastatic lymph nodes and to tumor sites. Furthermore, these cells migrate to spleen and the BM, where they encounter antigens presented by APCs and are clonally expanded. In our next experiments, we examined the anti-tumor activity of adoptively transferred BM-derived T cells from *ret* transgenic mice after 40 h of restimulation with DC pulsed with tumor lysate. The therapy with melanoma-antigen specific cells leads to a significant increase of the overall survival of tumor-bearing mice compared to mice treated with PBS. Although the tumors did not disappear, the tumor growth was significantly delayed. Moreover, mice in the treated group appeared more active and their fur was shiny compared with the untreated group where mice were moving very slow, had a sickly appearance and their fur was dull.

It has been recently reported that after the manipulation of the tumor microenvironment with PDE-5 inhibitor sildenafil, the amounts of MDSCs and their inhibitory functions were decreased (Meyer et al., 2011). Furthermore, a reduction of inflammatory mediators such as IL-6, IL-1 $\beta$ , VEGF and S100A9 was observed which led to a partial restoration of the  $\zeta$ -chain expression on T cells and a significantly increased survival. Therefore, we decided to treat tumor-bearing mice with sildenafil one week before the adoptive transfer. The survival of mice pretreated with sildenafil and AT of reactivated BM-derived T cells was significantly increased compared to the PBS group and the group treated with sildenafil alone. Since the amount of animals per group was low due to the

humble numbers of T cells in the murine BM, experiments have to be repeated to confirm these results.

When we used *in vitro* generated TRP-2-specific effector T cells from TRP-2 TCR transgenic mice for therapy, we were not able to observe the same effects as after the therapy with memory T cells even in the combination with sildenafil. Although TRP-2-specific T cells were found to migrate into tumor lesions, they might be inhibited by the immunosuppressive cells and mediators of the tumor microenvironment. Once the T cells are inhibited, they either undergo apoptosis or remain in an anergic state (Dong et al., 2002; Selenko-Gebauer et al., 2003).

BM-derived reactivated memory T cells from *ret* transgenic mice could be a promising tool for adoptive immunotherapy against melanoma since they are capable to migrate into tumor lesions and lymphatic organs. Their differentiation from  $T_{CM}$  into  $T_{EM}$  and  $T_{eff}$  cells within the tumor is implicated by the increase and decrease of the respective T cell subsets over time. Moreover, their anti-tumor reactivity could be determined by a significantly prolonged survival in tumor-bearing mice after adoptive transfer of these cells alone and in combination with the inhibition of the MDSC-induced immunosuppression.

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