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Gene expression profiles of T cells after adoptive transfer in a mouse model of pancreatic carcinoma

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1. Introduction

1.1 Adoptive T cell transfer for cancer immunotherapy

Cancer is one of the most deadly diseases in spite of significant advances in the fields of surgery, radiation therapy and chemotherapy [Siegel et al., 2014]. Based on an increasing understanding of tumor immunology, immunotherapy, which aims at stimulating or enhancing the function of the immune system to eradicate malignant cells, has emerged as an effective strategy for a number of cancer types [DeVita et al., 2012].

Among the rapidly emerging strategies for cancer immunotherapy, adoptive T cell transfer (ACT) is one of the most promising avenues. ACT transfers lymphocytes with enhanced functionality into patients to target tumor cells. It takes advantages of the natural ability of T cells to specifically recognize and eliminate targeted malignant cells, and has shown potent anti-tumor effects in defined patients [Rosenberg et al., 2015]. Currently, ACT may be classified in three different approaches: the use of autologous tumor-infiltrating lymphocytes (TILs) and the infusion of autologous T cells genetically engineered with high-affinity T cell receptors (TCRs) or with chimeric antigen receptors (CARs) [Gattinoni et al., 2006] (Figure 1).

1.1.1 Autologous tumor-infiltrating lymphocytes in the treatment of metastatic melanoma

For TIL therapy, lymphocytes are harvested from resected tumor specimens. These cells are expanded in the presence of stimulation factors, such as interleukin-2 (IL-2) or anti-CD3 antibodies, before transfer back into patients [Rosenberg et al., 2015]. A number of clinical trials have demonstrated complete and durable tumor regression in some patients with metastatic melanoma (Table 1).

However, successful application of TIL therapy has been so far restricted to the treatment of melanoma. Reasons include: a) the difficulty of generating TIL cultures due to low immune infiltrates [Clemente et al., 1996, Galon et al., 2006, Ino et al., 2013], b) the lack of readily accessible tissue in advanced tumor stages, c) the low specificity of TIL culture for the cancer cells [Dudley et al., 2003] and d) the risk that TIL may recognize only antigen lost to the cancer cell during the process of immunoediting [Dunn et al., 2002, Gyorki et al., 2013]. Accordingly, only a few clinical trials have used TILs for the treatment of epithelial cancers [Tran et al., 2014, Stevanovic et al., 2015].

1.1.2 Redirecting T cell specificity by genetic engineering with TCR or CAR

The high response rates to TIL therapy observed in patients with melanoma have provided clinical evidence that infusion of tumor-specific T cells can eradicate cancer cells. However, the limitations of TIL mentioned above prevent broad application of this approach. In contrast, a strategy which would in theory be applicable to every patient through *ex vivo* generation of the cellular products would have significant advantages over TIL while exploiting their efficacy. To enhance T cell specificity, autologous T cells isolated from peripheral blood can be genetically engineered with tumor-specific antigen receptors. The manipulated T cells can be expanded *ex vivo* for several weeks before reinfusion to patients [Kershaw et al., 2013]. Several approaches to engineer T cells for transgene expression have been investigated, but the most frequently used is currently virus-based transduction [June et al., 2009]. This viral transduction utilizes viral vectors derived from retroviruses or lentiviruses, which are able to integrate into the host genome and provide stable transgene expression over prolonged periods of time.

To redirect T cell specificity, two classes of receptors have been developed. High-affinity antigen-specific TCRs have the conventional structures of α- and β-chains, and can recognize epitopes of antigens presented by major histocompatibility complex (MHC) molecules [Kalos et al., 2013]. One strategy to generate such TCRs with high affinity is to select these from tumor-specific TIL populations. Such tumor antigen-specific TCRs can be sequenced using high-throughput sequencing [Li et al., 2005, Chervin et al., 2008] and cloned into vectors for gene transfer. TCR-based therapy was firstly used in the treatment of melanoma patients targeting melanocyte differentiation antigens (MDAs), such as melanoma-associated antigen recognized by T cells 1 (MART-1) and glycoprotein 100 (gp100) [Morgan et al., 2006, Johnson et al., 2009]. Cancer-testis antigens are other potential targets for TCR therapy [Robbins et al., 2011, Rapoport et al., 2015] with potentially less autoimmune toxicities than those observed in MDA-targeting TCR therapy. TCR-based T cell therapy also comes with a number of limitations. For example, tumor cells can escape T cells through MHC down-regulation, and mutagenesis might generate neo-epitopes that can result in unwanted immune responses in patients [Kalos et al., 2013]. In addition, the exogenous TCR might compete with endogenous TCR, which results in mispairing and suboptimal expression of exogenous TCR [Heemskerk et al., 2007, Govers et al., 2010].

In contrast, CARs [Gross et al., 1989] are hybrid antibody single-chain variable fragments coupled with T cell co-stimulatory signaling domains. These domains include CD28, CD134 (also known as OX40), or CD137 (also called 4-1BB) and T cell intracellular CD3 ζ signaling

chain [Barrett et al., 2014]. CARs can recognize antigens on the cell surface in a non-MHC-restricted manner. In addition, CAR T cells overcome the limitation generated by a lack of a second co-stimulatory signal in TCR T cells [Sadelain et al., 2013]. The most promising results obtained with CAR-based therapy have been seen when targeting CD19 in B cell malignancies, and tumor regression have been observed in clinical trials in both adult and pediatric patients (Table 1). There are also limitations to CAR therapy [Lipowska-Bhalla et al., 2012] which include the restriction to surface proteins.

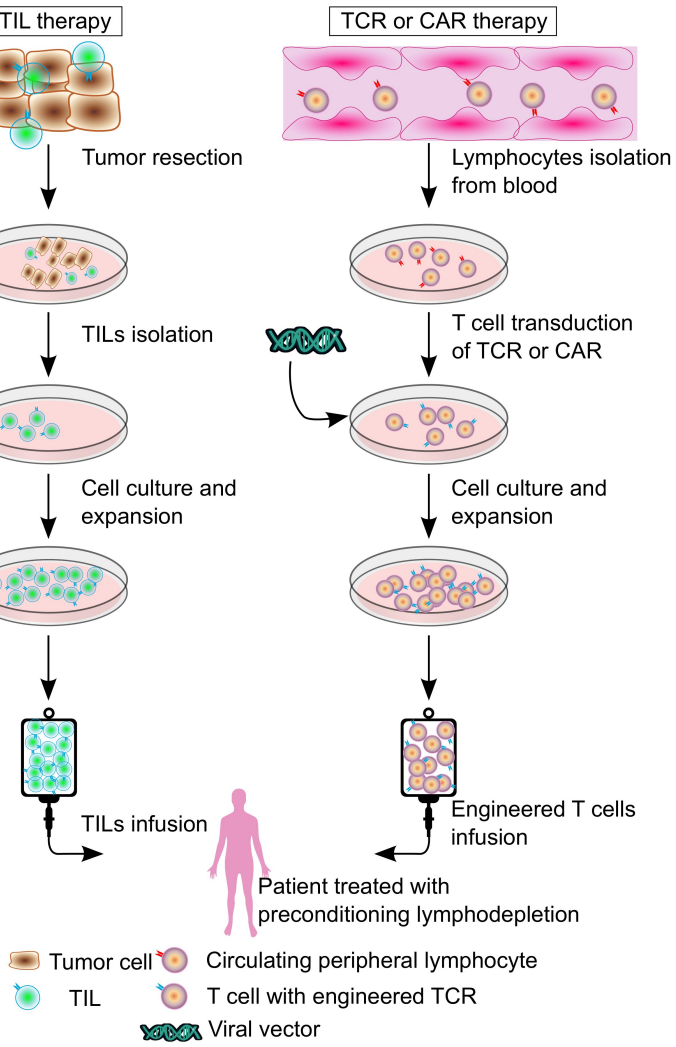

Figure 1: Comparison of schematic work flow for different types of T cell therapy (modified from Rosenberg, S. A., 2011). T cells are isolated from resected tumors or from peripheral blood. Tumor-infiltrating lymphocytes (TILs) are amplified in the presence of growth-stimulating cytokines (such as IL-2) and are infused back to patients. Autologous T cells are genetically engineered with antigen-specific TCRs or CARs, and are amplified to reach large numbers before infusion.

Table 1: Summary of successful clinical trials investigating T cell therapy in cancer.

Type of cell therapy	Cancer entity	Target	Number of patients	Overall response	References
TILs	Melanoma		20	60%	Rosenberg et al., 1988
	Melanoma		86	34%	Rosenberg et al., 1994
	Melanoma		13	46%	Dudley et al., 2002
	Melanoma		50	50% - 70%	Dudley et al., 2008
	Melanoma		93	22%	Rosenberg et al., 2011
	Melanoma		55	48%	Itzhaki et al., 2011
	Melanoma		6	60%	Ellebaek et al., 2012
	Melanoma		13	38%	Pilon-Thomas et al., 2012
	Melanoma		31	48%	Radvanyi et al., 2012
	Melanoma		57	40%	Besser et al., 2013
	Cervical cancer	Human papillomavirus (HPV)	9	33%	Stevanovic et al., 2015
TCRs	Melanoma	MART-1	15	13%	Morgan et al., 2006
	Melanoma	MART-1, gp100	36	30% for human TCR and 19% for mouse TCR	Johnson et al., 2009
	Melanoma and synovial cell	NY-ESO-1	11 for melanoma	45% for melanom	Robbins et al., 2011

	sarcoma		and 6 for synovial cell sarcoma	a and 67% for synovial cell sarcoma	
	Myeloma	NY-ESO-1	20	80%	Rapoport et al., 2015
CARs	B cell lymphoma	CD19	1	100%	Kochenderfer et al., 2010
	Chronic lymphocytic leukemia (CLL)	CD19	3	100%	Kalos et al., 2011
	Neuroblastoma	GD2	11	27%	Louis et al., 2011
	CLL and B cell lymphoma	CD19	8	75%	Kochenderfer et al., 2012
	Acute lymphoblastic leukemia (ALL)	CD19	5	100%	Brentjens et al., 2013
	ALL	CD19	2	100%	Grupp et al., 2013
	ALL	CD19	16	88%	Davila et al., 2014
	ALL	CD19	30	90%	Maude et al., 2014
	B cell lymphoma	CD19	15	80%	Kochenderfer et al., 2015
	ALL	CD19	21	67%	Lee et al., 2015

1.2 Limitations to T cell therapy and strategies to overcome these

In spite of promising results, there is no cancer type where ACT is an approved treatment. This is due to limited efficacy, as cancer regression could only be seen in a minority of patients with defined cancer entities [Rosenberg et al., 2015]. Current evidences suggest that both T cell intrinsic factors and extrinsic mechanisms arising from the tumor microenvironment significantly contribute to ACT failure and will need to be overcome to

enable ACT as therapeutics. [Rosenberg, S. A., 2011].

1.2.1 Identification of suitable tumor-specific target antigens

Ideal candidate antigens for ACT should be exclusively expressed on tumor cells and not found on normal tissues. A lack of specificity may result in severe autoimmune toxicities in patients.

Recently, neoepitopes arising from cancer-specific gene mutations have been identified as promising targets for ACT [Lu et al., 2013, Robbins et al., 2013]. Such mutations are rarely shared between patients, thus targeting cancer specific mutated epitopes would be an individualized therapy. In contrast, viral proteins may also fulfill the requirement of specificity as these would not be expressed or presented by healthy tissues. One example of oncogenic virus providing specific targets is HPV which drives cervical cancer [Piersma et al., 2008, van Steenwijk et al., 2010]. Epstein-Barr virus (EBV) antigens for lymphoma [Bollard et al., 2004], nasopharyngeal carcinoma [Comoli et al., 2005] and lymphoproliferative disorders [Heslop et al., 2010, Bollard et al., 2012] would be other examples for suitable viral motives.

1.2.2 Prolongation of T cell persistence

Poor persistence of T cells after ACT limits its efficacy. Based on data from both clinical trials and animal experiments, several crucial factors influencing the persistence of transferred T cells *in vivo* have been identified. These include, the phenotype and differentiation state of T cells *ex vivo* before transfer, T cell exhaustion state *in vivo* after transfer and potential host immune responses against infused cells [Kalos et al., 2013].

The differentiation state of T cells before transfer is inversely correlated with proliferation and persistence of T cells after transfer [Gattinoni et al., 2012]. T cells used in an early differentiation state, such as naive [Hinrichs et al., 2009], central memory [Klebanoff et al., 2005, Berger et al., 2008] or memory stem T cells [Gattinoni et al., 2011] might have longer persistance, enhanced proliferative potential and effective anti-tumor function. On the other hand, T cells with full effector function *in vitro* have impaired capacity to mediate tumor regression after transfer [Gattinoni et al., 2005]. In this respect, CD27 has been utilized as a predictive biomarker to select less differentiated T cells for ACT [Gattinoni et al., 2011, Hinrichs et al., 2011]. CD27 is mainly expressed by naive and memory T cells, but downregulated in late stage effector T cells. The cell culture conditions *in vitro* are also

important for the generation of less differentiated T cells. The addition of IL-7 and IL-15 to cell culture can induce and maintain T cells in a memory-like state [Cieri et al., 2013]. An additional feature of a successful cell product may be the collaboration of different cell populations. $CD4^+$ T cells, for example, are required for the formation of $CD8^+$ memory T cells [Sun et al., 2003, Sun et al., 2004]. For this reason, the transfer of mixed $CD4^+$ and $CD8^+$ T cells for ACT might be superior to the transfer of either population alone [Huang et al., 2002].

The exhaustion state of T cells after transfer is another important factor that hampers T cell persistence and function. Several mechanisms contribute to T cell exhaustion [Jiang et al., 2015], such as checkpoint pathway, immunosuppressive cells and transforming growth factor β (TGF-β). Checkpoint blockade strategy has been applied in combination with CAR therapy, using monoclonal antibodies targeting programmed cell death protein 1 (PD-1) [John et al., 2013] and cytotoxic T lymphocyte antigen 4 (CTLA-4) [Mahvi et al., 2015]. More recently, in our group, a PD-1-fusion receptor has been developed to genetically engineer T cells before transfer and render them insensitive to check point inhibitory pathways [Kobold et al., 2015].

Another strategy to enhance T cell persistence after transfer is to manipulate immunosuppressive immune cells in the tumor microenvironment. Regulatory T cells (Treg) predict a worse outcome of patients treated with TIL therapy [Yao et al., 2012]. Depleting these immunosuppressive cells may improve ACT. Lymphodepletion by total body irradiation (TBI) in addition to a cyclophosphamide chemotherapy regimen [Dudley et al., 2008] has been utilized for this purpose. This method can lead to decreased numbers of regulatory T cells (Tregs) [Yao et al., 2012], as well as increased levels of beneficial cytokines (such as IL-7 and IL-15) for effector T cells [Gattinoni et al., 2005, Dudley et al., 2008, Wrzesinski et al., 2010]. TBI can also enhance the function of innate immune cells by inducing microbial translocations from the gut, which activates dendritic cells and strengthens dendritic cell-mediated stimulation of adaptive immunity [Paulos et al., 2007]. Other strategies to reprogram the tumor microenvironment include engineering of T cells to express IL-12, which leads to activation of antigen presenting cells (APCs) in the tumor environment and enhanced anti-tumoral $CD8^+$ T cell response [Wagner et al., 2004, Zhang et al., 2012].

1.2.3 Improvement of T cell migration and trafficking into tumors

The successful migration and trafficking of T cells to the targeted tumor site is another important consideration for ACT success [Fisher et al., 2006]. The amount of transferred T cells migrating into tumor milieu correlates positively with clinical outcome [Fridman et al.,

2012], but the trafficking efficacy of transferred T cells to tumor tissues is very low [Fisher et al., 1989, Griffith et al., 1989]. It is thus necessary to develop strategies to improve T cell migration into the tumor site. Homing of effector T cells to tumor sites mainly depends on two factors: adhesion molecules [Bevilacqua, M. P., 1993] and specific chemokines secreted from tumor tissues [Balkwill, F., 2004].

Some adhesion molecules essential for T cell migration into tumors are down-regulated on tumor-infiltrating vascular endothelial cells [Piali et al., 1995, Griffioen et al., 1996]. The down-regulation of such molecules might be due to the increasing amounts of vascular endothelial growth factors and fibroblast growth factors secreted by tumor tissues. Those factors prevent T cells from leukocyte-vessel interaction and migration through blood vessels to tumor tissues. Strategies have been developed to overcome the endothelial barrier by administration of tumor necrosis factor-α (TNF-α) [Ten Hagen et al., 2008] and normalization of vasculature in the tumor milieu using anti-angiogenic antibodies to facilitate T cell infiltration into tumors [Jain, R. K., 2005, Chung et al., 2010, Huang et al., 2012].

A subset of chemokines are found to be abundant in tumor tissues and play critical roles in T cell infiltration. Based on chemokine profile studies, CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10 in metastatic melanoma [Harlin et al., 2009], CCL2 in several cancer cell lines [Brown et al., 2007], CXCL16 in breast cancer [Matsumura et al., 2008], colorectal cancer [Hojo et al., 2007], glioma [Ludwig et al., 2005], renal cell cancer [Gutwein et al., 2009] and pancreatic carcinoma [Meijer et al., 2008, Wente et al., 2008] may elicit T cell migration. On the other hand, some chemokine receptors expressed by TILs may have important functions in T cell recruitment, such as CXCR1 in melanoma [Sapoznik et al., 2012], CCR5, CCR6, CXCR3, and CXCR6 in renal cell carcinoma [Oldham et al., 2012] and CXCR3 in gastric carcinoma [Musha et al., 2005, Ohtani et al., 2009]. Strategies have been applied to redirect T cell trafficking into target sites by transducing appropriate chemokine receptors on T cells, such as CCR2 [Craddock et al., 2010, Moon et al., 2011, Asai et al., 2013], CXCR2 [Kershaw et al., 2002] and CCR4 [Di Stasi et al., 2009, Rapp et al., 2015] which all enhanced T cell infiltration and anti-tumor function of ACT.

1.2.4 Safety considerations of ACT

The major side effect of observed after ACT is a cytokine release syndrome, which is driven by massive T cell activation. CAR T cell trials were halted after some patients' deaths due to high levels of inflammatory molecules such as IL-6 [Morgan et al., 2010, Maude et al., 2014]. Autoimmune side effects against skin and eyes were observed in patients who received

high-avidity TCRs recognizing non-mutated self-tissue antigens, such as MART-1 and gp100 in patients suffering from melanoma [Morgan et al., 2006, Johnson et al., 2009] or carbonic anhydrase 9 for CAR therapy of patients with metastatic renal cell carcinoma [Lamers et al., 2013]. Autoimmune side effects observed stress the need to find better antigens for targets with tumor cell restriction, as discussed above. Furthermore, investigators have also explored novel strategies to control T cell activation, such as introducing inducible caspase 9 into the transferred T cells [Di Stasi et al., 2011, Budde et al., 2013, Gargett et al., 2014].

The genetic manipulation processes using viral elements to transduce T cells, may result in insertional oncogenesis or cellular transformation. This is another potential risk of ACT. However, integration-related insertional mutagenesis caused by retroviral transduction was so far only observed in hematopoietic precursor cells [Hacein-Bey-Abina et al., 2008], while no adverse effects have been demonstrated in mature T cells using retroviruses [Muul et al., 2003, Bushman, F. D., 2007, Scholler et al., 2012]. Consistent with what is seen with retroviral transduction, no safety issues have been reported regarding insertional mutation caused by lentiviral integration [Wang et al., 2009]. Alternatives to virus-based T cell transduction, are to introduce the messenger RNA (mRNA) for the given construct directly into the T cell by electroporation [Zhao et al., 2006], transposon [Perez et al., 2008, Hackett et al., 2010] or transcription activator-like effector nucleases [Reyon et al., 2012].

1.3 Objectives

1.3.1 Background

Few studies have addressed gene expression alterations of transferred T cells after transfer in the tumor microenvironment. The answer to this question might provide useful biomarkers to optimize ACT.

Most previous studies have focused on naturally arising T cells in tumor models [Klebanoff et al., 2006, Thompson et al., 2010]. However, the transferred T cells are different from naturally generated effector T cells, since they have undergone *ex vivo* manipulation, including *ex vivo* polyclonal stimulation, culture in the presence of growth factors, introduction of gene information by viral transduction and expression of exogenous immune receptor which would interact with endogenous TCRs [Burns et al., 2009]. On the other hand, the immune condition of the hosts is changed by lymphodepletion. Therefore, the biological features and behaviors of transferred T cells might be quite different from those of naturally occurring T cells.

One study compared gene expression profiles of TCR-engineered T cells before and after

infusion into patients with melanoma [Abate-Daga et al., 2013]. They found out that the transferred T cells detected in the peripheral blood have higher expression of inhibitory receptors than those before transfer. In this study, gene expression profiles of T cells have been only measured in peripheral blood cells. In contrast, T cell activity measured in the blood does not predict whether these T cells which are able to infiltrate the tumor or have anti-tumor efficacy. Further studues to characterize the intrinsic molecular signatures of transferred T cells by comparing the gene profiles at the tumor site and in lymphoid tissues are needed.

1.3.2 Aims of the thesis project

The aims of this thesis are:

1. to compare gene expression profiles of antigen specific T cells before ACT and after ACT in spleen and tumor, by utilizing reverse transcription polymerase chain reaction (RT-PCR) arrays involving genes important for T cell migration, activation and exhaustion;
2. to validate candidate genes that are significantly differentially regulated in the arrays;
3. to validate one candidate gene on protein level.

2. Materials and Methods

2.1 Materials

2.1.1 Technical equipment

Aeroject ultra tips (10 µl, 20 µl, 200 µl)	Ratiolab, Dreieich, Germany
Balance (LP 6209)	Sartorius, Göttingen, Germany
Bioanalyzer 2100	Agilent, Santa Clara, USA
Cell culture incubator (BD 6220)	Thermo Scientific Heraeus, Hanau, Germany
Cell culture laminar flow hoods	Thermo Scientific Hareaus, Hanau, Germany
Centrifuge 3L- R Mulifuge	Thermo Scientific Heraeus, Hanau, Germany
Centrifuge 460R	Andreas Hettich, Tuttlingen, Germany
Centrifuge 5415R	Eppendorf, Hamburg, Germany
Electrophoresis chamber	Bio Rad, Munich, Germany
96 - well - ELISA plate	Corning, New York, USA
FACS Canto II	Becton Dickinson, San Jose, USA
FACS AriaIII Cell Sorter	Becton Dickinson, San Jose, USA
Heating block Thermomixer 5436	Eppendorf, Hamburg, Germany
Transwell®-96 Permeable 5.0 µm Pore Polycarbonate Membrane	Corning, New York, USA
Light Cycler 480	Roche, Mannheim, Germany
LightCycler®480 Multiwell Plate 96	Roche, Mannheim, Germany
Microscopes Axiovert 40 C and HAL 100	Zeiss, Jena, Germany
Millex sterile filter unit (0.45 µm)	Millipore, Darmstadt, Germany
MilliQ water preparation equipment	Milliopore, Darmstadt, Germany
Minisart-plus sterile filter (0.2 µm)	Sartoorius AG, Goettingen, Germany
Mithras LB940 Multimode plate Reader	Berthold Tech, Bad Wildbad, Germany
NanoDrop Spectrophotometer	Thermo Scientific, Waltham, USA
pH meter	WTW, Weilheim, Germany
Refrigerators (4 °C, -20 °C, -80 °C)	Thermo Scientific, Waltham, USA
Rotina 420R centrifuge	Hettich, Massachusetts, USA
Shaker IKA-Vibrax VXR	IKA, Staufen, Germany
Shaking incubator	Innova, Hamburg, Germany
Thermocycler T3	Biometra, Göttingen, Germany
Thermomixer	Eppendorf, Hamburg, Germany

2.1.2 Chemicals, reagents and buffers

Agarose LE	Biozym, Hess. Oldendorf, Germany
Ampicillin	Sigma Aldrich, Steinheim, Germany
Bovine serum albumine (BSA)	Sigma Aldrich, Steinheim, Germany
Calcium chloride ($CaCl_2$)	Merck, Darmstadt, Germany
Collagenase, from *Clostridium histolyticum*	Sigma Aldrich, Steinheim, Germany
Deoxyribonuclease I, from bovine pancreas	Sigma Aldrich, Steinheim, Germany
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, Steinheim, Germany
6 × DNA gel loading dye	Fermentas, St. Leon-Rot, Germany
Easycoll separating solution	Biochrom, Berlin, Germany
Ethidium bromide (EB)	Sigma Aldrich, Steinheim, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, Steinheim, Germany
FACS Flow, FACS Safe	Becton Dickinson, San Jose, USA
Glycerol	Sigma Aldrich, Steinheim, Germany
Isoflurane (Forene®)	Abbott, Zug, Switzerland
Isopropanol (70 Vol %)	Apotheke Innenstadt, LMU Munich
LB medium (Lennox)	Carl Roth, Karlsruhe, Germany
LB-Agar (Lennox)	Carl Roth, Karlsruhe, Germany
Methanol	Sigma Aldrich, Steinheim, Germany
Protamine sulfate salt	Sigma Aldrich, Steinheim, Germany
RNase A	Qiagen, Hilden, Germany
RNase-Free Water	Qiagen, Hilden, Germany
Sodium azide (NaN_3)	Sigma, Aldrich, Steinheim, Germany
Sodium chloride (NaCl 0.9 %)	Apotheke Innenstadt, LMU Munich
Tween-20	Sigma, Aldrich, Steinheim, Germany
10 × Tris/Borate/EDTA (TBE) buffer	Apotheke Innenstadt, LMU Munich

ELISA wash buffer
0.05 % Tween 20
in PBS
pH 7.2 - 7.4

ELISA dilution buffer
1 % BSA
in PBS
pH 7.2 - 7.4

Easycoll separating solution
9 ml 44 % Easy-coll in PBS
6 ml 67 % Easy-coll in PBS

Agarose gel
1 % agarose
0.5 µg/ml EB
in 1 × TBE buffer

FACS buffer

1 % BSA

2 mM EDTA

0.1 % sodium azide

in PBS

2.1.3 T cell transduction reagents, buffers

Chloroquine diphosphate salt	Sigma Aldrich, Steinheim, Germany
Retronectin, Recombinant human fibronectin	Takara Biomedicals, Japan

2 × HEPES-buffered saline (HeBS) solution

275 mM natrium chlorid

10 mM potassium chloride

3.52 mM disodium hydrogen phosphate

40 mM HEPES acid in distilled water

pH 7.05 - 7.12

0.2 µm pore filter-sterilized

2.5 M calcium chloride

3.67 g $CaCl_2$

in 10 ml H_2O

0.2 µm pore filter sterilized

2.1.4 Kits

Bio-Rad DC Protein Assay	Bio-Rad, Munich, Germany
DNA gel loading ladder	Fermentas, St. Leon-Rot, Germany
DuoSet Mouse CCL1/TCA-3 ELISA	R and D systems, Wiesbaden, Germany
Gel and PCR clean-up system	Promega, Mannheim, Germany
Plasmid miniprep system	Fermentas, St. Leon-Rot, Germany
Plasmid maxiprep system	Promega, Mannheim, Germany
Restriction enzyme kit (EcoR I, Not I)	Fermentas, St. Leon-Rot, Germany
RNeasy Micro Kit	Qiagen, Hilden, Germany
RNeasy Mini Kit	Qiagen, Hilden, Germany

Real-time PCR

LightCycler® 480 Probes Master	Roche, Mannheim, Germany
RealTime ready cDNA Pre-Amp Master	Roche, Mannheim, Germany
RevertAid First Strand cDNA Synthesis Kit	Fermentas, St. Leon-Rot, Germany

PreAMP cDNA Synthesis Primer Mix (Mouse Chemokines and Receptors)	SABiosciences, Hilden, Germany
PreAMP cDNA Synthesis Primer Mix (Mouse T cell anergy and immune tolerance)	SABiosciences, Germany
RT^2 Profiler PCR Array (Mouse chemokines and receptors)	SABiosciences, Germany
RT^2 Profiler PCR Array (Mouse T cell anergy and immune tolerance)	SABiosciences, Germany
RT^2 qPCR Primer Assay (mouse Pdcd1)	SABiosciences, Germany
RT^2 SYBR Green qPCR Master Mix	SABiosciences, Germany

2.1.5 Cell culture medium, reagents and materials

β-Mercaptoethanol	Sigma Aldrich, Steinheim, Germany
Blasticidine S hydrochloride	Sigma Aldrich, Steinheim, Germany
Dulbecco's modified Eagle's medium (DMEM), high glucose	PAA, Pasching, Austria
Fetal calf serum (FCS)	GibcoBRL, Karlsruhe, Germany
HEPES (1 M)	Sigma Aldrich, Steinheim, Germany
L-glutamine 200 mM	PAA, Pasching, Austria
Phosphate-buffered saline (PBS)	PAA, Pasching, Austria
Penicillin/Streptomycin (100 fold)	PAA, Pasching, Austria
Puromycin dihydrochloride	Sigma Aldrich, Steinheim, Germany
Roswell Park Memorial Institute (RPMI) 1640 medium	PAA, Pasching, Austria
Sodium pyruvate	PAA, Pasching, Austria
Trypan blue	Sigma Aldrich, Steinheim, Germany
Trypsin (10 x)	PAA, Pasching, Austria

T cell medium	**Platinum-E cell line medium**
10 % FCS	10 % FCS
2 mM L-glutamine	2 mM L-glutamine
100 µg/ml streptomycin	100 µg/ml streptomycin
100 IU/ml penicillin	100 IU/ml penicillin
1 mM sodium pyruvate	1 µg/mL puromycin
1 mM HEPES	10 µg/mL blasticidin

50 µM β-Mercaptoethanol in DMEM medium (high glucose)
in RPMI 1640

Panc02-OVA cell line medium

10 % FCS
2 mM L-glutamine
100 µg/ml streptomycin
100 IU/ml penicillin
in DMEM medium (High Glucose)

Cytokines, functional grade antibodies and growth factors

Anti-Mouse CD3e (clone 145-2C11, Armenian Hamster, IgG)	eBioscience, Frankfurt, Germany
Anti-Mouse CD28 (clone 37.51, Golden Syrian Hamster, IgG)	eBioscience, Frankfurt, Germany
Dynabeads anti-mouse CD3/CD28	Life Technologies, Carlsbad, Canada
Interleukin-2, human recombinant	PeproTech, Hamburg, Germany
Interleukin-15, human recombinant	PeproTech, Hamburg, Germany
CCL1/I-309/TCA-3, mouse recombinant	R & D systems, Wiesbaden, Germany

Expendable plastic materials for cell culture experiments were purchased from Becton Dickinson (Heidelberg, Germany), Bibby Sterrilin (Stone, Staffordshire, Great Britain), Corning (New York, USA), Eppendorf (Hamburg, Germany), Falcon (Heidelberg, Germany), Greiner (Frickenhausen, Germany), Henke-Sass Wolf (Tuttlingen, Germany) or Sarstedt (Nümbrecht, Germany).

Special plastic materials for T cell transduction were purchased from the following distributors: Millex-HV sterile filter (0.45 µm pore) from Millipore (Darmstadt, Germany), Polystyrene falcon tube from Becton Dickinson (Heidelberg, Germany) and Syringe sterile filter (0.2 µm pore) from Corning (New York, USA).

2.1.6 FACS antibodies

Antibody	Distributor	Clone	Isotype
PE-Cy7 CD3	Biolegend	145-2C11	Armenian Hamster IgG
APC CD8	Biolegend	53-6.7	Rat IgG2a, κ
PerCP/Cy5.5 LAG-3	Biolegend	C9B7W	Rat IgG1, κ

APC PD-1	Biolegend	29F.1A12	Rat IgG2a, κ
PE-Cy7 PD-1	Biolegend	29F.1A12	Rat IgG2a, κ
Pacific Blue CD62L	Biolegend	MEL-14	Rat IgG2a, κ
Anti CCR8	GeneTex	Polyclone	Goat IgG
Goat IgG isotype	GeneTex	Polyclone	
Anti-Mouse CD16/CD32 (Fc Block)	BD Biosciences	2.4G2	Rat
Alexa Fluor 647 Anti-Goat	Jackson		Donkey

2.1.7 Primers for real-time PCR

Primers for the array were designed and provided by Qiagen on 96-well plates. Primers for real-time PCR to validate candidate genes using the probe method were designed by Probe Finder (version 2.48) from Roche Universal Probe Library as shown in Table 2.

Table 2: Primers for real-time PCR using specific probes.
BTLA: B- and T-lymphocyte attenuator, Cma1: Chymase 1, DGK α: Diacylglycerol kinase α, Eomes: Eomesodermin, GM-CSF: Granulocyte-macrophage colony-stimulating factor, HDAC9: Histone deacetylase 9, HPRT: Hypoxanthine-guanine phosphoribosyltransferase, PKC γ: Protein kinase C γ.

Description	Sequences		Probe
Mouse HPRT	Forward	5’-GGAGCGGTAGCACCTCCT-3’	#69
	Reverse	5’-AACCTGGTTCATCATCGCTAA-3’	
Mouse T cell anergy and immune tolerance			
Mouse PD-1	Forward	5’-TGCAGTTGAGCTGGCAAT-3’	#81
	Reverse	5’-GGCTGGGTAGAAGGTGAGG-3’	
Mouse 4-1BB	Forward	5’-GGCCTTCCAGTCCACCAT-3’	#46
	Reverse	5’-GTCCAGGAGTCATGCAGAGG-3’	
Mouse BTLA	Forward	5’-GGGAATTCTTCATCCTCCATC-3’	#50
	Reverse	5’-GTTGCACTGGACACTCTTCATC-3’	
Mouse CD40	Forward	5’-GAGTCAGACTAATGTCATCTGTGGTT-3’	#105
	Reverse	5’-ACCCCGAAAATGGTGATG-3’	
Mouse CD70	Forward	5’-GTCCTTCACACACGGACCA-3’	#25
	Reverse	5’-AGGCCATCTTGATGGATACG-3’	
Mouse Cma1	Forward	5’-TCTTCTTACTCTTCATCTGCTGCT-3’	#2
	Reverse	5’-GTGCCTCCAATGATCTCTCC-3’	
Mouse CTLA-4	Forward	5’-CAAGGCTTCTGGATCCTGTT-3’	#32
	Reverse	5’-GGGCAAATGTGCTGAGGT-3’	

Mouse DGK α	Forward	5'-TCCTCAGTTCCGGATATTGGT-3'	#22
	Reverse	5'-TGGTCTCTAGAACCCAGCCTAC-3'	
Mouse Eomes	Forward	5'-TCCAAGCGGTCAAGTATGC-3'	#21
	Reverse	5'-TAGCAACCAGCCATTTCCTC-3'	
Mouse GM-CSF	Forward	5'-GCATGTAGAGGCCATCAAAGA-3'	#79
	Reverse	5'-CGGGTCTGCACACATGTTA-3'	
Mouse HDAC9	Forward	5'-AATGCACAGTATGATCAGCTCAG-3'	#1
	Reverse	5'-GAGATCTGTCCTCAGGTCTAAAGG-3'	
Mouse IL-7R	Forward	5'-TCTAGCTCAGAAGCATTTGCAC-3'	#4
	Reverse	5'-CTAATCCAACAACAGGGAAAACA-3'	
Mouse L-selectin	Forward	5'-TGCTCTATTCAAGTTGGGAAAGT-3'	#6
(CD62L)	Reverse	5'-GGCTGTCACTCACAGATAGTGG-3'	
Mouse OX40	Forward	5'-GCCTGTCCGCCTACTCTTCT-3'	#10
	Reverse	5'-GTTTTTCCTTGCAGGGTGTG-3'	
Mouse PKC γ	Forward	5'-GTCGACTGGTGGTCTTTTGG-3'	#18
	Reverse	5'-CTCATCTTCCCCATCAAAGG-3'	
Mouse chemokines and receptors			
Mouse CCR8	Forward	5'-AGAAGAAAGGCTCGCTCAGA-3'	#4
	Reverse	5'-GGCTCCATCGTGTAATCCAT-3'	
Mouse CCR6	Forward	5'-TGGTTCGCCACTCTAATCAGT-3'	#108
	Reverse	5'-GCAGTTCAACCACACTCTCACT-3'	

2.1.8 Software

Blast	http://blast.ncbi.nlm.nih.gov/Blast.cgi
EndNote X 4	Thomson Reuters, Carlsbad, USA
FlowJo	Tree Star, Ashland, USA
GraphPad Prism 5.0 b	GraphPad, San Diego, USA
Lasergene 10	DNAStar, Madison, USA
ProbeFinder version 2.48	http://lifescience.roche.com/shop/CategoryDisplay?catalogId=10001&tab=&identifier=Universal+Probe+Library&langId=-1&storeId=15006
Real-time PCR analysis software	http://www.qiagen.com/de/products/genes%20and%20pathways/data-analysis-center-

overview-page/

2.2 Methods for cell culture

2.2.1 General culture conditions

All cell lines were cultured in tissue culture flasks at 37 °C in 95 % humidity and 5 % carbon dioxide (CO_2) atmosphere. Cell manipulations were performed with sterile reagents and materials under a laminar flow hood. Cell concentration and viability was determined by Trypan blue staining. Cell suspensions were mixed with 0.25 % Trypan blue in PBS at appropriate dilutions and counted in a cell counting chamber under the microscope. Cell number was calculated as follows: Cells / ml = (number of cells counted) × (dilution factor) × 10^4. Cells were split and supplemented with fresh medium to reach appropriate dilutions.

2.2.2 Tumor cell culture

The murine pancreatic carcinoma cell line Panc02-OVA stably expresses Ovalbumin (OVA). Panc02-OVA cells were cultured in DMEM complete medium and selected with 2 mg/ml G418. Cells were detached with 0.05 % trypsin / 0.02 % EDTA and split 1:5 every three days according to cell growth.

2.2.3 Platinum-E cell culture

The retrovirus packaging cell line Platinum-E (Plat-E) was generated based on the 293T cell line. Plat-E cells have potent packaging constructs with an EF1α promoter to ensure high-titer production of ecotropic retrovirus after transfection [Morita et al 2000]. The cells were cultured in cultured in DMEM medium containing 1 µg/mL puromycin and 10 µg/mL blasticidin for selection. Cells were split at the ratio of 1:5 every two to three days when the culture reached 70 - 90 % confluency.

2.2.4 Generation and cell culture of primary T cells

Primary T cells were generated from murine splenocytes. Spleen was filtered through a 40 µm cell strainer. Single cells were collected by centrifugation and were resuspended in erythrocyte lysis buffer and lysed for 2 minutes. Cells were washed once with RPMI complete medium and cultured in T cell medium containing 50 µM β-Mercaptoethanol, 1 µg/ml anti-CD3, 0.1 µg/ml anti-CD28 antibodies and 10 IU/ml IL-2 overnight. T cells could then be used for T cell transduction the next day.

2.3 Transduction of primary murine T cells

The murine T cell transduction protocol has been modified based on a protocol previously described [Leisegang et al., 2008, Lee et al., 2009].

2.3.1 Transfection using calcium phosphate-DNA precipitate method

Plat-E cells were seeded 1 - 2 × 10^6 cells per well in a 6-well plate. Cells were then cultured for 15 hours to reach 70 % - 80 % cell confluence on the day of transfection. The calcium phosphate and DNA precipitate is formed by slowly adding a solution containing calcium chloride and purified enhanced green fluorescent protein (GFP) plasmid DNA (18 µg per transfection) with a HEPES-buffered saline solution. 100 mM chloroquine was diluted 1:1000 directly into the calcium phosphate solution to inhibit DNA degradation by lysosomes. The precipitate was evenly distributed on the cells. The medium was replaced with 3 ml complete medium 6 hours after incubation at 37 ºC. 48 hours after transfection, viral supernatants were harvested and filtrated (0.45 µm pore size). Plat-E cells were cultured in T cell medium for another 24 hours for the second virus harvest.

2.3.2 Retroviral transduction of primary murine T lymphocytes

Each 24-well plate was prepared by incubation with 5 µg/mL retronectin diluted in sterile PBS overnight at 4 °C. Wells were blocked with 2 % BSA in sterile H_2O for 30 minutes and washed with 2 ml of 25 mM HEPES in PBS. In each pre-treated well, 1 ml of retrovirus supernatant was spun down (3000 g, 2 h, 4 °C). Then the supernatant was discarded and 10^6 T lymphocytes in 1 ml T cell medium (containing 8 µg/mL protamine sulfate, anti-CD3 - anti-CD28 beads and 10 IU/ml IL-2) were added to every well. T cells were spun down at 800 g for 30 min at 32 °C). Cells were co-cultured with viruses at 37 °C overnight. The second transduction was conducted by addition of another 1 ml virus supernatant to the cells and centrifugation (800 g, 90 min, 32 °C). After co-culture with virus for 6 hours at 37 °C, cells were washed and cultured in T cell medium supplemented with 50 ng/mL IL-15. T cells were split every two days to maintain a concentration of 10^6 cells/ml. Transduction efficiency could be analyzed after 4 - 5 days.

2.4 Animal experiment

2.4.1 Mice

OT-1 transgenic mice were obtained from the Jackson Laboratory. All $CD8^+$ T cells from OT-I mice have histocompatibility 2, Kb (H-2 Kb)-restricted T cell receptor (Vα2, Vβ5) that can specifically recognize the model antigen ovalbumin-derived SINFEKL peptide. Wild type C57BL/6 female mice were bought from Janvier (Le Genest-Saint-Isle, France).

All mice were maintained in a specific pathogen-free facility at the University hospital of Munich. Experimental procedures were performed when mice were at the age of 6 to 7 weeks. Mice were anesthetized with isoflurane for all interventions and all procedures were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany).

2.4.2 Tumor induction

10^6 Panc02-OVA cells were subcutaneously injected in the flank of wild type C57BL/6 female mice. Mice were monitored every day after tumor inoculation. 7 to 10 days after T cell injection, mice were sacrificed for further analysis, as indicated.

2.4.3 Adoptive T cell transfer

10 days after tumor inoculation, 10^7 retrovirally transduced OT-I T cells were injected into each Panc02-OVA tumor bearing wild type C57BL/6 mice in 0.2 ml of PBS by tail vein injection.

2.4.4 Organ and single cell preparation

Lymph nodes from cervical, brachial, axillary, inguinal and popliteal location were resected and pooled as peripheral lymph nodes. Lymph node were filtered through a 40 µm cell strainer. Tumor tissues were digested with 1 mg/ml collagenase and 0.05 mg/ml DNase I at 37 °C for 30 minutes. The digested tissues were sequentially pressed through a 100 µm and through a 40 µm cell strainer. Single cell suspensions were washed with PBS twice and then kept in PBS containing 0.5 % EDTA and 1 % FCS for further analysis.

To separate mononuclear cells (lymphocytes and monocytes) from tumor tissue density gradient centrifugation was used. In a 25 ml tube, 2 ml of tumor cell suspension was added to 15 ml solution containing two layers of Easycoll with different densities. The density of Easycoll is 1.124 g/ml. The upper layer of the separating solution contains 9 ml of 44 % Easycoll in PBS, and the lower layer contains 6 ml of 67 % Easycoll in PBS. Centrifugation was performed at 800 g, 30 min, room temperature without brake. Due to different density of

cells, mononuclear cells were enriched, which can be seen as a white layer between two layers of Easycoll. Mononuclear cells were harvested into a new tube and washed twice with PBS (400 g, 7 min, room temperature).

2.5 Quantitative real-time polymerase chain reaction

2.5.1 Isolation of cytoplasmic RNA

Cells were washed with PBS twice before RNA extraction. RNA isolation was performed according to the manufacturer´s protocol (Qiagen RNeasy Micro Kit) under DNA free conditions. RNA extraction for other cells was done following the protocol of Qiagen RNeasy Mini Kit. RNase free materials were used to avoid digestion of RNA. Precipitated RNA was resuspended in RNAse free water and exposed to DNase I at 22 °C for 20 minutes to avoid DNA contamination before extraction. Isolated RNA was kept on ice at all times. Concentrations of nucleic acids were determined by Spectrophotometer.

2.5.2 Reverse transcription and cDNA pre-amplification

Because of low RNA yields, isolated RNA was reverse-transcribed into cDNA (complementary deoxyribonucleic acid) and was pre-amplified before real-time PCR.

For Qiagen RT-PCR array using the SYBR green dye method (as described in Chapter 2.5.3), cDNA was prepared according to the protocol of the PreAMP cDNA Synthesis Primer Mix kit from Qiagen.

A second protocol was conducted to pre-amplify the cDNA template for real-time PCR using the fluorescent probe method (as described in Chapter 2.5.4) to validate candidate genes. Total RNA was reverse-transcribed into cDNA following instructions of the First Strand cDNA Synthesis Kit from Fermentas, then cDNA was pre-amplified using RealTime ready cDNA Pre-Amp Master from Rothe. The pre-amplification product was diluted 1:40 for RT PCR.

2.5.3 Real-time PCR array using the SYBR green method

To compare gene expression profiles of transferred T cells in lymphoid tissues and tumors, gene expression profiles were analyzed at mRNA level by real-time PCR array in sorted GFP^+-OT-1 T cells. The real-time PCR array is a set of commercially optimized real-time PCR primer assays on 96-well plate for a focused panel of genes and proper controls. Arrays

from Qiagen use a method involving non-specific fluorescent dye (SYBR green) as reporter which intercalate with double-stranded DNA. The specific binding of primers with cDNA template transcribed from mRNA and polymerization resulted in double-stranded DNA amplification. Then, fluorescence of SYBR green fluorophore could be detected using a thermal cycler and measurement was conducted after each amplification cycle.

Pre-amplified cDNA of spleen and tumor from one mouse was added into separate array plates containing the same primers for hybridization. Each of the two arrays (Mouse Chemokines and Receptors, Mouse T cell anergy and immune tolerance) was done three times with samples from three mice in the same experimental setting. RT PCR array was conducted according to the manufacturer's instructions. Measurement of fluorescence was plotted against the number of cycles on a logarithmic scale. A threshold for fluorescence levels was set, and the number of cycles at which the fluorescence reached the threshold is called threshold cycle (C_t). The C_t values were recorded as result for data analysis.

2.5.4 Real-time PCR using fluorescent probe method

To confirm results obtained from commercial RT PCR array, two additional experiments were conducted for candidate genes using fluorescent probes from Roche (as shown in Table 2), according to the manufacturer's instructions. The results were also recorded as C_t value.

2.5.5 Calculation

Relative gene expression was calculated online using the Qiagen web site, and analysis was based on the ΔΔCt method [Livak et al., 2001]. The ΔΔCt method is the normalization of raw threshold cycle data in the test sample with the gene of interest (GOI) to housekeeping genes (HKG), divided by normalized gene expression in the control sample. The formula to calculate fold change is shown below. The formula is based on the fact that both the GOI and the HKG are approximately 100% amplified, and the deviation of the amplification efficiency between GOI and HKG is less than 5%. In the experiment, the sample from tumor was set as "expect" and sample from spleen as "control". The Fold-change data is further used for statistical analysis.

$$\frac{\dfrac{2^{-\Delta C_t(\mathrm{GOI})\mathrm{expt}}}{2^{-\Delta C_t(\mathrm{HKG})\mathrm{expt}}}}{\dfrac{2^{-\Delta C_t(\mathrm{GOI})\mathrm{control}}}{2^{-\Delta C_t(\mathrm{HKG})\mathrm{control}}}} = \frac{2^{-[\Delta C_t(\mathrm{GOI})-\Delta C_t(\mathrm{HKG})]\mathrm{expt}}}{2^{-[\Delta C_t(\mathrm{GOI})-\Delta C_t(\mathrm{HKG})]\mathrm{control}}} = \frac{2^{-\Delta C_t\mathrm{expt}}}{2^{-\Delta C_t\mathrm{control}}} = 2^{-\Delta\Delta C_t}$$

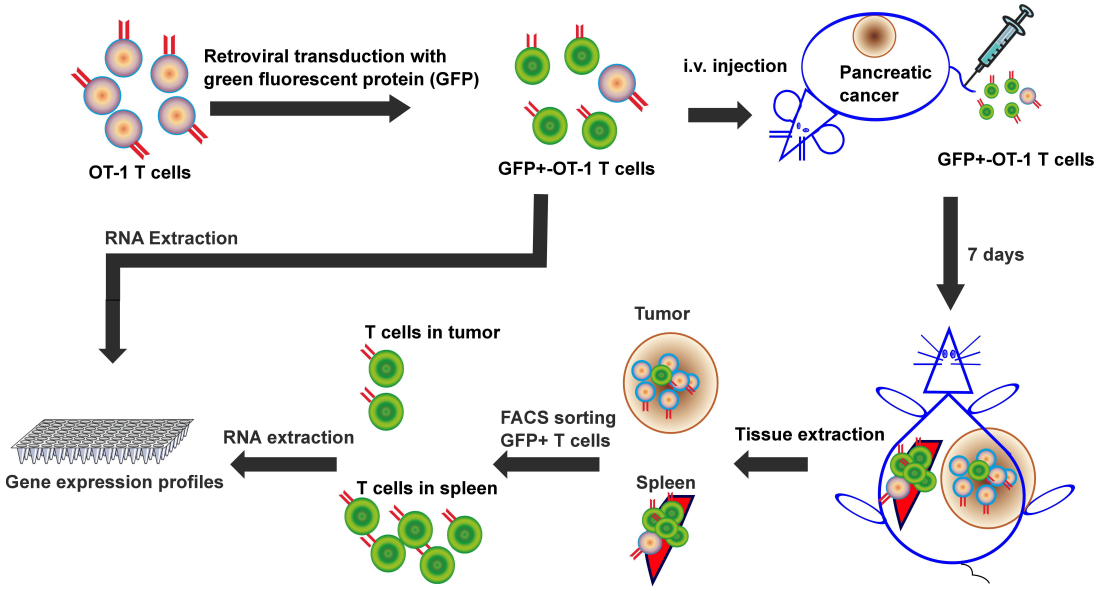

Figure 2: Schematic experimental design for gene expression profiles of transduced T cells. OT-1 T cells were retrovirally transduced with GFP, and GFP-transduced T cells were intravenously injected into mice bearing Panc02-OVA tumors. 7 days after injection, mice were sacrificed, and GFP^+-OT-1 T cells were sorted from tumor and spleen tissue. RNA was prepared from the sorted GFP^+-OT-1 T cells, and gene expression profiles of the sorted T cells were analyzed using RT-PCR array.

2.6 Flow cytometry analysis

2.6.1 Antibody staining for flow cytometry

2×10^6 splenocytes or mononuclear cells were distributed in 5 ml FACS tube and washed twice with 3 ml PBS/BSA buffer (400 g, 5 min, 4 °C). Supernatant and cell debris were discarded, and remaining cells were re-suspended in 50 µl staining buffer. Cells were pre-incubated with 1 µg anti-Mouse CD16/CD32 (Fc Block) on ice for 10 minutes prior to primary staining. Proper concentration of fluorescent dyes directly-conjugated antibodies for surface markers were added to each tube, mixed and incubated with cells at 4 °C for 30 minutes in the dark. Cells were washed twice with 3 ml FACS buffer (4 °C, 400 g, 5 minutes) and re-suspended in 100 µl buffer for analysis. Isotype control staining was performed in parallel to confirm the specificity of primary antibody binding when needed.

2.6.2 Analysis of cell surface markers

FACS Canto II configured with three lasers, which are blue (488 nm), red (633 nm) and violet (405 nm), was used to analyze cell viability and scatter properties. Data were analyzed with Flowjo software. Lymphocytes population was gated by forward scatter (FSC) and side

scatter (SSC), doublets were excluded further by FSC-A and FSC-H. T cells were gated in singlets as $CD3^+$ $CD8^+$ cells.

2.6.3 High speed cell sorting of GFP^+-OT-1 T cells

The main goal of cell sorting is to retrieve GFP^+-OT-1 T cells from a heterogeneous population of tumor tissue cells for real-time PCR array analysis. BD Aria cell sorter was kindly provided and conducted by PD Dr. med. Michael Hristov (Institution of prevention and epidemiology of cardiovascular disease, LMU, Munich) following FACS Aria user guidelines. GFP^+-OT-1 T cells were sorted for high-purity. The sorted cells were kept in PBS on ice for further RNA extraction.

2.7 Enzyme-linked immunosorbent assay

In the experiments, the sandwich ELISA kit of mouse CCL1 from R and D company was used to detect the chemokine in tumor and lymphoid tissues.

2.8 Statistical analysis

Variance of mean values is presented as standard error of the mean (SEM). Data in figures are shown as mean value ± SEM of minimum three replicates or independent experiments. Statistical analyses and graphical design were performed using GraphPad Prism software (version 5.0b). Differences of means are compared using the two-tailed Student's t-test. Comparison of three or more columns are analyzed by two-way analysis of variance (ANOVA) with the Bonferroni multiple comparison test. Significance was set at $p < 0.05$. The statistical analysis for RT-PCR array is based on the real-time PCR analysis software from Qiagen using raw C_t value. The differences of three replicates of normalized C_t values ($2^{(-\Delta Ct)}$) from independent RT-PCR array experiments were analyzed for each gene change in the control and treatment groups. Student's t-test is utilized for statistical analysis. Differences were considered significant with $p < 0.05$.

3. Results

3.1 Distribution of transferred T cells *in vivo*

To investigate whether T cells isolated from different donor lymphoid tissues have preferential homing to their organ of origin after transfer, T cells isolated from spleen and lymph nodes separately transduced with GFP following the same protocol were intravenously transferred into wild type C57BL/6 mice. 7 days after transfer, lymphocytes were isolated from lymphoid tissues and distribution of GFP^+-OT-1 T cells was compared in spleen and lymph nodes. No significant difference was found between groups (Figure 3), suggesting that the donor organ of T cell origin does not affect the homing of retrovirally transduced T cells.

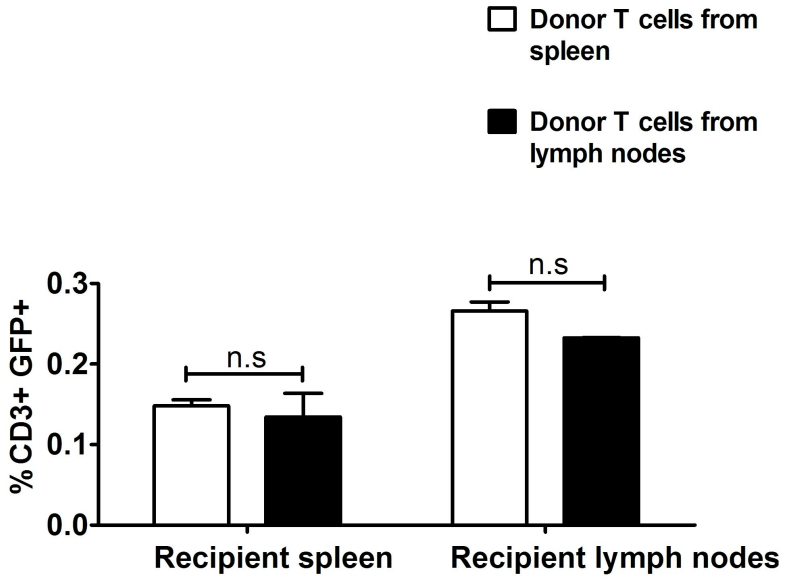

Figure 3: Distribution of transduced T cells *in vivo*. 5×10^6 GFP-transduced T cells were intravenously transferred into wild type C57BL/6 mice. On day 7, lymphocytes from lymph nodes and spleen were isolated and analyzed for the amount of $CD3^+$-GFP^+ cells within the live gate. Statistical analysis of four mice in each group are shown as mean ± SEM. N.S stands for not significant. The result is representative of two independent experiments.

3.2 Characteristics of transduced T cells

3.2.1 Phenotype of transduced T cells

Figure 4 shows representative transduction efficiency of 60 % to 70 % in cytotoxic $CD8^+$ T cells after transduction. To better understand the behavior of retrovirally transduced T cells, we next analyzed the expression of memory phenotypic markers (CD62L) and exhaustion markers (PD-1 and LAG-3) after transduction. About 15 % (14 % to 16%) of GFP^+-OT-1 T cells were PD-1 positive (Figure 5A), and about half (43 % to 50 %) of the GFP^+-OT-1 T cells

were CD62L positive (Figure 5B).

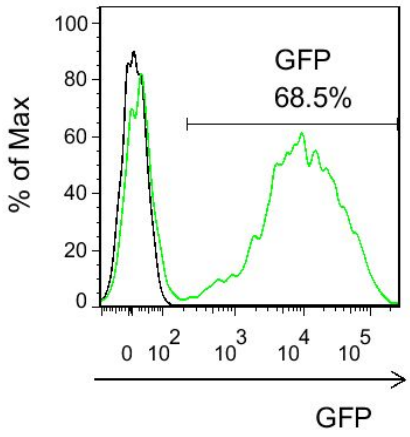

Figure 4: Representative FACS analysis of transduction efficiency for GFP in OT-1 T cells. The solid black line presents the background fluorescent signals of the untransduced T cells, and the solid green line presents the fluorescence from GFP-transduced T cells. The percentage of high fluorescent signals in the whole signals shows the amount of GFP^+ T cells in the GFP-transduced T cells. The results shown are representative of two independent experiments.

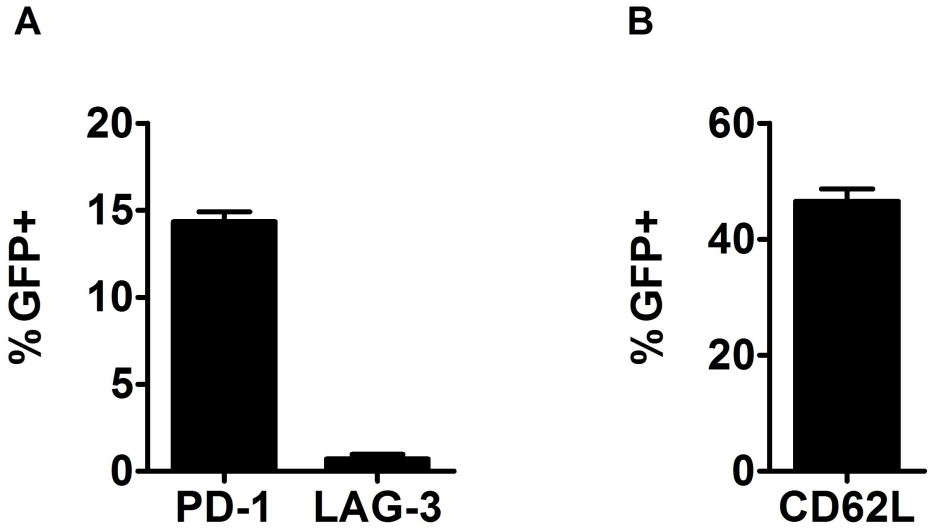

Figure 5: Phenotype GFP^+-OT-1 T cells before transfer. Figure 5A shows exhaustion markers for T cells, and Figure 5B shows the memory marker for T cells. T cells were stained with antibody targeting phenotype markers and detected by flow cytometry. Columns show percentage of positive cells pregated on GFP^+-OT-1 T cells. Data are presented as mean ± SEM with three mice in each group. The results shown are representative of two independent experiments.

3.2.2 Phenotype of transduced T cells *in vivo*

To further understand the biological behavior of the transferred T cells *in vivo*, surface markers were quantified by flow cytometry. As shown in Figure 6A, GFP$^+$-OT-1 T cells expressed high levels of the quiescence markers PD-1 (100 % positive, compared to 15 % before adoptive transfer in Figure 5A) and LAG3 (40 % positive, compared to less than 5 % before transfer) in the tumor tissues. T cells isolated from spleen were less exhausted (less than 20 % positive for PD-1, and less than 30 % positive for LAG-3). About 15 % (10 % to 18 %) of GFP$^+$-OT-1 T cells in the tumor were CD62L positive, whereas 30 % (25 % to 40 %) of GFP$^+$-OT-1 T cells in the spleen were CD62L positive (Figure 6B).

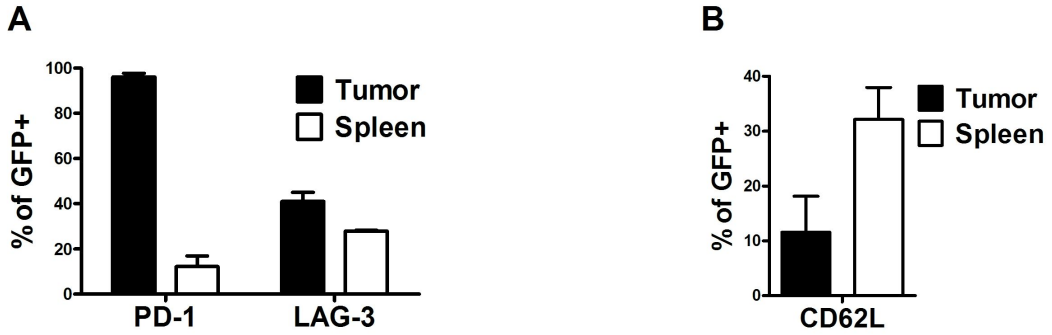

Figure 6: Phenotype of GFP$^+$-OT-1 T cells *in vivo*. GFP-transduced T cells were injected intravenously into mice bearing Panc02-OVA tumors. 7 days after adoptive transfer, the expression of surface markers from different compartments were analyzed on GFP$^+$-OT-1 T cells. Figure 6A shows the exhaustion markers expressed on GFP$^+$-OT-1 T cells. Figure 6B shows memory marker CD62L expressed on GFP$^+$-OT-1 T cells. Experiments were conducted twice with three mice in each group. The results are shown as mean ± SEM.

3.3 FACS sorting of GFP$^+$-OT-1 T cells from spleens and tumors

To investigate the gene expression profiles of transferred T cells within different compartment, 10^7 GFP-transduced T cells were transferred as indicated in 3.2.2. GFP$^+$-OT-1 T cells were sorted by flow cytometry for high purity (the purity of GFP$^+$-OT-1 T cells was 80 % to 90 % after sorting). The number of GFP$^+$ cells sorted was in average 100,000 from spleens and 30,000 from tumors (Figure 7).

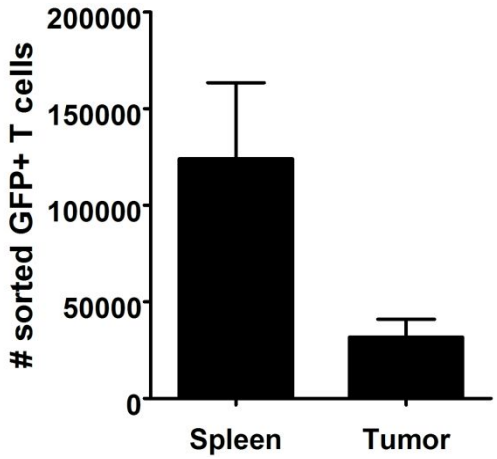

Figure 7: Quantification of FACS sorted GFP$^+$-OT-1 T cells from spleens and tumors.
Panc02-OVA cells were engrafted subcutaneously into wild type C57BL/6 mice followed, by intravenous transfer of 10^7 GFP-transduced T cells. Seven days after transfer, GFP$^+$-OT-1 T cells were sorted for high purity. Cell numbers of sorted GFP$^+$-OT-1 T cell are shown as mean ± SEM of n = 3 in one out of three experiments.

3.4 Exhaustion and anergy array analysis

The commercially available RT-PCR arrays were utilized to investigate differences in gene expression profiles (Figure 8). Results showed that transferred T cells in tumor tissue had a signature of stronger activation (Interferon-γ (IFN-γ), IL-2, IL-2 receptor α unit, OX40, 4-1BB) and a more prominent exhaustion phenotype (CTLA-4, PD-1) compared to T cells retrieved in the spleen (Table 3). Transferred T cells in the spleen showed a memory phenotype with relatively higher expression of IL7 receptor and CD62L (Table 4). Candidate genes were confirmed in additional independent experiments (Table 7 and Table 8).

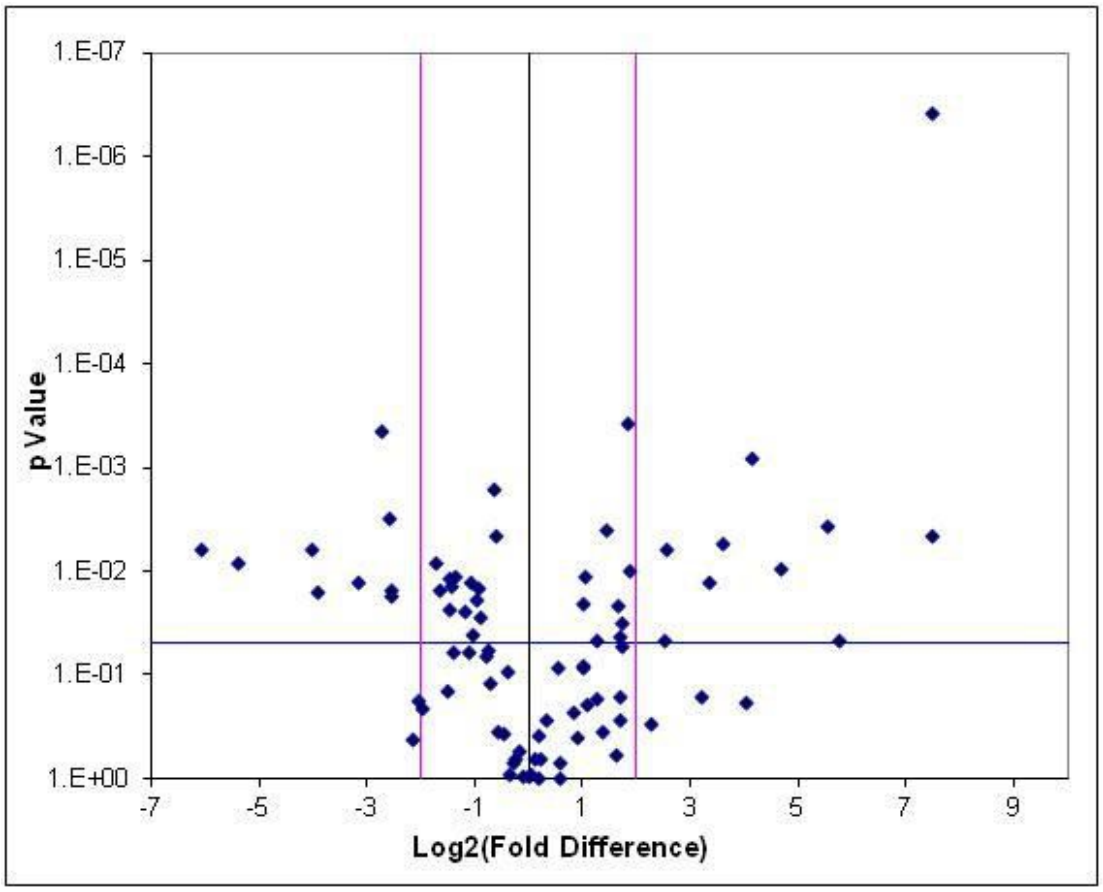

Figure 8: Volcano plots of anergy and tolerance RT-PCR gene expression changes. The x-axis plots the log-transformed fold changes, and the y-axis plots the p-values. The group of GFP^+-OT-1 T cells infiltrating into tumor site is defined as "treatment group", and that of spleen-residing GFP^+-OT-1 T cells as "control group". P-values were analyzed using Student's t-test of the replicate $2^{(-\text{ Delta Ct})}$ values for each gene in the control group and treatment groups, and p-values less than 0.05 are considered statistically significant. Fold change were calculated using ΔΔCt method as described in chapter 2.5.1, and values greater than two indicate up-regulation, and less than minus two indicate down-regulation. The data were analyzed based on three plates of the anergy and tolerance PCR array, and each array utilized the RNA extracted from one mouse.

Table 3: Genes of anergy and tolerance up-regulated in tumors vs. spleens.

Gene symbol	Fold regulation	p-value
CCL3	5.8	0.046
CD70	25.3	0.010
GM-CSF	53.8	0.046
CTLA-4	17.7	0.001
IFN-γ	5.9	0.006
IL-2	179.3	0.004
IL-2 receptor α unit	10.1	0.012

PD-1	176.0	0.000
OX40	12.0	0.005
4-1BB	46.8	0.004

Table 4: Genes of anergy and tolerance down-regulated in tumors vs. spleens.

Gene symbol	Fold regulation	p-value
BTLA	-6.7	0.000
CD40	-68.2	0.006
Chymase 1	-15.2	0.016
DGK α	-5.8	0.017
Eomes	-5.9	0.015
HDAC9	-133.2	0.026
IL15	-16.3	0.006
IL7 receptor	-6.0	0.003
PKC γ	-9.0	0.013
CD62L	-42.1	0.008

3.5 Chemokine and chemokine receptor array analysis

To understand the role of chemokine receptors that might help T cells to migrate into tumors, a chemokine and chemokine receptor RT-PCR array was utilized (Figure 9). Results showed that transferred tumor-infiltrating T cells had higher expression of CCR8 and CX3CR1 (Table 5), while T cells found in spleen had higher expression of other chemokine receptors (CCR6, CCR9, CCR like 1, CXCR3, CXCR5 and XCR1) (Table 6). Candidate genes were confirmed in additional independent experiments (Table 7 and Table 8).

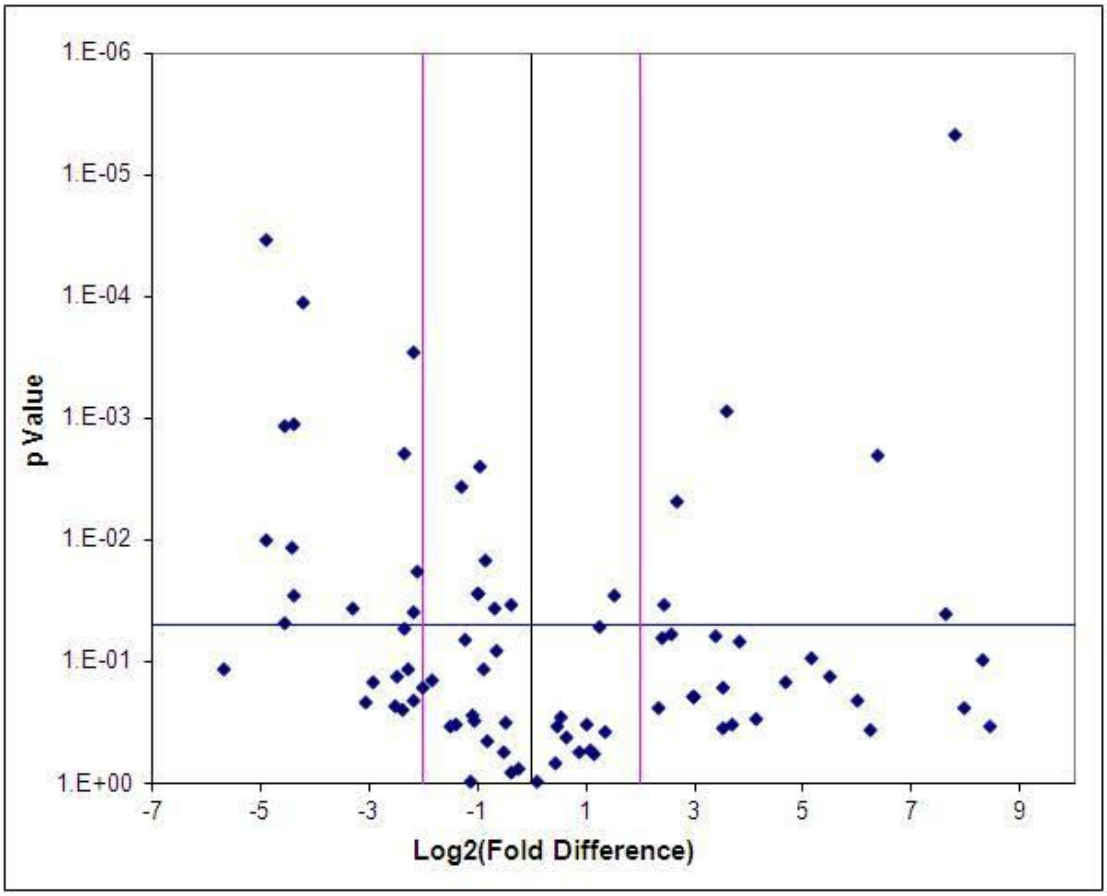

Figure 9: Volcano plots of chemokine and chemokine receptor RT-PCR array. Samples were prepared and data was analyzed as indicated above. Fold-difference values greater than two indicate an up-regulation, and less than minus two indicate a down-regulation. The p-values are calculated based on a Student's t-test of three replicates $2^{(-\Delta Ct)}$ values for each gene in the control and treatment groups. p-values less than 0.05 are considered statistically significant. Data was analyzed based on three plates of RT-PCR array for chemokine and chemokine receptor, whose RNA were extracted respectively from three mice.

Table 5: Genes of chemokines and chemokine receptors up-regulated in tumors vs. spleens.

Gene symbol	Fold regulation	p-value
CCL1	5644.6	0.018
CCR8	221.2	0.000
CCR like 2	12.0	0.001
CX3CR1	5.4	0.034
IFN-γ	6.2	0.005
TGFβ1	197.5	0.041
XCL1	81.3	0.002

Table 6: Genes of chemokines and chemokine receptors down-regulated in tumors vs. spleens.

Gene symbol	Fold regulation	p-value
Chemokine binding protein 2	-182.8	0.000
CCL19	-21.4	0.001
CCL22	-23.8	0.049
CCR6	-21.8	0.011
CCR9	-9.9	0.036
CCR like 1	-24.2	0.001
CXCR3	-4.6	0.000
CXCR5	-19.0	0.000
Duffy blood group chemokine receptor	-29.9	0.000
IL-16	-5.2	0.002
Pro-platelet basic protein (CXCL7)	-30.4	0.010
TLR4	-4.4	0.018
Thymidine phosphorylase	-21.3	0.028
XCR1	-4.6	0.039

3.6 Confirmation of candidate genes of T cells in tumor versus spleen

To confirm candidate genes up-regulated in the RT-PCR array, two additional mouse experiments were conducted following the same protocol as shown in Figure 2. RT-PCR was done using the fluorescent probe method (as described in chapter 2.5.4). Table 7 represents the summary of up-regulated genes in tumors compared with these found in spleen. Table 8 shows the genes which are down-regulated.

Table 7: Genes up-regulated in tumor vs. spleen. RNA was extracted from three different mice for each experiment. Data was analyzed as described before. Genes with more than 2-fold higher expression levels in the tumor-infiltrating T cells versus in those found in the spleen are shown.

Gene symbol	Fold regulation	p-value
PD-1	68.6	0.001
CD70	10.7	0.000
GM-CSF	222.1	0.026
4-1BB	44.9	0.011
CCR8	129.9	0.001

Table 8: Genes down-regulated in tumors vs. spleens. Two additional RT-PCRs were conducted as described before for candidate genes with down-regulation in the RT-PCR array. The genes which were more than -2-fold down-regulated in tumor-infiltrating T cells versus in those found in the spleen are shown.

Gene symbol	Fold regulation	p-value
HDAC9	-56.2	0.000
BTLA	-19.8	0.000
CD40	-98.4	0.000
Chymase 1	-59.6	0.012
CD62L	-25.2	0.000
Eomes	-5.2	0.043
CCBP2	-8.5	0.004
CCR6	-117.9	0.005
CCR9	-9.0	0.030
CXCR5	-24.5	0.001
CCR like 1	-693.0	0.023

3.7 Comparison of gene expressions in T cells before and after transfer

To understand how *in vitro* stimulation and expansion would affect gene expression, RNA of GFP-transduced T cells before transfer was also extracted. T cells infiltrating the tumor were found to have higher expression of activation (4-1BB) and exhaustion (PD-1) marker than those before transfer. Post-transfer T cells in tumors had also higher expression of CCR8 and CX3CR1 (Table 9). T cells found in spleen have higher expression of memory phenotype markers (IL-7 receptor and CD62L), as well as of epigenetic and metabolic related genes (HDAC9 and DGK α) (Table 10). PD-1 and CCR8 expression were also higher in T cells residing in spleen than those before transfer (Table 10).

Table 9: Genes up-regulated in tumors vs. prior to transfer. Total RNA was extracted from GFP-transduced T cells before transfer and from tumor-infiltrating GFP^+-OT-1 T cells after transfer. The genes which are up-regulated and have p-values less than 0.05 are shown in the table. Experiments were performed twice, and RNA of GFP^+-OT-1 T cells after transfer was extracted from three mice in each experiment.

Gene symbol	Fold regulation	p-value
PD-1	191.1	0.003
GM-CSF	51.9	0.044
CTLA-4	10.6	0.041
4-1BB	5.7	0.009

CCR8	546.9	0.040
CX3CR1	521.0	0.028

Table 10: Genes up-regulated in spleens vs. prior to transfer. Total RNA was extracted from GFP-transduced T cells before transfer and from GFP^+-OT-1 T cells in the spleen after transfer. Data was analyzed using the group of T cells before transfer as "control group". Genes which are down-regulated and have p-values less than 0.05 are shown in the table. The experiment was done twice, and the RNA for GFP^+-OT-1 T cells after transfer was extracted from three mice in each experiment.

Gene symbol	Fold regulation	p-value
HDAC9	29.8	0.004
PD-1	5.4	0.015
BTLA	27.3	0.001
CD40	229.7	0.004
Cma1	5.6	0.013
DGK α	14.7	0.000
PKC γ	18.3	0.003
CD62L	14.2	0.000
CCR8	16.4	0.003
CX3CR1	478.3	0.023
CCR9	6.2	0.049
TLR4	31.3	0.041

3.8 CCR8 is expressed on tumor-infiltrating GFP^+-OT-1 T cells

To confirm the expression at protein level, T cells were stained for CCR8 and analyzed by flow cytometry. In subcutaneous Panc02-OVA tumors, 25 to 80 % of transferred T cells infiltrating the tumor expressed CCR8, while less than 10 % of T cells in lymphoid tissues expressed CCR8 (Figure 10A). Similar results were found for GFP^-- T cell fraction which represents the endogeneous T cells (Figure 10B). To determine whether CCR8 expressing T cells have a different distribution in tumor draining and non-draining lymph nodes, ipsilateral and contralateral lymph nodes were isolated separately. No difference was found in CCR8 expression on T cells between these lymph nodes (Figure 11).

To investigate whether endogenous T cells also have higher CCR8 expression if found in the tumor, CCR8 expression was analyzed in tumor-bearing mice which had not received T cell transfer. T cells infiltrating the tumors had higher CCR8 expression (25 to 42 %) than those found in spleen (20 %) (Figure 12).

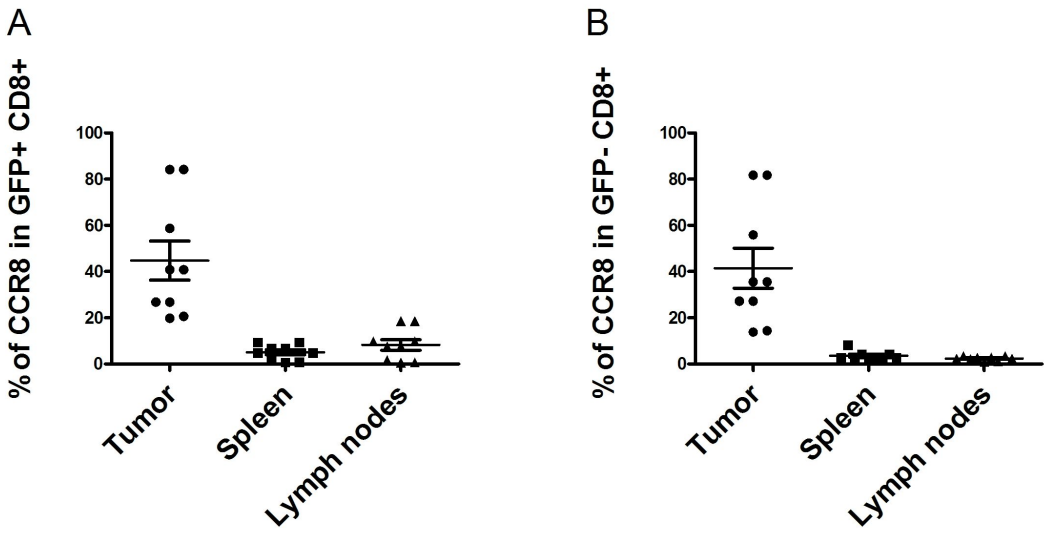

Figure 10: Adoptively transferred T cells infiltrating into tumors have higher CCR8 expression. GFP-transduced T cells were transferred intravenously into mice bearing Panc02-OVA tumors seven days after tumor inoculation. 14 days after tumor inoculation, spleen and tumor were isolated and lymph nodes were pooled to detect CCR8 expression on T cells. GFP^{+}-OT-1 T cells (Figure 10A) or GFP^{-}-OT-1 T cells (Figure 10B) cells were gated, and percentage of T cells expressing CCR8 was analyzed. Each dot represents one mouse, and three independent experiments were done with three mice in each group.

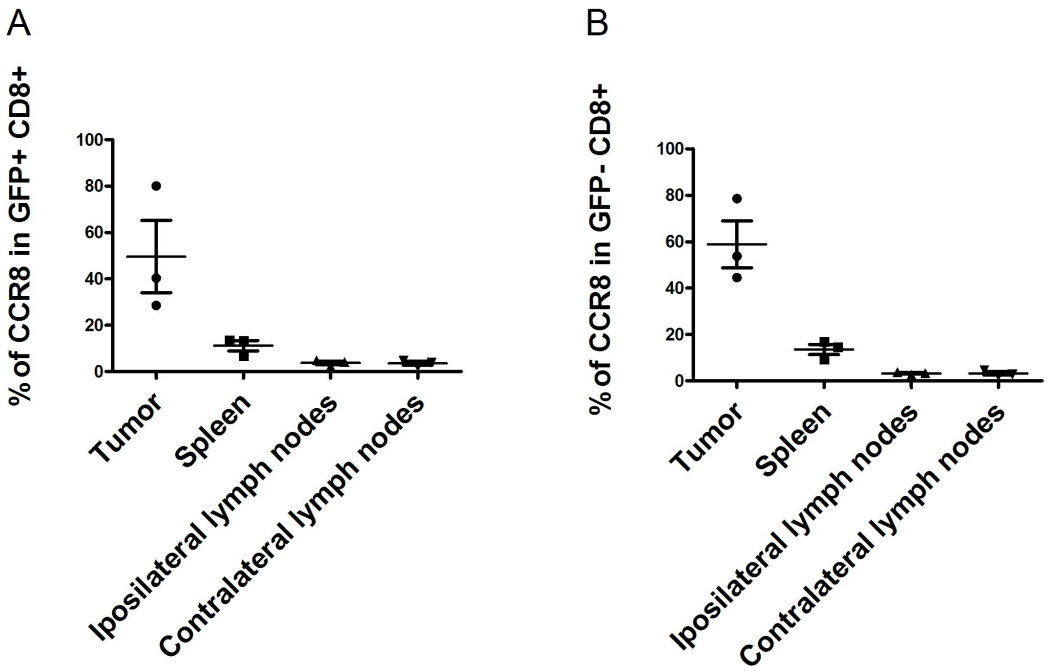

Figure 11: CCR8 expression on T cells in ipsilateral and contralateral lymph nodes. OT-1 T cells were transduced with GFP and GFP-transduced T cells were transferred into Panc02-OVA mice as

described before. Each dot represents one mouse, and two experiments were done with three mice in each group. Figure 11A and Figure 11B show CCR8 expressions on GFP^+-OT-1 T cells and GFP^--OT-1 T cells, respectively.

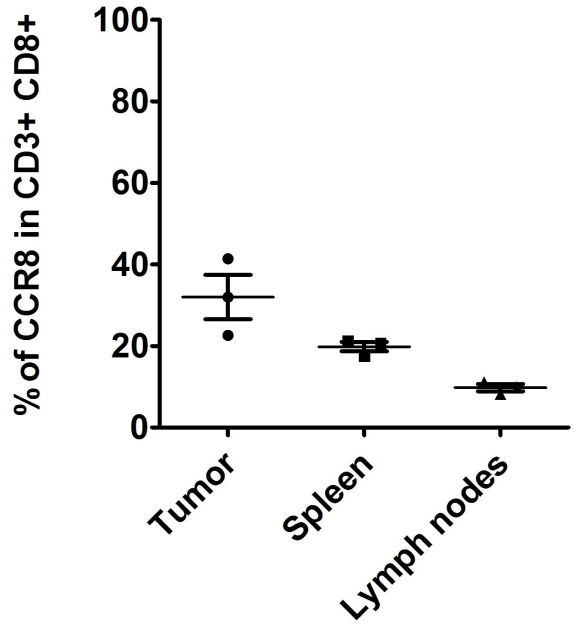

Figure 12: Endogeneous CCR8 expression on T cells in Panc02-OVA tumors. Panc02-OVA tumor cells were inoculated in wild type C57BL/6 mice as described before. Each dot represents one mouse, and one experiment was done with three mice in each group.

3.9 Concentration of CCL1 in different compartments

Because CCL1 is the specific ligand for CCR8, we analyzed the concentration of CCL1 in mice bearing Panc02-OVA tumors at different time points. The concentrations of CCL1 detected in ELISA in different organs ranged from 20 pg/ml to 1 ng/ml. As shown in Figure 13A, seven days after tumor inoculation, lymph nodes had the highest amount of CCL1 (10 pg/mg protein to 25 pg/mg protein), and tumor tissues contained the lowest amounts (6 pg/mg to 10 pg/mg) (Figure 13A); on day 14, CCL1 concentrations increased in tumors (10 pg/mg to 20 pg/mg), while the amount decreased in lymph nodes (5 pg/mg to 15 pg/mg), and stayed even until day 21. (Figure 13B and Figure 13C). There is no significantly different expression of CCL1 in ipsilateral and contralateral lymph nodes on day 14 (Figure 14).

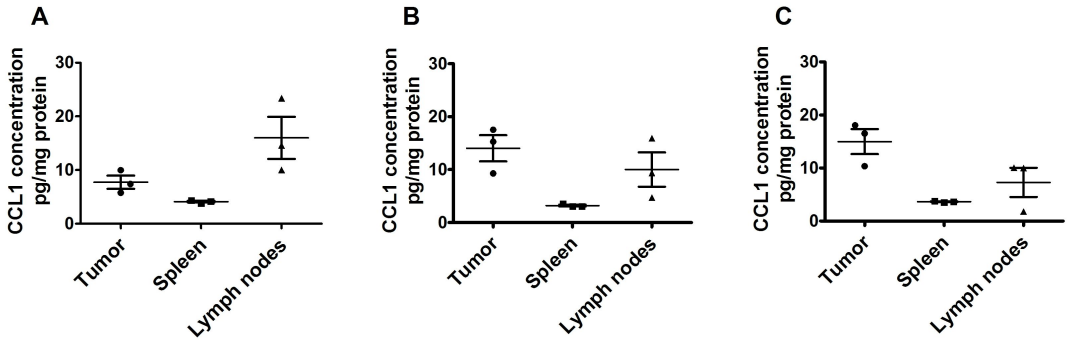

Figure 13: Expression of CCL1 in different tissue compartments over time. Panc02-OVA tumor cells were inocculated into wild type C57BL/6 mice. At different time points, spleen, tumor and lymph nodes were harvested and frozen. Frozen organs were mashed and protein lysate was prepared. CCL1 concentration was measured by ELISA and normalized to total amount of protein loaded (results are presented as pg/mg total protein of tissues). Each dot represents one mouse, and two experiments were done with three mice in each group. Figure 15A, 15B and 15C represent day seven, day 14 and day 21 after tumor inoculation, respectively.

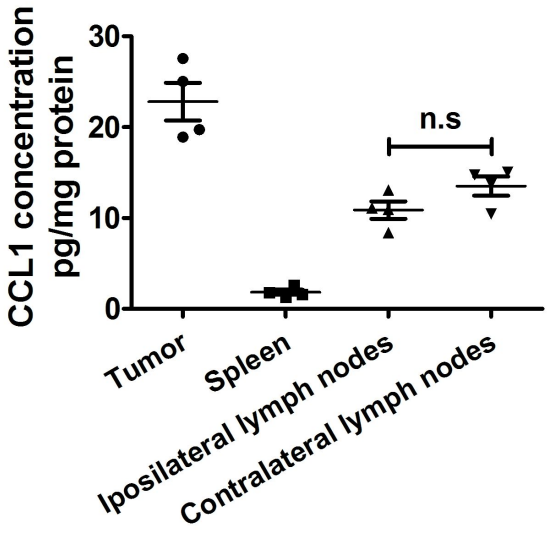

Figure 14: Expression of CCL1 in lymph nodes. 14 days after tumor injection, organs were harvested and protein lysate was prepared. CCL1 was detected using ELISA and concentration was normalized to the amount of protein used. Each dot represents one mouse, and two experiments were done with four mice in each group.

4. Discussion

In this thesis, RT-PCR-based arrays have revealed that tumor-infiltrating T cells after adoptive transfer display altered gene-expression profiles compared with those found in spleen. Pathways found to be differentially regulated in these T cells are associated with T cell activation and exhaustion, epigenetic regulation and migration. In the following paragraphs, the genes identified are discussed in detail.

4.1 Up-regulation of activation and exhaustion genes in tumor-infiltrating transferred T cells

Several genes for T cell activation were over-expressed in adoptively transferred T cells infiltrating the tumor, which are consistent with previous studies in naturally generating T cells, including IFN-γ [Schroder et al., 2004], GM-CSF [Min et al., 2010], IL-2 receptor [Schuh et al., 1998, Cheng et al., 2002], 4-1BB [Shuford et al., 1997], OX40 [Redmond et al., 2009] and CD70 [Tesselaar et al., 2003, Huang et al., 2006, Croft, M., 2009]. Similarly, genes for inhibitory receptors were also found to be upregulated in tumor-infiltrating GFP^+-OT-1 T cells, such as CTLA-4 and PD-1, which is in line with findings in tumor-specific T cells from cancer patients [Baitsch et al., 2011] and T cells in the context of ACT [Abate-Daga et al., 2013]. These indicate that gene-modified T cells that infiltrate tumors, interact with tumor cells and become activated. Overcoming these negative signals within the tumor milieu might be a potential strategy to enhance T cell therapy which is currently pursued by our group [Kobold et al., 2015].

Not all co-inhibitory molecules were detected with increased expression in tumor-infiltrating T cells after transfer. BTLA is structurally related to PD-1 and CTLA-4 and it interacts with B7 homologue to negatively regulate T cell activation [Haymaker et al., 2012, Isabelle Le Mercier et al., 2015]. In addition, BTLA can bind to herpes virus entry mediator and inhibit the anti-tumoral effect of $CD8^+$ T cells in melanoma patients [Derre et al., 2010, Fourcade et al., 2012], suggesting its potential as a target in cancer immunotherapy [Paulos et al., 2010]. In those previous studies, BTLA was found to be up-regulated on T cells in tumor tissues. However, in our experiments it was detected at higher levels in transferred T cells isolated from spleen, not on those found at the tumor site. This may be explained by the hypothesis that BTLA signaling may not be inhibitory under all conditions [Cheung et al., 2009, Sakoda et al., 2011]. Its expression might support the survival of transferred T cells based on the observation that BTLA expressing TILs have a central memory phenotype and have an enhanced proliferative response to IL-2 [Haymaker et al., 2015]. Furthermore, current

evidence suggests that expression of inhibitory receptors might not always be a marker of exhausted T cells, but may be more related to T cell differentiation or activation [Legat et al., 2013]. The role of BTLA and other exhaustion markers in adoptively transferred T cell still requires further investigation.

4.2 Expression of T memory maintenance genes in spleen-residing transferred T cells

Compared to those in tumor tissues, transferred T cells found in spleen had higher expression of IL-7 receptor and CD62L, which are markers for memory T cells [Huster et al., 2004]. This indicates a memory phenotype of transferred T cells residing in the spleen as opposed to those found at the tumor site. Another interesting finding is the significantly increased expression of CD40 on the transferred T cells in the spleen. CD40 is normally expressed on APCs and B cells. Expression on those cells is important for the activation of $CD4^+$ helper T cells and priming of $CD8^+$ T cell responses [Cella et al., 1996, Ridge et al., 1998, Schuurhuis et al., 2000]. CD40 expression on $CD8^+$ T cells is rare, but literature indicates that its expression on $CD8^+$ T cells plays a role for the generation of T cell memory [Bourgeois et al., 2002, Meunier et al., 2012]. Thus, our finding might provide an additional evidence for the hypothesis that transferred T cells have a memory phenotype in the spleen. Further investigation on the role of CD40 signaling on transferred T cells might be of interest and may give hints for the utilization of CD40 agonist in conjunction with T cell transfer.

4.3 Altered gene expression involving transcription factors and epigenetic modification

The T-box transcription factor Eomes was down-regulated in GFP^+-OT-1 T cells isolated from tumors. Eomes is important for the regulation of cytolytic functions of $CD8^+$ T cells via expression of perforine and granzyme B [Pearce et al., 2003] and interferon secretion [Glimcher et al., 2004, Atreya et al., 2007]. It also plays a crucial role in the maintenance of memory $CD8^+$ T cells by inducing IL-2 receptor β (CD122) expression [Intlekofer et al., 2005], which is important for T cells responding to cykokines such as IL-15 or IL-7. It also helps to maintain long-term survival of T cells without antigen stimulation as well as memory T cell function [Li et al., 2013].

On the other hand, Eomes is over-expressed in exhausted T cells during chronic infection [Wherry et al., 2007, Kao et al., 2011]. However, its role in regulating T cells in cancer is not yet fully understood. Some evidence suggests that Eomes plays a critical role in enhancing $CD8^+$ T cell activity to eradicate human colorectal cancer [Atreya et al., 2007]. In addition, T-bet and Eomes are important for tumor infiltration by $CD8^+$ T cells through the regulation

of CXCR3 expression and enhance T cell-mediated anti-tumoral immune responses [Zhu et al., 2010]. In contrast, suppression of Eomes expression in T cells before transfer leads to enhanced anti-tumoral function of adoptively transferred T cells [Hinrichs et al., 2008]. In the present study, Eomes was found of low expression in T cells isolated from tumor tissues. However, this finding does not allow for the identification of the signaling pathway involved in the regulation of gene expression in transferred T cells. Thus, it is necessary to further investigate the mechanism and dynamics of Eomes expression in transferred T cells in tumor models for a better understanding of its role in ACT [Waugh et al., 2015].

HDAC9 is another candidate gene found downregulated in transferred T cells gaining access to the tumor tissue. HDAC9 is a class IIA HDAC, which is a family of histone modification enzymes that remove acetyl from histone, and further epigenetically regulates DNA expression [Leipe et al., 1997]. It is mostly over-expressed in $CD4^+$ T cells, and has shown reverse correlation with stable Forkhead box protein 3 (Foxp3) expression and has suppressive function on Tregs [Li et al., 2007, Tao et al., 2007]. However these functions come from non-tumor mouse models which is in contrast to the one used in this study. Our observation may indicate that HDAC9 is involved in epigenetic modification of $CD8^+$ T cells in the context of ACT. Further studies should be extended to other member of HDACs in the regulation of gene expression in ACT, which are important for the application of histone deacetylase inhibitors (HDACis) in combination with this therapy [Vo et al., 2009, Lisiero et al., 2014].

4.4 Differences in gene expression in lipid signaling pathway and T cell activation

Genes involved in metabolism were found to be expressed at lower levels in the transferred T cells infiltrating the tumor. PKC γ and DGK α were found to be downregulated in infiltrating T cells. PKC γ is a member of the PKC family that can phosphorylate serine and threonine amino acid residues on the other proteins and control the function of these proteins. Its activation requires increasing concentration of second messengers like diacylglycerol (DAG) and calcium ions (Ca^{2+}) [Mellor et al., 1998]. In general, PKCs are involved in several cell signal transduction cascades, and are upregulated in activated T cells [Isakov et al., 1987, Berry et al., 1990]. Transduction of T cells with PKC γ can up-regulate IL-2 receptor expression, and adoptive transfer of those T cells can lead to tumor regression *in vivo* [Chen et al., 1994].

DGK α belongs to the DGK enzyme family that catalyze phosphorylation of DAG to phosphatidic acid and regulate lipid signaling. DKG correlates with T cell anergy and inhibits

TCR signaling [Olenchock et al., 2006, Zha et al., 2006], suggesting that pharmacologic inhibition of DGKs might be a strategy to improve T cell therapy [Riese et al., 2013, Wang et al., 2013]. However, its expression in different models is controversial: it is over-expressed in exhausted T cells after transfer in melanoma [Abate-Daga et al., 2013], whereas it is down-regulated in exhausted $CD8^+$ T cells in chronic infection [Wherry et al., 2007]. Furthermore, one study indicates that the expression of DGK α might play opposing roles during T cell primary expansion and memory phase [Shin et al., 2012]. The finding in this thesis might be interpreted as additional evidence to the hypothesis that adoptively transferred T cells found in spleen might have a memory T cell phenotype.

4.5 Different gene expression involving chemotaxis and migration in adoptively transferred T cells

Several genes for T cell migration are over-expressed in adoptively transferred T cells migrating into the tumor milieu, including CCR8, CX3CR1 and a chemokine receptor CC motif receptor like 2 (CCRL2). Among these, expression of CCR8 and CX3CR1 were confirmed by additional quantitative PCR.

CX3CR1 is predominantly expressed on $CD8^+$ T cells, $CD4^+$ T cells and natural killer cells (NK cells), and its interaction with CX3CL1 (also known as fractalkine) can mediate adhesion and migration of these immune cells [Imai et al., 1997]. IL-2 can up-regulate CX3CR1 expression on T cells. CX3CR1 is selectively expressed on cytotoxic T cells with enhanced expression of intracellular granzyme B and perforine [Nishimura et al., 2002]. CX3CL1 is mainly over-expressed in neurons, nerve fibers [Marchesi et al., 2008] and some types of cancer cells, such as glioblastoma [Erreni et al., 2010], colorectal cancer [Ohta et al., 2005] and breast cancer [Park et al., 2012]. High expression of CX3CL1 is positively associated with increased amounts of TIL and better prognostic outcome in colorectal cancer [Ohta et al., 2005] as well as breast cancer [Park et al., 2012]. CX3CL1 transduced lung cancer cells [Guo et al., 2003] and neuroblastoma [Zeng et al., 2007] attracted T cells and enhanced T cell mediated cytotoxicity. Thus, CX3CL1 - CX3CR1 may be considered as a potential therapeutic target for T cell therapy.

CCR8 was found at higher levels in tumor-infiltrating GFP^+-OT-1 T cells compared with those found in spleen by RT-PCR array. This was confirmed both by another pair of RT-PCR primers and by flow cytometry at the protein level. CCR8 is preferentially expressed in the thymus, and is upregulated in activated T cells, especially Th2 cells [DAmbrosio et al., 1998]. By interacting with its ligand CCL1, CCR8 plays a role in the chemotaxis of activated T

[Roos et al., 1997, Goya et al., 1998] and Treg cells [Iellem et al., 2001] to lymphoid tissues. In tumor models, CCR8 expression on melanoma and breast cancer cells may help tumor metastasis into the lymph nodes [Das et al., 2013], suggesting that CCR8 may be a potential therapeutic target in cancer. The CCR8 gene is located in close proximity with the CX3CR1 gene on chromosome 3 [DeVries et al., 2003], which would match our array data that CCR8 and CX3CR1 mRNA are simultaneously up-regulated in tumor-infiltrating GFP^+-OT-1 T cells. As pointed out previously, CX3CR1 expression on T cells correlates with T cells infiltration in tumors. However, the role of CCR8 expression on tumor infiltrating effector T cells has not yet been investigated. On the other hand, chemokine profiles can be variable in different types of tumors [Slaney et al., 2014]. Thus, it is worthwhile to further investigate the function of CCR8 expression on TIL and its potential consequences for T cell therapy.

4.6 Limitations of the methods used

RT-PCR array combines the quantitative advantage of RT- PCR and microarray profiling to compare gene expression, and it is the most reliable tool for analyzing gene expression. It is normally utilized to confirm candidate genes identified by DNA array [Etienne et al., 2004]. However, commercial RT-PCR based arrays lack the comprehensive screening range of whole genome profiling. This might lead to bias and miss important genes involved in ACT.

Secondly, the experimental design in this project was only focused on the comparison of gene expressions in different organs at specific time point. Therefore, genes with altered regulation in tumor-infiltrating T cells could not be simply interpreted whether the candidate genes are favorable factors that assist T cell migration, or these genes are consequently changed after T cells infiltrate into tumor site. Analyzing the dynamics of gene profiles [Wherry et al., 2007] and further function experiment at protein level might be of help to distinguish the role of candidate genes.

4.7 Outlook

Gene expression profiles of T cells after adoptive transfer within different compartments might give hints for further T cell manipulation to improve ACT. Further comprehensive gene profiling of T cells used in ACT might be needed. Candidate genes could be cloned to investigate their biological function and be used for gene engineering to enhance T cell function during ACT.

5. Summary

Adoptive transfer of T cells has emerged as a promising novel strategy in the treatment of cancer. The thesis project presented here investigated the molecular signatures of adoptively transferred murine T cells *in vivo*, which might reveal factors that influence effective T cell therapy.

A subcutaneous tumor model was established using the murine Panc02-OVA pancreatic cancer cell line in wild type C57BL/6 female mice. Following retroviral transduction, GFP-transduced OT-1 T cells were infused into tumor-bearing mice. One week later, lymphocytes from tumor and spleen were harvested, and GFP^+-OT-1 T cells were sorted by flow cytometry. mRNA was extracted from sorted GFP^+ T cells, and gene expressions were analyzed by RT-PCR arrays. Candidate genes were confirmed both at mRNA and protein level.

RT-PCR array of the transferred T cells infiltrated into tumors demonstrated a distinct gene expression profile including 1) over-expression of activation as well as exhaustion markers, such as PD-1 and CTLA-4; 2) expression of distinct transcription factors and epigenetic regulation genes; 3) changes in T cell receptor signaling pathway; 4) altered expression of chemokines and chemokine receptors, including increased expression of CCR8. CCL1, the ligand for CCR8, was also found upregulated in tumor tissue

In conclusion, RT-PCR arrays of tumor infiltrating T-cells revealed altered expression of factors involving T cell differentiation. These might distinguish effective T cells from ineffective T cells in this mouse model, and give hints for further T cell manipulation to improve the efficacy adoptive T-cell therapy.

6. Zusammenfassung

Der adoptive T-Zelltransfer ist ein vielversprechender Ansatz zur Tumorimmuntherapie. Ziel meiner Dissertation war es, die T-Zell-mRNA-Expressionsprofile nach adoptivem Zell-Transfer in Tumor tragende Mäuse zu analysieren.

OT-1 transgene T Zellen wurden mit GFP transduziert. Die transduzierten OT-1 T Zellen wurden daraufhin adoptiv in Wildtyp-Mäusen mit subkutanen Panc02-OVA-Pankreas-karzinomen, die das Modellantigen Ovalbumin (OVA) präsentieren, transferiert. Nach einer Woche wurden GFP-positive T Zellen aus den Tumoren und den Milzen mittels Durchflusszytometrie durch FACS *sorting* isoliert. Nachfolgend wurde die Gen-Expression der FACS-sortierten T-Zellen mittels RT-PCR Array quantifiziert. Die Expression von Kandidatengenen wurde auf Proteinebene mittels Durchflusszytometrie und ELISA bestätigt.

Die Tumor infiltrierenden T-Zellen zeigten: 1) Überexpression von aktivierenden als auch von inhibitorischen Rezeptoren, wie z. B. CTLA-4 und PD-1; 2) Expression von Transkriptionsfaktoren und Enzymen für epigenetische Regulationsvorgänge; 3) Veränderungen der TCR-Signaltransduktion; 4) erhöhte Expression des Chemokinrezeptors CCR8. Ergänzend dazu wurden erhöhte Spiegel von CCL1, dem Liganden für CCR8, im Tumorgewebe gemessen.

Die Genexpressionsanalyse von T-Zellen nach adoptivem Transfer im Mausmodell könnte zur Aufklärung von Veränderungen in T-Zellen nach Transfer beitragen. Dies kann die Grundlage für neue therapeutische Strategien zur Verbesserung der adoptiven T-Zell-Therapie bilden.

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8. Abbreviation list

A

ACT	Adoptive cell therapy
ALL	Acute lymphoblastic leukemia
APC	Allophycocyanin
APCs	Antigen presenting cells

B

BSA	Bovine serum albumine
BTLA	B- and T-lymphocyte attenuator

C

$CaCl_2$	Calcium chloride
CAR	Chimeric antigen receptor
CCBP2	Chemokine-binding protein 2
CCL	Chemokine (C-C motif) ligand
CCL2	Chemokine (C-C motif) ligand 2
CCR	Chemokine (C-C motif) receptor
CCRL	C-C chemokine receptor-like
CD	Cluster of differentiation
cDNA	Complementary DNA
CLL	Chronic lymphocytic leukemia
Cma1	Chymase 1
CO_2	Carbon dioxide
Ct	Threshold cycle
CTLA-4	Cytotoxic T lymphocyte antigen 4
CX3CL1	Chemokine (C-X3-C motif) ligand 1
CX3CR1	CX3C chemokine receptor 1
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor

D

DAG	Diacylglycerol

DGK α	Diacylglycerol kinase α
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide

E

E.coli	*Escherichia coli*
EB	Ethidium bromide
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EF1α	Elongation factor-1 α
ELISA	Enzyme-linked immunosorbent assay
Eomes	Eomesodermin

F

FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box protein 3
FSC	Forward scatter
FSC-A	Forward scatter-area
FSC-H	Forward scatter-height

G

GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOI	Gene of interest
gp100	Glycoprotein 100

H

HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HEPES acid	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HKG	Housekeeping genes
HPRT	Hypoxanthine-guanine phosphoribosyltransferase

HPV	Human papillomavirus

I

IFN-γ	Interferon-γ
IL	Interleukin

L

LAG-3	Lymphocyte-activation gene 3
LB	Lysogeny broth

M

MART-1	Melanoma antigen recognized by T cells 1
MDA	Melanocyte differentiation antigen
MHC	Major histocompatibility complex
mRNA	Messenger RNA

N

NCBI	National center for biotechnology information
NK cells	Natural killer cells

O

OVA	Ovalbumin

P

PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PI	Propidium iodide
PKC	Protein kinase C
Plat-E	Platinum-E
PKC γ	Protein kinase C γ

R

RNA	Ribonucleic acid

RNase	Ribonuclease
RPMI 1640 medium	Roswell park memorial institute 1640 medium
RT-PCR	Reverse transcription polymerase chain reaction

S

SEM	Standard error of the mean
SSC	Side scatter

T

TBI	Total body irradiation
TCR	T cell receptor
TGF-β	Transforming growth factor β
TILs	Tumor-infiltrating lymphocytes
TLR	Toll-like receptor
Tm	Melting temperature
TNF-α	Tumor necrosis factor-α
Tregs	Regulatory T cells

W

w/v	Weight per volume

X

XCL	Chemokine (C-X-C motif) ligand
XCR1	Chemokine (C-motif) receptor 1

9. Curriculum vitae

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10. Publications

Original publications

1. Kobold, S., Steffen, J., Chaloupka, M., Grassmann, S., Henkel, J., Castoldi, R., **Zeng, Y.,** Chmielewski, M., Schmollinger, J. C., Schnurr, M., Rothenfußer, S., Schendel, D. J., Abken, H., Sustmann, C., Niederfellner, G., Klein, C., Bourquin, C., and Endres, S. Selective bispecific T cell recruiting antibody and antitumor activity of adoptive T cell transfer. *Journal of the National Cancer Institute* 2014; 107:364.

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Conference abstract

1. Grassmann, S., Peter, P., **Zeng**, **Y.,** Schmollinger, J.C., Endres, S., and Kobold, S.
A new PD1-CD28 chimeric receptor overcomes PD-1-mediated immunosuppression in adoptive T cell therapy.
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1. **Zeng, Y.** 15./04./2013,
"A chimeric cytokine receptor for T cell therapy."
Doktorandenkolloqium at Graduiertenkolleg 1202 weekly seminar, Munich, Germany.
2. **Zeng, Y.,** Grassmann, S., Endres, S. and Kobold, S. 02./10.2012,
"Total body irradiation (TBI) increases tumor infiltration by T cells after adoptive cell transfer."
4th Autumn School Current Concept in Immunology, Bad Schandao, Germay.
3. **Zeng, Y.,** Endres, S. and Kobold, S. 24./03./2012,
"Fraudulent T cell Receptor---not all the things not good are bad.")
ICE School (Immunology Course Engadina), Bos-Cha, Switzerland

Poster

1. **Zeng, Y.,** Grassmann, S., Endres, S. and Kobold, S. 14./03.2013,
" Adoptively transferred antigen specific T cells exhibit an exhausted phenotype after total body irradiation in a murine model of gastric cancer-derived tumor."
7th cellular therapy international symposium, Erlangen, Germany.
2. **Zeng, Y.,** Endres, S. and Kobold, S. 05./05./2012,
"Transduction of T cell receptors for the immunotherapy of cancer", DoktaMed, Munich, Germany, Poster Award

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Eidesstattliche Versicherung

Zeng, Yi

Name, Vorname

Ich erkläre hiermit an Eides statt,
dass ich die vorliegende Dissertation mit dem Thema

Gene expression profiles of T cells
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