Division of Clinical Pharmacology
Prof. Dr. med. S. Endres

Munich Medical Research School
Ludwig-Maximilians-Universität München

Combination of C-C chemokine receptor 8 and dominant-negative TGF-β-receptor 2 expression in T cells for adoptive T cell therapy of solid cancer

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Bruno Loureiro Cadilha
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Direct supervision:	Prof. Dr. med. Sebastian Kobold
Thesis advisory committee:	Prof. Dr. med. Sebastian Kobold
	Prof. Dr. med. Marion Subklewe
	Prof. Dr. med. Hauke Winter
Thesis defense committee:	Prof. Dr. med. Sebastian Kobold
	Prof. Dr. med. Marion Subklewe
	Prof. Dr. med. Stefan Endres
	Prof. Dr. Wolfgang Zimmermann
Dean:	Prof. Dr. med. dent. Reinhard Hickel

Thesis defense on the 7th of April 2020

To my unconditionally supporting family and friends,

Obrigado

Bruno Loureiro Cadilha

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1 Introduction

1.1 Tumor immunology

Immunology, the study of the immune system, and oncology, the study of cancer, have been intertwined since the late 19th century, when the surgeon William Coley described that the injection of killed bacteria into sites of sarcoma would lead to tumor shrinkage (Coley 1991). Coley observed several tumor patients that developed into remissions after developing erysipelas, which prompted him to induce these reactions himself through the administration of a mixture of inactivated *Streptococcus pyogenes* and *Serratia marcescens* — Coley's toxin. Despite the reported successes, Coley's toxin was largely dismissed during his lifetime due to the advents of radiotherapy and chemotherapy. Coley was never able to pinpoint the anti-tumor effect of his toxin to the immune system, but his groundbreaking work set the stage for modern immuno-oncology. Currently, a broad spectrum of oncologic therapies are being studied on the basis of manipulating the immune system. A broadly accepted theory on the influence of the immune system on oncogenesis is termed cancer immunoediting, a several-step process first proposed by Schreiber, Old and Smyth (Schreiber et al. 2011). Mutations to genes with the potential to drive oncogenesis (such as tumor-suppressor genes and oncogenes) can occur in healthy tissues, enabling these cells to circumvent their intrinsic tumor-suppressive mechanisms. The immune system, through innate and adaptive mechanisms, enacts a tight control and elimination of aberrant clones — defining the elimination phase. Certain mutations might enable some oncogenic clones to be somewhat resistant to the immune system and prevail in the organism in a steady manner. Conversely, some mutations might also increase the anti-tumoral pressure imposed by the immune system — this equilibrium phase is thus characterised by an editing process that balances tumor immune destruction with persistence of some malignant clones. In some instances, the accumulation of an increased mutational load might confer malignant clones with the capacity to completely evade the immune system and further develop — characterising the escape phase (Schreiber et al. 2011).

Several methods have been devised to eliminate cancer through the immune system. Some of the most remarkable ones are antibodies directed against the cytotoxic T lymphocyte-associated protein 4 (CTLA-4) or the programmed cell death protein 1 (PD-1). These molecules naturally inhibit T cell function and in homeostatic conditions

prevent exacerbated reactions by the immune system. Tumors take advantage of these pathways to prevent the immune system to effectively mount anti-tumoral responses. Disinhibition through antibodies has the potential to reinvigorate T cells and drive anti-tumor effects in several solid tumors (Leach et al. 1996, Iwai et al. 2002). Nevertheless, a vast majority of solid tumor patients are either diagnosed at a stage where therapeutic strategies such as surgery, chemotherapy or radiotherapy cannot be effectively used and many have tumors that will be refractory to immune checkpoint blockade therapy. Recently, therapies that harness the anti-tumoral capacity of T effector (T_{eff}) cells have been developed and some modalities of these T cell therapies have fundamentally improved the treatment outcome in several hematological malignancies (June et al. 2018). These T cell-based therapies, may rely on the selection and expansion of tumor-specific T cells or even introduce genetic modifications in T cells of unknown specificity, which render them tumor-specific (Rosenberg et al. 2015). The success of T cell therapy in haematological malignancies is driving the field of immunotherapy with the aim of translating this success to the therapy of solid tumors (Lim et al. 2017).

1.2 Adoptive T cell therapy

Cancer immunotherapy seeks to manipulate the immune system in order to re-balance the pathologically disrupted equilibrium that leads to tumor formation (Hanahan et al. 2011). T cells have both the potential to promote tumor growth, mediated by T regulatory (T_{reg}) cells, as well as the potential to control it, mediated by T_{eff} cells (Savage et al. 2014). Adoptive T cell therapy (ACT) is a cancer immunotherapy that involves the harvest, expansion and re-infusion of T_{eff} cells into a patient (Melief 1992). There are different modalities of ACT that differ firstly in what concerns the tissue-source for the T cell harvest, and secondly in the need to genetically engineer the harvested T cells to confer them with tumor specificity (Rosenberg et al. 2015).

T cells can be harvested directly from the tumor tissue. In such an instance, the ACT modality is referred to as tumor infiltrating lymphocyte (TIL) therapy. TIL therapy requires an *ex vivo* selection process for tumor-specificity that takes place before expansion and re-infusion into the patient. TIL therapy has the advantage of being an ACT modality that does not require genetic engineering. Nevertheless, it requires the

existence of this cell population in the tumor and that the tumor in question is accessible for a biopsy that enables T cell isolation in sufficient amounts (Rosenberg et al. 1988).

Alternatively, if T cells are isolated from peripheral blood by apheresis, they will be of an unknown specificity and will thus require the introduction of genetic constructs that enable them to recognise the tumor. This can be performed through T cell receptor (TCR) engineering or chimeric antigen receptor (CAR) engineering of T cells (Benmebarek et al. 2019). A TCR induces physiological T cell activation and cytotoxic response through conventional binding to an antigen presented by a major histocompatibility complex (MHC) on a tumor cell, whereas a CAR can virtually target any surface molecule presented by a tumor cell. This is possible due to the architecture of the CAR itself. In its most simple form a CAR combines a single chain variable fragment (scFv) of an antibody to enable target-recognition, chained to a hinge and transmembrane domains that allow for CD3ζ domain activation that will trigger a T cell response (Eshhar et al. 1993). Currently, CAR products with clinical approval add a co-stimulatory domain (either CD28 or 4-1BB) to the original design of the molecule (Kruger et al. 2019). Nevertheless, CAR design varies greatly within pre-clinical settings. Some of the most notable limitations of CAR T cells are exhaustion, activation-induced cell death, tonic signaling, tumor antigen loss and toxicity. All the elements of the CAR can be fine-tuned in an effort to reduce these limitations and ultimately improve therapeutic efficacy (Stoiber et al. 2019). ACT modalities and the modular structure of a CAR are depicted in Figure 1.

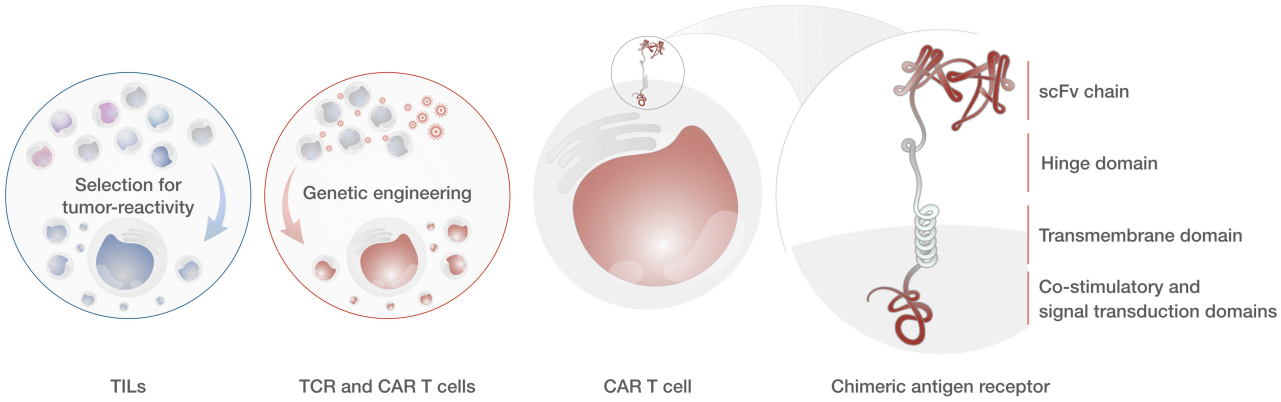

Figure 1 ACT modalities and structure of a CAR.

Adapted from Kruger et al. 2019 and Stoiber et al. 2019.

CD19 targeted CAR T cell therapy is the most promising modality of ACT with successful results in acute lymphocytic leukemia (ALL) (Maude et al. 2014), diffuse large B cell lymphoma (DLBCL) (Kochenderfer et al. 2015), and chronic lymphocytic leukaemia (CLL) (Turtle et al. 2017). For example in ALL, the study from Parker and colleagues reports complete remission in 83% of patients and a median overall survival of 12.9 months which compared favourably to the 7.7 months median overall survival of adult ALL patients treated with the anti-CD3/anti-CD19 bispecific antibody blinatumomab (Park et al. 2018). The promising results of the clinical trials ELIANA, JULIET and ZUMA-1 have led to the approval of two distinct CAR T cell products in several hematological malignancies (Neelapu et al. 2017, Maude et al. 2018, Bishop et al. 2019, Locke et al. 2019, Schuster et al. 2019). Multiple solid-tumor-directed CAR T cell therapies are currently undergoing clinical trials, nevertheless, results show that this therapy is still incapable of producing sustainable responses in solid tumors (Brown et al. 2016, Beatty et al. 2018).

1.3 ACT limitations in solid tumors

Despite the success in hematological malignancies, solid tumors remain utterly refractory to CAR T cell therapy (June et al. 2018). Two recent clinical trials have now reported results of CAR T cell therapy in glioblastoma and pancreatic cancer. Brown et al. conducted a trial where one patient with recurrent multifocal glioblastoma was infused for a total of 10 cycles of CAR T cell therapy from day 112 to day 298 after enrolment, without grade 3 or higher neurotoxic adverse events. For 7.5 months the CAR T cell therapy, which was directed against IL13Rα2, could bring about a clinical response that consisted of regression of tumor lesions and was accompanied by increased cytokine levels and immune cell numbers in the cerebrospinal fluid. Nevertheless, tumor lesions were never completely cleared and continued to progress eventually (Brown et al. 2016). The phase I study by Beatty et al. assessed safety and efficacy of anti-mesothelin CAR T cells in pancreatic ductal adenocarcinoma (PDAC). A total of six patients with chemotherapy-refractory metastatic PDAC were treated three times weekly for three weeks with CAR T cells. The therapy was completely uneventful in regard to toxicity, despite appropriate expansion of the therapy product (as measured by surrogate molecules). Nevertheless, progression-free survival was only observed for two patients and in both cases the response was ephemeral (less than six months)

(Beatty et al. 2018). This trial relied on the transient expression of CAR in T cells, through mRNA electroporation. Therefore, optimisations could still be made, such as the stable incorporation of the CAR molecule into the T cell genome through retro- or lentiviral vectors which would be expected to lead to longer-lasting therapeutic effects. In both trials, antigens that were expressed at high levels in the tumors were used as targets for the CAR T cells. However, in both cases the therapeutic efficacy was poor. It is thus clear that in order to enact powerful anti-tumor responses in solid tumors, CAR T cells need to overcome further biological limitations. Appropriate trafficking to the tumor site and resistance to the tumor microenvironment-induced suppression are major hurdles to the employment of T cell therapies in solid tumors (Lim et al. 2017).

Having high numbers of T cells infiltrating the tumor site correlates with better clinical outcomes (Slaney et al. 2014). When trying to overcome this tumor access hurdle, several strategies may be employed, aimed at targeting the anarchic tumor vasculature (Kadambi et al. 2001), skewing the tumor environment into a more pro-inflammatory state (Di Pilato et al. 2019), inducing chemokine expression patterns favourable for T_{eff} cell recruitment (Hong et al. 2011) or modifying T cell therapy to express chemokine receptors that match the ligand profile of the solid tumor (Di Stasi et al. 2009, Rapp et al. 2015). Improving the recruitment of ACT products into solid tumors is necessary for effectiveness (Lim et al. 2017).

Given an appropriate infiltration of the tumor site, several cell-to-cell and soluble factor interactions will cause immunosuppression, leading to a sub-optimal T cell cytotoxic and proliferative capacity (Rabinovich et al. 2007). Several cell populations besides the tumor cells themselves are capable of driving T cells into anergic states. Myeloid-derived suppressor cells (MDSCs), T_{reg} cells and even dendritic cells (DCs) have the potential to drive immunosuppression (Rabinovich et al. 2007, Steinman et al. 2003, Sakaguchi et al. 2008). TGF-β, IL-10 and VEGF are immunosuppressive cytokines secreted in the tumor micro-environment (Gorelik et al. 2002, Gerlini et al. 2004, Kurte et al. 2004). Checkpoint inhibitor molecules such as CTLA-4 and PD-1 can, through cell-to-cell interactions, drive immunosuppression of T cells (Leach et al. 1996, Iwai et al. 2002). The tumor microenvironment and its suppressive characteristics are now incorporated in scores with the aim of better stratifying patients regarding their prognosis and treatment options (Galon et al. 2006, Galon et al. 2014). Several

antibody therapies targeting the aforementioned checkpoint inhibitors are now approved in the clinics (Snyder et al. 2014, Tumeh et al. 2014). Currently, there are several approaches that aim at introducing immunosuppressive resistance mechanisms in genetically engineered T cells which result in consistent improvements to ACT effectiveness (Kobold et al. 2015, Ren et al. 2017, Bollard et al. 2018).

1.4 Chemokine receptors to guide T cell infiltration

Chemoattraction refers to the unidirectional movement of a cell in response to a chemical gradient of ligand. Chemokines are some of the molecules capable of triggering chemoattraction upon binding to chemokine receptors (Zlotnik et al. 2012). Chemokine receptors are class A, rhodopsin-like, members of the G-protein coupled receptors (GPCR) family. They are also referred to as heptahelicoidal receptors (Mantovani et al. 2004, Locati et al. 2005). Chemokine receptors and chemokine ligands have a high degree of promiscuity that provides this system with a high degree of robustness (Johnson et al. 2004). Murine chemokine receptors and ligands are depicted in Table 1.

Among the different functions of chemokine receptors, there is the capacity to direct and position cells during immune responses (Luster 1998, von Andrian et al. 2000).

Table 1 Murine chemokine receptor and ligand family nomenclature.

Adapted from Zlotnik et. al 2012.

C-C										
CCR1	CCR2	CCR3	CCR4	CCR5	CCR6	CCR7	CCR8	CCR9	CCR10	CCR11
CCL3 CCL4 CCL5 CCL6 CCL7 CCL9 CCL10	CCL2 CCL7 CCL12	CCL4 CCL5 CCL6 CCL7 CCL9 CCL10 CCL11 CCL24	CCL2 CCL3 CCL5 CCL17 CCL22	CCL2 CCL3 CCL4 CCL5	CCL20	CCL19 CCL21	CCL1 CCL8	CCL25	CCL27 CCL28	CCL19 CCL21 CCL25

C-X-C							X-C	C-X3-C
CXCR1	CXCR2	CXCR3	CXCR4	CXCR5	CXCR6	CXCR7	XCR1	CX3CR1
CXCL1 CXCL7 LIX	CXCL1 CXCL2 CXCL3 CXCL7 LIX	CXCL4 CXCL9 CXCL10 CXCL11	CXCL12	CXCL13	CXCL16	CXCL11 CXCL12	XCL1	CX3CL1

Intracellularly, the binding of a chemokine ligand to its receptor will trigger a cascade of events that culminate in a cytosolic calcium elevation. This event will then induce the necessary up-regulation and organisation of the cellular machinery required for chemotaxis (Cabrera-Vera et al. 2003).

Tumors can shape their surroundings through specific chemokine ligand up- or down-regulation, excluding T_{eff} cells from their vicinity and thereby averting immunological surveillance (Curiel et al. 2004, Harlin et al. 2009, Facciabene et al. 2011). Therefore, a tumor chemokine receptor and ligand screen may be able to reveal an axis whose targeting may restore T_{eff} cell infiltration capacity (Cadilha et al. 2017). Strategies involving the overexpression of chemokine receptors in T cells have unveiled that C-C chemokine receptor 4 (CCR4) can improve the recruitment of ACT products in non-Hodgkin lymphoma (Di Stasi et al. 2009) and pancreatic solid tumors (Rapp et al. 2015), consequently leading to superior anti-tumor control. Similarly, also the C-X-C chemokine receptor 2 (CXCR2) could be used to improve ACT anti-tumor control in colon and melanoma tumor models (Peng et al. 2010). Both CCR4 and CXCR2 are currently listed in clinical trials aimed at improving ACT in solid tumors (respectively, NCT03602157 and NCT01740557).

CCR4 and CXCR2 have shown that chemokine receptors have the potential to improve T cell infiltration into some solid tumors entities, but have yet to reach clinical application. The wide gamut of existing chemokine receptors and their diverse roles besides migration, demands that further chemokine receptors are probed for their capacity to improve ACT, either by improving its effectiveness in the already studied tumor entities, or by enabling ACT for a wider scope of tumor entities. Furthermore, one should consider that combining chemokine receptor engineering with ACT might not be sufficient to render T cells effective in solid tumors. Therefore, it is of utmost importance to simultaneously tackle solid tumors with strategies that relieve T cells from immunosuppression.

1.5 TGF-β resistance for improved T cell proliferation

TGF-β signaling is involved in various homeostatic and pathological processes ranging from cell growth to differentiation and apoptosis. Upon TGF-β-binding to its receptors, these receptors will be phosphorylated, which will trigger subsequent phosphorylation of

Smad2 and Smad3 that will then be able to bind to Smad4. These Smad complexes will be able to act in the nucleus as transcription factors (Massague 2008).

Regarding tumorigenesis, the TGF-β pathway can be highjacked to promote tumor growth, invasion, metastasis and tumor angiogenesis (Massague 2008, Ikushima et al. 2010). Besides this, TGF-β acts as a major enforcer of immune tolerance by inhibiting the development and functions of the innate immune system (macrophages, antigen presenting cells and Natural Killer (NK) cells) and the adaptive immune system (cytotoxic T cells, T helper (Th) 1 and Th2 cells). Immune-suppression can be exerted either directly by tumor cells or by T_{reg} cells that are activated by TGF-β (and consequently produce TGF-β as well) (Massague 2008, Moutsopoulos et al. 2008, Yang et al. 2010).

It has been shown that in the Panc02 pancreatic carcinoma C57BL/6 tumor model high levels of TGF-β exist, and furthermore that treatment with siRNA to silence this molecule had an impact on controlling pancreatic tumor growth in a $CD8^+$ T cell-dependent manner (Ellermeier et al. 2013).

A dominant negative version of the TGF-β-R2 (DN-TGF-β-R2 or DNR) has been created and described by Massague and others (Wieser et al. 1993, Bollard et al. 2002). This receptor has a stop codon after the tenth intracellular amino acid, therefore it does not induce downstream signaling upon ligand binding (depicted in Figure 2). The effects of this receptor are not only beneficial to the cell that expresses the DNR but also to cells in the vicinity as DNR-bearing cells will scavenge TGF-β preventing its

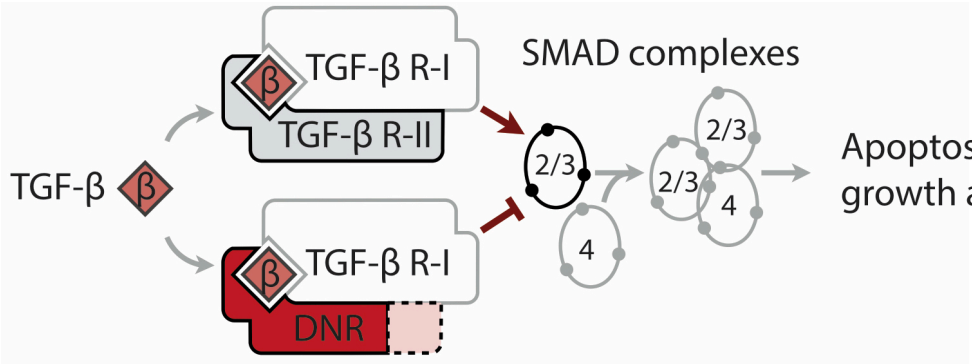

Figure 2 Schematic representation of the DNR.

Upon binding to TGF-β, the lack of an intracellular domain will prevent the activation of the intracellular SMAD complex, thus preventing the proliferation arrest on protected T cells.

Adapted from Massagué 2008.

effects on cells lacking DNR expression. Recently this receptor has been combined in an anti-PSMA CAR T cell showing improved results in prostate cancer (Kloss et al. 2018) and one clinical trial (NCT00368082) in Hodgkin lymphoma has reported promising results (Bollard et al. 2018).

The well-established safety profile and advanced stage of development place the DNR as a very interesting candidate to genetically engineer into tumor-specific T cells. Its ability to shield T cells from TGF-β make it a strong combination partner for chemokine receptor-engineered T cell therapy.

1.6 Preliminary data — Panc02 tumor infiltrating lymphocytes significantly up-regulate CCR8

Our group has previously shown that C-C chemokine receptor 8 (CCR8) was significantly up-regulated in adoptively transferred OT-I T cells retrieved from Panc02-OVA tumors when compared to adoptively transferred OT-I T cells retrieved from the spleen (Zeng 2017). CCR8 is not known to be expressed in T_{eff} cells. Conversely, T_{reg} cells have been described to express CCR8. $CCR8^+$ T_{reg} cells exhibit an increased immunosuppressive capacity (Barsheshet et al. 2017). Accumulation of $CCR8^+$ T_{reg} cells in breast tumors correlates with a worse prognosis (Plitas et al. 2016). These results prompted us to research the use of CCR8 to promote the migration of T_{eff} cells into pancreatic solid tumors in order to counteract the natural recruitment of T_{reg} cells in these tumors.

1.7 Research hypothesis and aims of the work

The present work investigates if the forced expression of CCR8 in tumor-specific T cells can improve their accumulation in solid tumors and if the DNR can confer T cells with TGF-β resistance (depicted in Figure 3). The following aims have been set as experimental goals:

I. Transcriptomic and proteomic characterisation of murine and human solid tumors for CCL1 and TGF-β expression.

II. *In vitro* and *in vivo* functional characterisation of murine and human antigen-specific T cells modified to express CCR8, DNR or the combination of both receptors.

Together, CCR8-DNR-transduced tumor-specific T cells will be assessed for their anti-tumor effects in pancreatic tumor models.

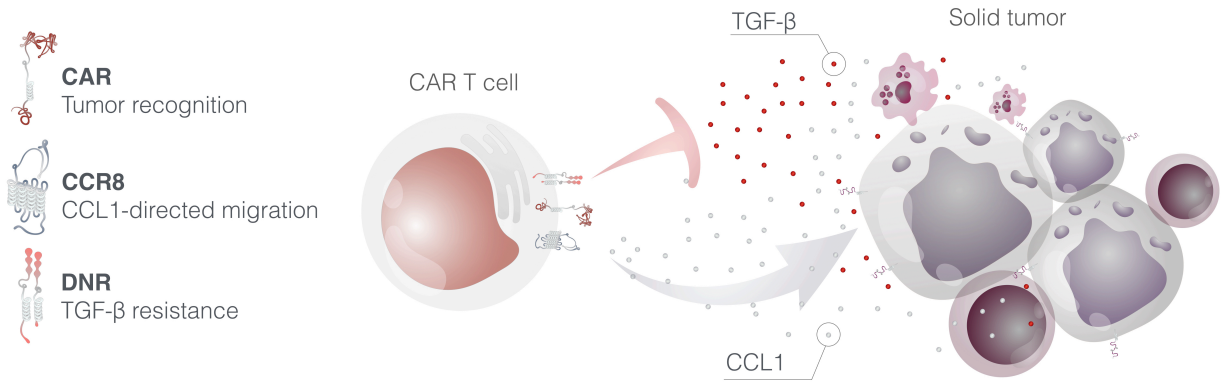

Figure 3 Schematic representation of the research hypothesis.

Do CCR8-DNR CAR T cells have an improved capacity to infiltrate and proliferate in pancreatic tumor models, thus improving anti-tumor efficacy?

2 Material and methods

2.1 Technical devices and reagents

Table 2 Technical devices

Alpha Imager HP gel imager	Alpha Innotech	Kasendorf, Germany
Cell culture flow HeraSAFE KS	Heraeus, ThermoFischerScientific	Massachusetts, USA
Centrifuge Rotina 420R	Hettich GmbH	Tuttlingen, Germany
Clinical Cryostat CM 1950	Leica Biosystems	Wetzlar, Germany
CO_2 – Incubator (BD6220)	Heraeus, ThermoFischerScientific	Massachusetts, USA
FACS Canto II	BD Biosciences	New Jersey, USA
Innova44 Thermoshaker	New Brunswick Scientific, Eppendorf	Hamburg, Germany
Leica TCS SP5 confocal system	Leica Microsystems	Wetzlar, Germany
Light microscope Axiovert 40C	Zeiss	New York, USA
LightCycler480 System	Roche	Mannheim, Deutschland
Nanodrop 2000c	ThermoFischerScientific	Massachusetts, USA
PowerPac™ Universal Power Supply	Bio-Rad Laboratories	Munich, Germany
T3 Thermocycler	Biometra	Göttingen, Germany

Table 3 Reagents

Albumin fraction V (BSA)	Sigma-Aldrich	Steinheim, Germany
Ammonium chloride	Merck	Darmstadt, Germany
BD Pharm lyse lysing buffer (10x)	BD Biosciences	New Jersey USA
Blasticidin	InvivoGen	California, USA
Calcium chloride	Sigma-Aldrich	Steinheim, Germany
Chloroform	Sigma-Aldrich	Steinheim, Germany
Colagenase D	Sigma-Aldrich	Steinheim, Germany
Count Bright, counting beads	LifeTechnologies	California, USA
DC™ Protein Assay	Bio-Rad Laboratories	Munich, Germany
Dimethylsulfoxid (DMSO)	Sigma-Aldrich	Steinheim, Germany
DNase I	Roche	Mannheim, Germany
Dulbecco's modified Eagles medium DMEM	PAA	Pasching, Austria
Dulbecco's Phosphate Buffered Saline (PBS)	PAA	Pasching, Austria
Dynabeads mouse T-activator CD3/CD28	Invitrogen (ThermoFischerScientific)	Massachusetts, USA
EcoRI	NEB	Massachusetts, USA
Ethanol 100%	Carl Roth GmbH	Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	Steinheim, Germany
FACSFlow, FACSSafe	BD Biosciences	New Jersey USA
GeneJet plasmid mini prep kit	ThermoFischerScientific	Massachusetts, USA
Heparin-sodium 2.500 IE / 5 ml	Braun AG	Melsungen, Germany
HEPES buffer 1 M	Sigma-Aldrich	Steinheim, Germany
High glucose FBS	Gibco Products	New York, USA
Human serum	Sigma-Aldrich	Steinheim, Germany

Isofluoran	CP PHARMA	Burgdorf, Germany
LB agar	Carl Roth GmbH	Karlsruhe, Germany
LB medium	Carl Roth GmbH	Karlsruhe, Germany
LE agarose	Biozym	Hessisch Oldendorf, Germany
L-glutamin 200mM	PAA	Pasching, Austria
MEM non-essential amino acids (NEAA, 100x)	Gibco Products	New York, USA
Mouse CCL1/TCA-3 DuoSet ELISA	RnD Systems	Minneapolis, USA
Sodium pyruvate	PAA	Pasching, Austria
NotI	NEB	Massachusetts, USA
peqGOLD TriFast	PEQ LABS	Erlangen, Germany
Q5 enzyme	NEB	Massachusetts, USA
Penicillin/ Streptomycin (100x)	PAA	Pasching, Austria
Puromycin	InvivoGen	California, USA
RetroNectin	TaKaRa	Kyoto, Japan
RevertAid first strand cDNA synthesis kit	ThermoFischerScientific	Massachusetts, USA
Roswell Park Memory Institute (RPMI)	PAA	Pasching, Austria
SERVA DNA Stain Clear G	SERVA	Heidlberg, Germany
Sodium chloride	Sigma-Aldrich	Steinheim, Germany
Sulphuric acid	Apotheke Innenstadt	LMU Munich, Germany
Tissue freezing medium	Leica biosystems	Nussloch, Germany
Trypan blue	Sigma-Aldrich	Steinheim, Germany
Trypsin (10x)	PAA	Pasching, Austria
Tween 20	Carl Roth GmbH	Karlsruhe, Germany
β-Mercaptoethanol	Sigma-Aldrich	Steinheim, Germany

Table 4 Cell culture media

Medium	Composition	Medium	Composition	Medium	Composition
Murine T cell	RPMI 10% FBS 2 mM L-Glutamine 100 IU/ml Penicillin 100 µg/ml Streptomycin 1 mM Sodium Pyruvate 1 mM HEPES 50 µM β-Mercaptoethanol	**Tumor/ECO**	DMEM 10% FBS 4 mM L-Glutamine 100 IU/ml Penicillin 100 µg/ml Streptomycin	**Human T cell**	VLE RPMI 2% Human Serum 2 mM L-Glutamine 100 IU/ml Penicillin 100 µg/ml Streptomycin 1 mM Sodium Pyruvate 1% MEM-NEAA 50 µM β-Mercaptoethanol
Migration	RPMI BSA 1%	**Freezing**	90 % FBS 10 % DMSO		

2.2 Cell lines

The Panc02 cell lines have previously been modified with retroviral vectors to express either the chicken-derived ovalbumin antigen (UNIPROT entry P01012) (Panc02-OVA) or the EpCAM protein (UNIPROT entry Q99JW5) as a target antigen (Panc02-EpCAM), as described by Karches et al. (Karches et al. 2019). For this work's purpose, the murine CCL1 chemokine (UNIPROT entry P10146) (Panc02-CCL1) or a blue

fluorescent protein tagged to a histone protein (Panc02-H2B-Cerulean) have also been retrovirally introduced into the Panc02 tumor model. The SUIT-2 tumor cell lines have been modified with retroviral vectors to express the full length human mesothelin protein (UNIPROT entry Q13421) as a target antigen (SUIT-2-MSLN) (previously described by Karches et al. (Karches et al. 2019)) and the full length human CCL1 chemokine (UNIPROT entry P22362) (generated for this work, SUIT-2-MSLN-CCL1). Tumor cell lines transduced with OVA, EpCAM or mesothelin have been tested by flow cytometry for protein detection (anti-mouse H-2K^{b} bound to SIINFEKL (25-D1.16), anti-mouse CD326 (G8.8) or anti-human mesothelin (MN)) and tested against antigen-specific T cells for IFN-ɣ release, measured by ELISA. Supernatants of tumor cell lines transduced with either murine or human CCL1 have been tested by ELISA. Tumor cell lines transduced with the Cerulean fluorescent protein have been tested by flow cytometry. 293Vec-Galv, 293Vec-Eco and 293Vec-RD114 were a kind gift of Manuel Caruso, Québec, Canada and have been previously described (Ghani et al. 2009). For virus production retroviral pMP71 (kindly provided by C. Baum, Hannover) vectors carrying the sequence of the relevant receptor were stably introduced in packaging cell lines. Single cell clones were generated and indirectly screened for highest level of virus production by determining transduction efficiency of primary T cells. This method was used to generate the producer cell lines 293Vec-RD114 for GFP, mCherry, CCR8, DNR, CAR-MSLN, CCR8-CAR-MSLN, DNR-CAR-MSLN and CCR8-DNR-CAR-MSLN. Producer cell lines 293Vec-Eco were generated for GFP, mCherry, H2B-Cerulean, CCR8, DNR, CCR8-DNR, CAR-EpCAM, CCR8-CAR-EpCAM, DNR-CAR-EpCAM and CCR8-DNR-CAR-EpCAM.

Murine T cells have been isolated from splenocytes from donor mice. Spleens were mashed though 100 μm strainers and treated with erythrocyte lysis buffer. Cells were then counted and cultured for 24 hours with murine TCM and 1 μg/ml anti-CD3 and anti-CD28 antibodies (eBioscience, clones 145-2C11 and 37.51, respectively). Afterwards T cells would either be transduced with previously described protocols (Karches et al. 2019) and then expanded or directly expanded with T cell medium supplemented with human IL-15 (Peprotech) every second day. Human T cells have been isolated from healthy donor peripheral mononuclear cells after a MACS CD3 positive selection (Miltenyi Biotec) and 24 hour stimulation with human TCM and 8.25 µl per 10^{6} cells of human anti-CD3-anti-CD28 dynabeads (eBioscience). Cells could then

be transduced using previously described protocols (Karches et al. 2019) or directly taken into culture with human TCM in concentrations of 10^6 T cells per ml medium.

Table 5 Cell lines

Panc02	**Tumor/ECO medium**	Panc02 Pancreas carcinoma C57BL/6 tumor model.	Prof. Dr. med. C. Bruns (Klinikum der Uniklinik Köln, Deutschland)
SUIT-2	**Tumor/ECO medium**	Human pancreatic ductal adenocarcinoma cell line derived form a liver metastasis.	Acquired from ATCC.
293Vec-Galv	**Tumor/ECO medium**	Amphitropic packaging cell line.	Prof. Dr. Manuel Caruso (Québec, Canada)
293Vec-Eco	**Tumor/ECO medium**	Ecotropic packaging cell line.	
293Vec-RD114	**Tumor/ECO medium**	Amphitropic packaging cell line.	

2.3 Animal experiments

C57BL/6RJ and NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice were purchased from Charles River. OT-I mice were bred at the animal facilities at the Klinikum der Universität München or Massachusetts General Hospital (MGH). Animals were housed in specific pathogen-free facilities. All experimental studies were approved and performed in accordance with guidelines and regulations implemented by the Regierung von Oberbayern and the MGH Institutional Animal Care and Use Committee (IACUC). All experiments were carried out randomized and performed blinded and with adequate controls. In accordance to the animal experiment application, tumor growth and health status of mice were monitored every other day. For survival analyses, general health condition, weight loss of more than 15% from baseline weight and tumor area greater then 225 mm^2 were taken as surrogates for survival and recorded in Kaplan Meyer plots.

2.4 Tumor challenge experiments

All tumor cell lines were subcutaneously (s. c.) injected in 100 μl PBS into the flanks of mice. Animals were randomized into treatment groups according to tumor volumes. Tumor volumes were determined before and every other day after treatment was started and calculated as $V = (length \times width^2)/2$. For ACT studies, 10^7 T cells were injected intravenously (i. v.) in 100 μl PBS once average group tumor volumes had reached at least 50 mm^3.

2.5 Primers, PCR and RT-PCR

All DNA constructs (primers for murine or human cDNA cloning are described in Table 7) were generated by overlap extension PCR (Heckman et al. 2007) and recombinant expression cloning into the retroviral pMP71 vector (Sommermeyer et al. 2006) using standard molecular cloning protocols (Heckman et al. 2007).

cDNA was synthesized using the Superscript II kit (Life Technologies) from murine or human RNA isolated from peripheral blood mononuclear cells (PBMC). Polymerase chain reaction (PCR) primers for real-time PCR (RT-PCR) were designed automatically from the NCBI GenBank sequences in the assay design center from the Roche Universal ProbeLibrary and have been previously published (Rapp et al. 2015). Real-time PCR was performed using a Kapa Probe Universal MasterMix (VWR) in a LightCycler 480 instrument (Roche Diagnostics).

Table 7 Primer list for cDNA cloning

mCCR8	Forward	5'- ATGGATTACACGATGGAGCC -3'
	Reverse	5'- TCACAAGATGTCATCCAGGG -3'
hCCR8	Forward	5'- ATGGATTATACACTTGACCTCAGTGT -3'
	Reverse	5'- TCACAAAATGTAGTCTACGCTGGA -3'
DNR	Forward	5'- ATGGGTCGGGGGC -3'
	Reverse	5'- CTACAGCTTCTGCTGCC -3'

2.6 Migration assays

Cell migration was evaluated using transwell plates (Corning) as previously described (Rapp et al. 2015). 5 x 10^5 T cells were placed onto a 3 μm pore membrane in the upper chamber of a transwell plate with the lower chamber containing different concentrations of recombinant murine or human CCL1 (Biolegend). After 3 hours incubation at 37°C the migrated cells in the lower chamber were analyzed by flow cytometry.

2.7 xCelligence assays

For impedance-based real-time killing assays using an xCELLigence system (ACEA Bioscience, USA), 10^4 tumor cells were seeded per well in a 96-well plate. Cell number was monitored over the time frame of 10 hours for every 20 minutes. 10^5 T cells transduced with the indicated receptors were added to the tumor cells. Impedance

values (representing tumor adhesion to the assay plate, thus inversely correlated with T cell mediated killing) were quantified for 20 hours every 20 minutes.

2.8 Proliferation assays

Proliferation was measured by a flow cytometry-based assay that compared fold proliferation of T cells over a period of 48 hours normalized to the number of T cells per condition upon assay start. Recombinant human TGF-β (Cell Signaling Technology) or vehicle solution was added to concentrations of 20 ng/ml to test for proliferation arrest of T cells cultured with murine T cell medium supplemented with IL-15.

2.9 Flow cytometry and preparation thereof

Lymph nodes and spleens were passed through 30 μm cell strainers. Spleens were then processed for erythrocyte lysis. Tumors were digested with 1.5 mg/ml collagenase IV and 50 U/ml DNAse I for 30 minutes at 37ºC under agitation. Dead cells were stained using the fixable viability violet dye Zombie Red or Violet (Biolegend) for 15 minutes at room temperature, followed by blocking of Fc receptors with TruStain fcX (Biolegend) for 20 minutes at 4ºC. Following, cell surface proteins were stained for 20 minutes at 4˚C with anti-CD4 (GK1.5), anti-CD45.1 (A20), anti-CD45.2 (104) anti-CD8 (53-6.7), anti-CD90.1 (OX-7), anti-CD90.2 (30-H12), CD62L (MEL-14) and CD44 (IM7) (all from Biolegend) or anti-c-myc (SH1-26E7.1.6, Miltenyi Biotec) for detection of CAR constructs. Nuclear proteins were stained for 60 minutes at room temperature after permeabilization and fixation (Mouse regulatory T cell staining Kit; eBioscience) using anti-FOXP3 (MF-14, Biolegend) and anti-TGF-β (TW7-16B4, Biolegend). Cells were analyzed on Canto or LSRFortessa flow cytometers (BD Biosciences), and data were analyzed with FlowJo software version 9.9.5 or version 10.3.

2.10 Immunofluorescence

Tissue samples obtained from tumors were embedded and frozen in OCT. Sections of 5 μm were stained with a primary goat anti-CCL1 antibody (R&D Systems) and an Alexa Fluor 488 (Life Technologies) secondary antibody and DAPI (Vectashield) according to previously described standard procedures (Das et al. 2013).

2.11 Multi-photon intra-vital microscopy

Multi-photon intra-vital microscopy experiments were performed during a seven-month rotation in the laboratory of Prof. Dr. Thorsten Mempel at the Massachusetts General Hospital in Boston. CCR8-GFP-transduced T cells were compared to mCherry mock-transduced T cells. Tumor cells expressed the H2B-Cerulean fluorescent protein. Tumors were implanted in the back of mice after removal of hair. Engrafted tumors were framed within a dorsal skin-fold chamber, implanted by means of an aseptic surgical procedure under anaesthesia, as previously described (Bauer et al. 2014). Vessels were identified through an i. v. administered Qtracker 655 non-targeted quantum dots (Invitrogen). Mice were monitored daily for tumor growth as well as for pain and local or systemic inflammatory signs. Imaging took take place every other day, under anaesthesia. Multiphoton excitation was done with a MaiTai Ti:sapphire laser (Spectra-Physics) tuned to 950 nm to excite all fluorescent probes used. Sections with 4- to 5- µm z spacing were acquired on an Ultima multiphoton microscope (Prairie Technologies) every 60 seconds, for 60 to 90 minutes. Time sequences with a field of view (FOV) of approximately 500 μm, and depths of 28 FOV spaced in the z-axis were acquired. Emitted fluorescence was detected through 460/50, 525/50, 595/50, and 660/40 band-pass filters and nondescanned detectors to generate 4-color images. Quantification was performed with the Imaris software (Bitplane).

2.12 TCGA data analysis

We utilized the bioinformatics tool UCSC Xena. TCGA (The Cancer Genome Atlas) RNA sequencing datasets were analyzed in comparison to GTEx (Genotype-Tissue Expression) healthy tissue reference datasets concerning the expression of multiple genes. Correlations were assessed through r^2, goodness of fit estimated by the Pearson's squared. mRNA normalization was estimated by the TCGA using the RSEM (RNA-seq by expectation maximization) method.

2.13 Statistical analysis

Two-tailed student's t-test was used for comparisons between two groups, while two-way ANOVA with Bonferroni post-test (multiple time-points) or one-way ANOVA with Tukey post-test (single time-points) were used for comparisons across multiple groups. A log-rank (Mantel-Cox) test was used to compare survival curves. All statistical tests were performed with GraphPad Prism 8 software, and significance was set to $p < 0.05$ and represented as * < 0.05, ** < 0.01 and *** < 0.001. Investigators were blinded to allocation during experiments and outcome assessment.

3 Results

3.1 CCL1 is produced in murine pancreatic solid tumors by activated T cells

The accumulation of CCR8+ cells at the tumor site described in the preliminary data might be the consequence of directed migration by these cells to the tumor site mediated by the ligands, CCL1 and CCL8 (in the murine system). Both have been described to trigger calcium influx upon binding to CCR8 (Islam et al. 2011). Out of the two, CCL1 is particularly interesting due to its capacity to potentiate the immunosuppressive function of CCR8+ T_{reg} cells. We could detect the expression of these two ligands following a RT-PCR expression-level profiling of all known chemokine ligands in Panc02-tumors (Figure 4).

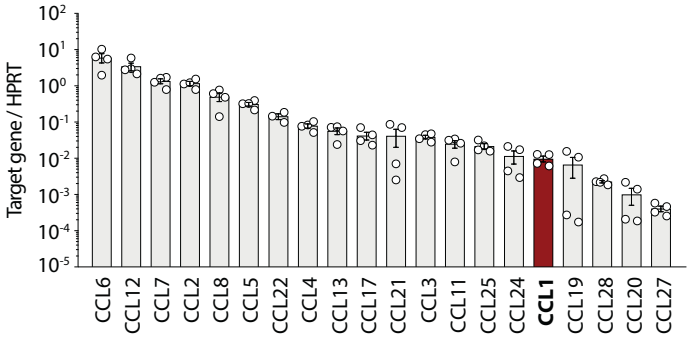

Figure 4 CCL1 is present on mRNA level in murine Panc02-OVA tumors

RT-PCR gene analysis of explanted Panc02-OVA tumors. mRNAs for all known 40 chemokine ligands in the murine system were analysed. C-C chemokine ligands are depicted. 1x10^{6} tumor cells were implanted on four C57BL/6 mice, sub-cutaneously, in 100 μl PBS. 10 days after tumor implantation mice were sacrificed and tumors explanted and snap-frozen with liquid nitrogen. Samples were then processed for RNA isolation and then first-strand cDNA generation. The results are depicted as relative expression levels to HPRT (n = 4 biological replicates).

The presence of this ligand in Panc02 tumors suggests that there is a causality for the accumulation of CCR8+ lymphocytes at the tumor site compared to the spleen. This should be further validated through a comparison of protein expression levels of CCL1 in the tumor and other tissues. Therefore, we analysed the expression of CCL1 in protein lysates of Panc02 tumors and several other healthy tissues in tumor bearing mice and healthy controls. We were able to observe that at protein level, tumors produce significant more CCL1 than healthy tissues (Figure 5). Furthermore, although not significantly, there appeared to be an increased level of CCL1 at lymph nodes ipsilateral to the tumor.

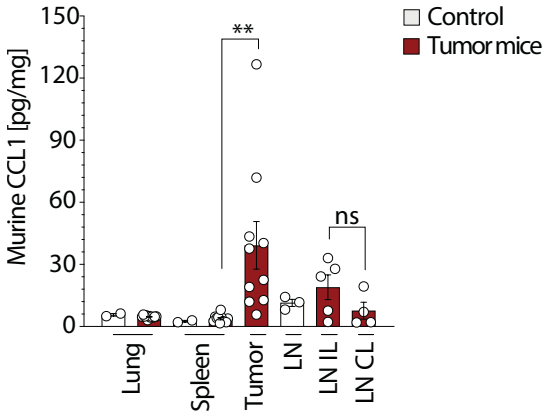

Figure 5 CCL1 is present on protein level in murine Panc02-OVA tumors in higher levels than control healthy tissues.

ELISA for murine CCL1 in organ lysates. Protein lysates have been quantified via Bradford assay, and the samples have been diluted so that whenever possible 1 mg of protein would be tested per ELISA well, and further diluted if the amount of protein would not be enough to meet this criteria until a maximal dilution of 1:100. The ELISA results have been calculated using a 4PL non-linear regression and values that have not fit the standard curve have either been further diluted (if the values were too high) or considered as non-detectable if too low. The control group has an n = 3 mice and the tumor group has n = 10 mice. ns stands for non-significant $p > 0.05$. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001. Experiments show mean values ± SEM, p-values are based on two-sided unpaired t-test. Data shown is pooled from 3 independent experiments. IL, ipsilateral; CL, contralateral; LN, lymph node.

Thus the presence of CCL1 in the tumor tissue was confirmed, however, the source of this cytokine was still unknown. According to literature, activated T cells are the major source for CCL1 (Miller et al. 1992) but dendritic cells, langerhans cells or even endothelial cells might be able to produce this chemokine ligand if stimulated under the right conditions (Gombert et al. 2005). We could demonstrate that activated $CD4^+$ or $CD8^+$ T cells, *in vitro*, can produce CCL1 upon activation with antibodies (Figure 6A) or through TCR activation (Figure 6B). In contrast, Panc02 tumor cells do not seem capable of producing this chemokine in our experimental setup (Figure 6B).

The presence of CCL1 in Panc02 tumors at mRNA and protein level suggests that a recruitment mechanism based on CCR8 may exist in these tumors. According to previous studies in mouse and human, T_{reg} cells have been shown to have specific sub-populations that are $CCR8^+$ (Barsheshet et al. 2017, Plitas et al. 2016). Thus, it is plausible to assume that such a recruitment axis can be utilised by T_{reg} cells to infiltrate solid tumors and enact immunosuppressive functions. This axis may therefore be exploited to enable the access of $CCR8^+$ T_{eff} cells to the tumor site.

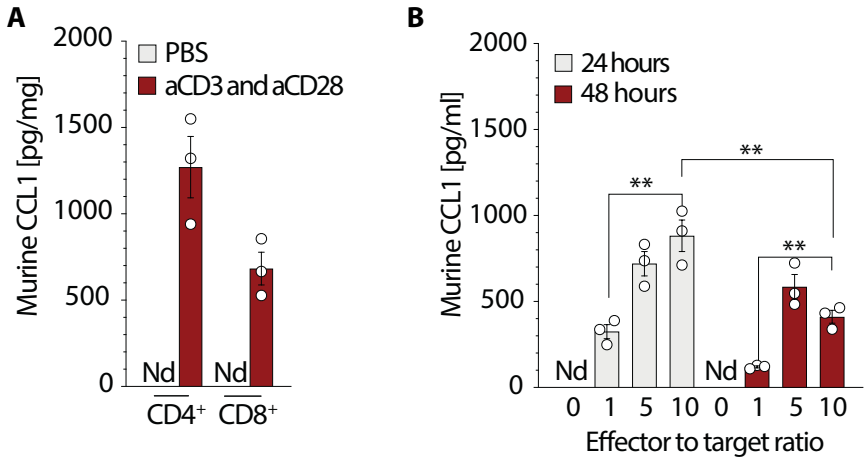

Figure 6 CCL1 is produced by activated T cells.

A ELISA for murine CCL1 on CD4+ or CD8+ MACS-enriched T cells after a 24-hour stimulation with anti-CD3 and anti-CD28 antibodies or vehicle solutions only (representative of n = 3 independent experiments). **B** ELISA for murine CCL1 of supernatants generated upon 24- or 48-hour culture of different effector to target ratios, namely 0, 1, 5 or 10 OT-1 T cells to 1 tumor target cell (a constant number of 20,000 Panc02-OVA tumor cells was used) (representative of n = 3 independent experiments). Nd stands for non-detectable. Experiments show mean values ± SEM of triplicates and are representative of three independent experiments. p-values are based on two-sided unpaired t-test. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001.

3.2 CCR8-transduced T cells migrate to CCL1 *in vitro*

While T_{eff} cells do not express CCR8 it is possible to genetically engineer them to introduce T_{reg} cell-like trafficking properties. We have therefore cloned the CCR8 cDNA in a retroviral expression plasmid and linked it to a 2a-GFP cassette to enable the assessment of genetic engineering efficiency by flow cytometry and fluorescence microscopy, without the need for antibody staining due to their sub-optimal affinity to G-protein coupled receptors, such as chemokine receptors. Forced expression of CCR8-GFP could be readily detected by flow cytometry (Figure 7A) and CCR8-transduced T cells have increased migratory capacity to gradients of murine CCL1 *in vitro* (Figure 7B).

We then aimed at characterising the migratory capacity of these CCR8-transduced T cells compared to mCherry-transduced T cells (control) *in vivo.* For this purpose, we implanted Panc02 tumors s. c. in C57BL/6 mice and assessed them by multiphoton intravital microscopy (MP-IVM) and flow cytometry.

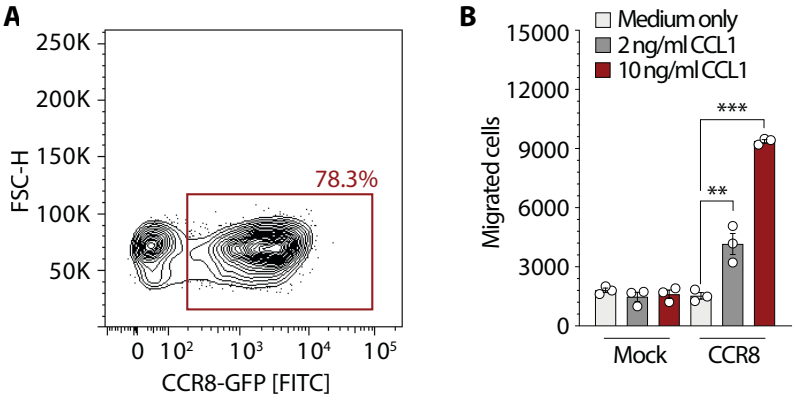

Figure 7 CCR8 can be functionally expressed in murine primary T cells.

A Through retroviral transduction the genetic construct CCR8-GFP can be ectopically expressed in primary murine T cells. **B** Boyden chamber migration assay. Migration is represented by the total number of migrated cells to the bottom well after a 6 hour incubation time, normalised with counting beads. Experiments show mean values ± SEM of triplicates and are representative of three independent experiments. p-values are based on two-sided unpaired t-test. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001.

3.3 CCR8-transduced T cells accumulate in Panc02 tumors *in vivo*

To enable visualisation of our tumor cells *in vivo* we have transduced Panc02 tumors with a H2B-Cerulean construct. H2B is a protein that localises to the nucleus, while Cerulean is a fluorescent protein that possesses a similar excitation and emission spectrum as Pacific Blue. Furthermore, we have used Qtracker 655 non-targeted quantum dots (Invitrogen) (delivered i. v.) to visualise blood vessels. Mice were then imaged (Figure 8A). Sequences were analysed by comparing infiltration (in absolute cell numbers) between CCR8-transduced and control T cells, which revealed an increased accumulation of CCR8-transduced T cells compared to control T cells (Figure 8B). Further analysis of cell tracking during the timespan of the imaging revealed a trend towards an increased motility, however, without reaching statistical significance (Figure 8C). This set of experiments was performed with TCR repertoire unrestricted T cells to prevent an antigen-recognition-mediated bias in the accumulation of T cells at the tumor site.

MP-IVM results can accurately and with great detail depict events, however, only in the examined sections of the tumor. Therefore it was deemed relevant to further characterize our CCR8-transduced T cells through flow cytometry to gain a comprehensive picture of the whole tumor.

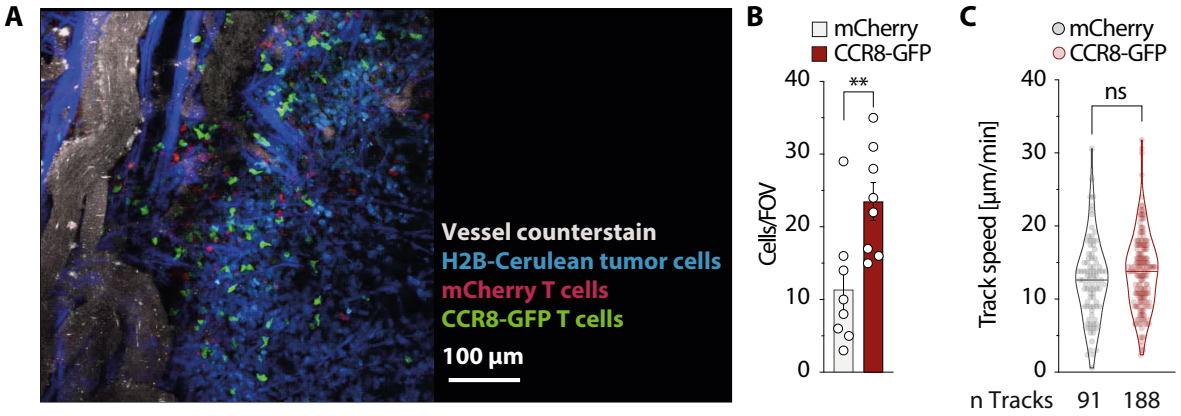

Figure 8 CCR8-transduced T cells have a higher *in vivo* infiltration capacity than control T cells without differences in their speed.

A MP-IVM of Panc02-H2B-Cerulean tumors was performed through a dorsal skin-fold chamber. 5 x 10^6 CCR8-GFP-transduced T cells were co-injected with 5 x 10^6 mCherry-transduced T cells (representative of n = 7 mice). **B** Quantification of cells per field of view (n = 7 mice). **C** Tracking of cell speed (n = 7 mice). p-values are based on two-sided unpaired t-test. ns stands for non-significant $p > 0.05$. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001.

3.4 Improved *in vivo* CCR8-transduced T cell migration is CCL1 dependent

Flow cytometry five days post T cell transfer to mice that were treated identically to the ones used for MP-IVM, was also employed in the assessment of two other models, Panc02-WT and Panc02-CCL1, to characterise the influence of increased gradients of CCL1 on the accumulation of CCR8-transduced T cells *in vivo* (Figure 9A). Compared to lymphoid tissue, there was an increased accumulation of CCR8-transduced T cells in

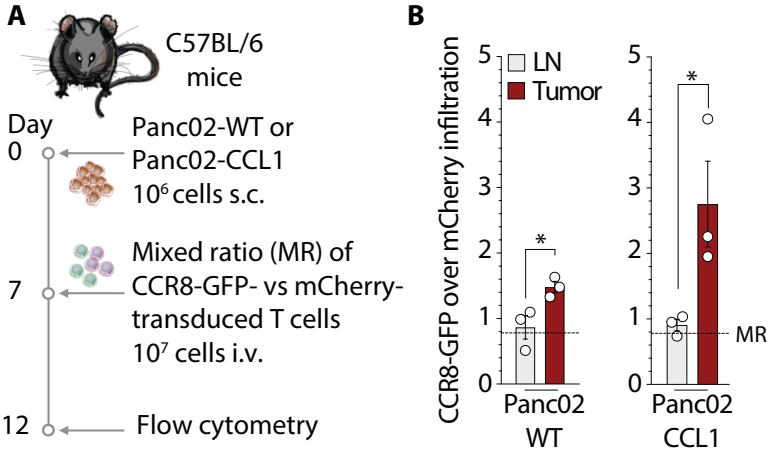

Figure 9 CCR8-transduced T cells infiltrate Panc02 tumors in a CCL1-dependent manner.

A Experimental layout for Panc02 tracking experiments. 5 x 10^6 CCR8-GFP-transduced T cells were co-injected with 5 x 10^6 mCherry-transduced T cells. **B** Flow cytometry tracking showing live CD45.1$^+$ Panc02 or Panc02-CCL1 infiltrating T cells fluorescently labeled with mCherry (control) or GFP (CCR8-transduced) (n = 3 mice). LN stands for lymph node. p-values are based on two-sided unpaired t-test. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001.

the Panc02 tumors and this effect was amplified in CCL1-transduced tumors, indicating that the increased accumulation of CCR8-transduced T cells is CCL1-dependent (Figure 9B).

3.5 Antigen-specific CCR8-transduced T cells have improved anti-tumoral effects

The improved accumulation of CCR8-transduced T cells can be used to increase the anti-tumor effects of antigen-specific T cells in solid tumors. To address this hypothesis, we introduced the CCR8 gene in CD8+ OVA-specific T cells (OT-I) and tested these cells in Panc02-OVA tumors (Figure 10A). Upon ACT of CCR8-transduced OT-I T cells, Panc02-OVA tumor growth was controlled to a greater extent compared to the tumors of animals treated with OT-I T cells alone (Figure 10B). Tumor rejection also took place in a small fraction of animals, leading to improved survival and cures (Figure 10C).

In this tumor challenge setting CCL1-expression levels also seemed to have an impact on the extent of the anti-tumoral effects observed. The previous experimental findings have been recapitulated in Panc02-OVA-CCL1 tumor challenges (Figure 11A). The increased expression of CCL1 in this tumor model led to tumors that developed in a more aggressive manner than wild-type Panc02-OVA tumors (Figure 11B and 11C).

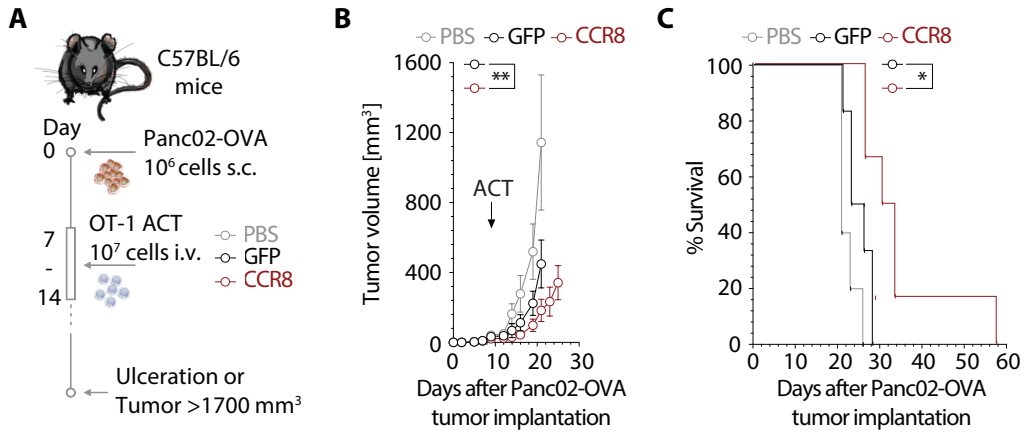

Figure 10 Tumor-specific CCR8-transduced T cells show improved Panc02-OVA-tumor growth-control and survival.

A Experimental layout for Panc02-OVA tumor challenge experiments. **B** Growth curves of Panc02-OVA tumors in C57BL/6 mice that were treated with a single i. v. injection of PBS, or 10^7 GFP-transduced or CCR8-transduced OT-1 T cells (n = 5 mice per group). **C** Tumor survival curves of a tumor challenge experiment with Panc02-OVA tumors. Mice were treated with a single i. v. injection of PBS, or 10^7 GFP-transduced or CCR8-transduced OT-1 T cells (n = 5 mice per group). Experiments show mean values ± SEM and are representative of three independent experiments. Analysis of differences between groups for **B** was performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. Comparison of survival rates for **C** was performed with the Log-rank (Mantel-Cox) test. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001.

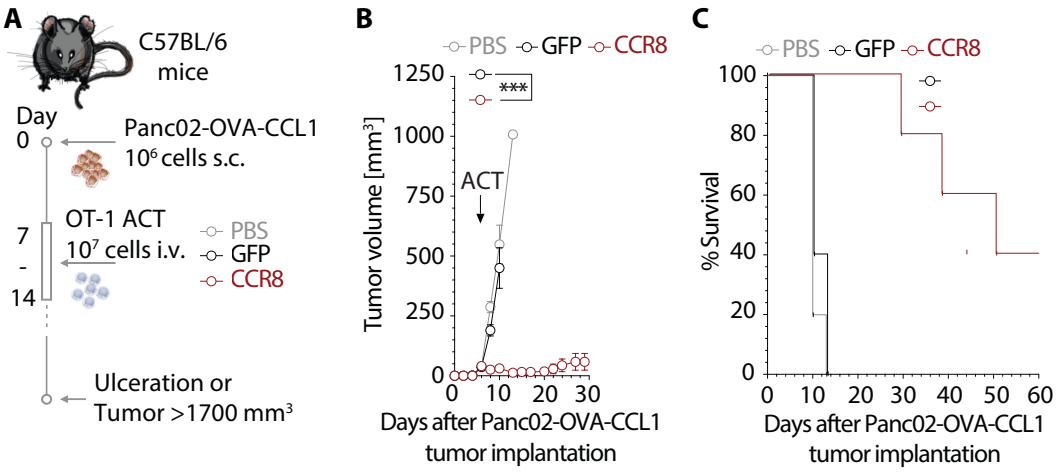

Figure 11 Ectopic expression of CCL1 in Panc-02-OVA tumors allows for improved tumor growth control and improved survival by tumor-specific CCR8-transduced T cells.

A Experimental layout for Panc02-OVA-CCL1 tumor challenge experiments. **B** Growth curves of Panc02-OVA-CCL1 tumors in C57BL/6 mice that were treated with a single i. v. injection of PBS, or 10^7 GFP-transduced or CCR8-transduced OT-1 T cells (n = 5 mice per group). **C** Tumor survival curves of a tumor challenge experiment with Panc02-OVA-CCL1 tumors. Mice were treated with a single i. v. injection of PBS, or 10^7 GFP-transduced or CCR8-transduced OT-1 T cells (n = 5 mice per group). Experiments show mean values ± SEM and are representative of two independent experiments. Analysis of differences between groups for **B** was performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. Comparison of survival rates for **C** was performed with the Log-rank (Mantel-Cox) test. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001.

Despite the fast growth rate of these tumors, treatment with CCR8-transduced OT-I T cells resulted not only in an improved stunting of tumor growth (Figure 11B) but also led to improved overall survival and a higher number of tumor-free mice (Figure 11C).

The effectiveness of CCR8 transduction in improving the anti-tumor effects of ACT in this murine syngeneic model is promising. Nevertheless, despite the improved accumulation there still was no complete tumor clearance in several mice. Thus, it can be postulated that relieving immunosuppression could also play a role in further improving the therapeutic effectiveness of the approach. These results also set the demand to translate these findings, both to models that more closely recapitulate human immunobiology, and to other CAR T cell therapy-resistant solid tumors.

3.6 T_{reg} cells and TGF-β contribute towards immunosuppression in Panc02 tumors

Immunosuppression is one of the limitations hindering the translation of ACT in solid tumors (Cadilha et al. 2017). Pancreatic tumors are known to have several immunosuppressive mechanisms such as a milieu rich in T_{reg} cells or cytokines such as TGF-β (Ellermeier et al. 2013). Upon *ex vivo* analysis of subcutaneous Panc02 tumors

through flow cytometry we were able to observe that compared to healthy tissue, Panc02 tumors had a much higher ratio of T_{reg} cells over $CD4^+$ T cells (Figure 12A). Furthermore, the percentage of eT_{reg} cells, a population which under inflammatory conditions is known to be immunosuppressive was also increased in tumor tissues (Figure 12B) and was nearly entirely positive for TGF-β (Figure 12C) which was again in contrast to healthy tissues where only half of eT_{reg} cells were positive for TGF-β. This cytokine is not only produced by eT_{reg} cells in this tumor model as Panc02 tumor cells also produced TGF-β as seen in an *in vitro* culture experiment which revealed increased levels of TGF-β accumulation in medium supernatant over the course of 5 days (Figure 12D).

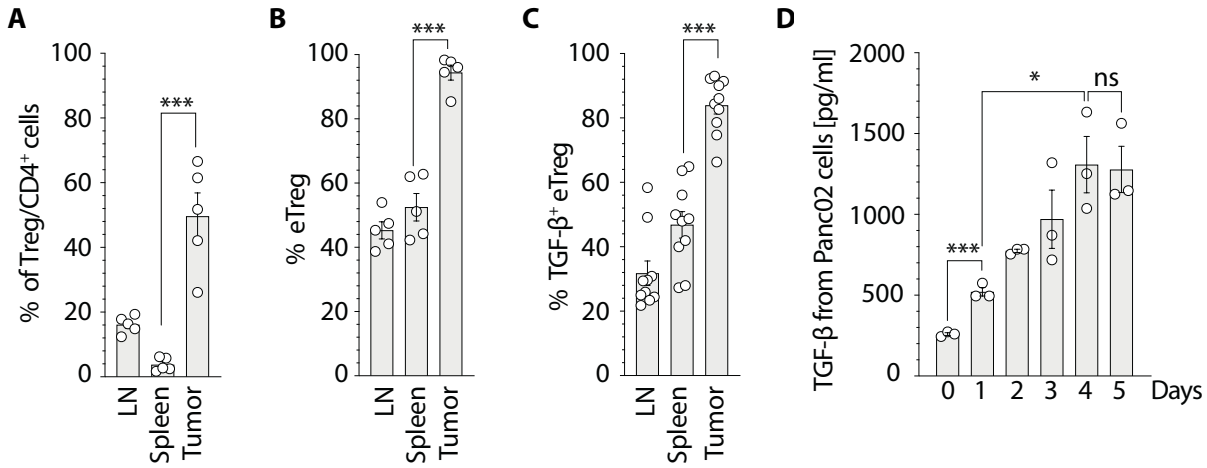

Figure 12 T_{reg} cells accumulate in Panc02 tumors and increment to the amount of TGF-β already produced by tumor cells themselves.

A *Ex vivo* flow cytometry quantification of T_{reg} to $CD4^+$ cell ratio in Panc02 tumors compared to healthy tissues (n = 5 mice). **B** *Ex vivo* percentage of eT_{reg} cells (defined by $CD44^{hi}$ $CD62L^{hi}$) in Panc02 tumors compared to healthy tissues, analysed by flow cytometry (n = 5 mice). **C** *Ex vivo p*ercentage of TGF-β^+ eT_{reg} cells in Panc02 tumors compared to healthy tissues, analysed by flow cytometry (n = 10 mice). **D** *In vitro* protein levels of TGF-β in the supernatant of Panc02 tumor cell cultures after several days of culture. Experiments show mean values ± SEM and are representative of at least two independent experiments. p-values are based on two-sided unpaired t-test. ns stands for non-significant $p > 0.05$. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001.

3.7 DNR-transduced T cells show improved proliferative capacity

The effects of TGF-β in T_{eff} cells have been extensively characterised and reviewed (Massague 2008). Several approaches on how to bypass the deleterious effects of this cytokine have been published, but perhaps the strategy that has produced the most promising pre-clinical and clinical data up until now has been a decoy receptor for TGF-β that does not trigger the TGF-β receptor singling pathway upon ligand binding.

The DNR has shown promising results in models for Hodgkin lymphoma and prostate cancer (Bollard et al. 2002, Kloss et al. 2018).

We could show that DNR can be expressed in primary murine T cells (Figure 13A). Its expression renders DNR-transduced T cells capable of proliferating despite the presence of TGF-β *in vitro,* whereas control T cells will suffer from hampered proliferation (Figure 13 B). *In vivo*, with an experimental layout for tracking of DNR-transduced T cells compared to control T cells (Figure 13C), there is no preferential accumulation of these cells in tumor tissue or lymphatic tissue. Nevertheless, across both tissues it can be appreciated that DNR-transduced T cells showed an increased proliferative capacity with an averages of a 2.5 fold higher expansion rate than control T cells (Figure 13D).

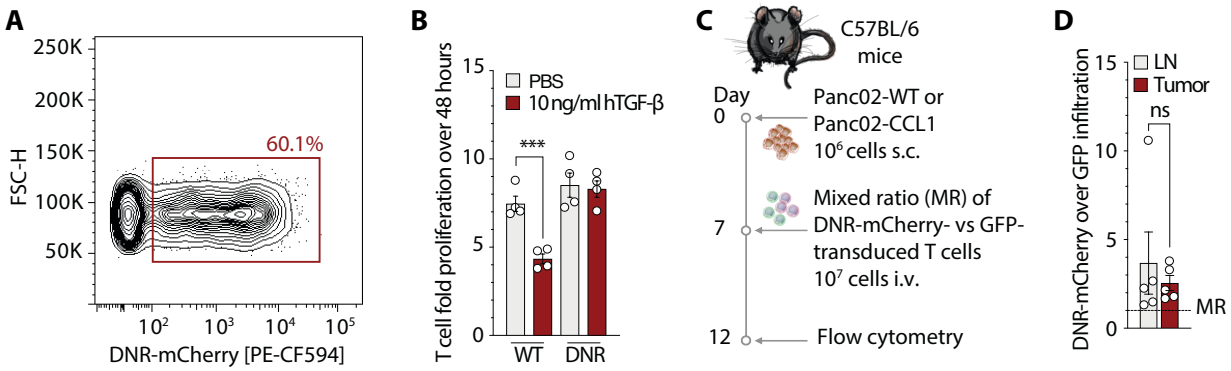

Figure 13 DNR-transduced T cells have exhibit superior proliferative capacity despite presence of TGF-β, *in vitro* and *in vivo*.

A The genetic construct DNR-mCherry can be expressed in primary murine T cells through retroviral transduction. **B** *In vitro* proliferation experiment of DNR-transduced T cells compared to WT T cells in the presence of 10 ng/ml TGF-β or vehicle solution. **C** Experimental layout for Panc02 tracking experiments. 5 x 10^6 DNR-mCherry-transduced T cells were co-injected with 5 x 10^6 GFP-transduced T cells. **D** Flow cytometry tracking showing live CD45.1+ Panc02 infiltrating T cells fluorescently labeled with GFP (control) or mCherry (DNR-transduced) (n = 5 mice). LN stands for lymph node. Experiments show mean values ± SEM and are representative of at least three independent experiments. p-values are based on two-sided unpaired t-test. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001.

3.8 Antigen-specific DNR-transduced T cells show improved anti-tumoral effects

The improved proliferative capacity of DNR-transduced T cells — that led to combination with TCR specific T cells and even CAR T cells in the above mentioned tumor entities — prompted us to assess wether this therapy could also improve anti-tumor efficacy in pancreatic solid tumors. Tumor challenge experiments were performed with antigen-specific T cells (Figure 14A). DNR-transduced T cells showed

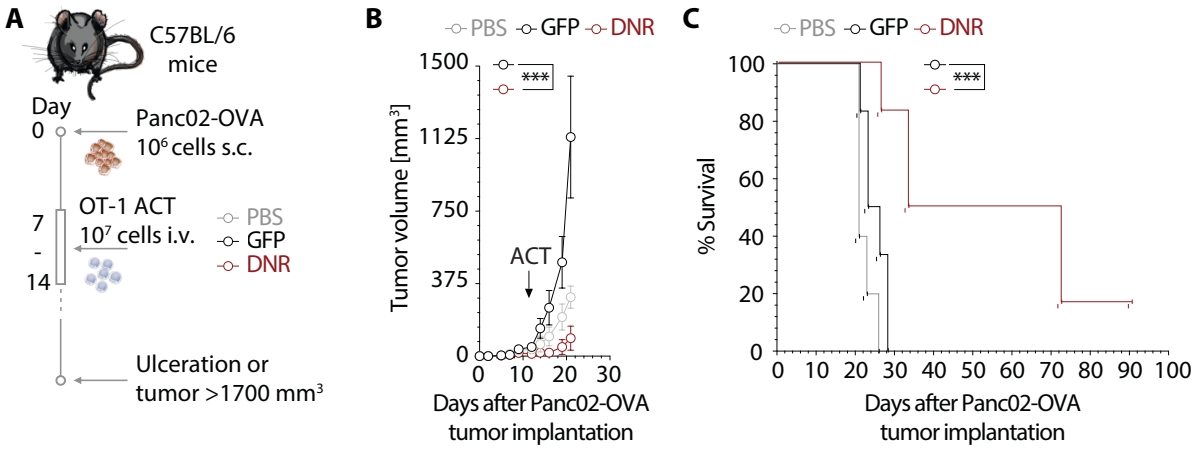

Figure 14 Tumor-specific DNR-transduced T cells show improved Panc02-OVA tumor growth control and survival.

A Experimental layout for Panc02-OVA tumor challenge experiments. **B** Growth curves of Panc02-OVA tumors in C57BL/6 mice that were treated with a single i. v. injection of PBS, or 10^7 GFP-transduced or DNR-transduced OT-1 T cells (n = 5 mice per group). **C** Tumor survival curves of a tumor challenge experiment with Panc02-OVA tumors. Mice were treated with a single i. v. injection of PBS, or 10^7 GFP-transduced or DNR-transduced OT-1 T cells (n = 5 mice per group). Experiments show mean values ± SEM and are representative of three independent experiments. Analysis of differences between groups for **B** was performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. Comparison of survival rates for **C** was performed with the Log-rank (Mantel-Cox) test. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001.

improved tumor growth control (Figure 14B) and improved survival (Figure 14C) when compared to control T cells.

3.9 CCR8 and DNR synergize for improved anti-tumor CAR T cell efficacy

The potential for synergy of the CCR8 and DNR stems from the fact that both receptors individually target separate pitfalls hampering the successful translation of ACT to solid tumors. Individually both receptors show stark mechanistic effects that lead to guided-migration and shielding from immunosuppression, respectively. We have decided to test the combination of both receptors in an antigen-specific system that could more closely compare to clinical trial settings. Thus, given the major advances in cellular engineering and CAR T cell development with relevant targets for solid tumors such as glioblastoma, prostate, and even pancreatic tumors, we took advantage of a CAR against the EpCAM antigen, previously described and tested against Panc02 tumors over-expressing the EpCAM antigen (Karches et al. 2019).

We have tested the synergy of CCR8-DNR-transduced T cells with tumor challenge experiments (Figure 15A) and were able to reiterate previous findings showing that both

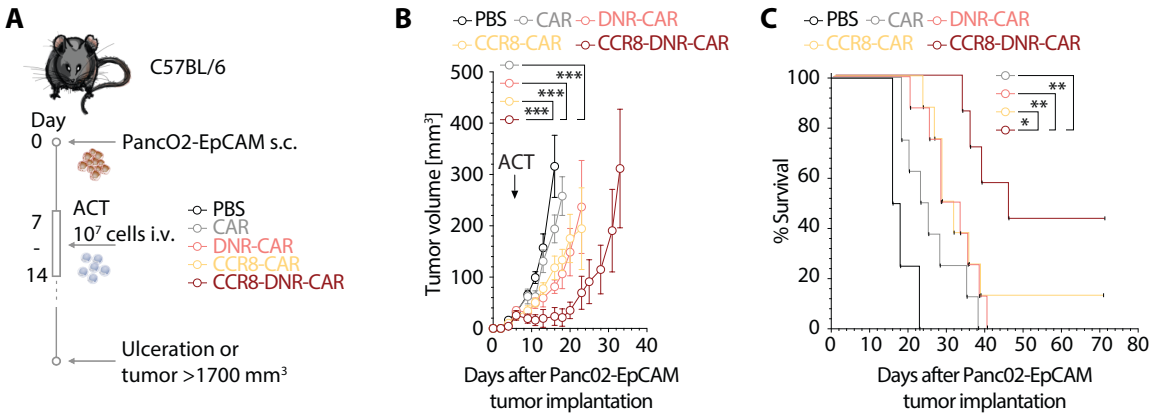

Figure 15 CCR8 and DNR synergize for improved anti-tumor CAR T cell efficacy.

A Experimental layout for Panc02-EpCAM tumor challenge experiments. **B** Growth curves of Panc02-EpCAM tumors in C57Bl/6 mice that were treated with a single i. v. injection of PBS, or 10^7 CAR-, DNR-CAR-, CCR8-CAR- or CCR8-DNR-CAR-transduced T cells (n = 8 mice per group, except for the CCR8-DNR-CAR group that only had 7 mice). **C** Tumor survival curves of a tumor challenge experiment with Panc02-EpCAM tumors. Mice were treated with a single i. v. injection of PBS, or 10^7 CAR-, DNR-CAR-, CCR8-CAR- or CCR8-DNR-CAR-transduced T cells (n = 8 mice per group, except for the CCR8-DNR-CAR group that only had 7 mice). Experiments show mean values ± SEM and are representative of three independent experiments. Analysis of differences between groups for **B** was performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. Comparison of survival rates for **C** was performed with the Log-rank (Mantel-Cox) test. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001.

receptors individually had superior anti-tumor effects than conventional CAR T cells. When the expression of both CCR8 and DNR in CAR T cells was combined however, it resulted in a synergistic anti-tumor effect leading to improved tumor growth control (Figure 15B) and increased overall survival with 43% of tumor-challenged mice completely clearing their tumors. Being able to tackle infiltration and immunosuppression simultaneously to improve CAR T cell therapy efficacy is novel and enabled the treatment of these Panc02 tumors. We thus deemed it relevant to assess the relevance of these two receptors in the human system to potentially translate this strategy to improve CAR T cell efficacy for pancreatic tumor patients in the future.

3.10 CCR8 and TGFB1 strongly correlate with FOXP3 in several human solid tumor models

A prerequisite for this strategy to have a meaningful impact in human solid tumors is the presence of CCR8$^+$ T_{reg} cells in human solid tumor tissue. Such has been recently demonstrated in breast tumors and is associated with poor prognosis (Plitas et al. 2016).

We have conducted a similar analysis on the TCGA database and probed several human solid tumors, including pancreatic ductal adenocarcinoma, for their expression levels of CCR8, TGFB1 and FOXP3. We could find strong correlations of CCR8 and FOXP3 in several human solid tumors ($r^2 > 0.5$), a signature suggesting the presence of CCR8$^+$ T_{reg} cells (Figure 16A). Furthermore, we could also find a strong correlation in pancreatic ductal adenocarcinoma for TGFB1 and FOXP3 ($r^2 = 0.5275$) indicating that the presence of TGF-β is also linked to the presence of T_{reg} cells in the tumor microenvironment of these patients (Figure 16B). While this correlation was not as strong in the other solid tumors analyses, despite high levels of mRNA expression of CCR8, TGFB1 and FOXP3 (Figure 17) still suggest that there can be advantages of utilising a strategy to relieve TGF-β-immunosuppression in these solid tumor entities.

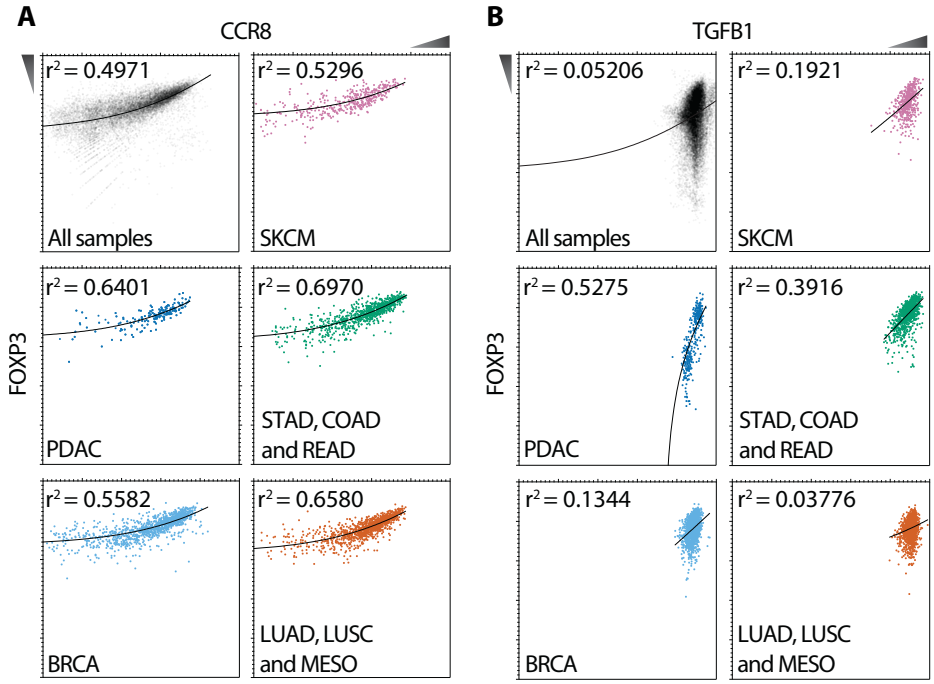

Figure 16 CCR8 and TGFB strongly correlate with FOXP3 in several human solid tumors.

A Correlation of CCR8 and FOXP3 and **B** correlation of TGFB1 and FOXP3 in different tissues. r^2 was used to evaluate the fit estimated by the Pearson's squared method. PDAC pancreatic adenocarcinoma, BRCA breast invasive carcinoma, STAD stomach adenocarcinoma, COAD colon adenocarcinoma, READ rectum adenocarcinoma, NSE not-sun exposed, SE sun exposed, SKCM skin cutaneous melanoma, LUAD lung adenocarcinoma, LUSC lung squamous cell carcinoma, MESO mesothelioma. Samples analysed for pancreas tissue n = 167, PDAC n = 183, breast mammary tissue n = 179, BRCA n = 1212, stomach n = 175, STAD n = 450, colon sigmoid n = 141, colon transverse n = 167, COAD n = 331, READ n = 103, skin NSE n = 233, skin SE n = 324, SKCM n = 470, lung n = 288, LUAD n = 574, LUSC n = 548 and MESO n = 87. All scales are depicted in a $\log_2$ scale (minimum 0.5 and maximum 16, major tick interval of 1 power of 2) and mRNA normalization was estimated by the TCGA using the RSEM (RNA-seq by expectation maximization) method.

At this point we have also investigated if CCR8 would be the optimal candidate to mimic T_{reg} cell migration with T_{eff} cells in the context of solid tumors. Previously, CCR4 has

been described as a promising chemokine receptor to improve T cell accumulation in ovarian cancer (Curiel et al. 2004) and Hodgkin lymphoma (Di Stasi et al. 2009). CCR4 is also expressed in T_{reg} cells (Lee et al. 2005). The increased immunosuppressive phenotype of T_{reg} cells has so far only been linked to CCR8^{+} T_{reg} cells, which implies a functional advantage of using CCR8 instead of CCR4 as binding of CCR8-transduced T_{eff} cells to CCL1 will reduce this ligand's availability for CCR8^{+} T_{reg} cells. Taking advantage of the dataset from TCGA we also probed several healthy tissues for the expression of the main ligands for CCR4 and CCR8. From this one might be able to predict off-tumor accumulation of CCR4-transduced and CCR8-transduced T cells in healthy tissue, thus predicting potential toxicity from these therapeutic modalities.

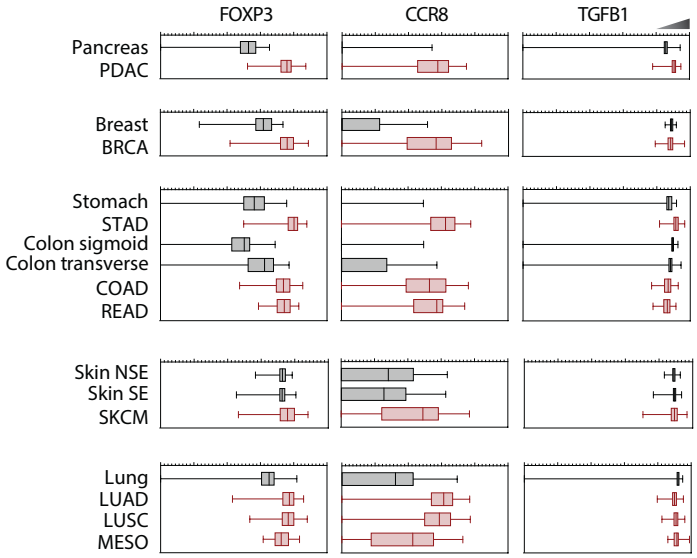

Figure 17 FOXP3, CCR8 and TGFB1 are highly expressed in several human solid tumors compared to healthy tissue.

Expression levels of FOXP3, CCR8 and TGFB1 in different tissues. PDAC pancreatic adenocarcinoma, BRCA breast invasive carcinoma, STAC stomach adenocarcinoma, COAD colon adenocarcinoma, READ rectum adenocarcinoma, NSE not-sun exposed, SE sun exposed, SKCM skin cutaneous melanoma, LUAD lung adenocarcinoma, LUSC lung squamous cell carcinoma, MESO mesothelioma. Samples analysed for pancreas tissue n = 167, PDAC n = 183, breast mammary tissue n = 179, BRCA n = 1212, stomach n = 175, STAD n = 450, colon sigmoid n = 141, colon transverse n = 167, COAD n = 331, READ n = 103, skin NSE n = 233, skin SE n = 324, SKCM n = 470, lung n = 288, LUAD n = 574, LUSC n = 548 and MESO n = 87. All scales are depicted in a $\log_2$ scale (minimum 0.5 and maximum 16, major tick interval of 1 power of 2) and mRNA normalization was estimated by the TCGA using the RSEM (RNA-seq by expectation maximization) method.

3.11 CCR8 ACT has safer ligand-driven infiltration profile than CCR4 ACT in humans

Upon comparison of CCL22 and CCL1 expression levels in several healthy human tissues we were able to observe that CCL22 is expressed at higher levels than CCL1 throughout the whole human body with the exception of the testis (Figure 18). Furthermore, the majority of samples probed did not express CCL1 or only very little

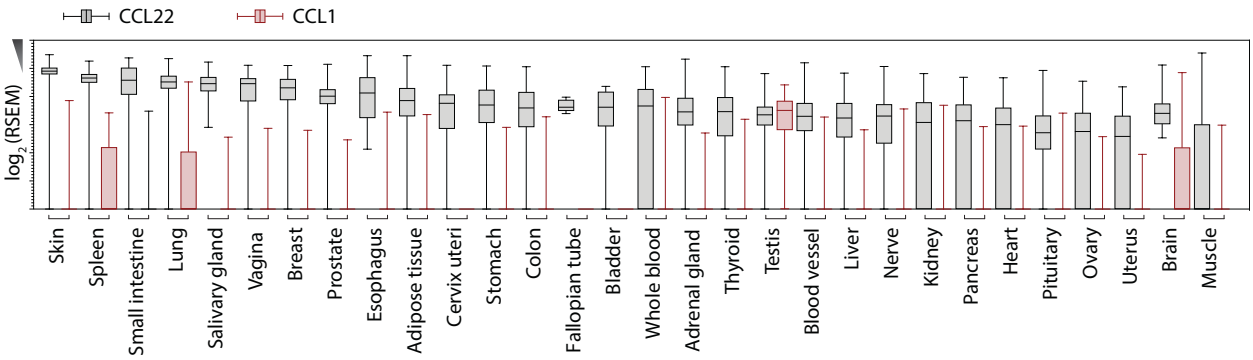

Figure 18 CCL1 is barely expressed in healthy human tissues compared to CCL22.

Boxplots comparing CCL22 and CCL1 gene expression levels in several healthy tissues. Samples analyzed for skin n = 557, spleen n = 101, small intestine n = 93, lung n = 289, salivary gland n = 56, vagina n = 86, breast n = 180, prostate n = 101, esophagus n = 557, adipose tissue n = 516, cervix uteri n = 11, stomach n = 175, colon n = 309, fallopian tube n = 6, bladder n = 10, whole blood n = 338, adrenal gland n = 129, thyroid n = 280, testis n = 166, blood vessel n = 557, liver n = 111, nerve n = 279, kidney n = 29, pancreas n = 168, heart n = 378, pituitary n = 108, ovary n = 89, uterus n = 79, brain n = 557 and muscle n = 397. Scales are depicted in a $\log_2$ scale (minimum 0.5 and maximum 16, major tick interval of 1 power of 2) and mRNA normalization was estimated by the TCGA using the RSEM (RNA-seq by expectation maximization) method.

(Figure 18). These results indicate a favourable safety profile for CCR8 ACT compared to CCR4 ACT. Despite the fact that a chemokine receptor by itself will not be able to trigger T cell activation, and thus potential harmful cytokine release, the increased accumulation of T cells at off-tumor sites might result in serious adverse effects due to the fact that most targets currently used in the clinic are tumor-associated antigens rather than tumor-specific antigens.

Lastly, comparing healthy pancreatic tumor tissues to pancreatic ductal adenocarcinoma, it is appreciable that the main ligands to CCR8 are produced at higher levels in tumor tissue than in healthy tissue, further supporting the hypothesis of superior efficacy of CCR8 ACT for pancreatic solid tumors (Figure 19).

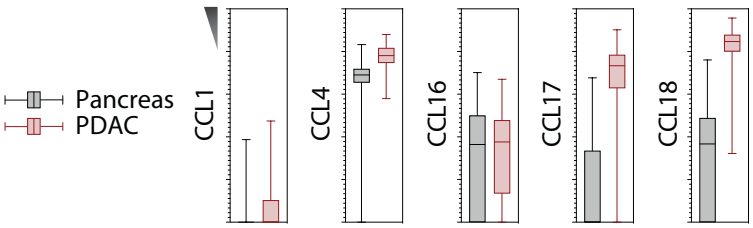

Figure 19 CCR8 ligands are unregulated in PDAC when compared to healthy pancreatic tissue.

Boxplots comparing CCR8 ligands' gene expression levels in PDAC to healthy pancreatic tissue. PDAC stands for pancreatic ductal adenocarcinoma. Samples analysed for pancreas tissue n = 167 and for PDAC n = 183. All scales are depicted in a $\log_2$ scale (minimum 0.5 and maximum 16, major tick interval of 1 power of 2) and mRNA normalization was estimated by the TCGA using the RSEM (RNA-seq by expectation maximization) method.

3.12 CCR8 and DNR are functional *in vitro* in human T cells

Following the rationale of testing each receptor individually in human cells we have generated retroviral constructs to ectopically express both CCR8 and DNR cDNAs in human cells and retrovirally transduced them into primary human T cells. We tested the approach with anti-mesothelin CAR T cells that we have previously characterised. We were able to demonstrate increased migration of CCR8-transduced T cells towards human CCL1 *in vitro* (Figure 20A) compared to the T cells that lacked the CCR8 cDNA. Similarly, also the introduction of DNR rendered transduced T cells capable of proliferating in an unhampered manner independently of the presence of TGF-β in culture media (Figure 20B). The combination of CCR8-DNR-CAR T cells also proved to be functional in increasing migration and TGF-β-immunosuppression resistance. Thus we deemed the platform ready for testing in an *in vivo* model of pancreatic cancer.

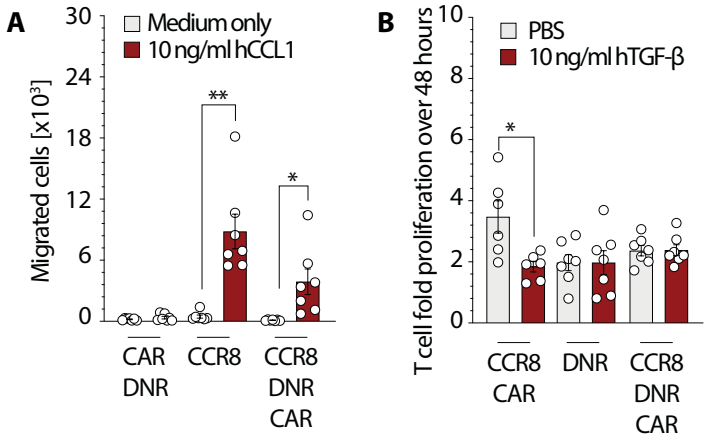

Figure 20 CCR8 improves CCL1-directed migration of human T cells and DNR shields human T cells from TGF-β-immunosuppression, *in vitro*.

A Boyden chamber assay to assess the *in vitro* migration of PBMC to recombinant human CCL1, measured through flow cytometry (n = 7 healthy donors). **B** PBMC fold expansion over 48 hours with or without TGF-β in the culture medium measured through flow cytometry (n = 7 healthy donors). Experiments show mean values ± SEM, of distinct healthy donors n = 7. p-values are based on two-sided unpaired t-test. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001.

3.13 CCR8 and DNR synergise for improved human anti-tumor CAR T cell efficacy

For the purpose of testing this strategy for anti-tumor efficacy in tumor challenges we have worked with the SUIT-2 human pancreatic ductal adenocarcinoma cell line. We have genetically modified this cell line to express human mesothelin and human CCL1.

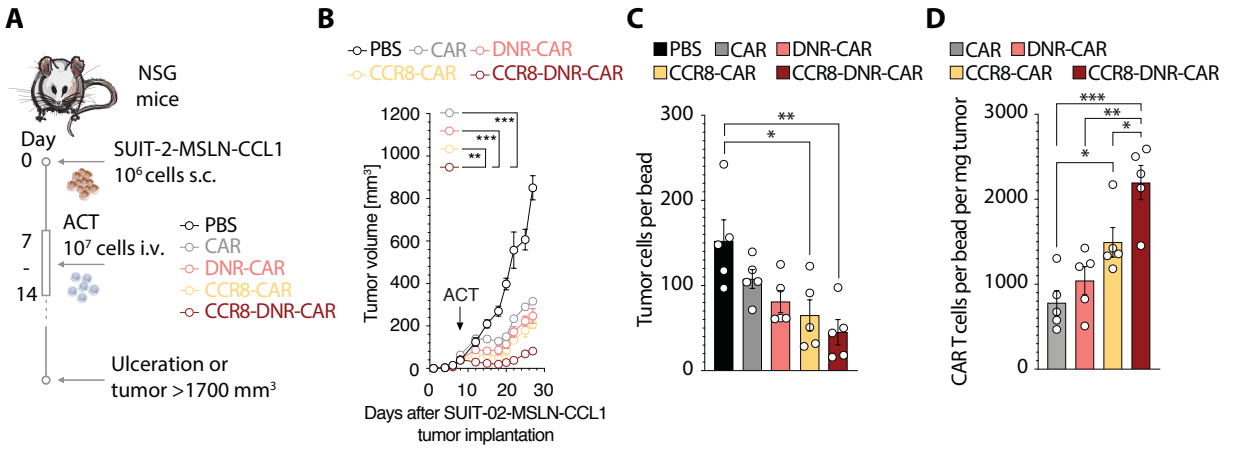

Figure 21 CCR8 improves CCL1-directed migration of human T cells and DNR shields human T cells from TGF-β-immunosuppression, *in vivo*.

A Experimental layout for an ACT experiment with SUIT-2-MSLN-CCL1 in NSG mice. **B** Growth curves of SUIT-2-MSLN-CCL1 human tumors in NSG mice that were treated with a single i. v. injection of PBS, or 10^7 CAR-transduced, DNR-CAR-transduced, CCR8-CAR-transduced or CCR8-DNR-CAR-transduced T cells (n = 5 mice per group). **C** Tumor cells per bead quantified through flow cytometry, *ex vivo*, after termination of experiment on day 27 after tumor implantation (n = 5 mice). **D** CAR T cells per bead, normalized to tumor weight in milligram, quantified through flow cytometry, *ex vivo*, after experiment termination on day 27 after tumor implantation (n = 5 mice). Analyses of differences between groups for **B** were performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. p-values for **C** and **D** are based on two-sided unpaired t-test. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001.

In vivo experiments were performed with NSG mice to allow for successful implantation of human tumors in mice. After tumor implantation, mice were treated with one dose of ACT once tumors reached a volume of 50 mm^3 (Figure 21A). We observed that CAR T cell treatment was significantly better than PBS treatment but could only slightly stunt tumor growth. The addition of CCR8 and DNR individually improved efficacy of CAR T cell therapy and further synergized when combined to deliver the best outcome with regards for tumor control (Figure 21B). Flow cytometry analysis of these mice upon experiment termination revealed significantly reduced tumor cell counts for the CCR8-DNR-CAR T cell therapy group (Figure 21C) and the CAR T cell accumulation at the tumor site was significantly increased compared to all groups in the CCR8-DNR-CAR T cell therapy group (Figure 21D).

At the tumor site, we found a preferential accumulation of $CD8^+$ T cells compared to the spleen where most of the cells were $CD4^+$ T cells. $CD4^+$ and $CD8^+$ T cells were skewed towards a central memory phenotype at the tumor site and in the spleen. This corroborates the notion that *in vivo* central memory T cells may better persist in tumor bearing animals (Figure 22).

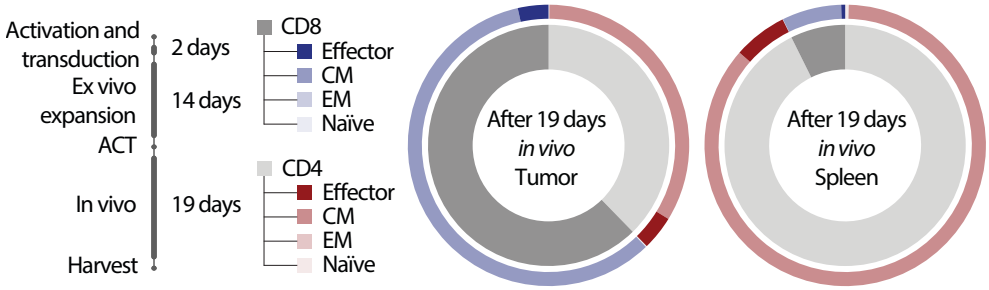

Figure 22 *Ex vivo* central memory CAR T cells are the main drivers of anti-tumor efficacy.

Flow cytometry of $CD8^+$ and $CD4^+$ expression in live, $CD45^+CD3^+$ SUIT-2-mesothelin-CCL1 infiltrating cells, phenotypically differentiated according to their expression of CD45RO and CCR7, allowing for differentiation of effector memory (EM, $CCR7^{low}$ $CDR45RO^{high}$), central memory (CM, $CCR7^{high}$ $CDR45RO^{high}$), effector ($CCR7^{low}$ $CDR45RO^{low}$) or naïve T cells ($CCR7^{high}$ $CDR45RO^{low}$). T cell product from one healthy human donor. Data for Tumor and Spleen infiltrates after 19 days *in vivo* are averages of n = 5.

4 Discussion

4.1 Summary of the results

The results presented herein reveal that the combination of CCR8 and DNR can enable effective adoptive T cell therapy in solid tumors. The ligand for CCR8, CCL1, was present in the Panc02 tumor model. CCR8 transduction in T cells enabled the migration of these T cells *in vitro*, and *in vivo*, towards CCL1.This increased the accumulation of CCR8-transduced T cells in the tumor, thus augmenting anti-tumor efficacy. Furthermore, Panc02 tumors were found to be infiltrated by T_{reg} cells, *in vivo*, and TGF-β was detected in these cells, as well as being produced by the Panc02 tumor cells themselves. DNR-transduced T cells were effective in improving the anti-tumor efficacy of ACT in the Panc02 tumor model, counteracting the immunosuppressive effects of TGF-β. The combination of these two receptors showed synergy in the Panc02 syngeneic tumor model and also in the SUIT-2 xenograft tumor model, showing the potential of this combination in a human-like setting. Analysis of TCGA database revealed scarce CCL1 expression across healthy tissues and increased expression in tumor tissues. Furthermore, this analysis could also correlate a higher expression of CCR8 and TGFB1 with an increased expression of FOXP3, indicating that several human solid tumor entities are highly infiltrated by T_{reg} cells and could benefit from this combination therapy.

4.2 General considerations for the functionality of the proposed enhancements for ACT

CCR8-CCL1 is a pivotal receptor-ligand pair for the immunosuppressive capacity of T_{reg} cells and is associated with poor disease prognosis in breast tumors (Barsheshet et al. 2017, Plitas et al. 2016). CCR8 expression in T_{reg} cells may therefore be a key determinant to enable the access of this cell population to $CCL1^{+}$ solid tumors. On the contrary, CCR8 is not expressed in T_{eff} cells. Thus, forced expression of CCR8 in T_{eff} cells can confer T_{reg} cell migration properties to T_{eff} cells, enabling them to migrate into CCL1-positive solid tumors.

Besides improving tumor infiltration by T_{eff} cells, there is also a fundamental need for their activity and proliferation within the tumor. TGF-β is a hallmark of the immunosuppressive tumor milieu in pancreatic and other solid tumors (Principe et al.

2016). It can control T cell homeostasis by inhibiting both T cell proliferation and activation (Gorelik et al. 2002). It can also drive tumor growth and promote metastasis formation (Massague 2008). Additionally, it can inhibit T_{eff} cell activity through an anti-cytotoxic program of transcriptional repression, down-regulating the expression of perforin, granzyme A, granzyme B, Fas ligand and IFN-ɣ (Thomas et al. 2005). The DNR can act as a TGF-β shield for transduced T cells, conferring them the capacity to proliferate independently of the deleterious effects of this cytokine.

Tackling these two individual mechanisms enables an improved migratory capacity of ACT towards solid tumors and, when on site, increased proliferation and cytotoxicity. A transcriptomic and proteomic characterisation of solid tumors for CCL1 and TGF-β expression is required to further validate this therapeutic combination.

4.3 The CCL1-CCR8 axis for improved migration of T cells into solid tumors

The high amount of $CCR8^+$ T_{reg} cells in breast tumors (Plitas et al. 2016) suggests that this chemokine receptor is a mechanism by which T_{reg} cells can access the tumor site. Our animal experiments revealed that in the Panc02 pancreatic tumor model, CCL1 is present at mRNA and protein level, justifying the increased accumulation of $CCR8^+$ cells in the tumor mircroenvironement. Likewise, our TCGA analysis confirms both the correlation of CCR8 and FOXP3 and the correlation of T_{reg} cells with poor prognosis. Furthermore, it expands it to several solid tumors, such as colorectal, lung and pancreatic cancer. Interestingly, a recent study from Beatty et al. using CAR T cells in pancreatic cancer could not achieve complete responses in the primary tumor site (Beatty et al. 2018). This study, using an anti-mesothelin CAR, could perhaps report improved results if combined with the herein proposed CCR8 recruitment mechanism to the tumor site.

$CCR8^+$ T_{reg} cells have been proposed to be master drivers of immune regulation owing to the fact that the specific binding of CCL1 to CCR8 increases the suppressive capacity of T_{reg} cells. The CCL1-CCR8 axis is however incapable of inducing a switch from $FOXP3^-$ to $FOXP3^+$ (Barsheshet et al. 2017), therefore forced expression of CCR8 in T_{eff} cells will not be able to induce a conversion from effector to suppressive functions. Furthermore, the lack of CCR8 expression in T_{eff} cells increases the likelihood of an improved migration upon forced expression.

Transduction of different chemokine receptors has been used in the past to improve adoptive T cell therapies for solid tumor entities (Di Stasi et al. 2009, Kershaw et al. 2002). Out of the receptors used, CCR4 is the only other chemokine receptor expressed in T_{reg} cells, thus making it an approach that is directly comparable to the strategy presented in this study. In mouse studies, CCR4 transduction has been proven to improve ACT anti-tumor efficacy in pancreatic tumors (Rapp et al. 2015). In another study, mouse and human models of Hodgkin lymphoma were used to show that CCR4 transduction improves CAR T cell efficacy depending on the availability of CCL22 (Di Stasi et al. 2009). $CCR4^+$ and $CCR8^+$ ACT high-jack two distinct axes – CCL22 and CCL1, respectively – that normally enable T_{reg} cell trafficking to solid tumors.

The CCL1-CCR8 axis is more specific and can directly counterbalance T_{reg} cell infiltration in solid tumors, making it a superior choice for chemokine receptor-enhanced ACT in solid tumors when compared to the CCL22-CCR4 axis.

Considering tumor specificity and potential safety concerns, our TCGA analysis on the expression levels of CCL1 and CCL22 reveals that CCL22 is more broadly expressed in different healthy tissues. This may lead to the redirection of $CCR4^+$ CAR T cells into off-target $CCL22^+$ tissue, on one hand diminishing the anti-tumor effect and on the other hand increasing the risk for off-tumor toxicity. On the contrary, CCL1 is virtually not expressed in any healthy tissue.

On another note, both CCR4 and CCR8 can enable T_{reg} cell access to solid tumors. Upon effective ingress of tumor-specific T cells into the tumor site, T cell activation will trigger CCL1 production. This will result in an increased $CCR8^+$ T_{reg} cell accumulation at the tumor site with the potential to dampen effective ACT. Such can only be counterbalanced with a CCR8-transduced ACT product.

This work shows that CCR8-transduced T cells have improved migratory capacity towards CCL1 gradients, *in vitro* and *in vivo*, in murine and human models of pancreatic cancer. This resulted in improved anti-tumor efficacy of ACT. It is clear that an adequate patient screening for ligand expression in tumor biopsies may dictate the chemokine receptor that should be used to improve ACT for this patient. There are nevertheless mechanistic arguments as well as general safety considerations that might influence the receptor choice in the cases where both ligands are present.

4.4 DNR as a strategy to alleviate TGF-β-induced immunosuppression

Our *in vitro* and *in vivo* mouse experiments clearly show that TGF-β is present in Panc02 tumors and identifies two main sources for this cytokine, tumor cells and T_{reg} cells. Furthermore, eT_{reg} cells were herein shown to be present in the pro-inflammatory tumor microenvironment and were nearly all positive for TGF-β staining, which was in stark contrast to the same population in healthy tissues. TGF-β is extremely immunosuppressive. On one hand, due to its direct effects on T cells, recapitulated by us through the proliferation experiments in presence of TGF-β, on the other hand due to its capacity of potentiating immunosuppression by dendritic cells and T_{reg} cells through other cell-to-cell interactions and the release of further immunosuppressive cytokines (Rabinovich et al. 2007, Massague 2008). IL-10, another T_{reg} cell-derived cytokine, can also be found in abundance in the tumor microenvironment. IL-10 has been described to impair dendritic cell functionality and hamper T_{eff} cell cytotoxicity. Nevertheless, this cytokine may also play a role in promoting tumor rejection instead of inducing immunosuppression (Rabinovich et al. 2007), therefore we deemed TGF-β a more interesting cytokine to target.

Through our TCGA analysis, we were able to show that besides FOXP3 and CCR8 (as well as its ligands), TGFB1 is up-regulated in human pancreatic adenocarcinoma compared to healthy pancreatic tissue, verifying the strong correlation between the expression of these genes.

The DNR is a well-characterised receptor and binding by TGF-β will not trigger downstream signaling in the cell. Moreover, TGF-β scavenging, will prevent its detrimental effects on other T_{eff} cells (Bollard et al. 2002). Such a receptor harbours multiple advantages when compared to anti-TGF-β antibody therapy as the effect with DNR is limited to the proximity of the DNR-expressing cell. Furthermore, there is an added layer of safety as T cells will also require engineering for antigen-specificity to recognize the target tumor cells. Thus, endogenous T cells with different TCR specificities will remain unaffected, reducing the risk of self-reactivity (Bollard et al. 2018). The usage of DNR instead of genome-editing technologies such as CRISPR/Cas9 for relieving T cell immunosuppression was considered. The knowledge on safety and efficacy of genome editing technologies is however still limited and more evidence will be needed for a comprehensive assessment (Pineda et al. 2019). The

advanced stage of testing in clinical trials — where DNR enhanced anti-tumor efficacy of anti-PSMA CAR T cells for prostate cancer patients (Kloss et al. 2018) — as well as its safety profile, make it an well-established tool for the relief of T cell immunosuppression (Bollard et al. 2018). Our *in vivo* results, in syngeneic and xenograft pancreatic tumor models, showed the anti-tumor efficacy of this approach. This highlighted the potential relevance of combining the CCL1-CCR8 axis with TGF-β-shielding in pancreatic tumors and provided a rationale for testing it, in a more advanced stage, against other tumor entities with comparable expression profile.

4.5 The CCR8-DNR combination synergises to improve ACT

Our study goes on to test the combination of CCR8 and DNR in CAR T cells in syngeneic and xenograft pancreatic tumor models. The DNR has been previously used to improve the efficacy of CAR T cells in solid tumors (Kloss et al. 2018).However, never has a DNR-transduced CAR T cell been equipped with a chemokine receptor to improve tumor trafficking, nor has the CCR8 receptor ever been a target of cancer therapy. Combinatorial approaches of enhanced migration and immunosuppression resistance are mentioned, among other mechanisms, by Lim and June as ways to improve ACT effectiveness in solid tumors (Lim et al. 2017)

The synergy from this combination was evident and enabled complete tumor clearance and improved overall survival in mice with established tumors upon administration of a single therapy. Currently, clinical trials with CAR T cell therapy for solid tumors rely on multiple administrations of CAR T cells to achieve limited anti-tumor responses (Brown et al. 2016, Beatty et al. 2018). Our pre-clinical results show an enhanced CAR T cell therapy that can induce tumor rejections with a single treatment. Furthermore, we postulate that increasing the times the therapy is administered can further increase anti-tumor efficacy, should it fail to achieve a complete response. The CCR8 and DNR combination has the potential to become a universal platform to improve efficacy of ACT in several solid tumors and will benefit from a thorough patient screening to select patients with a favourable tumor cytokine expression profile.

4.6 Targeting tumor antigens and the safety considerations associated

Antigen-specificity remains a major hurdle and a pivotal requirement to ensure the feasibility of any tumor-targeted T cell therapy (Hinrichs et al. 2013). The current targets utilised in clinical trials such as mesothelin or IL13Rα2 have not shown any severe toxicity events, nevertheless this can be attributed to the fact that the CAR T cells could not unleash a full blown anti-tumor response due to poor infiltration or due to immunosuppression mechanisms at the tumor site. Upon improved trafficking and accumulation of CAR T cell therapy into solid tumors, one will decrease the potential for off-tumor toxicity, however there is an increased risk for massive tumor lysis, T cell activation and therefore cytokine release syndrome. In our study, targeting the model antigen EpCAM in a murine tumor model did not reveal any signs of toxicity. However, C57BL/6 mice are typically highly resistant to immune-mediated side effects which may only occur with additional modifications to the treatment schedule (Cheadle et al. 2014). Thus, toxicity associated with CCR8- and DNR-transduced CAR T cells could not be comprehensively assessed in this model. Nonetheless, the addition of chemokine receptors or DNR to T cells have not been associated with acute toxicity events, such as cytokine release syndrome and tumor lysis syndromes which have been observed for CAR T cell therapy (Brudno et al. 2016).

Autoimmunity has been described for DNR-based T cell therapy (Gorelik et al. 2000) and transgenic mice expressing DNR have been reported to develop lymphoproliferative disorder (Lucas et al. 2000). The settings where these observations were made will hardly be found in a CAR T cell therapy patient. Autoimmunity can occur, and is likely to occur, in settings of unknown antigen-specificity. With CAR T cells, current manufacturing protocols insert the CAR gene without removing the endogenous TCR. Nevertheless, there are several approaches being developed to insert the CAR gene in the TCR α constant (TRAC) locus (Eyquem et al. 2017). Integrating such an approach in the manufacturing protocol of CAR T cells will prove pivotal in the future to decrease the possibility of autoimmunity derived from unknown antigen-specificity. On another note, lymphoproliferative disorders caused by DNR have only been reported in transgenic mice, and so far, have not been reproduced in other animal models. Currently, a clinical trial is assessing the safety and efficacy of DNR transduction in Epstein-Barr virus-specific T cells for lymphoma (NCT00368082) (Bollard et al. 2018).

It remains to be proved in settings of ACT, if DNR-transduced T cells have the potential to develop into a lymphoproliferative disorder.

4.7 CCR8-DNR potential for combination with different ACT modalities

Poor access to the tumor site and tumor associated immunosuppression are common mechanisms that will reduce ACT effectiveness. TIL, TCR and CAR therapy rely on the existence of antigen specificity, either pre-existing (TIL) or genetically engineered into the T cell product (TCR and CAR).

Kershaw et al. were pioneers showing the potential of inducing forced expression of CXCR2 on T cells to redirect their migration (Kershaw et al. 2002). Following in their footsteps, Idorn et al. have described that TIL can be effectively engineered to transiently express CXCR2 (Idorn et al. 2016) demonstrating the feasibility of TIL engineering to improve anti-tumor efficacy. Within this work both TCR-transgenic T cells and CAR T cells were further engineered to express CCR8, demonstrating not only that the newly identified target CCR8 can improve ACT effectiveness, but can also do so in TCR and CAR engineered T cells and very likely such would also work with TIL ACT.

The DNR has been proven to work when engineered in Epstein-Barr virus-specific T cells in a study by Bollard et al. setting the precedent for the use of this receptor as a strategy to shield T cells from TGF-β induced immunosuppression (Bollard et al. 2002). Recently this same strategy was used to enable the efficacy of anti-PMSA CAR T cells (Kloss et al. 2018). Within this work, TCR-transgenic T cells and CAR T cells were engineered to express DNR and had improved anti-tumor efficacy.

The CCR8-DNR has yet to show incompatibility with any form of ACT. The synergistic mechanisms of action of the individual receptors complements tumor-specificity of TIL, TCR and CAR therapies, to enable stronger anti-tumoral effects. An appropriate patient screen for expression of the respective ligands will determine the possibility of employment of the CCR8-DNR as means of complementing any known form of ACT.

5 Conclusions and outlook

Adoptive T cell therapy has come a long way into reaching clinical approval for haematological malignancies with the anti-CD19 CAR T cell products. This therapy changed the clinical outcome of refractory and relapsed patients with some haematological malignancies, and it heralds the potential to be truly transformative for solid tumor patients (Lim et al. 2017).

The target selection for the use of T cell therapies in solid tumors have been for long, and still are, main drivers of research in immuno-oncology (Hinrichs et al. 2013). The advent of checkpoint blockade inhibitors expanded on the observation that TILs can already effectively target tumor cells (Schietinger et al. 2012, Robbins et al. 2013). With an adequate release from immunosuppression, the immune system can effectively drive tumor regression in several tumor entities (Mardiana et al. 2019). Access of immune cells to the tumor site nevertheless remains an unmet need in solid tumors (Cadilha et al. 2017). Infiltration and proliferation are two crucial pitfalls that ACT will need to overcome to begin inducing significant response rates in solid tumor patients (Lim et al. 2017).

Within this work we have addressed these two limitations and we were able to identify a chemokine receptor, CCR8 that can effectively improve migration towards pancreatic solid tumors. The use of this receptor in ACT is novel and tackles a critical axis of tumor immune evasion as it directly counteracts T_{reg} cells competing for ligand access and enabling comparable T_{eff} cell trafficking to pancreatic tumors. Analysis of genetic signatures of several other solid tumor entities such as colon carcinoma and breast cancer reveal the potential utility of CCR8 to enable ACT in these tumor entities.

We have concluded that despite significant increases in anti-tumor efficacy upon CCR8 transduction for ACT, therapy still remained sub-optimal and could benefit from additional improvements.

The well-characterised DNR success in recent pre-clinical and clinical trials make it one of the best established genetic engineering receptors for the relief of immunosuppression to date (Bollard et al. 2002, Kloss et al. 2018). Targeting TGF-β from within a genetically modified cellular product result in the localised scavenging of the ligand within close proximity of the cellular product, as opposed to systemic antibody

therapy. The impact of this functionally binding, but signaling-deficient receptor is twofold. On one hand the binding to TGF-β prevents effects of this cytokine in adjacent cells, on the other hand binding of the ligand to DNR-transduced T cells will not trigger immunosuppressive pathways. The relevance of this mode of action also spans beyond pancreatic cancers into virtually all solid tumors analysed within this study. Combination of the DNR with CCR8 synergised into a potent anti-tumor ACT that far surpassed the effectiveness of only antigen-specific T cells or cells that were equipped with only one of the receptors, CCR8 or DNR.

Much headway remains to be made regarding the validation of this platform and eventually testing in patients. We believe that with curative treatment options for pancreatic solid tumors being an unmet medical need, this constituted an appropriate tumor entity to investigate the efficacy of this platform. Nevertheless, like pancreatic cancer, the incidence of other solid malignancies such as lung, colon and breast tumors is also rising (Ferlay et al. 2019), and currently available therapies are still sub-optimal due to both lack of effectiveness and considerable toxicity. CAR T cell therapy is currently not an approved therapy for any solid tumor, mostly due to lack of effectiveness, despite available targets showing potential in pre-clinical studies (Brown et al. 2016, Beatty et al. 2018). Likewise, neither TIL nor TCR therapies have reached approval for solid tumors. Our CCR8-DNR platform has the capacity to enable a more adequate assessment of how targeting these antigens can be effective.

For the current CAR T cell indications, toxicity is still an issue that requires much optimisation. Toxicity will inevitably occur as the therapy becomes available in a broader range of centers. Therefore, the safety profile of this therapy must be further enhanced for example by use of a platform like the one we have described or abrogation of toxicity with suicide gene induction.

These findings warrant further investigation to extend testing of CCR8-DNR-CAR T cells in other solid tumor malignancies and could be translated into a small clinical trial to establish the safety of this approach and enable CAR T cell therapy for solid tumor patients.

6 Abstract

Across cancer types, T regulatory (T_{reg}) cells contribute to cancer progression through immune suppression. The C-C chemokine receptor 8 (CCR8) was shown to be expressed in T_{reg} cells and the presence of this cell population in solid tumors is associated with poor prognosis. CCR8 may enable T cell access to the tumor site, however, in contrast to T_{reg} cells, T effector (T_{eff}) cells lack endogenous expression of CCR8. Furthermore, T_{reg} cells can inhibit T_{eff} cells through, among others, the release of transforming growth factor β (TGF-β). Not only do T_{reg} cells contribute to TGF-β production, they can also activate latent TGF-β. In such an inhibitory environment CAR T cells have poor persistence and limited effector functions. These issues represent bottlenecks to CAR T cell therapy translation. Here we show that genetically engineering CAR T cells to express CCR8 improves their migration into solid tumors. Simultaneous engineering with a dominant-negative TGF-β-receptor 2 synergized well, allowing rejection of tumors that are otherwise resistant to CAR T cell therapy. Both in murine syngeneic and human xenograft tumor models, we demonstrate the capacity of these enhanced CAR T cells to stunt solid tumor growth and to improve survival. Mechanistically, we use flow cytometry to delineate the improved function of these enhanced CAR T cells. Our results demonstrate the viability of two additional genetic modifications on CAR T cells and open an unprecedented avenue for effective CAR T cell therapy in solid tumors.

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8 List of abbreviations

A

Acute lymphocytic leukemia	ALL
Adoptive T cell therapy	ACT

C

C-C chemokine ligand	CCL
C-C chemokine receptor 4	CCR4
C-C chemokine receptor 8	CCR8
$CD8^+$ OVA-specific T cells	OT-I
C-X-C chemokine receptor 2	CXCR2
Chimeric antigen receptor	CAR
Chronic lymphocytic leukaemia	CLL
Cytotoxic T lymphocyte-associated protein 4	CTLA-4

D

Dendritic cells	DCs
Diffuse large B cell lymphoma	DLBCL
Dominant negative version of the TGF-β-R2	DN-TGF-β-R2 or DNR

G

G-protein coupled receptors	GPCR

I

Injected intravenously	i. v.

M

Major histocompatibility complex	MHC
Mesothelin	MN
Multiphoton intravital microscopy	MP-IVM
Myeloid-derived suppressor cells	MDSCs

N

Natural Killer	NK

P

Pancreatic ductal adenocarcinoma	PDAC
Peripheral blood mononuclear cells	PBMC
Polymerase chain reaction	PCR
Programmed cell death protein 1	PD-1

R

Real-time PCR	RT-PCR

S

Single chain variable fragment	scFv
Subcutaneously	s. c.

T

T cell receptor	TCR
TCGA	The Cancer Genome Atlas
TCR α constant	TRAC
T effector	T_{eff}
T helper	Th
Transforming growth factor β	TGF-β
Tumor infiltrating lymphocyte	TIL

W

Wild-type	WT

9 Curriculum vitae

Bruno Loureiro Cadilha

Nationality: Portuguese

Born the 26th of April 1990 in Lisboa

Native Portuguese speaker and fluent in English, French, Spanish and German

Highschool

2005 to 2008	Escola Secundária de Gago Coutinho Alverca do Ribatejo, Portugal

University

2008 to 2014	Master of Medicine Faculdade de Medicina da Universidade de Lisboa, Portugal

Doctoral studies

2014 to 2020	Doctor of Philosophy in Medical Research Ludwig-Maximilians-Universität München, Deutschland

Internships

2017 to 2018	Mempel laboratory, Massachusetts General Hospital, Harvard Medical School, Boston, United States of America
2016	Straten laboratory, Herlev Hospital, Copenhagen University, Copenhagen, Denmark

Teaching activities

Since 2018	Immuntherapie maligner Erkrankungen, Modul 23, Klinikum der Ludwig-Maximilians-Universität München

Doctoral students supervised

Since 2020	Hannah Obeck, Dr. med. candidate
Since 2019	Theo Lorenzini, Dr. med. candidate
Since 2018	Duc Huynh, Ph. D. candidate
2016 to 2017	Klara Dorman, Dr. med. candidate (Experiments concluded)

Patent submissions

EP19161708	CCR8-transduced T cells for targeted tumor therapy. Kobold S, Endres S, Cadilha BL. 8th March 2019

Awards

m4 Award BioM	Pre-seed funding program of the Bavarian State Ministry of Economic Affairs, State Development and Energy. € 0.5 Million to Kobold S, Endres S, Cadilha BL.
BMS 2019	Young Scientists Immuno Oncology 2019. € 5000 to Cadilha BL.

Original research articles

2018

1. Rataj F, Kraus FBT, Chaloupka M, Grassmann S, Heise C, **Cadilha BL**, Duewell P, Endres S, Kobold S.
   PD1-CD28 fusion protein enables CD4+ T cell help for adoptive T cell therapy in models of pancreatic cancer and non-Hodgkin lymphoma.
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2019

2. Rataj F, Jacobi S, Stoiber S, Asang F, Ogonek J, Tokarew N, **Cadilha BL**, Puijenbroek E, Heise C, Düwell P, Endres S, Klein C, Kobold S.
   High affinity CD16-polymorphism and Fc-engineered antibodies enable activity of CD16-chimeric antigen receptor-modified T cells for cancer therapy.
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4. Liu X, Li J, **Cadilha BL**, Markota A, Voigt C, Huang Z, Lin PP, Wang DD, Dai J, Kranz G, Krandick A, Libl D, Zitzelsberger H, Zagorski I, Braselmann H, Pan M, Zhu S, Huang Y, Niedermeyer S, Reichel CA, Uhl B, Briukhovetska D, Gosálvez JS, Kobold S, Gires O, Wang H.
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   Bispecific antibodies activate synthetic agonistic receptor T cells for tumor therapy.
   Clinical Cancer Research 2019; 25:5890-5900. **JIF 10.2**

Review articles

2017

1. **Cadilha B**, Dorman K, Rataj F, Endres S, Kobold S.
   Enabling T cell recruitment to tumours as a strategy for improving adoptive T cell therapy.
   European Oncology and Haematology 2017; 13:66-73. **JIF pending**

2019

2. Benmebarek MR, Karches CH, **Cadilha BL**, Lesch S, Endres S, Kobold S.
   Killing mechanisms of chimeric antigen receptor (CAR) T cells.
   International Journal of Molecular Sciences 2019; 14:20(6). **JIF 3.7**

3. Stoiber S, **Cadilha BL**, Benmebarek MR, Lesch S, Endres S, Kobold S.
   Limitations in the design of chimeric antigen receptors for cancer therapy.
   Cells 2019; 17:8(5). **JIF 4.8**

4. Kruger S, Ilmer M, Kobold S, **Cadilha BL**, Endres S, Ormanns S, Schuebbe G, Renz BW, D'Haese JG, Schloesser H, Heinemann V, Subklewe M, Boeck S, Werner J, von Bergwelt-Baildon M.
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   Determinants of response and resistance to CAR T cell therapy.
   Seminars in Cancer Biology 2019; in press. **JIF 9.7**
   * Authors contributed equally.

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