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Generation of Prostaglandin E2-resistant Chimeric Antigen Receptor T cells

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Abstract

While chimeric antigen receptor (CAR) T cell therapy has revolutionized the treatment of blood-borne malignancies, so far it could not be successfully translated into the treatment of patients with solid cancer. A reason for this is the immunosuppressive microenvironment created by many solid tumors. One factor contributing to the tumor-induced immunosuppression is prostaglandin E_2 (PGE_2). PGE_2 can bind to its receptors EP2 and EP4 on T cells and exerts an inhibitory function that so far is not well understood.

In this thesis, it is shown that PGE_2 indeed does diminish the efficacy of CAR T cell therapy, mainly by reducing proliferation and persistence of CAR T cells in a PGE_2-rich environment. CRISPR/Cas9-mediated double knockout of its receptors EP2 and EP4, but not single knockouts of the respective receptors alone, was able to rescue CAR T cell proliferation and persistence in the presence of PGE_2, thus increasing CAR T cell numbers. Although no differences in CAR T cell activation could be observed in the presence of PGE_2, the increase in T cell numbers achieved by using EP2 and EP4 knockout CAR T cells was sufficient to improve CAR T cell mediated tumor cell lysis *in vitro*. Improved tumor control and increased survival mediated by EP2 and EP4 knockout T cells could be confirmed *in* vivo in an OT-I model. A tracking experiment confirmed that EP2 and EP4 knockout OT-I T cells indeed persist successfully in the tumor microenvironment as opposed to wild type OT-I T cells.

In summary, this work highlights the feasibility and potential of EP2 and EP4 knockout T cells in the setting of adoptive cell transfer therapy and encourages further development of this strategy.

List of abbreviations

°C	degrees Celsius
ACT	adoptive cell transfer
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAR	chimeric antigen receptor
Cas9	CRISPR-associated protein 9
CD	cluster of differentiation
CO_2	carbon dioxide
COX	cyclooxygenase
CREB	cAMP response element-binding protein
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	clustered regularly interspaced short palindromic repeats ribonucleic acid
CRS	cytokine release syndrome
DMEM	Dulbecco's modified eagle medium
EdU	5-ethynyl-2′-deoxyuridine
EpCAM (-FC)	epithelial cell adhesion molecule (Fc-tagged)
Fc	crystallisable fragment
FCS	fetal calf serum
FDA	Food and Drug Administration
gRNA	guide ribonucleic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFNγ	interferon-γ
LAG-3	lymphocyte-activation gene 3
L-Glu	L-glutamin
MDSC	myeloid-derived suppressor cell
NK cell	natural killer cell
PBS	phosphate buffered saline
PD-1	programmed cell death protein 1
PGE_2	prostaglandin E_2
PGES	prostaglandin E synthase
PGH_2	prostaglandin H_2

PKA	protein kinase A
PLA_2	phospholipase A_2
RNA	ribonucleic acid
RNP	ribonucleoprotein
RPMI	Roswell Park Memorial Institute
TCR	T cell receptor
TGF-β	transforming growth factor beta
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TME	tumor microenvironment
tracrRNA	trans-activating clustered regularly interspaced short palindromic repeats ribonucleic acid
T_{reg}	regulatory T cell
x g	times of earth gravitation

1. Introduction

1.1 Chimeric antigen receptor T cell therapy in solid tumors

Helping the immune system fight cancer, broadly called tumor immunotherapy, has completely transformed cancer treatment in recent years[1,2]. Therein, many of the successful treatment strategies are focusing on an improvement of the anti-tumor T cell response. For example, immune checkpoint inhibition with programmed cell death protein 1 (PD-1) blockade[2] as well as using chimeric antigen receptor (CAR) T cell in adoptive cellular therapy[2] have shown great success and were granted approval for different tumor entities by the Food and Drug Administration (FDA)[1,2] as well as the European Medicines Agency[3,4]. Nevertheless, many cancer types, especially solid tumors, remain unresponsive to immunotherapy, highlighting the need for further advancements in the field[5].

1.1.1 Chimeric antigen receptor T cells

Chimeric antigen receptor (CAR) T cells have shown outstanding response rates in blood-borne malignancies with the first product achieving FDA approval in 2017[1,2]. In short, CAR T cells are an adoptive cell transfer (ACT) therapy, where T cells are isolated from the patients' peripheral blood, which subsequently are equipped with a CAR targeting the tumor, expanded *ex vivo* and finally are re-infused into the patient[5].

CAR design

A CAR is a fully synthetic receptor consisting of an extracellular antibody-derived single chain variable fragment targeting a tumor surface antigen, and a transmembrane and intracellular domain capable of inducing T cell activation upon antigen binding by the extracellular part of the construct[5]. The detailed components may vary depending on CAR generation and general design, but commonly used transmembrane domains include CD4, CD8α, CD28 and CD3ζ[5]. The most widely used intracellular costimulatory domains are derived from CD28 and 4-1BB, but also CD27- and inducible T cell co-stimulator-derived domains have been investigated[5].

CAR-transgene delivery

Most CAR constructs are delivered to the T cell by γ-retroviral or lentiviral vectors, although transposon systems and targeted integration into the genome using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system have been employed as well[5]. All these approaches lead to stable integration of the transgene into the genome, which, depending on the delivery method (random or targeted integration), harbours risks concerning insertional mutagenesis and dysregulation of genes adjacent to the integration site[5]. In contrast to

that, CAR constructs can also be delivered by nucleofection with ribonucleic acid (RNA), which has no risk of insertional mutagenesis but suffers from the drawback of only transient expression of the CAR due to rapid RNA degradation limiting its usefulness in a clinical setting[5].

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 mediated gene knockouts

The recently developed gene editing technology CRISPR/Cas9 has made genetic engineering broadly available and thus revolutionized translational research since its discovery in 2012[6,7]. Therefore, it is not surprising that by now the CRISPR/Cas9 system is already widely tested in the attempt to create improved CAR T cell products for cancer therapy[6]. These strategies include for example knockouts of endogenous T cell receptor (TCR) chains to reduce the likelihood of graft versus host disease or the knockout of immune checkpoint inhibitors to make CAR T cells more resistant against exhaustion[6]. Although none of them have gained FDA approval until now, many of these strategies are currently employed in phase I and II clinical trials[6]. Briefly, the Cas9 nuclease forms a ribonucleo protein (RNP) complex by taking up a guide RNA (gRNA) consisting of a tracrRNA that facilitates the binding to Cas9 and a crRNA, which consists of a sequence complementary with the tracrRNA to facilitate uptake into the RNP and a sequence complementary to the targeted gene locus[6,7]. This crRNA can be specifically designed for nearly every target sequence and by aligning with it facilitates specific cutting of the Cas9 nuclease at the targeted locus[6]. Error-prone DNA repair of the cutting site by the cells own repair mechanisms then can lead to (frameshift) mutations leading to a knockout of the target gene[6]. Alternatively, donor DNA can be provided to do precise gene insertions or corrections after the cutting event[6].

1.1.2 Challenges of CAR T cell therapy in solid tumors

CAR T cell therapy still faces significant limitations[5,8]. On one hand, universal problems of CAR T cell therapy independent of the tumor entity as for example toxicities like cytokine release syndrome (CRS) and neurotoxicity[5] or antigen loss and subsequent tumor escape[8] still limit their applicability and success[8]. On the other hand, CAR T cells lack efficiency especially in solid tumors[8]. Reasons for this primarily lie in the additional hurdles a solid tumor including its microenvironment present[8]. One of the main challenges is achieving proper infiltration of CAR T cells into the tumor[8], but even if an accumulation of CAR T cells can be achieved, poor persistence of the CAR T cells accompanied by exhaustion and a loss of function still impair the success of CAR T cell therapy[8].

Commonly known immunosuppressive molecules in the tumor microenvironment severely limiting T cell function are immune checkpoint molecules like PD-1, T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) and lymphocyte-activation gene

3 (LAG-3)[8]. Immune checkpoint molecules can lead to exhaustion and death of CAR T cells upon binding to their ligands, which are often highly expressed by tumor cells[8]. Additionally, tumor cells as well as tumor-associated cells like myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages, cancer-associated fibroblasts or regulatory T cells (T_{reg}) produce a range of soluble factors inhibiting T cell function[8]. One example for this is TGF-β, which further promotes T_{reg} function and has direct T cell inhibitory effects[9]. Apart from cytokines shaping the anti-tumor response of T and other immune cells, there is also a range of metabolites released from tumor and tumor-associated cells[8]. These include for example adenosine and prostaglandin E_2 (PGE_2), both of which have been reported to inhibit T cell function[8] but their role in CAR T cell therapy is unclear. This work is thus focussed on characterizing and overcoming the influence of PGE_2 on CAR T cell function.

1.2 Prostaglandin E_2

1.2.1 Synthesis

Prostaglandin E_2 (PGE_2), the most abundant prostaglandin in the human body, is a principal mediator of inflammation and can be produced by nearly all cell types in the body[10]: Inflammatory stimuli lead to calcium influx, which results in the translocation of phospholipase A_2 (PLA_2) from the cytoplasm to the nuclear membrane, where it hydrolyses membrane phospholipids to release arachidonic acid[10]. Arachidonic acid is further processed to prostaglandin H_2 (PGH_2) via the unstable intermediate prostaglandin G_2 by cyclooxygenase (COX)-1 or COX-2[10]. Upon stimulation, prostaglandin E synthase (PGES) also gets translocated to the nuclear membrane, where it coordinates with COX-1 to convert PGH_2 to PGE_2[10]. Alternatively, PGH_2 can also be converted to PGE_2 by PGES-1[10]. While PLA_2, COX-1 and PGES are constitutively expressed, COX-2 and PGES-1 expression is regulated and needs to be induced first[10]. Therefore, COX-2 is considered to be the rate-limiting enzyme during PGE_2 synthesis[10]. PGE_2 can then exit the cell by diffusion or may be secreted via the multi-drug resistance protein 4 transporter[10] and thus trigger signalling pathways on nearby cells.

1.2.2 Signalling

Extracellular PGE_2 can bind to its receptors EP1, EP2, EP3 or EP4, which are all G protein-coupled receptors[11,12]. EP1 mainly functions via the increase of free calcium in the cell[11,12] by coupling to $G_{\alpha q}$, which activates the phopholipase C - inositol-1,4,5-trisphosphate pathway[12]. EP2 and EP4 couple to $G\alpha_S$ and activate adenylate cyclase leading to increased concentrations of cyclic adenosine monophosphate (cAMP)[11,12]. cAMP subsequently activates protein kinase A (PKA) which then leads to phosphorylation of cAMP response element-binding protein (CREB)[12]. EP2 and EP4

therefore, by sharing a signalling pathway, act redundantly in some situations, although also distinct roles are described for each receptor[11]. This might either be due to selective expression of only one of the receptors on the respective cell type or the ability of EP4, but not EP2, to activate phosphatidylinositol 3-kinase, probably via G_i[11], leading to nuclear factor 'kappa-light-chain-enhancer' of activated B-cells-mediated transcription programs[12]. EP3 couples to $G\alpha_i$ to either inhibit (EP3α, EP3β)[11,12] or activate (EP3γ)[12] adenylate cyclase, depending on its isoform[11,12]. EP1-EP4 are expressed on multiple cell types important for shaping the tumor microenvironment, including MDSCs, macrophages, dendritic cells, natural (NK) cells or T cells in different combinations[13]. Thus, PGE_2 has been described as a potent modulator of the TME.

1.2.3 Effect of PGE_2 in the tumor microenvironment

As described in chapter 1.1.2, the immunosuppressive microenvironment created by many tumors is one of the main challenges to overcome to enable successful CAR T cell therapy in solid tumors. A factor contributing to this is PGE_2. High abundance of PGE_2 and its rate-limiting synthesis enzyme COX-2 are associated with a bad prognosis and decreased survival in several cancer types[14]. Although PGE_2 exerts direct pro-tumorigenic effects by promoting proliferation, invasiveness, and apoptosis-resistance of tumor cells as well as increasing angiogenesis[15], recent studies have shown that the pro-tumorigenic effect of PGE_2 is primarily caused by its immunosuppressive functions[16-19]. Genetic ablation of cyclooxygenases or PGE_2 synthases restores susceptibility to immune control in murine melanoma, breast, and colorectal cancer models by shifting the tumor inflammatory profile towards classical anti-cancer immune pathways also conserved in human melanoma samples[18]. The anti-tumorigenic effects of PGE_2-reduction were characterized by increased interferon-γ signalling and improved efficacy of checkpoint inhibition mediated by increased cytotoxic T cell responses[16,17].

1.2.4 Effect of PGE_2 on T cells

Indeed, inhibition of T cell function by PGE_2 is described as early as 1979[20] and recently several different mechanisms of action have been proposed. Results range from indirect effects of PGE_2 on NK cells leading to a shift in the NK – classical dendritic cell - T cell axis reducing the cytotoxic potential of T cells in the tumor microenvironment[16,17,19] to direct T cell inhibitory effects. Several studies report that PGE_2 induces a shift towards the tumor-promoting T helper cell subpopulations Th2 and Th17 and high levels of PGE_2 are also associated with an increase in regulatory T cell numbers[14]. Furthermore, there are several publications highlighting PGE_2 mediated suppression of cytotoxic T cells. The reported mechanisms range from upregulation of inhibitory receptors and checkpoint molecules[21-25] over direct inhibition of TCR signalling and impaired cytotoxic function[26-28] to reduced proliferation and survival[20,29] of T cells. Currently, there is little

known on the effects of PGE_2 on CAR T cells, but it must be assumed that adoptively transferred T cells will be at least equally affected[30]. Some reports even lead to the assumption that protection from PGE_2 might be especially important in ACT, as the sensitivity towards PGE_2 increased over time in tumor infiltrating lymphocytes when expanded *ex vivo*[26]. Thus, it is not surprising that targeting the COX-2 - PGE_2 axis therapeutically in cancer patients has become a widely investigated strategy.

1.2.5 Therapeutic strategies interfering with the PGE_2 axis

The results of genetic ablation of cyclooxygenases could be mimicked by therapeutic COX-2 - PGE_2 pathway inhibition by using COX-inhibitors[16]. However, although COX-inhibition has shown some positive effects in cancer therapy, it has also been associated with adverse events such as gastrointestinal irritations and cardiovascular events[31,32], leaving a need for alternative strategies to target the COX-2 - PGE_2 axis. Recent studies are investigating more selective inhibitors of the COX-2 - PGE_2 axis, for example by targeting the PGE_2 receptors EP2 and EP4 instead of completely blocking prostaglandin synthesis[16,33]. Nevertheless, also more targeted inhibition of the multifunctional metabolite PGE_2 by small molecule inhibitors might remain problematic in a systemic setting. Therefore, there are approaches to further limit the effects of anti- PGE_2 therapy by restricting it spatially to the tumor site, for example by PGE_2 degradation locally in the tumor microenvironment mediated by oncolytic virus therapy [34].
In contrast, an advantage of ATC therapies such as CAR T cells is that they offer possibilities to restrict PGE_2 signalling in a well-defined and controllable population of effector cells without interfering in the systemic networks of prostaglandin signalling.

PGE_2 signalling is mediated via its receptors EP1, EP2, EP3 and EP4. While all four receptors were detected in T cells[28,35], there is still some controversy concerning the importance of EP1 and EP3 on T lymphocytes, and many publications have identified EP2 and EP4 as the main mediators of PGE_2 signalling in T cells[21,26,28,29,33,35]. So far, two different approaches are published to interfere with this pathway in an ACT setting. The first strategy is to stop the effect of PGE_2 by overexpressing phosphodiesterase 4A using retroviral transduction in primary T cells and thereby abrogating cAMP signaling[36]. The other approach constructed a peptide blocking the localization of PKA to the immune synapse to be expressed in CAR T cells, thereby stopping PGE_2 signalling at the PKA stage[30].

However, while showing promising results, both approaches suffer from the fact that the cAMP - PKA axis is not exclusive to PGE_2 signalling but regulates a wide variety of cellular processes. On one hand, as the cAMP - PKA axis is mainly associated with inhibition in T cells, this offers protection from several immunosuppressive stimuli, for example adenosine, additionally to PGE_2[26,30,36]. On the other hand, blocking the whole cAMP - PKA axis might lead to the unwanted interference with other cellular processes.

1.3 Hypothesis and aims of the work

Our laboratory could previously demonstrate that the immunosuppressive TME inhibits effective CAR T cell therapy in solid tumors and that such suppressive effects can be successfully counteracted by targeted genetic engineering. Examples for this are PD-1 – CD28 switch receptors[37] turning inhibitory to activating signals or a TGF-β dominant negative receptor blocking TGF-β signaling[38].

The aim of this work was to improve the efficacy of CAR T cells in PGE_2-rich solid tumors by knocking out the receptors EP2 and EP4 on CAR T cells by using the CRISPR/Cas9 system. Thereby, we aimed to achieve protection of the transferred CAR T cells exclusively without interfering with the systemic PGE_2 metabolism, while additionally protecting the CAR T cells only from PGE_2, without targeting other signalling pathways. Thus, we hoped to maximize the effect of PGE_2-protection while minimizing the risk for unwanted side-effects and hoped to achieve a pronounced improvement of CAR T cell therapy in the treatment of solid tumors.

Therefore, the following goals have been defined:

1. **Determination of the effect of PGE_2 on the effectivity of CAR T cell therapy in solid tumors.**
   In this work, a second-generation CAR targeting murine epithelial cell adhesion molecule (EpCAM)[39] was delivered to primary murine T cells via a retroviral transgene delivery system, which has been described previously[39]. It was then planned to expose such generated CAR T cells to PGE_2 and characterize its influence on basic CAR T cell functions such as survival, expansion, activation and killing capacity.

2. **Development of a protocol for the successful generation of anti-EpCAM-CAR T cells with EP2 and/or EP4 knockout**.
   To protect CAR T cells from the suppressive effect observed in the first part of this work, it was next planned to establish a protocol for the double knockout of EP2 and EP4 using the CRISPR/Cas9 system.

3. **Functional characterization of wild type and $EP2^{-/-}$ and/or $EP4^{-/-}$ anti-EpCAM-CAR T cells in the presence of PGE_2 *in vitro* and *in vivo*.**
   Finally, it was planned to functionally characterize survival, expansion, activation and killing capacity of such generated $EP2^{-/-}EP4^{-/-}$ CAR T cells *in vitro*. In the case of successful protection against PGE_2 *in vitro*, it was planned to evaluate whether these results also translate into improved *in vivo* efficacy.

2. Material and Methods

2.1 Material

2.1.1 Reagents

Table 1: Reagents

Reagent	Manufacturer
Alt-R Cas9 Electroporation Enhancer	Integrated DNA Technologies (IDT), Coralville, USA
ALT-R CRISPR tracrRNA	Integrated DNA Technologies (IDT), Coralville, USA
Alt-R S.p. Cas9 Nuclease V3	Integrated DNA Technologies (IDT), Coralville, USA
anti-Mo CD28	Thermo Fisher Scientific, Waltham, USA
anti-Mo CD3	Thermo Fisher Scientific, Waltham, USA
BD Cytofix™ Fixation Buffer	BD Biosciences, Franklin Lakes, USA
BD GolgiPlug™	BD Biosciences, Franklin Lakes, USA
BD GolgiStop™	BD Biosciences, Franklin Lakes, USA
BD Phosflow™ Perm Buffer III	BD Biosciences, Franklin Lakes, USA
Bovine Serum Albumin (BSA)	Carl Roth, Karlsruhe, Germany
Collagenase from clostridium histolyticum	Sigma Aldrich, Steinheim, Germany
CountBright™ Absolute Counting Beads	Thermo Fisher Scientific, Waltham, USA
DMEM	Gibco Products, Grand Island, USA
DMEMF/12 (1 : 1) (1 X)	Gibco Products, Grand Island, USA
DNase I recombinant	Sigma Aldrich, Steinheim, Germany
Dynabeads™ Mouse T-Activator CD3/CD28	Gibco Products, Grand Island, USA
Ethanol 100 %	VWR Chemicals (BDH Prolabs), Fontenay-sous-Bois France
Fetal calf serum (FCS)	Gibco Products, Grand Island, USA
Heparin-Natrium-25000	Ratiopharm, Ulm, Germany
Interleukin-15	Miltenyi, Bergisch-Gladbach, Germany
Interleukin-2	Novartis, Basel, Switzerland
L-Glutamine	Sigma Aldrich, Steinheim, Germany
PBS	Sigma Aldrich, Steinheim, Germany
Penicillin/Streptomycin (Pen/Strep)	Sigma Aldrich, Steinheim, Germany
Recombinant Mouse EpCAM/TROP1 Fc Chimera Protein, CF	R&D Systems, Minneapolis, USA
Retronectin	Takara Bio Europe, St-Germain-en-Laye, France
RPMI 1640	Sigma Aldrich, Steinheim, Germany
Trypan blue	Carl Roth, Karlsruhe, Germany
Trypsin-EDTA solution (10x)	Sigma Aldrich, Steinheim, Germany
UltraComp eBeads™ Plus	Thermo Fisher Scientific, Waltham, USA
β-Mercaptoethanol	Carl Roth, Karlsruhe, Germany

Table 2: Antibodies and stains

Antibody/Stain	Clone	Dilution	Manufacturer
Alexa Fluor 700 anti-CD4	GK1.5	1:100	BioLegend, San Diego, USA
Alexa Fluor® 647 Mouse Anti-CREB (pS133) / ATF-1 (pS63)	J151-21	1:100	BD Biosciences, Franklin Lakes, USA
APC anti-rat CD90/mouse CD90.1 (Thy-1.1)	OX-7	1:100	BioLegend, San Diego, USA
BV510 anti-CD69	H1.2F3	1:100	BioLegend, San Diego, USA
BV605 anti-CD366 (Tim-3)	RMT3-23	1:100	BioLegend, San Diego, USA
BV711 anti-CD45.1	A20	1:100	BioLegend, San Diego, USA
BV785 anti-CD8	53-6.7	1:100	BioLegend, San Diego, USA
FITC anti-CD8	53-6.7	1:100	BioLegend, San Diego, USA
FITC anti-TCR Vα2	B20.1	1:100	BioLegend, San Diego, USA
Fixable Viability Dye eFluor™ 780	-	1:1000	Invitrogen
Pacific Blue anti-CD3	17A2	1:100	BioLegend, San Diego, USA
Pacific Blue anti-CD4	GK1.5	1:100	BioLegend, San Diego, USA
PE anti-CD279 (PD-1)	RMP1-30	1:100	BioLegend, San Diego, USA
PE anti-CD95	15A7	1:100	eBioscience, San Diego, USA
PE/Dazzle594 anti-CD62L	MEL-14	1:100	BioLegend, San Diego, USA
PerCP-Cy5.5 anti-CD223 (LAG-3)	C9B7W	1:100	BioLegend, San Diego, USA
PerCP-Cy5.5 anti-mouse/human-CD44	IM7	1:100	BioLegend, San Diego, USA

2.1.2 Consumables

Table 3: Consumables

Consumables	Manufacturer
6 well plates (cell culture)	Sarstedt, Nürmbrecht, Germany
96 well plates (cell culture)	Sarstedt, Nürmbrecht, Germany
Cell culture flasks	Costa Corning, New York, USA
E-plate 96	ACEA Biosciences Inc., San Diego, USA
Filtropur S 0,2 &0,45 µm	Sarstedt, Nümbrecht, Germany
MACS® SmartStrainers (30, 70, 100 µm)	Miltenyi, Bergisch-Gladbach, Germany
P3 Primary Cell 4D-Nucleofector™ X Kit L	Lonza, Basel, Switzerland
P3 Primary Cell 4D-Nucleofector™ X Kit S	Lonza, Basel, Switzerland
PCR tubes (0,2 ml)	Sarstedt, Nürmbrecht, Germany
Pipet Tips (10, 20, 200, 1000 µl)	Sarstedt, Nürmbrecht, Germany
SafeSeal SurPhob Spitzen (10, 20, 200, 1000 µl)	Biozym, Hessisch Oldendorf, Germany
Serological pipets (25 ml)	Greiner Bio-One, Frickenhausen, Germany
Serological pipets (5 ml, 10 ml)	Costa Corning, New York, USA
Syringes (2 ml, 5 ml, 10 ml, 20 ml)	Becton-Dickinson, Franklin Lakes, NJ, USA
TC Plate 24 Well, Suspension, F	Sarstedt, Nümbrecht, Germany
Tubes (1,5 ml, 2 ml)	Sarstedt, Nürmbrecht, Germany
Tubes (15 ml, 50 ml)	Greiner Bio-One, Frickenhausen, Germany

2.1.3 Devices

Table 4: Devices

Device	Manufacturer
4D Nucleofector™ Core Unit	Lonza, Basel, Switzerland
4D Nucleofector™ X Unit	Lonza, Basel, Switzerland
Axiovert 40C Microscope	Zeiss, Jena, Germany
BD FACSCanto™ II	BD Biosciences, Franklin Lakes, USA
BD LRSFortessa™ Cell Analyzer	BD Biosciences, Franklin Lakes, USA
Berthold Tristar 3	Berthold, Bad Wildbad, Germany
CO2 Incubator (BBD 6220)	Heraeus, Hanau, Germany
CytoFLEX LX Flow Cytometer	Beckman Coulter, Brea, USA
Eppenford Research Plus Pipets	Eppendorf, Hamburg, Germany
Heracell 240i	Heraeus, Hanau, Germany
Heracell150	Heraeus, Hanau, Germany
Herasafe KS	Heraeus, Hanau, Germany
Integra Pipet Boy 2	Integra Biosciences, Zizers, Switzerland
MagRack 6	Cytiva, Marlborough, USA
Multifuge 3 L-R	Heraeus, Hanau, Germany
Multifuge X3R	Heraeus, Hanau, Germany
Neubauer Haemocytometer	Optik Labor Frischknecht, Balgach, Germany
Primovert Microscope	Zeiss, Jena, Germany
Rotina 420 R	Hettich, Tuttlingen, Germany
Table Top Centrifuge Fresco 17	Heraeus, Hanau, Germany
Thermocycler Gene Touch	BIOER, Hangzhou, China
xCELLigence RTCA MP	ACEA Biosciences Inc., San Diego, USA
xCELLigence RTCA SP	ACEA Biosciences Inc., San Diego, USA

2.1.4 Software

Table 5: Software

Software	Manufacturer
BD FACSDiva v8.0.1	BD Biosciences, Franklin Lakes, USA
CytExpert v2.4	Beckman Coulter, Brea, USA
EndNote vX9.3.3	Clarivate Analytics, Philadelphia, USA
FlowJo v10.8.1	BD Biosciences, Franklin Lakes, USA
GraphPad PRISM® v9.5.1	GraphPad Software, La Jolla, USA
Microsoft Office 365	Microsoft, Redmond, WA, USA
MicroWin 2000	Berthold Technologies, Bad Wildbad, Germany
MikroWin v5.24	Mikrotek Laborsysteme GmbH, Overath, Germany
RTCA Software Pro v2.6.1	ACEA Biosciences Inc., San Diego, USA

2.1.5 Kits

Table 6: Kits

Kit	Manufacturer
EdU Assay/EdU Staining Proliferation Kit (iFluor488)	Abcam, Cambridge, UK
Foxp3/Transcription Factor Staining Buffer Set	eBioscience, San Diego, USA
P3 Primary Cell 4D-Nucleofector™ X Kit L	Lonza, Basel, Switzerland
P3 Primary Cell 4D-Nucleofector™ X Kit S	Lonza, Basel, Switzerland

2.1.6 gRNA

Table 7: crRNA Sequences

Target	Sequence	Manufacturer
PTGER2	/AlTR1/rGrUrArGrArArGrUrArArGrGrGrUrArCrCrCrGrArGrUrUr UrUrArGrArGrCrUrArUrGrCrU/AlTR2/	IDTDNA
PTGER2	/AlTR1/rCrCrUrGrCrCrGrCrUrGrCrUrCrArArCrUrArCrGrGrUrUr UrUrArGrArGrCrUrArUrGrCrU/AlTR2/	IDTDNA
PTGER4	/AlTR1/rArCrArGrGrCrCrArCrCrGrArArGrCrUrArCrCrGrGrUrUr UrUrArGrArGrCrUrArUrGrCrU/AlTR2/	IDTDNA
PTGER4	/AlTR1/rCrCrArGrCrCrGrCrUrUrGrUrCrCrArCrGrUrArGrGrUrUr UrUrArGrArGrCrUrArUrGrCrU/AlTR2/	IDTDNA

2.2 Animal work

2.2.1 Mouse strains and mouse handling

Mouse strains used in animal studies:

Male C57BL/6RJ mice were purchased from Charles River and were housed in specific pathogen free conditions. All animal experiments were approved by the Regierung von Oberbayern (reference number ROB-55.2-2532.Vet_02-20-208) and were executed according to its guidelines. As specified in the animal experiment application, all mice were inspected at least every second or third day, if necessary, every day, by monitoring the overall condition of the mice, taking the weight, and measuring the tumor size with a caliper. For this, the mouse was restrained by hand and the tumor size was measured across its widest side and in a 90-degree angle to the first measurement.

Survival analyses were performed randomized and blinded. Survival was defined by humane surrogate parameters corresponding to termination criteria, which were specified in the animal experiment application. In detail, the overall health condition of the mice, weight loss of more than 15 % from their highest bodyweight, ulceration of the tumor, tumor area greater than 225 mm^2 or the longest tumor diameter greater than 15 mm were considered as termination criteria.

Mouse strains used for organ donation:
Male C57BL/6RJ mice were purchased from Charles River, mice transgenic for the ovalbumin-specific T cell receptor OT-I (obtained from The Jackson Laboratory, stock number 003831) and mice with the congenic marker CD90.1 (kind gift from Dr. Reinhard Obst (LMU)) were bred at the animal facility of the LMU Klinikum and were housed in specific pathogen free conditions. Spleens of CD45.1-CD4^{Cre}Ptger2$^{-/-}$Ptger4$^{fl/fl}$ mice for T cell isolation were a kind gift from Dr. Jan Böttcher (Technical University of Munich).

2.2.2 Subcutaneous injection of tumor cells

To ensure the cells are in an exponential growth phase at injection time, D4M.3A-SIINFEKL cells were split 1:2 the day before the injection. On the day of injection, the cells were detached with trypsin. The tumor cells were washed with phosphate buffered saline (PBS) three times and were adjusted to 10^7 cells/ml (10^6 cells/mouse in 100 µl PBS). The cells were kept on ice until the injection.
Before the injection, the tumor cells were resuspended and then loaded into a 1 ml syringe without any remaining air. While the mouse was restrained by hand, the injection site on the right flank was disinfected. The syringe was driven through the skin and by lifting the skin inserted further between skin and peritoneum. Approximately 1 cm away from the intruding site, 100 µl of the cell suspension were injected.

2.2.3 Intravenous injection of T cells

T cell preparation for survival studies:
T cells were washed in PBS three times and the cell number was adjusted to 10^8 cells/ml in PBS (10^7 cells/mouse in 100 µl PBS). The T cells were kept on ice until the injection.

T cell preparation for tracking experiments:
To facilitate T cell tracking, CD45.1-CD4^{Cre}Ptger2$^{-/-}$Ptger4$^{fl/fl}$ mice and CD90.1 mice were used as organ donors. After transduction with the OT-I receptor and expansion of the T cells, they were washed three times with PBS, and mixed in a 1:1 live cell ratio. The cell number was adjusted to 1 x 10^8 cells/ml in PBS (1 x 10^7 cells/mouse in 100 µl PBS, thereof 5 x 10^6 CD45.1-CD4^{Cre}Ptger2$^{-/-}$Ptger4$^{fl/fl}$ and 5 x 10^6 CD90.1 T cells). The T cells were kept on ice until the injection.

Intravenous injection:
Before the injection, the T cells were resuspended and then loaded into a 1 ml syringe without any remaining air. The mouse was placed in a restrainer and the injection site on the tail was disinfected. The needle was inserted into the tail vain and 100 µl of the cell suspension were injected. After pulling out the needle, a piece of tissue was pressed on the injection site for several seconds to stop the bleeding.

2.2.4 Survival studies

Male C57BL/6RJ mice were subcutaneously (s.c.) injected with 10^6 D4M.3A-SIINFEKL cells in 100 µl PBS in the right flank (see chapter 2.2.2). After six days, when most tumors reached a size between 4 x 4 to 6 x 6 mm, mice were randomized according to tumor size and were treated with 10^7 T cells in 100 µl PBS by intravenous (i.v.) injection (see chapter 2.2.3). The tumor size was measured and termination criteria as pre-defined in the animal experiment application and described in chapter 2.2.1 were taken as a surrogate for survival.

2.2.5 *In vivo* T cell tracking experiments

Male C57BL/6RJ mice were subcutaneously (s.c.) injected with 10^6 D4M.3A-SIINFEKL cells in 100 µl PBS in the right flank (see chapter 2.2.2). After eight days, mice were treated with 10^7 T cells in 100 µl PBS by intravenous (i.v.) injection (see chapter 2.2.3). 2 and 5 days post T cell injection, 5 mice each were injected with 5-ethynyl-2'-deoxyuridine (EdU) in PBS i.p. and 4 h after injection the mice were sacrificed and single cell suspension were prepared from tumor, spleen, lymph nodes and blood (see chapter 2.2.6). After 9 and 14 days post T cell injection, mice were sacrificed and single cell suspensions were prepared without prior EdU injection. On days 2 and 5 post T cell injection, the single cell suspensions were separated into three aliquots and stained with one of the panels listed in Table 8 each. On days 9 and 14 post T cell injection, the single cell suspension was divided into two aliquots and stained with the activation/exhaustion and differentiation panel, respectively. Surface stainings for all panels were done as described in chapter 2.4.1.1, for the EdU panel on day 2 and 5 post T cell injection, an EdU staining was done additionally with the EdU Assay/EdU Staining Proliferation Kit (iFluor488) (abcam) according to the manufacturer's instructions. All samples were analyzed on a BD LRSFortessa™ Cell Analyzer.

Table 8: Panel composition for *in vivo* tracking of T cells

Proliferation panel		**Activation/Exhaustion panel**		**Differentiation panel**	
Marker	**Color**	**Marker**	**Color**	**Marker**	**Color**
FVD	eFluor780	FVD	eFluor780	FVD	eFluor780
CD45.1	BV711	CD45.1	BV711	CD45.1	BV711
CD90.1	APC	CD90.1	APC	CD90.1	APC
OT1 TCR	PerCP-Cy5.5	OT1-TCR	FITC	OT1-TCR	FITC
CD3	PB	CD3	PB	CD3	PB
CD4	AF700	CD4	AF700	CD4	AF700
CD8	BV786	CD8	BV786	CD8	BV786
EdU	FITC	CD69	BV510	CD44	PerCP-Cy5.5
		PD1	PE	CD62L	PE-CF594
		TIM3	BV605	CD95	PE
		LAG3	PerCP-Cy5.5		

2.2.6 Preparation of organ single cell suspensions

Blood was taken from the vena facialis before sacrificing the animal. For all other investigated organs, organ donor or experimental mice were killed by cervical dislocation. The mice were opened, and the desired organs were removed.

Spleen and lymph nodes:
Spleens were removed from the body and stored in PBS on ice until further use. To obtain a single cell suspension, spleens were passed through a 100 µm strainer stacked on top of a 30 µm strainer in a 50 ml tube using a syringe plunger and rinsing with 30 ml PBS. Cells were centrifuged at 400 x g for 5 min, the supernatant was discarded, and the cells were resuspended in 2 ml of erythrocyte lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, 100 µM $C_{10}H_{16}N_2O_8$, pH = 7.2) for 2 min. The tubes were filled up with PBS to stop erythrocyte lysis. After centrifugation at 400 x g for 5 min, the supernatant was discarded and the splenocytes were resuspended in the desired amount of PBS.

Tumor:
Tumors were removed from the body and stored in PBS on ice until further preparation. For this, tumors were cut thoroughly using scalpels and were then treated with 1 mg/ml collagenase (Sigma Aldrich, Germany) and 0.05 mg/ml DNase (Sigma Aldrich, Germany) for 30 min at 37 °C. The digested cells were further processed through strainers and erythrocyte lysis as described above for spleens and lymph nodes.

Blood:
Blood was collected from the vena facialis in Eppendorf tubes containing Heparin. The blood was then diluted in 10 ml of erythrocyte lysis buffer and incubated for 10 min at

room temperature. The tubes were filled up with PBS to stop erythrocyte lysis. After centrifugation at 400 x g for 5 min, the supernatant was discarded and the splenocytes were resuspended in the desired amount of PBS.

2.3 Cell culture methods

2.3.1 Cell lines and culture conditions

The packaging cell line 293VecEco-anti-EpCAM-CAR-mCherry has been described before[40] and 293VecEco-OT1-TCRαβ have been generated in the same manner. Panc02-OVA-EpCAM cells have been described previously as well[40,41]. D4M.3A-SIINFEKL-H2B-cerulean were a kind gift of Thorsten Mempel (Massachusetts General Hospital)[42].
All cell lines and primary cells used for this work were incubated at standard conditions (37 °C, 5 % CO_2, 95 % humidity). Detailed culture conditions are listed in Table 9.

Table 9: Culture conditions and origin of cell lines

Cell line	Culture medium	Origin
293VecEco-anti-EpCAM-CAR-mCherry	DMEM + 10 % FCS + 2 % L-Glu + 1 % Penicillin/Streptomycin	Producer cell line modified from 293VecEco (gift from Prof. Dr. Manuel Caruso, Québec, Canada)
293VecEco-OT1-TCRαβ	DMEM + 10 % FCS + 2 % L-Glu + 1 % Penicillin/Streptomycin	Producer cell line modified from 293VecEco (gift from Prof. Dr. Manuel Caruso, Québec, Canada)
Panc02-OVA-EpCAM	DMEM + 10 % FCS + 1 % L-Glu + 1 % Penicillin/Streptomycin	Chemically induced pancreatic cancer cell line[41]
D4M.3A-SIINFEKL-H2B-cerulean	DMEM/F12 + 10 % FCS + 1 % L-Glu + 1 % Penicillin/Streptomycin	$BRAF^{V600E}$ x $PTEN^{null}$ melanoma cell line (gift from Prof. Dr. Thorsten Mempel, MGH)
Primary murine T cells	RPMI + 10 % FCS + 1 % L-Glu + 1 % Penicillin/Streptomycin + 1 % Sodium Pyruvate + 0,1 % HEPES	splenocytes obtained from organ donor mice (C57BL/6RJ, CD90.1 or CD45.1-$CD4^{Cre}Ptger2^{-/-}Ptger4^{fl/fl}$)

Suspension cells were adjusted to 10^6 cells per ml every two to three days. Adherent tumor cell lines were split every two to three days by trypsinization after washing away residual medium with PBS. Producer cell lines (293VecEco) were split twice a week after physical detachment.

2.3.2 Cell number determination

To determinate cell numbers, a Neubauer haemocytometer was used. For this, cells were detached by trypsination. The obtained single cell suspension was diluted 1:10 in trypan blue and 10 µl were loaded into a Neubauer haemocytometer. Cells were counted in all squares and the cell concentration was calculated with formula 1:

Formula 1:

$$\frac{Sum\ of\ cells\ in\ all\ squares}{Number\ of\ counted\ squares} \cdot dilution\ factor \cdot 10^4\ \frac{cells}{ml} = cells\ per\ ml\ cell\ suspension$$

2.3.3 Production of virus supernatant for the retroviral transduction of T cells

Two to three days before the planned harvest date of the virus, 293VecEco cells were seeded into a cell culture flask. When 80-90 % confluence was reached the virus-containing supernatant was removed from the cells and centrifuged at 400 x g for 5 min to remove residual cell contaminations. For transduction of T cells for *in vivo* experiments, instead of centrifuging the virus-containing supernatant was filtered using a 45 µm sterile filter. The supernatant was used for transductions immediately or was stored at -20 °C until further use.

2.3.4 Retroviral transduction of primary murine T cells

Splenocytes were isolated as described in 2.2.6 and resuspended at 2 Mio cells per ml in basic murine T cell medium containing 50 ng/ml β-mercaptoethanol and were stimulated with 1 ng/ml IL-2 and anti-CD3 antibodies (1:1000 dilution) as well as anti-CD28 antibody in a 1:10.000 dilution for 24 h. In parallel, a sterile non-tissue culture coated 24-well plate was coated over night at 4 °C by adding 400 µl of a 1:50 dilution of RetroNectin in PBS per well. On the next day, the RetroNectin was removed from the plate and each well was blocked with 500 µl of blocking buffer (2 % BSA in PBS, sterile filtered). After incubation for 30 min at room temperature, the blocking buffer was removed and the wells were washed once with 1 ml of washing buffer (1:40 dilution of HEPES in PBS). 2 ml of virus-containing supernatant (preparation described in 2.3.3) were added to each washed well and the plate was centrifuged at 3000 x g for 2 h at 4 °C. In the meantime, pre-activated T cells from the previous day were adjusted to 10^6 cells per ml in fresh basic murine T cell medium containing 50 nM β-mercaptoethanol and 1 ng/ml IL-2. After centrifugation, the supernatant was removed from the wells and 1 ml of the T cell suspension was added to each well. Additional wells were filled to produce untransduced control cells. 10 µl Dynabeads™ Mouse T-Activator CD3/CD28 were added and the plate was centrifuged at 800 x g for 30 min at 32 °C. Cells were

incubated under standard conditions over night. On the following day, T cells were either CRISPRed or expansion of the CAR T cells was started.

2.3.5 Expansion of murine T cells

After transduction or CRISPRing, murine T cells were expanded every second day. For this, the cell number was adjusted to 10^6 cells per ml in fresh basic murine T cell medium containing 50 µM β-mercaptoethanol and 50 ng/ml IL-15.

2.3.6 Tracking CAR T cell-mediated tumor cell killing kinetics in an xCELLigence assay

2,5 x 10^4 T cells were plated in 100 µl T cell expansion medium. The T cells were treated with 250 ng/ml PGE_2 or the respective volume of vehicle solution and were incubated for 48 h at standard conditions. The wells of an xCELLigence E-Plate 96 were filled with 50 µl of DMEM + 10 % FCS + 1 % L-Glu + 1 % Pen/Strep (DMEM3+) and a baseline measurement in the xCELLigence RTCA analyzer was taken. After that, 2,5 x 10^4 tumor cells were seeded in 100 µl DMEM3+ into each well. This step was timed in a way that the tumor cells would be in the exponential growth phase when the T cells reached an PGE_2-treatment time of 48 h. After 48 h of incubation with PGE_2 or vehicle solution, the T cells were centrifuged at 400 x g for 5 min. The expansion medium was removed and replaced by 100 µl DMEM3+ + 2 % Sodium Pyruvate + 0,2 % HEPES, again containing 250 ng/ml PGE_2 or vehicle solution. Then, T cells were transferred on top of the already plated tumor cells. The Cell Index was measured at least once every 60 min until the Cell Index of the tumor only control was decreasing.

2.3.7 Assessment of CAR T cell proliferation and survival

For each time point (0 h, 24 h, 48 h and 72 h), 10^5 T cells were plated into a 96-U-well plate in triplicates in expansion medium. CAR T cells were treated with 250 ng/ml PGE_2 or the respective volume of vehicle solution. 3 hours before each time point 15 µM EdU were added to each well. After 3 hours of incubation, a live cell staining with the Fixable Viability Dye eFlour™ 780 was performed as described in detail in 2.4.1.1. Subsequently, cells were fixed, permeabilized and then stained for EdU with the EdU Assay/EdU Staining Proliferation Kit (iFluor488) (abcam) according to the manufacturer's instructions. 2,5 µl CountBright™ Absolute Counting beads were added to each sample directly before acquiring the sample at a BD FACSCanto II or Cytoflex LX Cytometer.

2.3.8 Determination of CAR T cell activation capacity

One day before assay start, 1 µg/ml recombinant Fc-tagged EpCAM or 1 % BSA were coated to a 96-flat-well in 50 µl PBS. T cells were pre-treated with 250 ng/ml PGE_2 or vehicle solution for 8 h in murine or human T cell expansion medium. Before adding the T cells, the coated plate was washed once with PBS. 2 x 10^5 T cells were then added to each coated well in T cell medium containing BD GolgiStop (1:1400), BD GolgiPlug (1:1000) and PGE_2 or vehicle solution. After incubation for 16 h, a live cell staining was done with Fixable Viability Dye eFlour™ 780 as well as a surface stains for CD4-Pacific Blue and CD8-FITC (see 2.4.1.1), subsequently cells were fixed, permeabilized and intracellularly stained for IFNγ-PE-Cy7 (see section 2.4.1.2). Samples were analyzed on a BD LRSFortessa™ Cell Analyzer or Cytoflex LX.

2.3.9 Assessing EP2 and EP4 downstream signaling by monitoring CREB phosphorylation

To avoid incubation times after PGE_2 stimulation that can lead to a loss of the induced phosphorylation until fixation of the cells, the surface stain with Fixable Viability Dye eFlour™ 780, anti-CD4 and anti-CD8 was done as described in 2.4.1.1 before the start of the assay. Stained cells were counted and at least 2 x 10^5 cells were plated in a 96-U-well plate in 100 µl basic T cell medium with or without 1600 ng/ml PGE_2. T cells were incubated for 60 min at standard conditions and were subsequently stained for CREB phosphorylation as described in 2.4.1.3.

2.3.10 CRISPR/Cas9-mediated knockout of EP2 and EP4 in CAR T cells

CRISPRing of CAR T cells was always performed on the day after the first transduction hit. Two different gRNAs were used in combination for each of the receptors EP2 and EP4, the sequences are listed in Table 7. trRNA and crRNA were mixed in equal amounts and hybridized by incubation for 5 min at 95 °C and subsequent cooling to room temperature over 10 min. Three parts of the thus observed gRNA were then mixed with one part of Alt-R® Cas9 Electroporation Enhancer (stock concentration 25 µM) and one part of Alt-R® S.p. Cas9 Nuclease V3 and were incubated for 15 min at room temperature to form the ribonucleoprotein complex (RNP). In the meantime, the Dynabeads™ Mouse T-Activator CD3/CD28 beads were removed from the cells using a magnetic rack, the cells were then washed in PBS and adjusted to the desired cell number in electroporation buffer P3 (Lonza). For a small-scale production 1-2 x 10^6 CAR T cells were resuspended in 20 µl of electroporation buffer and were then mixed with 3 µl of each RNP (2 RNPs for EP2 and 2 RNPs for EP4, 4 RNPs in total). The mixture was nucleofected in a 20 µL 16-well Nucleocuvette™ Strip. For large-scale productions 5-10 x 10^6 CAR T cells were resuspended in 80 µl electroporation buffer and were then

mixed with 5 µl of each RNP (4 RNPs, 20 µl total). The mixture was nucleofected in a 100 µL NucleocuvetteTM Vessel. Irrespective of the production scale, for the pulse-program CM137 was used with P3 as the selected buffer setting. After nucleofection, pre-warmed basic T cell medium was added to the strip-well (100 µl) or the cuvette (1 ml) and the cells were transferred into a 24-well (small-scale) or to a 6-well (large-scale). The strip-well/cuvette were rinsed again twice with the same amount of basic T cell medium. The amount of basic T cell medium was adjusted to 500 µl (24-well) or 4 ml (6-well) and after resting of the cells for 30 min at 37 °C the cell number was adjusted to 10^6 cells per ml and 10 µl Dynabeads™ Mouse T-Activator CD3/CD28 beads were added per each 10^6 cells. Expansion of the CAR T cells was started as described in 2.3.5.

2.4 Immunological methods

2.4.1 Flow cytometry

2.4.1.1 Surface staining

Cells were centrifuged for 5 min at 400 x g and pellets were resuspended in 50 µl staining master mix containing eFluor780 FVD (1:5000 for *in vitro* and 1:1000 for *in vivo* samples) and 1:100 dilutions of the respective antibodies in PBS. Samples were stained for 10 min at 4 °C in the dark. 150 µl PBS were added and cells were centrifuged at 400 x g for 5 min. Hereafter, cells were either directly analyzed in a flow cytometer or it was proceeded with one of the intracellular stains described in 2.4.1.2, 2.4.1.3 or 2.3.7.

2.4.1.2 Intracellular staining

Intracellular stainings were done with the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set according to the manufacturers' instructions.

2.4.1.3 Staining of phosphorylation-specific sites

After stimulation T cells were fixed for 10 min at 37 °C by adding 100 µl BD Cytofix™ Fixation Buffer directly into the wells without removing the medium first. Fixed cells were washed once with 200 µl PBS and were then permeabilized with 200 µl ice-cold BD Phosflow™ Perm Buffer III for 30 min on ice in the dark. Cells were centrifuged for 5 min at 400 x g. The supernatant was discarded and cells were washed with 200 µl PBS twice. After discarding the supernatant, pellets were resuspended in 50 µl Alexa Fluor® 647 Mouse Anti-CREB (pS133)/ATF-1 (pS63) diluted 1:20 in PBS.

3. Results

The inhibition of T cells by PGE_2 is described since the 1970ies[20], although it is not well understood until today. Currently, there is little known on the effects of PGE_2 on CAR T cells, but we hypothesized that adoptively transferred T cells will be at least equally affected as one other publication already demonstrates[30].

3.1 PGE_2 reduced CAR T cell performance *in vitro*

Under normal conditions, anti-EpCAM CAR T cells are capable to completely control the pancreatic tumor cell line Panc02-OVA-EpCAM *in vitro*. However, pre-treatment of murine anti-EpCAM CAR T cells with 250 ng/ml PGE_2 for 48 h fully impaired the killing of the tumor cells by the CAR T cells (Figure 1).

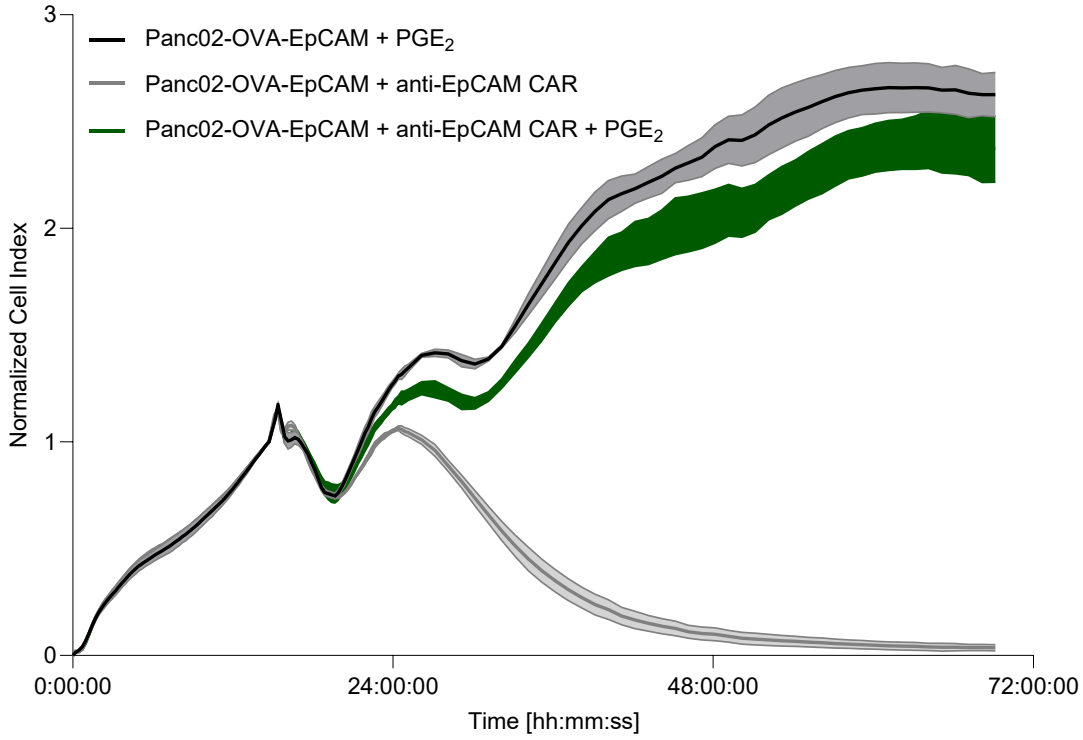

Figure 1: Prostaglandin E_2 pre-treatment of CAR T cells severely inhibited *in vitro* tumor cell control. 5 x 10^4 anti-EpCAM CAR T cells were treated with 250 ng/ml of PGE_2 or vehicle solution in expansion medium for 48 h. 2,5 x 10^4 Panc02-OVA-EpCAM cells were seeded into an xCELLigence RTCA analyzer. After CAR T cell pre-treatment, the expansion medium was replaced by cytokine-free murine T cell medium containing 250 ng/ml PGE_2 or vehicle solution. T cells were transferred to the tumor cells and the Cell Index was observed over time (shown is a representative of n = 4 independent repetitions).

As the incubation time for 48 h in the presence of expansion medium allows the CAR T cells to proliferate during the pre-incubation time, it is possible that the difference observed between vehicle and PGE_2-treated CAR T cells may not only be due to an altered killing capacity but may also reflect changes in cell numbers. Therefore, the

influence of PGE_2 on the survival and proliferation of CAR T cells has to be considered additionally.

3.1.1 The inhibition of CAR T cell performance by PGE_2 was caused by impaired T cell survival but not activation

To control for changes of the cell numbers caused by the PGE_2 pre-treatment, anti-EpCAM-CAR T cells were expanded under normal conditions in the presence of PGE_2 or vehicle solution. Cell numbers, percentage of proliferating cells and viability were monitored over time. Indeed, the addition of PGE_2 to the expansion medium led to a reduced number of living T cells within 48 h. This is explained by a shutdown of the proliferative capacity after already 24 h which is followed by a decrease in the viability after 48 h, suggesting bad persistence and survival of CAR T cells in a PGE_2-rich environment.

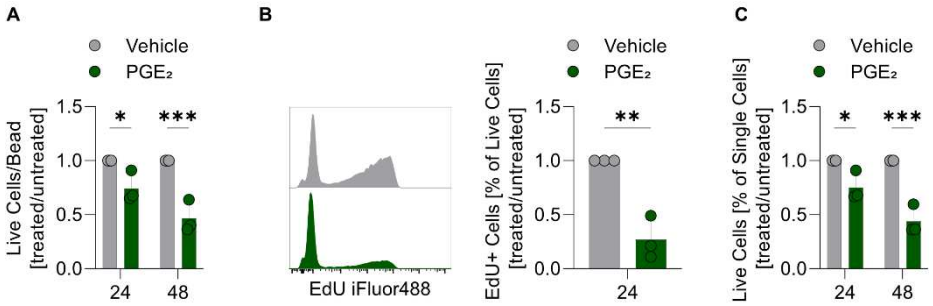

Figure 2: PGE_2 reduces anti-EpCAM-CAR T cell numbers *in vitro* by impairing T cell survival and proliferation. 10^5 anti-EpCAM-CAR T cells were cultured in expansion medium containing 250 ng/ml PGE_2 or vehicle solution for 24 and 48 h. At the respective time points, T cell numbers (A), EdU incorporation as a surrogate for proliferation (B) and viability (C) were measured (n = 3 independent repetitions, significance was calculated with a 2way ANOVA (A, C) or unpaired two-tailed Student's t test (B)).

In contrast to survival and proliferation, the activation of CAR T cells by their antigen, measured as IFNγ production on single cell level, did not seem to be changed by PGE_2-treatment.

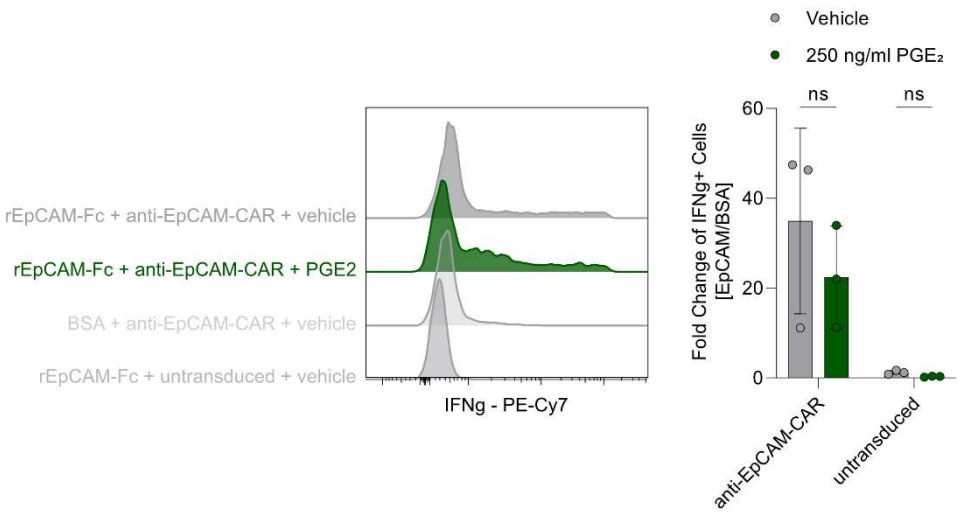

Figure 3: PGE_2-stimulation does not influence CAR T cell activation. 10^5 anti-EpCAM CAR T cells were pre-treated for 8 h with 250 ng/ml PGE_2 or vehicle solution, stimulated with recombinant EpCAM-Fc (rEpCAM-Fc) and stained for IFNγ production. Depicted is a representative IFNγ staining and the pooled data from n = 3 independent repetitions. Significance was calculated with a 2way ANOVA and Tukey's multiple comparisons test.

Although detailed mechanistic information is still missing, it seems that the cause for the impaired function of anti-EpCAM-CAR T cells observed in 3.1.1 is rather caused by differences in the CAR T cell numbers than by an impaired killing capacity.

3.1.2 PGE_2 acted on CAR T cells via its receptors EP2 and EP4

Although expression of EP1 and EP3 have been described on T cells, EP2 and EP4 have been identified as the main mediators of PGE_2-singnaling on T cells[21,26,28,29,33,35]. Therefore, EP2 and EP4 signalling were monitored by looking at their pathway activation downstream of the receptors. Upon PGE_2 stimulation via EP2 and EP4, the cAMP level in T cells increases, which leads to PKA activation and ultimately to phosphorylation of the transcription factor cAMP response element-binding protein (CREB).

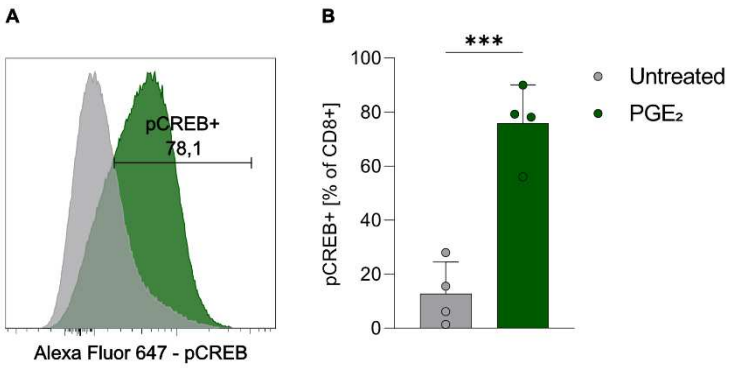

Figure 4: PGE_2 stimulation leads to CREB phosphorylation. 2 x 10^5 anti-EpCAM CAR T cells were stimulated with 1600 ng/ml PGE_2 for 60 min and subsequently stained for CREB phosphorylation. (A) MFI change caused by PGE_2 stimulation (representative experiment of n = 4 independent repetitions) (B). CD8+ pCREB+ % cells (pooled data of 4 independent repetitions, significance was calculated with an unpaired, two-tailed Student's t test.

Indeed, signaling downstream of EP2 and EP4 could be observed upon PGE_2 stimulation.

3.2 Knockout of EP2 and EP4 protected CAR T cells from PGE_2

As described in the previous chapter, PGE_2 has pronounced inhibitory effects on CAR T cells *in vitro*, which are accompanied by pathway activation downstream of its receptors EP2 and EP4. Therefore, a double knockout of both receptors might be able to rescue CAR T cells in a PGE_2-rich tumor microenvironment. Thus, to generate those $EP2^{-/-}EP4^{-/-}$ CAR T cells, the CRISPR/Cas9 system was used. For this, T cells were first isolated from mouse splenocytes and then transduced with the anti-EpCAM-CAR as described in chapters 2.2.6 and 2.3.4. 24 h after the transduction, EP2 and EP4 were knocked out from the anti-EpCAM-CAR T cells by CRISPR/Cas9 (see 2.3.9).

3.2.1 Double knockout of EP2 and EP4 in CAR T cells was feasible using the CRISPR/Cas9 system

To validate the efficiency of the EP2 and/or EP4 knockout, downstream pathway activation was monitored by measuring cAMP production and CREB phosphorylation. Upon stimulation with PGE_2, only $EP2^{-/-}EP4^{-/-}$ CAR T cells, but not $EP2^{-/-}$ or $EP4^{-/-}$ CAR T cells showed a complete shutdown of their downstream signaling pathway, demonstrating the need for and feasibility of an EP2 and EP4 double knockout.

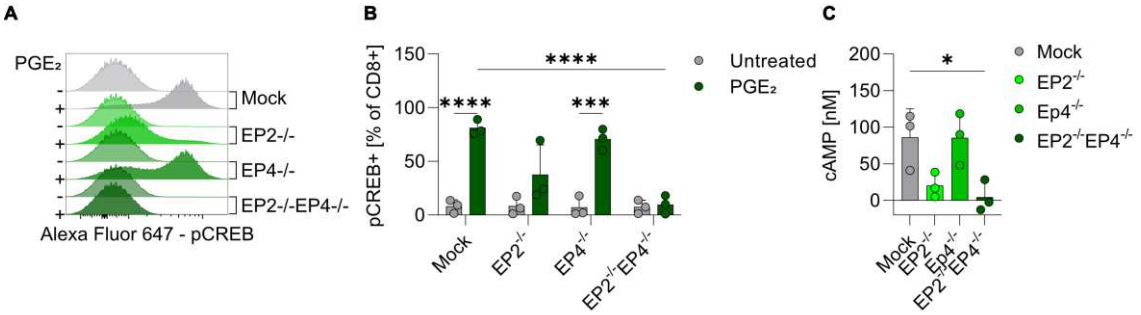

Figure 5: $EP2^{-/-}EP4^{-/-}$ CAR T cells do not show downstream pathway activation upon PGE_2 stimulation. At least 2 x 10^5 were stimulated with 1600 ng/ml PGE_2 for 60 min and subsequently stained for CREB phosphorylation. (A) MFI change caused by PGE_2 stimulation (representative experiment of n = 3 independent repetitions). (B) pCREB+ cells as percentage of CD8+ cells (n = 3 independent repetitions, significance was calculated with a 2way ANOVA with Tukey's multiple comparison correction). (C) 10^5 anti-EpCAM-CAR T cells with EP2 and/or EP4 knockout or mock-CRISPRed cells were stimulated with 2000 ng/ml PGE_2 for 60 min (n = 3 independent repetitions, significance was calculated with ordinary one-way ANOVA with Dunnett's multiple comparison correction).

3.2.2 $EP2^{-/-}EP4^{-/-}$ CAR T cells were protected from PGE_2 *in vitro*

In the next step, it was investigated whether $EP2^{-/-}EP4^{-/-}$ CAR T cells that showed a complete inhibition of the intracellular pathway activation upon PGE_2 stimulation were also showing improved *in vitro* efficacy in the presence of PGE_2. Therefore, the anti-EpCAM-CAR T cell performance was assessed as described in chapter 3.1.

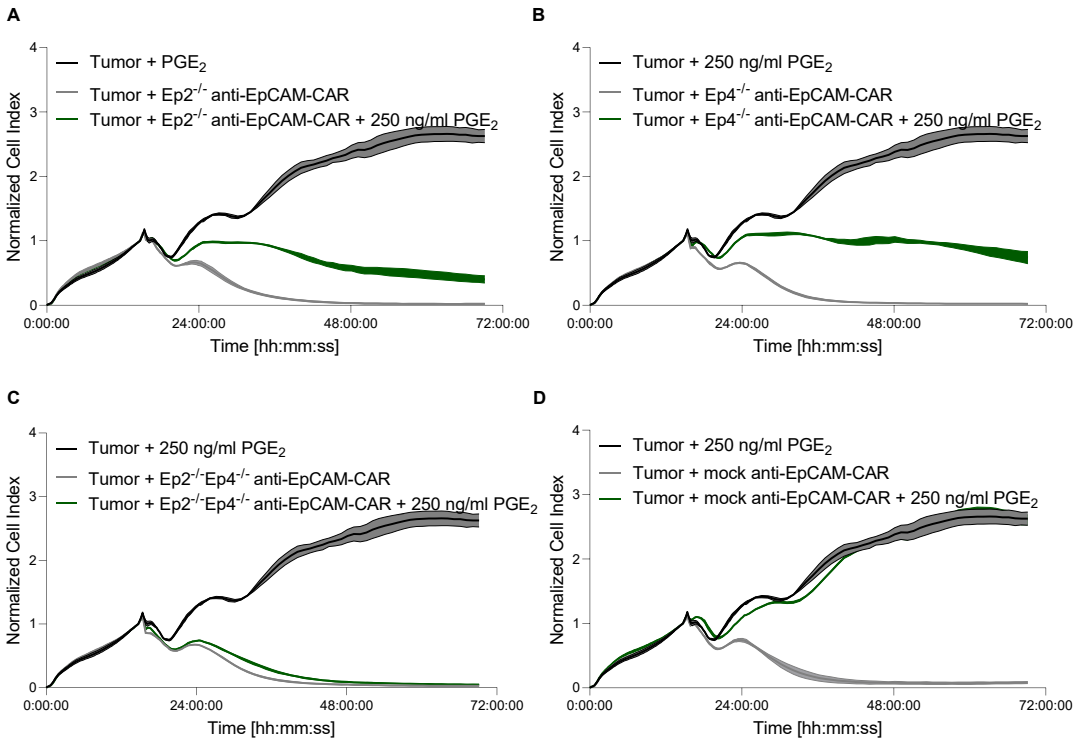

Figure 6: EP2 and EP4 double knockout is necessary to fully restore anti-EpCAM-CAR T cell *in vitro* function. 5 x 10^4 anti-EpCAM CAR T cells were treated with 250 ng/ml of PGE_2 or vehicle solution in expansion medium for 48 h. 2,5 x 10^4 Panc02-OVA-EpCAM cells (tumor) were seeded into an xCELLigence RTCA analyzer. After CAR T cell pre-treatment, the expansion medium was replaced by cytokine-free murine T cell medium containing 250 ng/ml PGE_2 or vehicle solution. T cells were transferred to the tumor cells and the Cell Index was observed over time for (A) $EP2^{-/-}$ anti-EpCAM-CAR T cells, (B) $EP4^{-/-}$ anti-EpCAM-CAR T cells, (C) $EP2^{-/-}EP4^{-/-}$ anti-EpCAM-CAR T cells and (D) mock-CRISPRed anti-EpCAM-CAR T cells (shown is a representative of n = 3 independent repetitions).

Without PGE_2 pre-treatment, the anti-EpCAM CAR T cells were all able to control the Panc02-OVA-EpCAM tumor cell growth. However, when pre-treated with 250 ng/ml PGE_2, the mock-CRISPRed CAR T cells showed severely impaired functionality as already observed for unCRISPRed CAR T cells in 3.1. EP2 and EP4 single knockout anti-EpCAM-CAR T cells already had a visibly improved functionality compared to mock-CRISPRed CAR T cells in the presence of PGE_2. Nevertheless, the double knockout of both EP2 and EP4 was necessary to fully restore CAR T cell function to the level of untreated CAR T cells.

This again is explained by the reduction of proliferation and survival of T cells by PGE_2. As already described for wild type CAR T cells, the mock-CRISPR CAR T cells showed a decrease in cell number, proliferation, and viability in the presence of PGE_2. Single knockout of EP2 or EP4 led to a small but mostly not significant increase in cell numbers caused by slightly, but not significantly improved proliferation and viability. $EP2^{-/-}EP4^{-/-}$ anti-EpCAM-CAR T cells, however, showed an increase in their viability, accompanied by improved proliferation and higher cell numbers after 24 h.

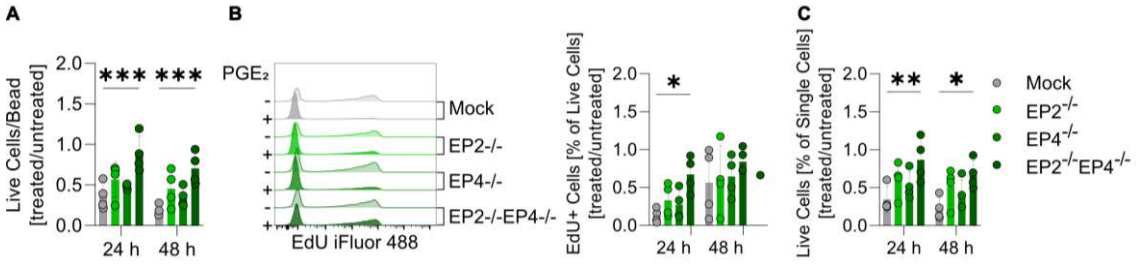

Figure 7: EP2$^{-/-}$EP4$^{-/-}$ anti-EpCAM-CAR T cells show improved expansion and viability in the presence of PGE$_2$. 10^5 anti-EpCAM-CAR T cells were cultured in expansion medium containing 250 ng/ml PGE$_2$ or vehicle solution for 24 and 48 h. At the respective time points, T cell numbers (A), EdU incorporation as a surrogate for proliferation (B) and viability (C) were measured (n = 3 independent repetitions, significance was calculated with a 2way ANOVA).

As already described in chapter 3.1, CAR T cell activation, measured as the percentage of cells producing IFNγ in response to antigen-stimulation, was not affected by PGE$_2$ in all CRISPR conditions irrespective of the EP2 and/or EP4 knockouts.

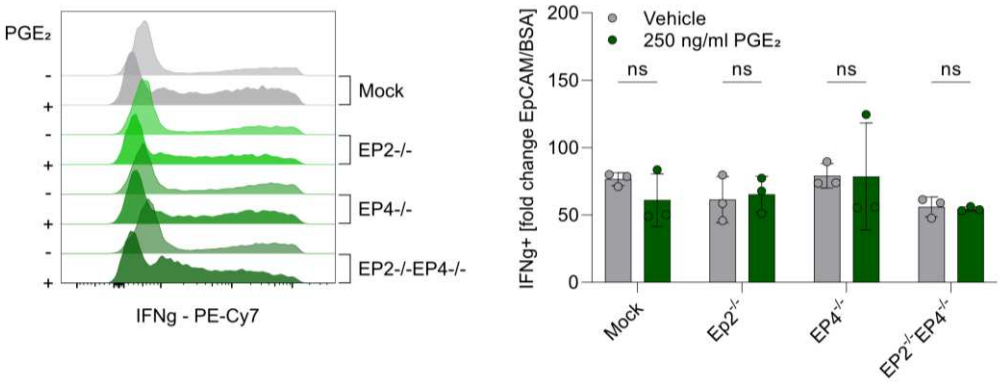

Figure 8: PGE$_2$-stimulation does not influence CAR T cell activation independent of the EP2 or EP4 knockouts. 10^5 anti-EpCAM CAR T cells were pre-treated for 8 h with 250 ng/ml PGE$_2$ or vehicle solution, stimulated with recombinant EpCAM-Fc (rEpCAM-Fc) and stained for IFNγ production. Depicted is a representative IFNγ staining and the pooled data from n = 3 independent repetitions. Significance was calculated with a 2way ANOVA and Tukey's multiple comparisons test.

3.3 EP2 and EP4 knockout improved the therapeutic effect of OT-I T cells *in vivo*

As described in the previous chapters, EP2$^{-/-}$EP4$^{-/-}$ anti-EpCAM-CAR T cells clearly showed superior function in a PGE$_2$-rich environment *in vitro*. However, the addition of external PGE$_2$ in high concentration hardly mimics a complete tumor microenvironment and thus it remains unclear whether the EP2 and EP4 double knockout will indeed be sufficient to improve the therapeutic efficacy of CAR T cells *in vivo* in a solid tumor model. To test whether a double knockout of EP2 and EP4 can be successfully done in a larger scale and be effective *in vivo*, T cells isolated from a mouse transgenic for the OT-I receptor specific for the ovalbumin peptide SIINFEKL were used for the generation of

EP2$^{-/-}$EP4$^{-/-}$ OT-I T cells. These cells were subsequently used to treat mice bearing subcutaneous D4M.3A-SIINFEKL-H2B-cerulean tumors.

3.3.1 EP2$^{-/-}$EP4$^{-/-}$ OT-I T cells facilitated better tumor control and prolonged mouse survival

Unmodified OT-I T cells increased the survival time and showed a visible, though not significant, reduction in the tumor volume in comparison to the PBS placebo treatment. However, eventually all mice still succumbed to the tumor over time. In contrast, EP2$^{-/-}$ EP4$^{-/-}$ OT-I T cells as well as EP2$^{-/-}$ OT-I T cells increased the survival time and tumor size compared to the therapy with wild type OT-I T cells. While EP4$^{-/-}$ OT-I T cells did not improve survival compared to the wild type OT-I T cells, still a shift towards improved survival and a reduction in the tumor size could be achieved.

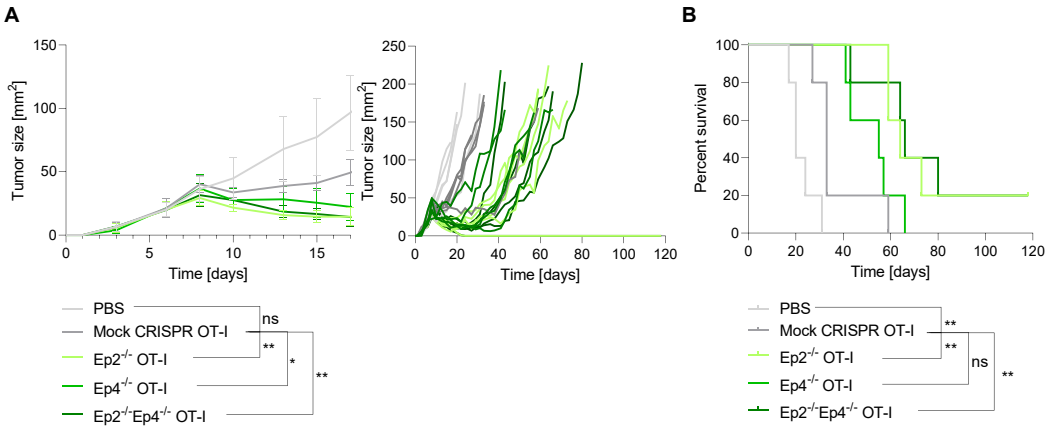

Figure 9: EP2$^{-/-}$EP4$^{-/-}$ OT-I T cells facilitated better tumor control and prolonged mouse survival. Mice were injected with 10^6 D4M.3A-SIINFEKL-cerulean cells s.c. on day 0. On day 6, mice were treated with 10^7 OT-I T cells with EP2 and/or EP4 knockout, wild type OT-I T cells or PBS. (A) Tumor growth (n = 5 mice per group, significance calculated with a 2way ANOVA and Tukey's multiple comparisons test) and (B) survival (n = 5 mice per group, significance calculated with a Log-rank (Mantel-Cox) test) are shown.

Thus, in the used melanoma model, the double knockout of EP2 and EP4 as well as the EP2 single knockout improved the efficacy of OT-I T cells *in vivo*.

3.3.2 Improved tumor control by EP2$^{-/-}$EP4$^{-/-}$ OT-I T cells was mediated through improved persistence in the tumor microenvironment

While the superior function of EP2$^{-/-}$EP4$^{-/-}$ T cells for the therapy of solid tumors producing PGE_2 could be confirmed in an *in vivo* melanoma model, many questions remain open concerning the underlying mechanism of action. To get some insights into the T cells kinetics and phenotype, the congenic markers CD45.1 and CD90.1 were used to track wild type and EP2$^{-/-}$EP4$^{-/-}$ OT-I T cells over four time points. Those markers facilitate the

possibility to distinguish the wild type from the EP2$^{-/-}$EP4$^{-/-}$ OT-I T cells as well as the injected T cell product from endogenous T cells. Therefore, wild type and EP2$^{-/-}$EP4$^{-/-}$ OT-I T cells can be monitored in the same tumor ruling out tumor-to-tumor differences in the tumor microenvironment. To get a more diverse overview of the behavior of wild type and EP2$^{-/-}$EP4$^{-/-}$ OT-I T cells in the tumor microenvironment, infiltration of T cells into and persistence in the tumor were monitored as well as proliferation, differentiation, activation, and exhaustion of the OT-I T cells.

In accordance with the *in vitro* data, EP2$^{-/-}$EP4$^{-/-}$ OT-I T cells showed an improved persistence in the tumor microenvironment, especially on the later time points. However, the EdU data suggests that the increased percentage of EP2$^{-/-}$EP4$^{-/-}$ OT-I T cells in the total T cell population in the tumor is not caused by an increased proliferation of the knockout T cells, suggesting that EP2$^{-/-}$EP4$^{-/-}$ OT-I T cells show improved persistence in the TME compared to WT OT-I T cells.

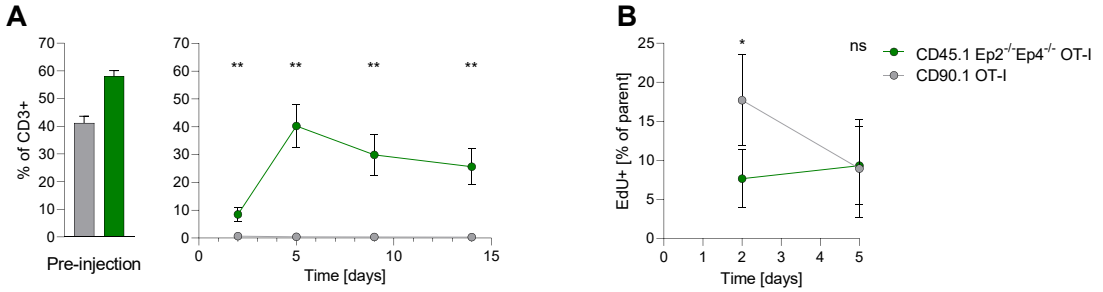

Figure 10: Improved persistence of EP2$^{-/-}$EP4$^{-/-}$ T cells in the tumor microenvironment. Mice were injected with 10^6 D4M.3A-SIINFEKL-cerulean cells s.c. on day 0. On day 8, mice were treated with 10^7 OT-I T cells consisting of wild type and EP2$^{-/-}$EP4$^{-/-}$ OT-I T cells in a 1:1 ratio. EP2$^{-/-}$EP4$^{-/-}$ OT-I T cells and wild type OT-I T cells were tracked by their congenic markers CD45.1 and CD90.1, respectively, over time. (A) Composition of the CD3+ T cells pre-injection and in the TME (n = 5 mice per group, significance calculated with a 2way ANOVA and Šídák's multiple comparisons test) and (B) proliferating T cells in the TME (n = 4 mice per group on day 2 and n=5 mice per group on day 5, significance calculated with a mixed-effects analysis and Šídák's multiple comparisons test).

To identify the reason for the inferior tumor control of wild type OT-I T cells in the TME, T cell activation and exhaustion as well as the differentiation state of the T cells were analyzed. However, due to the extremely low numbers of wild type OT-I T cells found in the tumor, this analysis proved to be challenging and results should be interpreted with care.

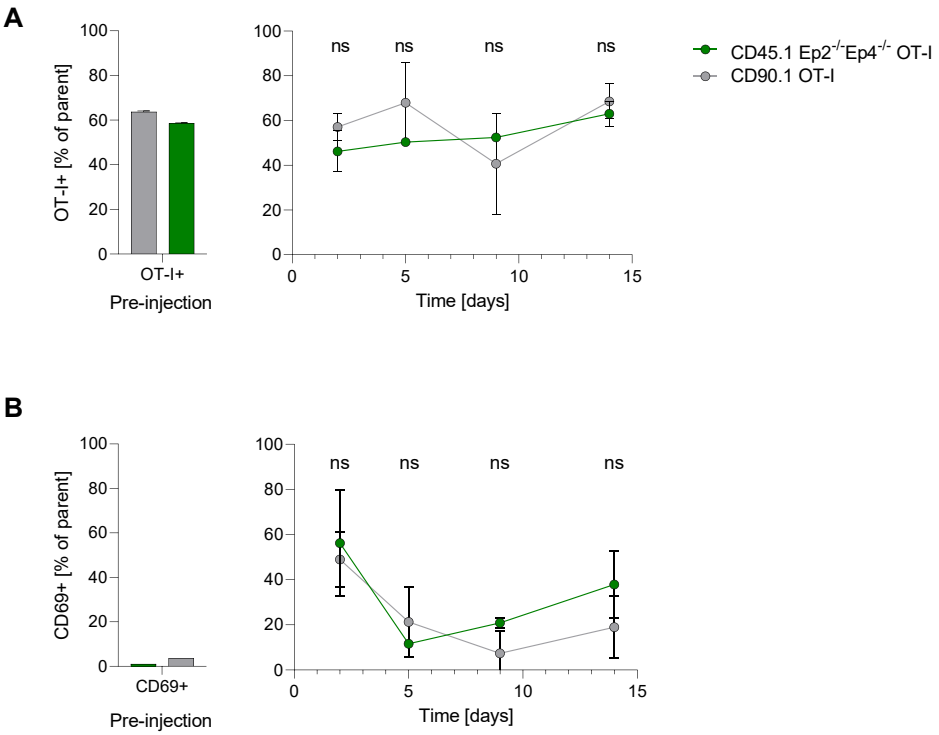

Figure 11: Wild type and EP2$^{-/-}$EP4$^{-/-}$ OT-I T cells show a similar activation capacity *in vivo*. Mice were injected with 10^6 D4M.3A-SIINFEKL-cerulean cells s.c. on day 0. On day 8, mice were treated with 10^7 OT-I T cells consisting of wild type and EP2$^{-/-}$EP4$^{-/-}$ OT-I T cells in a 1:1 ratio. OT-I (A) and CD69 (B) expression were analyzed in the tumor on day 2, 5, 9 and 14 (n = 5 mice per time point, significance calculated with a 2way ANOVA and Šídák's multiple comparisons test).

Neither OT-I expression nor CD69 levels were altered between wild type and EP2$^{-/-}$EP4$^{-/-}$ T cells, which, although it is not a perfect surrogate, indicates there is likely no difference in the cytotoxic capacity or activation of the cells, as was already observed *in vitro*.

When looking at exhausted T cells, which were defined as triple positive for PD1, TIM3 and LAG3, it is noticeable that they do only occur in the tumor and not in any of the peripheral organs analyzed and are elevated in the EP2$^{-/-}$EP4$^{-/-}$ T cells, especially at the later time points, when wild type T cells are barely detectable. Most likely, wild type T cells are excluded from the tumor as shown in Figure 10 before they can exert tumor control or subsequently become exhausted.

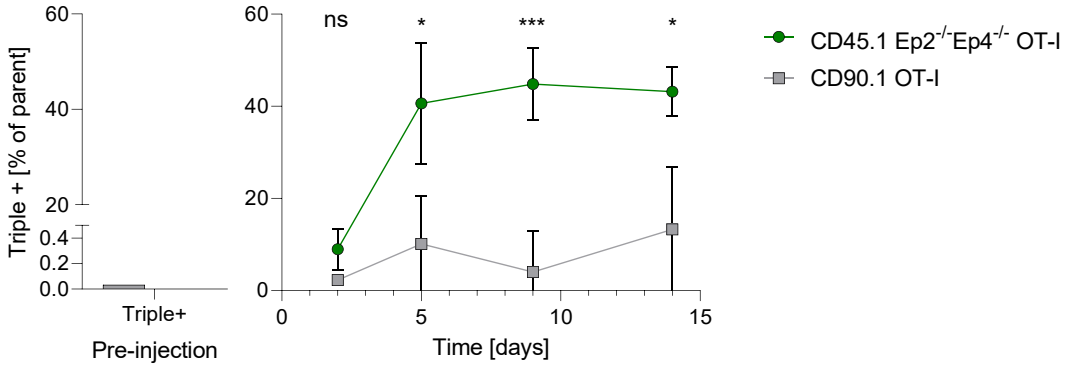

Figure 12: EP2$^{-/-}$EP4$^{-/-}$ T cells become exhausted in the tumor microenvironment. Mice were injected with 10^6 D4M.3A-SIINFEKL-cerulean cells s.c. on day 0. On day 8, mice were treated with 10^7 OT-I T cells consisting of wild type and EP2$^{-/-}$EP4$^{-/-}$ OT-I T cells in a 1:1 ratio. Percentages of PD-1, TIM3 and LAG3 triple positive, exhausted T cells are shown (n = 5 mice per group, significance was calculated with a 2way ANOVA and Šídák's multiple comparisons test).

Another important factor influencing tumor control is the differentiation state of the T cells. Traditionally, the protocol used for manufacturing of the OT-I T cells leads to a very high percentage of CD8+ T cells. However, a profound recovery of the CD4+ T cell population could be observed only in the tumor and only for the EP2$^{-/-}$EP4$^{-/-}$ T cells, indicating that the improved tumor control might at least partly be linked to an improved T helper cell response.

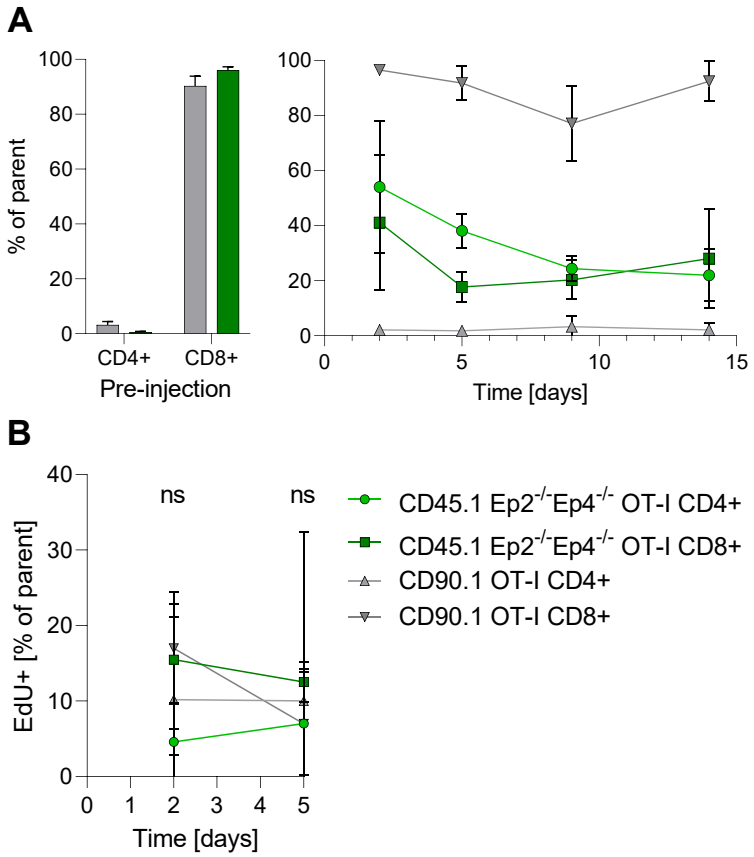

Figure 13: CD45.1 EP2-/-EP4-/- CD4+ population recovers in the tumor microenvironment. Mice were injected with 10^6 D4M.3A-SIINFEKL-cerulean cells s.c. on day 0. On day 8, mice were treated with 10^7 OT-I T cells consisting of wild type and $EP2^{-/-}EP4^{-/-}$ OT-I T cells in a 1:1 ratio. (A) CD4/CD8 composition of CD45.1 $EP2^{-/-}EP4^{-/-}$ OT-I T cells(n = 4 mice per group on day 2 and n=5 mice per group on day 5, significance calculated with a mixed-effects analysis and Tukey's multiple comparisons test)

4. Discussion

4.1 Summary of the results

The data presented in this work highlights the possible advantage of the double knockout of EP2 and EP4 in ACT by enhancing T cell survival and persistence in the TME. It was shown in this work that CAR T cells fail to expand and show decreased viability in the presence of PGE_2, which leads to insufficient tumor control *in vitro*, although no difference in CAR T cell activation could be observed. Single and double knockouts of EP2 and EP4 using the CRISPR/Cas9 system could be successfully achieved. A double knockout of EP2 and EP4, but not the respective single knockouts, could improve this phenotype in PGE_2-rich conditions. Although PGE_2 concentrations are likely much lower *in vivo*[24], the improved efficacy of $EP2^{-/-}EP4^{-/-}$ T cells could also be observed *in vivo* in an OT-I-based model. $EP2^{-/-}EP4^{-/-}$ T cells facilitated improved tumor control and extended the survival in a murine melanoma model. A tracking experiment revealed that improved persistence of the $EP2^{-/-}EP4^{-/-}$ T cells compared to wild type T cells is likely the main driver of improved tumor control.

4.2 Functionality considerations concerning the proposed strategy

Clinical trials showing a reduction in cancer occurrence and metastasis using non-steroid anti-inflammatory drugs (NSAIDs), mainly known for inhibiting cyclooxygenases and thus PGE_2 synthesis, highlight the importance of PGE_2 as a tumorigenic factor[31,43-45]. Animal studies could show that the pro-tumorigenic potential of PGE_2 mainly derives from its immunosuppressive properties, as genetic ablation of its synthesis enzymes led to a re-established control of the tumor growth by the immune system[17-19]. These results could be transferred into a therapeutic strategy using COX inhibitors in a pre-clinical setting[16]. Additionally, the PGE_2 axis has recently been proposed as a novel immune checkpoint[16] and many strategies using inhibitors for enzymes in the PGE_2 synthesis cascade or of its receptors are currently employed in pre-clinical[16,46-48] and clinical studies[49-61].

However, several studies using COX inhibitors for cancer treatment in the clinics have failed due to lack of efficacy[50-52,56-58] or toxicity problems[50,51,58], leaving a need for alternative strategies to target the PGE_2 axis in cancer therapy effectively and safely.

A way to improve safety is to not use small molecule inhibitors systemically blocking the PGE_2 axis in the whole body, but to restrict the therapy effects to the tumor site or specific cell populations. As T cells have been described to suffer from PGE_2 influence[24,26] and considering the so far unrealized potential of CAR T cell therapy in solid tumors, CAR T cells present an attractive target for a controlled disruption of the PGE_2 axis. In this work, it could be confirmed that CAR T cells indeed would benefit from protection against PGE_2, which is in line with other people's work[30]. As PGE_2 is described to mainly act via

EP2 and EP4 on T cells[21,26,28,29,33,35], knocking out these receptors is a suitable strategy to restrict PGE_2 inhibition to the tumor site that has not been explored yet. It is known that EP2 and EP4 signaling involves production of cAMP, which has been described to suppress T cell function intensively[26,30,36]. Other strategies aiming to reduce cAMP levels, like inhibition of adenosine-induced signaling[62], have shown promising results in CAR T cell therapy already. Also, a construct limiting PKA activation and thus disrupting the cAMP signaling cascade in CAR T cells showed promising pre-clinical results[30], highlighting the likelihood to achieve similar effects by blocking the PGE_2 pathway.

This work provides a proof of concept that knockout of the EP2 and EP4 receptors is feasible and gives first evidence that it has potential to be a successful strategy in cancer treatment. Improved persistence in the presence of PGE_2 could be demonstrated *in vitro* and *in vivo* in murine tumor models. These findings are in line with reports about similar strategies interfering in the PGE_2 - cAMP axis and expected to be very relevant in an ACT context due to sensitization to the PGE_2 pathway during *ex vivo* expansion[26,30].

In this work, no alterations in the killing capacity of CAR T cells in the presence of PGE_2 could be observed, although this has been reported by others in the T cell context[26,30]. The differences might be explained by differences in experimental design, as for example the relatively high concentration of PGE_2 used in the *in vitro* experiments in this work. However, the failed expansion in presence of PGE_2 *in vitro* and more importantly the complete exclusion of CAR T cells from the tumor microenvironment observed in the tracking experiment *in vivo* shows that persistence in the TME, not failure in tumor cell killing, is likely to be the main driver for the T cell dysfunction.
As the add-on of an EP2 and EP4 double knockout to CAR T cell therapy will only protect them against PGE_2, it should be noted that this strategy by nature will only improve CAR T cell efficiency in PGE_2-rich tumors. High expression of COX enzymes has been reported in several cancer entities[14,44], suggesting that a wide range of patients might benefit from the proposed strategy. Additionally, it could be shown in this work that the improved functionality is independent of the tumor-targeting receptor used, namely an anti-EpCAM-CAR and the OVA-specific OT-I TCR. Although this needs to be further tested, it is likely that the strategy of knocking out EP2 and EP4 could be flexibly combined with various CARs targeting different antigens to adjust the strategies to different tumor entities.

In summary, the data presented in this work is encouraging further exploitation of the proposed strategy, but to draw better conclusions about its functionality, crucial data, as for example successful translation into a human system, is still missing.

4.3 Safety considerations concerning the proposed strategy

Concerning the safety of the proposed strategy, known side effects of both PGE_2-blockade as well as CAR T cell therapy and genetic engineering using the CRISPR/Cas9 editing must be considered.

Clinical trials using COX inhibitors have reported severe toxicities, which have led to the discontinuation of the treatment in several patients, overall hampering analysis of COX-inhibitor effectivity in cancer therapy[50,51,58,63]. The most common side effect leading to treatment discontinuation was skin rashes, which were speculated to be due to allergic reactions against the COX-inhibitors[50,51,64]. Further, the use of COX-inhibitors is associated with gastrointestinal adverse effects[64]. Those are partly attributed to the acidic nature of the COX-inhibitors directly injuring the gastrointestinal tract[64]. Additionally, COX-inhibitors also inhibit the synthesis of protective prostaglandins and are thus hampering the gastric mucosa integrity[64]. Also, COX-inhibitors have been associated with an enhanced risk for cardiovascular events[63,64]. Mechanistically, COX-inhibitors lead to a misbalance between thromboxane and prostacyclin leading to a pre-disposition for thrombosis, hypertension and atherosclerosis[63]. In summary, the side effects of COX-inhibitors in cancer treatment can all be attributed to their activity in healthy tissues outside of the tumor and could therefore be avoided by using a non-systematic approach to block PGE_2. Additionally, discontinuation of COX-inhibitors due to their side effects has probably prevented them from being effective in several trials[50,51,58], highlighting the need for alternative strategies. The strategy proposed in this work, namely knocking out EP2 and EP4 only on the CAR T cells, does not influence normal PGE_2 signaling throughout the body, which will reduce the risk of side effects caused by aberrant PGE_2 signaling.

Nevertheless, the general safety concerns regarding CAR T cell therapy like CRS and neurotoxicity as well as on-target off-tumor effects[5] still apply in a setting of EP2 and EP4 knockout and will be dependent on the respective CAR used. As the PGE_2 axis has recently been proposed as a new immune checkpoint[16], releasing this natural break in the T cell response might further increase the risk of an uncontrolled immune responses. However, the first clinical trials investigating the CRISPR/Cas9-based knockout of the strong checkpoint inhibitor PD-1 in cancer patients did not observe abnormally high toxicities[65]. The large number of other clinical trials that have been started since then and are currently still ongoing, suggesting that releasing CAR T cells from inhibitory pathways as PD-1 or PGE_2 could be done safely in theory[65-68]. While no special effort was made in this work to assess safety so far, it can be noted that none of the mice in either of the two animal studies showed any signs of toxicity, as evidenced by lack of weight loss or behavioral changes.

Further safety considerations must be made concerning the risks derived from CRISPR/Cas9-based gene editing in the clinics. By now, several clinical trials using

CRISPR/Cas9 for gene editing in T cell therapies have published results and overall showed an acceptable safety profile[66-68], Adverse events did not exceed those expected for T cell therapies without gene editing and none of the patients experienced cytokine release syndrome[66-68]. The main concern associated with the CRISPR/Cas9 system were off-target editing rates, which were reported to be between 0,05 - 4 % in clinical trials[66]. A variety of strategies has been developed to minimize off-target editing[69-71]. First, as the Cas9 nuclease can tolerate up to three mismatches between sgRNA and genomic DNA[71], using *in silico* prediction of possible off-targets during sgRNA design is useful to reduce unspecific binding[69,71]. Additionally, it is recommended to use a delivery method with a quick turnover of the Cas9 nuclease to minimize off-target cleavage by avoiding prolonged Cas9 activity[71]. Examples for this are the delivery of Cas9-encoding mRNA opposed to a vector stably expressing Cas9 or the use of recombinant Cas9 in the form of an RNP[71]. Further, it would be possible to use novel engineered versions of the Cas9 protein that were designed to minimize off-target editing by various methods[71] or to modify the sgRNA by adjusting its length or inducing chemical modifications to increase their specificity[71]. No effort was made so far to determine off-target editing rates in this work, but minimizing off-target rates was considered during the development of the workflow. *In silico* off-target prediction was done during the design of the gRNA and a transient delivery option, namely the use of recombinant Cas9 protein in an RNP, was chosen.

All in all, safety considerations need to be considered in future experiments, especially when transitioning into a human system. However, additional safety strategies as for example including suicide switches or similar approaches could be applied, if necessary.

4.4 Comparison of the proposed strategy to other strategies targeting the EP2/EP4- PGE_2 axis

A preventive effect of cyclooxygenase inhibition on cancer development was discovered for several cancer types[31,43-45] and has led to several trials in different solid tumor entities testing if COX inhibition might also show effects in a therapeutic setting. The results, however, have been disappointing so far. In some cases, treatment had to be discontinued due to side effects like gastrointestinal irritation, rashes or cardiovascular events[63]. Shifting from unspecific COX inhibitors to COX inhibitors selectively targeting COX-2 and not COX-1 improved, but not eliminated, side effects but seems to lack effectiveness in the therapy of solid tumors[50-52,56-58]. Currently, several clinical trials using EP2 and EP4 inhibitors are recruiting patients[59-61], but results are still pending. In any case, in pre-clinical models, selectively blocking EP2 and EP4 together with checkpoint inhibition improved tumor control and thus encourages further investigation[16].

Concerning the combination of CAR T cell therapy and PGE_2-inhibition, this was suggested by Dinh et al. for anti-CD19-CAR therapy and celecoxib for multiple myeloma

in 2017[72]. This approach was tested by Yang et al. in an *in vitro* setting by treating anti-CD19-CAR T cells with celecoxib or aspirin. The study could show a negative effect off both inhibitors on T cell viability and function in high concentrations[73], however it remains unclear whether the observed effects will also occur *in vivo*. However, the possibility of toxic effects on CAR T cells as well as possible side effects of the COX inhibitors itself suggests using other means to protect CAR T cells from PGE_2.

One proposed strategy doing this is the overexpression of phosphodiesterase 4A, an enzyme degrading cAMP, in T cells, which could reverse the inhibitory effects of PGE_2 *in vitro*[36]. However, it was not tested in a therapeutic setting *in vivo*. The second strategy targeting PGE_2 signaling in ACT is to co-express a peptide blocking the localization of PKA to the immune synapse and thereby impairing PGE_2 intracellular signaling, which improved tumor control of the CAR T cells *in vitro* and *in vivo*[30].

In summary, COX inhibition has proven its anti-tumor potential by preventing cancer development[31,43-45] but lacks efficiency in later stages of cancer when used in combination with conventional therapy as for example chemotherapy or radiation[50-52,56-58]. However, given the evidence provided in this thesis and by others, CAR T cells can greatly benefit from protection against PGE_2[30,36,62]. However, given the safety problems with systemic inhibition of the PGE_2 pathway, more selective strategies are preferable. The strategy of knocking out EP2 and EP4 on CAR T cells so far is the only presented strategy that is specific to PGE_2 and not other cAMP-inducing agonists as for example adenosine and shows comparable pre-clinical efficacy encouraging further research.

4.5 Study limitations and outlook

High expression of COX-2 and thus likely high levels of PGE_2 have been found in many solid tumors such as colorectal, liver, pancreatic, breast and lung cancer[74], highlighting the clinical need for PGE_2-resistant CAR T cells for the treatment of solid tumors.

To achieve this, first, it is necessary to be able to translate the double knockout of EP2 and EP4 into a human CAR T cell setting. Thus, the establishment of a protocol for the generation of human $EP2^{-/-}EP4^{-/-}$ CAR T cells is currently in progress. Preliminary data shows the technical feasibility of knocking out EP2 and EP4 in human CAR T cells, but in-depth characterization of behavior, functionality, and safety of the human $EP2^{-/-}EP4^{-/-}$ CAR T cells *in vitro* and *in vivo* are still pending.
Furthermore, to be able to apply this strategy to a variety of tumor types with high COX expression, it needs to be verified that the approach can work in a modular way. In that case, the CAR could be chosen to match the antigen-profile of the respective tumor type. This was already considered during the protocol design, so that in theory the transduction with the CAR and the EP2 and EP4 knockouts are technically done independent of each other. Improved function of $EP2^{-/-}EP4^{-/-}$ T cells could already be demonstrated in this

work with both the anti-EpCAM-CAR and the OT-I receptor, providing a proof-of-feasibility for adapting the approach to different tumor antigens. However, further testing is needed to verify that the approach will indeed be successful in several tumor types using different CARs *in vivo*.

Concerning the applicability to different tumor types, improved efficacy of the $EP2^{-/-}EP4^{-/-}$ T cells could already be shown in this work in a melanoma and a PDAC model, both of which were confirmed to produce PGE_2. While this further supports the feasibility of transferring the strategy to different tumor entities, this also suggests that screening for high levels of PGE_2 might be a valuable strategy to select patients for treatment. By doing so, the costs and risks associated with the CRISPR/Cas9 editing could be limited to patients with a high likelihood to benefit. So far, this has been tried by correlating pre- and post-therapy levels of urinary PGE-M, the main metabolite of PGE_2, in NSCLC patients receiving celecoxib[55]. While a positive correlation could be observed between treatment benefit and decreasing PGE-M levels upon celecoxib therapy, high initial PGE-M levels did not indicate better responses to celecoxib[55]. This was attributed to the observation that tumors with high levels of PGE_2 are usually associated with a more aggressive disease and worse prognosis[55] and thus harder to treat. Nevertheless, although initial PGE-M levels failed to be predictive of the treatment outcome using COX-inhibitors, they might still be useful for the stratification of patients for CAR T cell therapy with EP2 and EP4 knockout and should be considered in future study design.

In the end, a better understanding of the mechanism by which EP2 and EP4 double knockout CAR T cells facilitate improved tumor control would be beneficial to strategically select possible combination partners to further enhance the therapeutic effectiveness. Although it is known since 1979 that PGE_2 has a negative influence on T cell function[20], up until today very little is known concerning the exact mechanism. Having access to CAR T cells resistant to PGE_2, provides a possibility to further investigate the underlying mechanism, which can give valuable information that can be used to further improve the proposed therapeutic strategy. First steps have been taken in that direction and revealed that PGE_2-resistant CAR T cells, while showing better persistence in the tumor, are still prone to exhaustion, suggesting checkpoint inhibition as a good combination partner. However, a repetition of the tracking experiment and additional mechanistic research are still needed to draw educated conclusions.

Finally, as already discussed in chapter 4.3, several safety considerations like CRISPR off target editing efficiency still must be addressed.

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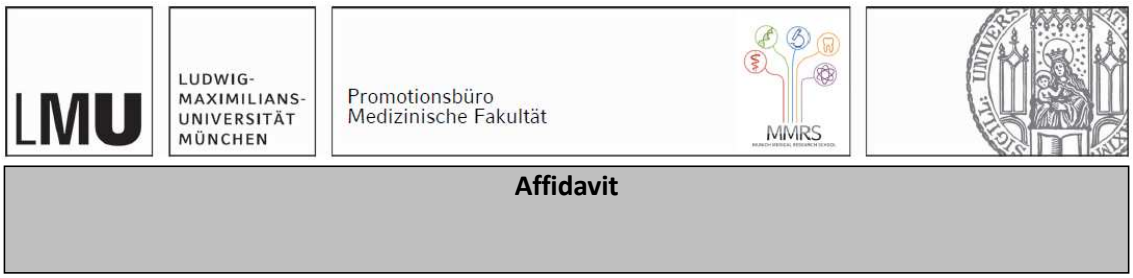
Affidavit



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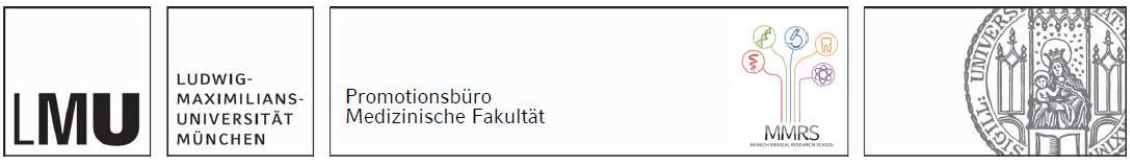

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Publications

Original Articles

1. Seifert M, Benmebarek MR, Briukhovetska D, Märkl F, **Dörr J**, Cadilha BL, Jobst J, Stock S, Andreu-Sanz D, Lorenzini T, Grünmeier R, Oner A, Obeck H, Majed L, Dhoqina D, Feinendegen M, Gottschlich A, Zhang J, Schindler U, Endres S, Kobold S
   Impact of the selective $A2_AR$ and $A2_BR$ dual antagonist AB928/etrumadenant on CAR T cell function
   British Journal of Cancer 2022; 127:2175–2185 **JIF 8.8**

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Review Articles

1. Briukhovetska D*, **Dörr J***, Endres S, Libby P, Dinarello CA, Kobold S
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