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Synthetic myeloid activating receptors enable effective adoptive cellular therapy of pancreatic cancer models

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1 Introduction

1.1 Cellular therapies

Immune cell therapies are defined as the use of autologous or allogenic immune cells against disease. In these therapies, immune cells are derived from primary patient material, modified to confer advantage or specificity against disease, and reintroduced into either the same patient or another patient for therapeutic effect. They are typically targeted against various types of cancer, though cellular therapies against pathogens such as bacteria or viruses do exist. Beginning with the introduction of dendritic cell (DC) vaccines against cancer in the 1990's, the clinical use and relevance of cellular therapies increased over time until the recent blockbuster successes of T cell therapies against hematological malignancies spurned a surge in cellular therapeutic research and implementation. There are currently seven approved cellular therapies: one DC vaccine – Sipuleucel-T for the treatment of prostate cancer [1], and six T cell therapies – idecabtagene vicleucel, ciltacabtagene autoleucel, lisocabtagene maraleucel, tisagen lecleucel, brexucabtagene autoleucel, and axicabtagene ciloleucel for the treatment of a variety of B-cell lymphomas and leukemias [2]. Currently, much scientific effort is exerted in implementing novel cellular therapeutic strategies utilizing dendritic cells, T cells, NK cells, and macrophages to improve solid cancer outcomes [3].

1.2 Dendritic cells and DC vaccines

Dendritic cells are the premier antigen presenting cell of the immune system and occupy an important niche on the border of the innate and the adoptive immune systems [4]. Being one of the main cell types in peripheral tissues that normally respond to pathogen infection, they have the capacity to recognize foreign pathogen-associated molecular pattern molecules (PAMP) and damage associated molecular pattern molecules (DAMP) and initiate innate immune responses [5]. Additionally, dendritic cells can acquire antigen via pinocytosis (uptake) and phagocytosis of cells from their environment during this process. Upon maturation and migration to lymph nodes, they present these antigens to T cells and B cells to mobilize an antigen specific adaptive immune response [5]. It is these specific functions that were exploited to form the first DC vaccines. A DC vaccine is comprised of mature dendritic cells or of dendritic cells

that were differentiated from blood monocytes and after pulsing (loading) with cancer-specific peptides, reintroduced into the patient to elicit an adaptive immune response against tumors which express that peptide [6-8]. The specificity of the vaccine is dependent on the peptides that are used and can be targeted to tumor-associated antigens or neoantigens, or combinations of them. Lymphocytes that are responsive to and stimulated by these dendritic cells are licensed to proliferate and form the basis of the effector function of the dendritic cell vaccine. DC vaccines have demonstrated feasibility both in safety and efficacy in clinical trials against pediatric solid tumors and other forms of solid tumors [6-9]. The approved DC vaccine Sipuleucel T improved outcomes for prostate cancer patients but suffered problems with cost and efficacy [7]. Its marketing authorization was withdrawn in the European Union. Despite this, DC vaccines still present substantial potential for the treatment of cancers and are currently intensively investigated.

1.3 T cell-based cellular therapies

The other main branch of cellular therapy that has achieved FDA and EMA approval against cancers are T cell-based cellular therapies. Despite being newer to clinical application than DC vaccines, the breadth of research as well as the clinical impact of T cell therapies dwarfs that of all other cellular therapies. These therapies can generally be grouped into three main categories: tumor infiltrating lymphocyte (TIL) therapy, T cell receptor (TCR) T cell therapy, and chimeric antigen receptor (CAR) T cell therapy. TIL are a heterogenous population of immune cells, composed mostly of T cells, that are extracted from patient tumors with the presumption being that this T cell population is enriched for tumor-antigen specific T cells that have infiltrated and remained in the tumor [10, 11]. These cells are then expanded *ex vivo* and used as therapy against the patient's tumor, in essence amplifying the body's own natural immune response. Despite the issue of high variability within the actual TIL products across patients, TIL therapy achieved meaningful response rates especially against melanomas [11]. Though recent advances and successes in antibody therapy – most notably immune checkpoint blockade (ICB) – have changed the calculus of melanoma treatment, autologous TIL therapy after ICB failure interestingly maintained robust positive

response rates in a large portion of patients [11]. Advances in TIL therapeutics research have culminated in the therapy achieving regulatory approval in the Netherlands with simultaneous pending approval in the EU [12].

While TIL are derived directly from patient material, TCR T cell therapy differs in that genetic engineering is involved to produce the final T cell product. TCR naturally occur on T cells, with the average human having unique TCR in the order of billions [13]. Of particular interest are TCR which are reactive against various tumor-associated antigens or neoantigens, which coincidentally arise naturally in aforementioned TIL populations, and from which a large majority of tumor specific TCR are derived [14]. These TCR can then be maintained as is or genetically improved for better function, and then transduced onto patient blood-derived T-cells [15, 16]. Engineered T cells are then expanded and reintroduced into the patient to target their tumors. Two main points underly the basis of TCR usage and serve as both advantage and disadvantage: 1) TCR function is HLA-type restricted, and thus a TCR engineered for and effective for one patient's tumor may not be easily transferrable to the next, as HLA-types vary broadly across populations and within populations, and 2) TCR-T cells have the ability to target even proteins that occur intracellularly due to the unique mechanisms underlying antigen presentation in normal somatic cells that present self-antigens [16]. TCR therapy is thus related to TIL therapy in their common dependency on tumor antigen-specific T cells, with the difference being that TCR therapy selects for certain effective TCR and thus ensures a homogenous therapeutic product with a predictable anti-tumoral mechanism. This, however, does not ensure therapeutic success, given that TCR T cell therapy has no regulatory approvals to date. Issues arise with the cost of therapy, which increases given that TCR skew towards being a personalized treatment, and with efficacy, where TCR T cell clinical trials have displayed mixed-bag results across many different types of cancers, with some displaying therapy-induced fatalities [16]. Here also, given the potential of a personalized and naturally derived therapy, much effort is made to find better TCR, improve TCR sequences, and develop methods of application that may enable effective TCR tumor treatment [15, 16].

The latest but most consequential T cell-based cellular therapy is CAR T cell therapy, which comprises the highest number of regulatory approvals as well as largest

impact on clinical treatment [17]. CAR are fully synthetic receptors that comprise of a single chain variable fragment (scFv) derived from antibodies that confers antigen specificity and intracellular T cell receptor domains that activate T cells when antigen is bound [18]. While this is known as a 1st generation CAR, proceeding iterations add intracellular domains of T cell costimulatory molecules onto the cell (2nd generation), and even additional costimulatory or activating domains (3rd generation) [18]. Further iterations enhance CAR with activation-dependent (TRUCK) or activation-independent (armored CAR) release of T-cell stimulatory cytokines such as IL-12 and IL-15 to improve the anti-tumoral performance of CAR T cells [19-21]. Unlike TCR, CAR are not HLA-restricted as antigen specificity is conferred via an antibody-based mechanism, though this also brings with it the important limitation of only being able to target cell surface antigens [18]. Though this lessens the potential pool of targetable tumor-associated or neoantigens and presents as a limiting factor for CAR T cell, the advent of CAR T cell therapy has brought with it the most significant paradigm shifts in cellular therapy, culminating in six approved therapies. CAR T cell treatment, in cases of relapsed and refractory B-cell hematological malignancies, has evolved the last-line standard-of-care and has conferred significantly improved life expectancies in these patients [2, 17]. CAR T cell treatment for acute myeloid leukemia and solid tumors, however, remains decidedly underwhelming and an area of active research [22, 23]. Sharing the same burdens with other types of cellular therapies when used against solid tumors, CAR T cells struggle when obstructed by the tumor microenvironment (TME), a suppressive and restrictive structure of particular importance in these types of tumors [22, 23].

1.4 The suppressive tumor microenvironment

While cellular therapies have excelled in the treatment of various B cell malignancies, where malignant cells are more accessible for engagement, the TME present in solid tumors and the similar bone marrow niches in AML frustrate the effective function of cellular therapies [24]. The TME is composed of tumor cells as well populations of recruited immune cells, fibroblasts, extracellular matrix (ECM), blood vessels, and secreted factors [3, 24]. As a whole, the TME inhibits cellular therapies via

robust contact- and cytokine-mediated immunosuppression, physical exclusion, and metabolic downregulation [24]. Furthermore, it has pro-tumorigenic functions such as the promotion of angiogenesis to support tumor growth and the elements of the TME function to ease metastasis of tumors [24, 25]. TME composition begins with the malignant tumor cells however, and tumor cells from a range of solid tumors secrete chemotactic factors that draw in immune cells, fibroblasts, mesenchymal stems cells (MSC) and other cells that become tumor-associated cells via interaction with the tumor. The chemokines C-C motif ligand (CCL)2, CCL5, and C-X-C motif chemokine (CXCL)5 recruit myeloid-derived suppressor cells (MDSC), a poorly defined but very immunosuppressive population of myeloid cells, into the tumor [3, 26]. CCL2 and CCL3 secreted by tumors recruit tumor-associated monocytes, which eventually differentiate into the strongly immunosuppressive, M2-like, tumor-associated macrophages (TAM) through TME exposure [3, 27]. Regulatory T cells (T-reg) also traffic to the TME through CCL1, CCL5, CCL17, and CCL22 signaling where they contribute to suppression [3, 28]. Mast cells and neutrophils also migrate the TME and contribute to suppression through cytokine secretion [29, 30]. These tumor and tumor-associated suppressive cells exert tremendous suppressive pressure on possible inflammatory or cytotoxic cells that infiltrate into the tumor. Contact-dependent immune suppression by these cells is mediated by inhibitory markers such as programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), “don’t eat me” signals such as CD47 [31] and CD24 [32], and direct T-reg-mediated antigen-dependent and -independent inhibition [3, 28, 33]. In addition, tumor cells and associated cells also form a cytokine milieu composed of factors such as interleukin (IL)-10, IL-4, and transforming growth factor-β (TGF-β) that further suppresses and exhausts possible anti-tumor responses [24]. Though the TME does inhibit immune activation through a variety of other mechanisms, for the sake of brevity and scope of this thesis, the TME explanation will focus on contact- and cytokine-dependent suppression mediated by tumor cells and tumor-associated cells. The influence of TME elements not discussed here will be mentioned in the discussion section.

1.5 Cellular therapies to address the TME

Cellular therapy research has evolved to meet the challenges posed by the TME [3]. The aforementioned TRUCK T cells and armored CAR T cells, for instance, were designed to directly counteract TME suppression by supplying CAR T cells with their own stimulatory signals so that they may persist inside the tumor despite the suppressive environment. The cytokines they secrete also work to inflame the TME, downregulating inhibitory signals and possibly reversing the suppressive environment to be more immune permissive [19-21]. A related strategy to address the TME engineers cellular therapies to secrete immunoinflammatory chemokines such as CCL21 and CCL19 that attract endogenous dendritic cells, T cells, and NK cells with the aims of spurring an endogenous immune response against the tumor [34-36]. Another strategy of enhancing cellular therapies against the TME targets tumor-associated immune cells directly: anti-CD123 CAR targets both Hodgkin lymphoma cells as well as the TAM that present frequently in that tumor indication, enabling a dual pronged approach that leads to impressive efficacy [37]. Similar concepts have targeted CSF1R on TAM [38], TR2 and NKG2D on MDSC [39, 40], CD25 on T-reg [41], and FAP on cancer-associated fibroblasts (CAF) [42]. Some of these efforts have led to clinical trials, though none have yet shown conclusively convincing results. Thus, cellular therapy research constantly is evolving to meet the challenges posed by the solid tumors and the TME.

1.6 Myeloid CAR, CAR for phagocytosis, and CAR-Macrophages

While CAR therapy initially was conceived and designed for implementation in T cells, the engineering of CAR onto macrophages again advanced the progress of cellular therapy. The intracellular domain that provides primary T cell activation in CAR – the CD3ζ – was shown to be homologous to the intracellular FcRγ chain that largely regulates macrophage antibody-dependent cellular phagocytosis (ADCP) [43]. Both were indeed interchangeable in 1st generation CAR before the addition of costimulatory domains [43]. Engineering of 1st generation CAR on macrophages (CAR-M) enabled an antigen-specific phagocytotic function that, when targeted against tumor cells, led to levels of cytotoxicity on the scale of that shown by CAR T cells [44]. Related concepts include chimeric antigen receptors for phagocytosis (CAR-P), which utilize receptors fusing the multiple EGF-like-domains 10 (Megf10) intracellular domain or FcRɣ to an

extracellular scFv to induce antigen-specific phagocytosis and inflammation [45]. In addition to a phagocytotic anti-tumor effect, both CAR-M and CAR-P were shown to elicit a level of TME remodeling marked by increased expression of pro-inflammatory markers on tumor-associated cells, and upregulation of inflammation-associated pathways within the tumor [44, 45]. This inflammatory signaling is linked to the innate immune properties of macrophages and dendritic cells as the premier “first-responders” in the periphery, where their activation by foreign molecules leads to localized inflammation and the beginnings of immune mobilization. CAR-M additionally were shown to maintain an inflammatory M1 phenotype intratumorally, which alleviates the concern of the plasticity of transduced macrophages in whether they would revert to an M2-like phenotype when exposed to suppressive signaling [44]. This phenotypic maintenance was shown to be intrinsic to the adenovirus-based transduction method used to genetically engineer the macrophages, where residual levels of adenoviral genes stimulate the constitutive M1 phenotype. Lastly, the use of macrophages, as a primary intratumoral effector enables the possibility of antigen presentation of tumor antigens to the endogenous immune system, leading to further immune mobilization against non-CAR-directed tumor antigen. CAR-M were shown to at least be able to cross-present MHC-I restricted antigen after phagocytosis of antigen-expressing tumor cells, though MHC-II presentation was not demonstrated [44]. Altogether, CAR-M represent a step forward in cellular therapy addressing issues presented by the TME, demonstrating promising results in HER2+ breast cancer models, as well as advancement to clinical trials.

1.7 SMARt-DC targeting tumors and the TME in PDAC

The principles cited above propose that cellular therapies can be further powered for anti-tumor efficacy by shifting the immunosuppressive TME to be more immunopermissive. Already, modified CAR T cell concepts with added cytokine or chemokine expression to boost endogenous cells have increased potential for tumor clearance [35, 36]. CAR-M and CAR-P demonstrate further that myeloid cellular therapy is feasible and adds benefit in enabling antigen cross-presentation and possible epitope spreading [44, 45]. In accordance with these concepts, we have hypothesized in this

thesis, that a novel cellular therapy – synthetic myeloid activating receptors (SMARt) transduced into dendritic cells – that takes advantage of these points would better function and outcome.

The SMARt receptor is a CAR-like receptor which is composed of an scFv that confers specificity, combined with the transmembrane and intracellular domains of the CD40 protein, a prominent signaling molecule in the activation, maturation, and licensing function of dendritic cells [46]. We selected dendritic cells for similar reasons that justify macrophage usage: functionality as an effector of inflammatory innate immune signaling, phagocytotic capability that may be cytotoxic to tumor cells directly, and most importantly, for their main function of presenting antigen and promotion of T cell-based immune responses [47, 48]. We selected pancreatic adenocarcinoma (PDAC) as the target tumor indication for a range of reasons: its status as one of the most difficult to treat cancers with abysmal life expectancies post-diagnosis and lack of treatment options, the central role that the suppressive TME plays in PDAC development and pathogenesis, and the characteristic high metastatic potential and burden of PDAC [49-51] that might be addressed by the successful function of SMARt-DC. The proposed mechanism of action of these SMARt-DC against PDAC is threefold and each shall be investigated in this thesis:

1) antigen-specific accumulation and CD40 activation of the dendritic cells in antigen-expressing tumors,
2) CD40 signaling-mediated dendritic cell maturation and inflammatory remodeling of the TME,
3) antigen uptake and subsequent mobilization of endogenous immune responses in the lymph nodes.

To elaborate, circulating CAR T cells have been shown to be anchored in tumors through the binding of their scFv to antigen-expressing tumors, an effect that coincides parallel with CAR-mediated T cell activation [18]. We surmise that SMARt-DC will behave similarly, as extracellularly the SMARt construct is identical to CAR, and ultimately this results in transduced dendritic cell infiltration and localization. As CD40

signaling canonically initiates on homodimerization of its intracellular domain, it is postulated that upon antigen binding of the extracellular scFv, the intracellular CD40 domains would correspondingly aggregate and initiate CD40 signaling downstream, in a similar manner proposed for CAR and their CD3z domain [52, 53].

This CD40 activation would lead to secretion of inflammatory cytokines and chemokines such as IL-1, IL-6, IL-8, IL-10, IL-12, tumor necrosis factor-alpha (TNF-α), macrophage inflammatory protein-1 alpha (MIP-1α), CXCL10, among others, into the tumor [54]. MIP-1α, CXCL10, and CXCL11 build migratory gradients for cytotoxic T cells and NK cells to infiltrate the tumor, leading to higher numbers of potential anti-tumor effectors [55-57]. The other secreted inflammatory cytokines such as IL-1β, IL-6, IL-10, IL-12, and TNF-α can mediate direct antitumor effects such as apoptosis [58-62]. Many of the above cytokines will also have effects on intratumoral immune cells. IL-12 for instance is a potent enhancer for T and NK cell survival, proliferation, and cytotoxicity [61, 63]. IL-10 likewise has been recently shown to reverse the terminal exhaustion of intratumoral T cells, reinvigorating them for anti-tumor function [64]. The sum effect of this SMARt-DC-mediated cytokine release results in a remodeled TME marked by increased numbers of anti-tumor effectors, reduced suppression by both tumors and tumor-associated populations, and some degree of recovery in function of infiltrated tumor-specific cytotoxic T cells.

The activation of CD40 and subsequent inflammation furthermore matures the dendritic cell from its initial immature state to a mature immunogenic phenotype similar to that described for inflammatory maturation in pattern recognition receptor (PRR)-activated DC [65]. For the final proposed portion of their function, we postulate that SMARt-DC would pinocytose or phagocytose tumor antigen during intratumoral SMARt-mediated activation. The resulting mature SMARt-DC would in theory eventually migrate to lymph nodes and cross-present MHC-I-restricted tumor antigen to CD8 T cells and present antigen to CD4 and B cells in an MHC-II dependent manner.

In this thesis dissertation, the entirety of the development of SMARt-DC will be discussed, as well as experimental results, which underpin the feasibility of the utilization of these cells as an adoptive cellular therapy, either as monotherapy or in

combination with other treatments. The following concepts were determined to be relevant and comprise central parts of the scientific investigation:

1) Interactions between SMARt-DC and antigen-bearing tumor cells in respect to cytotoxicity, cytokine release, and phagocytosis,

2) Synergies with CAR T cells,

3) The contribution of all of the above to anti-tumor efficacy in *in vitro* and *in vivo* tumor models.

2 Material and methods

2.1 Technical devices and reagents

Table 1: Technical devices

Device	Manufacturer	Location
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
CO2 – Incubator (BD6220)	Heraeus, ThermoFischerScientific	Massachusetts, USA
FACS Canto II	BD Biosciences	New Jersey, USA
FACS Fortessa	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	BioRad Laboratories	Munich, Germany
T3 Thermocycler	Biometra	Göttingen, Germany
Isolight System	Isoplexis	Connecticut, USA
Zellscanner	Zellkraftwerk	Leipzig, Germany
Xcelligence	Aligent	California, USA
Plate Reader	Berthold	Bad Wildbad, Germany

Table 2: Reagents

Reagent	Manufacturer	Location
Albumin fraction V (BSA)	Sigma-Aldrich	Steinheim, Germany
BD Pharm lyse lysing buffer (10 x)	BD Biosciences	New Jersey USA
Blasticidin	InvivoGen	California, USA
Calcium chloride	Sigma-Aldrich	Steinheim, Germany
Cesium chloride	Sigma-Aldrich	Steinheim, Germany
Collagenase D	Sigma-Aldrich	Steinheim, Germany
Count Bright, counting beads	Life Technologies	California, USA
CD14 Microbeads	Miltenyi	Bergisch-Gladbach, Germany
CD3 Microbeads	Miltenyi	Bergisch-Gladbach, Germany
CD40-TRAF6 Signaling Inhibitor, 6877002	Sigma-Aldrich	Steinheim, Germany
Codeplex Human Adaptive Immune Chips	Isoplexis	Connecticut, USA
Dimethylsulfoxide (DMSO)	Sigma-Aldrich	Steinheim, Germany
DNase-I	Roche	Mannheim, Germany
DQ-Ovalbumin	Invitrogen	Massachusetts, USA
Dulbecco's Modified Eagle Medium DMEM	Sigma-Aldrich	Steinheim, Germany
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma-Aldrich	Steinheim, Germany
Dynabeads human T-activator CD3⁄CD28	Sigma-Aldritch	Steinheim, Germany
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
Fetal Calf Serum	Gibco Products	New York, USA
Gateway LR Clonase II	Invitrogen	Massachusetts, USA
GeneJet plasmid mini prep kit	ThermoFischerScientific	Massachusetts, USA
Heparinsodium 2.500 IE/w5 ml	Braun AG	Melsungen, Germany
HEPES buffer 1 M	Sigma-Aldrich	Steinheim, Germany
High glucose FBS	Gibco Products	New York, USA
Histopaque-1077	Sigma-Aldrich	Steinheim, Germany
Human serum	Sigma-Aldrich	Steinheim, Germany
Human TNF-α DuoSet ELISA	RnD Systems	Minneapolis, USA
Human IL-2 ELISA	BD Biosciences	New Jersey, USA
Human IL-10 DuoSet ELISA	RnD Systems	Minneapolis, USA
Human IL-12 DuoSet ELISA	RnD Systems	Minneapolis, USA
Human CXCL10/IP-10 DuoSet ELISA	RnD Systems	Minneapolis, USA
Human Interferon-γ ELISA	BD Biosciences	New Jersey, USA
LB agar	Carl Roth GmbH	Karlsruhe, Germany
LB medium	Carl Roth GmbH	Karlsruhe, Germany
LE agarose	Biozym	Hessisch Oldendorf, Germany
L-glutamine 200 mM	Sigma-Aldrich	Steinheim, Germany
Lipofectamine 2000	Invitrogen	Massachusetts, USA
Liquid DAB+ Substrate Chromogen System	Agilent	California, USA
MACS LS Columns	Miltenyi	Bergisch-Gladbach, Germany

Magnesium chloride	Sigma-Aldrich	Steinheim, Germany
Matrigel matrix	Corning	New York, USA
MEM non-essential amino acids (NEAA, 100 x)	Gibco Products	New York, USA
MluI	NEB	Massachusetts, USA
NotI	NEB	Massachusetts, USA
PacI	NEB	Massachusetts, USA
Q5 Enzyme	NEB	Massachusetts, USA
Penicillin/Streptomycin	PAA	Pasching, Austria
Phorbol myristate acetate	Sigma-Aldrich	Steinheim, Germany
Puromycin	InvivoGen	California, USA
Recombinant Human GM-CSF	PeproTech	New Jersey, USA
Recombinant hIL-2	PeproTech	New Jersey, USA
Recombinant hIL-4	PeproTech	New Jersey, USA
Recombinant hIL-6	PeproTech	New Jersey, USA
Recombinant hIL-13	PeproTech	New Jersey, USA
Recombinant hIL-15	PeproTech	New Jersey, USA
Recombinant hLIF	PeproTech	New Jersey, USA
Recombinant hM-CSF	PeproTech	New Jersey, USA
Recombinant hTGF-β	PeproTech	New Jersey, USA
Recombinant hMesothelin	ACRO Biosystems	Delaware, USA
RetroNectin	TaKaRa	Kyoto, Japan
RevertAid First Strand cDNA Synthesis kit	ThermoFischerScientific	Massachusetts, USA
Roswell Park Memory Institute-1640 (RPMI)	Sigma-Aldrich	Steinheim, Germany
SERVA DNA Stain Clear G	SERVA	Heidelberg, Germany
Slide-A-Lyzer Dialysis Cassette G2	ThermoFischerScientific	Massachusetts, USA

Sodium chloride	Sigma-Aldrich	Steinheim, Germany
Sodium pyruvate	PAA	Pasching, Austria
Sulfuric acid	Carl Roth GmbH	Karlsruhe, Germany
Tissue Freezing Medium	Leica Biosystems	Nussloch, Germany
Tris-HCl	Carl Roth GmbH	Karlsruhe, Germany
Tris-Base	Sigma-Aldrich	Steinheim, Germany
Trypan blue	Sigma-Aldrich	Steinheim, Germany
Trypsin (10 x)	PAA	Pasching, Austria
Tween 20	Carl Roth GmbH	Karlsruhe, Germany
T4 DNA Ligase	NEB	Massachusetts, USA
β-Mercaptoethanol	Sigma-Aldrich	Steinheim, Germany
Cell culture flasks (T25 to T175)	Corning	New York, USA
Cell culture plates (6 to 96 well)	Corning	New York, USA
ELISA microplates (96 well)	Corning	New York, USA
Eppendorf tubes (0.5 ml, 1.5 ml, 2.0 ml)	Sarstedt	Nümbrecht, Germany
FACS tubes	BD Biosciences	New Jersey, USA
Nylon filter SmartStrainer (100 µm, 30 µm)	Miltenyi Biotec	Bergisch Gladbach, Germany
Pipetboy	Hirschmann Laborgeräte	Eberstadt, Germany

Table 3: Antibodies

Antibody	**Clone**	**Manufacturer**	**Concentration Used**
TruStain FcX Human	Polyclonal	Biolegend	1:100

Anti-CD45RA (Human)	HI100	Biolegend	1:100
Anti-HLA-DR (Human)	L243	Biolegend	1:100
Anti-CD8a (Human)	SK1	Biolegend	1:100
Anti-PD-1 (Human)	EH12.2H7	Biolegend	1:100
Anti-CCR7 (Human)	G043H7	Biolegend	1:100
Anti-CD3e (Human)	HIT3a	Biolegend	1:100
Anti-CD4 (Human)	A16.1A1	Biolegend	1:100
Anti-CD14 (Human)	HCD14	Biolegend	1:100
Anti-CD80 (Human)	2D10	Biolegend	1:100
Anti-CD86 (Human)	IT2.2	Biolegend	1:100
Anti-CD83 (Human)	HB15e	Biolegend	1:100
Anti-CD1c (Human)	L161	Biolegend	1:100
Anti-CD11c (Human)	3.9	Biolegend	1:100
Anti-CD163 (Human)	GHI/61	Biolegend	1:100
Anti-CD68 (Human)	Y1/82A	Biolegend	1:100

Anti-LAG3 (Human)	11C3C65	Biolegend	1:100
Anti-TIM3 (Human)	F38-2E2	Biolegend	1:100
Anti-DCSIGN (Human)	9E9A8	Biolegend	1:100
Anti-CD206 (Human)	15-2	Biolegend	1:100
Anti-cmyc Tag (Human)	SH1-26E7.1.3	Miltenyi Biotec	1:100
Anti-EpCAM (Human)	9C4	Biolegend	1:100
Anti-MSLN (Human)	420411	RD Systems	1:100
Anti-Hexon	Polyclonal	ThermoFisher	1:200

2.2 Synthetic myeloid activating receptor (SMART) and chimeric antigen receptor (CAR) cloning

The intracellular and transmembrane domains of the SMART receptor are taken directly from the human CD40 protein (UniProt P25942). For the purposes of this study, SMARt receptors targeting human MSLN (UniProt Q13421) and EpCAM (UniProt P16422) were utilized which combined the aforementioned domains with an extracellular single chain variable fragment consisting of the localization signal peptide from human CD8 protein (UniProt P01732), MSLN- or EpCAM-specific CDRs (Roche), a G4S linker, the c-myc epitope tag, and a spacer region derived from human CD8 protein. CAR constructs directed against MSLN and EGFR (UniProt P00533) were used in this study. The CAR constructs are composed extracellularly of scFvs containing the localization signal peptide from CD8, MSLN-specific (Roche) or EGFR-specific (Cetuximab/C225) CDR, a G4S linker, the c-myc epitope tag, and a spacer region derived from human CD8 protein. Intracellularly, both CAR consist of the human CD28

(UniProt P10747) transmembrane domain, the CD28 intracellular domain, and the zeta chain of the human CD3 protein (UniProt P20963). Both are second generation CAR and are identical in sequence except the CDR sequences. Additionally, the extracellular scFv of the MSLN-directed SMARt and CAR are identical. The SMART receptor sequence was cloned into the pENTR-Twist (Twist Biosciences) vector with a EF1a promoter preceding the recombinant receptor. CAR constructs were cloned into the retroviral vector pMP71 [66] which utilizes viral LTR regions as a promoter when integrated. Construct sequence synthesis and cloning into vectors was conducted by Twist Biosciences.

2.3 Cell lines and primary cells

Table 4: Cell lines

Cell Line	Culture Medium	Cell Type	Source
Miapaca2	DMEM 3+	Pancreas carcinoma	ATCC
BxPC3	DMEM 3+	Pancreas adenocarcinoma	ATCC
Suit2	DMEM 3+	Pancreas adenocarcinoma	ATCC
MSTO-211H	RPMI 3+	Biphasic mesothelioma	ATCC
K-562	RPMI 3+	Chronic Myelogenous Leukemia	ATCC
THP-1	RPMI 3+	Acute Monocytic Leukemia	ATCC
293A	DMEM 4+	Adenoviral packaging cell line derived from HEK293	ThermoFisherScientific
293T	DMEM 4+	Lentiviral packaging cell line derived from HEK293	ATCC

293Vec-Galv	DMEM 4+	Retroviral packaging cell line derived from HEK293	Prof. Dr. Manuel Caruso (Quebec, Canada)
293Vec-RD114	DMEM 4+	Retroviral packaging cell line derived from HEK293	Prof. Dr. Manuel Caruso (Quebec, Canada)

Table 5: Cell culture media and supplements

Complete DMEM medium (DMEM 3+)	
DMEM	500 ml
FBS	50 ml
Penicillin	1 IU/ml
Streptomycin	100 µg/ml
L-Glutamine	2 mM

Complete RPMI medium (RPMI 3+)	
RPMI 1640	500 ml
FBS	50 ml
Penicillin	1 IU/ml
Streptomycin	100 µg/ml
L-Glutamine	2 mM

Human T cell medium (hTCM)	
RPMI 1640	500 ml
Human Serum	12.5 ml
Penicillin	1 IU/ml
Streptomycin	100 µg/ml
L-Glutamine	2 mM
Sodium pyruvate	1 mM
NEAA (100%)	1 %

Producer DMEM medium (DMEM 4+)	
DMEM	500 ml
FBS	50 ml
Penicillin	1 IU/ml
Streptomycin	100 µg/ml
L-Glutamine	4 mM

2.3.1 Cell lines

The human pancreatic cell lines Miapaca2, BxPC3, Suit2, the human mesothelioma cell line MSTO-211H, and the human leukemia cell line K-562 were used in the study. Miapaca2, Suit2, MSTO-211H, and K-562, were retrovirally transduced to overexpress human mesothelin to create the Miapaca2-MSLN and Suit2-MSLN cell lines, respectively. Additionally, certain original and overexpressing cell lines were additionally retrovirally transduced to co-express green fluorescent protein (GFP) and firefly luciferase (ffLuc) for the purposes of FACS tracking or luciferase-based killing

assays. The gene encoding full-length hMSLN was cloned into the MCS of the lentiviral vector obtained from System Bioscience, pCDH-EF1a-MCS-T2A-Puro cDNA. The cells were then selected with puromycin at 5 µg/mL for 2 to 3 days. Surviving cells were stained and confirmed with surface expression of hMSLN. Cells were transduced with lentivirus encoding GFP-ffLuc, cloned into the MCS of the lentiviral vector obtained from System Bioscience, pCDH-CMV-MCSEF1a-Neo. The cells were then selected with 5 µg/mL of G418 for 2 to 3 days. Cells were cultured as described above in 37C and 95% humidity. All human cell lines were short tandem repeat profiled in house to verify their origin. Cells were used for a period no longer than 2 months.

2.3.2 THP-1-derived dendritic cells

For THP-1 derived dendritic cell experiments, THP-1 cells were first transduced via standard protocol to express SMARt-MSLN, live cell polyclonally-bulk-sorted, and differentiated into dendritic cells using GM-CSF and IL-4 as described previously [67].

2.3.3 Primary human monocyte-derived dendritic cells and T cells

Primary human monocytes and T cells were isolated from either fresh heparinized blood or from donor buffy coats provided by the Bayerische Blutspendedienst. Leukocytes were isolated via standard ficoll (Histopaque 1077) gradient centrifugation. Cells were MACSed first with human CD14 selection beads (Miltenyi) via manufacturer's protocol. Effluent was subsequently reMACSed with human CD3 beads via manufacturer's protocol. CD14+ cells were then adjusted to $1x10^6$ cells/ml in RPMI3+ supplemented with 1000U/ml rhGMCSF and 500U/ml rhIL-4 and plated in 6-well plates at $4 x 10^6$ cells/well. On the third day, medium was refreshed with cytokines, and the now differentiated monocyte-derived dendritic cells were transduced on the fifth day. CD3+ human T cells were adjusted to 10^6 cells/ml in hTCM supplemented with 5 ng/ml IL-15, 0.2 µg/ml IL-2, 50 µM β-mercaptoethanol and human T-Activator anti-CD3- anti-CD28 Dynabeads (33 µl per $4x10^6$ cells). Next, the T cells were cultured in 6-well plates at $4x10^6$ cells/well. After two days of cell culture, the cells were used for transduction.

2.3.4 M2 macrophages suppression assays

Primary human monocytes were isolated from either fresh heparinized blood or from donor buffy coats provided by the Bayerische Blutspendedienst in the same manner as described above. Isolated monocytes were cultured in 50ng/ml M-CSF and plated in 6-well plates at $4x10^6$ cells/well to induce M2-like immunosuppressive macrophage phenotype. Cytokines were refreshed on the third day and cells were further stimulated into M2A, M2C, and M2D phenotypes on the 7^{th} day. M2 macrophages were further differentiated thus: M2A, 100 ng/ml M-CSF; 20 ng/ml IL-13 and 20 ng/ml IL-4 (2 d), M2C, 100 ng/ml M-CSF (7 d); 10 ng/ml IL-10 and 10 ng/ml TGF-β1 (2 d), amd M2D, 100 ng/ml M-CSF (7 d); 50 ng/ml IL-6; 20 ng/ml LIF (2 d). These cells were then used in suppression assays of cytotoxic capacity.

2.4 Virus Production and Transduction

2.4.1 Retrovirus Production

The vector pMP71 (kindly provided by C. Baum, Hannover) was used as the backbone for all CAR constructs, resulting pMP71-CAR-MSLN and pMP71-CAR-HER2. These plasmids were transfected into the 293Vec-Galv packaging cell line. The resulting supernatant after 3 days was used to transduce the 293Vec-RD114, which resulted in the 293Vec-RD114-CAR-MSLN and 293Vec-RD114-CAR-HER2 cell lines, both stably secreting retroviral particles encoding the respective CAR. Single cell clones were selected and screened for receptor expression levels, then screened for levels of virus production by determining the transduction efficiency of primary T cells. Clones which resulted in highest transduction efficiencies were thus frozen and used for all subsequent transductions. All producer cell lines grown in DMEM 4+.

2.4.2 Adenovirus Production

The shuttle vector containing the recombinant receptor pENTR-SMART-MSLN was combined with the adenoviral vector pAD-DEST-PL (ThermoFischer) using the LR Recombinase II reaction (ThermoFischer) to recombine the sequences between the L and R recombination sites on both vectors. Successful recombination of pAD-SMART-MSLN would result in the replacement of the cytotoxic *ccdB* gene between the L and R

sites in the original backbone pAD-DEST-PL vector with the sequences for the EF1a promoter and the SMART receptor from the shuttle vector, and thus recombined clones were selected from bacterial colonies growing on agar plates in the presence of ampicillin (encoded on pAD-DEST-PL backbone, outside of L-R region). The recombinant pAD-SMART-MSLN plasmid was then verified via restriction digest with EcoRI and MluI for expected band at ~1300bp. Proceeding adenoviral generation steps follow standard adenoviral production protocols (ThermoFischer). The adenoviral vector was then restricted with PacI to expose the LTR regions flanking the PacI site and transfected with Lipofectamine 2000 according to manufacturer's protocol into 293A cells. Virus was then expanded and harvested from 293A cell culture and purified on cesium chloride gradients via standard protocols. Purified virus titer was measured relative to a virus standard using an anti-Hexon-HRP antibody on virus infected 293A cells which were processed with a liquid DAB chromogen system and quantified via light microscopy.

2.4.3 Retroviral Transduction

293Vec-RD114 virus-producing cell lines were used to generate supernatants for human cell transduction. Virus supernatant is harvested from the producer cells, put through a 0.45μm filter, and either used fresh or frozen. The retrovirus supernatant was then loaded onto 24-well culture plates, which were previously coated with 6.25 μg/ml RetroNectin in 4°C overnight. This is proceeded by spinoculation of the virus onto the plate at 37°C for 1.5 hours at 3000G. Retroviral supernatant is then discarded, and $1x10^6$ activated human T cells in 1ml complete hTCM supplemented with IL-2, IL-15, and β-mercaptoethanol were seeded each well. T cells were checked for their transduction efficiency using flow cytometry analysis two days post transduction and further expanded in hTCM supplemented with IL-2, IL-15 and β-mercaptoethanol. All assays utilize T cells with transduction efficiencies greater than 50%.

2.4.4 Adenoviral Transduction

Differentiated monocyte-derived dendritic cells (mdDC) are harvested on day 5 and resuspended $1x10^6$ cells/ml in RPMI with rhGM-CSF and rhIL-4. Virus is added to

the mdDC at an MOI of 1000. After two days, transduction efficiency is measured using flow cytometry analysis. All assays utilize dendritic cell transduction efficiencies greater than 40%. Accutase is utilized for removal of final dendritic cell product as well as harvesting the cells pre-transduction.

2.5 Cytotoxicity assays

Cytotoxicity assays were carried out according to a previously published protocol (Karches et al. 2019). DC and T cells were co-cultured with tumor cells at various indicated effector to target ratios. Real-time electrical impedance, a function of cell adhesion and healthiness, was measured with the xCELLigence device. Impedance is represented by cell index values. All measurements of impedance and calculations of cell index are based on the RTCA software version 1. A separate and additional luciferase-based killing assay was used additionally to measure killing at individual timepoints. The BioGlo cytotoxicity assay (Promega, USA) system was used where indicated according to the manufacturer's protocol, and utilized with cell lines engineered to express ffLuc. All luciferase values were read on a microplate reader (Berthold Mithras) measuring luminescence.

2.6 Cytokine release assays

Cytokine release assays were carried out according to a previously published protocol (Karches et al. 2019). Human T cell and dendritic cell stimulation assays were set up at indicated effector-to-target ratios. CXCL10, IFN-γ, IL-10, IL-12, and TNF-α were quantified by enzyme-linked immunosorbent assay (ELISA). CXCL10, IL-10, IL-12, and TNF-α ELISA were done using the DuoSet system (RD Systems) and the IFN-γ ELISA was done using the OptiEIA system (BD Biosciences). All ELISA were performed according to the manufacturer`s protocols. Absorbance at 450 nm was measured with a microplate reader (Berthold Mithras), with background correction at 595nm. Protein concentrations were calculated using standard curves established with recombinant proteins included in each kit. A multiplexed proteomics assay measuring cytokine release was also conducted using the Codeplex Human Adaptive Immune system and analyzed using Isospeak software.

2.7 Animal Experimentation

NSG mice (NOD.Cg-PrkdcscidIl2rgtm1WjI/SzJ) were purchased from Charles River. The MiaPaCa-MSLN xenograft model was established by subcutaneously injecting $1x10^6$ cells into the right flank. $5x10^6$ dendritic cells were intravenously injected into the tail vein, follow by a brief rest period, and then $1x10^7$ T cells were injected intravenously into the opposite vein on the tail as indicated. Mice and tumors were scored three times a week. All animal experiments were approved by the local regulatory agency (Regierung von Oberbayern) and adhered to the NIH guide for the care and use of laboratory animals. Endpoints were registered by an observer blinded to the treatment groups as previously defined [68].

2.8 Single cell analysis by flow cytometry

Flow cytometry was carried out according to a previously published protocol (Voigt et al. 2017). To achieve single cell suspensions from tissue, spleens were passed through 30 µm cell strainers, following an erythrocyte lysis. Tumors were digested with 1.5 µg/ml collagenase IV and 50 U/ml DNAse I for 30 minutes at 37ºC and then passed through 30 µm cell strainers. Lymph nodes were passed through 30 µm cell strainers. Analysis was carried out with a BD FACS Fortessa (BD Bioscience, Germany). FACS data was analyzed using FlowJo V10. For the separation of live cells of specific subpopulations, single cell suspensions were prepared and stained as described above. Cells of interest were sorted into a 1.5 ml tube containing FBS using BD FACS Aria II run on FACSDiva Software. FACS-sorted cells were cultivated or lysed for further studies.

2.8.1 Flow cytometry staining

Dead cells were stained using the fixable viability dye eFluor 780 (eBioscience) for 15 minutes at RT. This was followed by the blocking of Fc receptors with TruStain fcX Human (Biolegend) for 20 minutes at 4ºC. For phenotype analysis surface staining was performed by anti-human CD45RA (clone H1100, Biolegend), anti-human HLA-DR (clone L243, Biolegend), anti-human CD8a (clone SK1, Biolegend), anti-human PD1

(clone EH12.2H7, Biolegend), anti-human CCR7 (clone G043H7, Biolegend), anti-human CD3e (clone HIT3a, Biolegend), anti-human CD4 (clone A16.1A1, Biolegend), anti-human CD14 (clone HCD14, Biolegend), anti-human CD80 (clone 2D10, Biolegend), anti-human CD86 (clone IT2.2, Biolegend), anti-human CD83 (clone HB15e, Biolegend), anti-human CD1c (clone L161, Biolegend), anti-human CD11c (clone 3.9, Biolegend), anti-human CD163 (clone GHI/61, Biolegend), anti-human CD68 (clone Y1/82A, Biolegend), anti-human LAG-3 (clone 11C3C65, Biolegend), anti-human TIM-3 (clone F38-2E2, Biolegend), anti-human DC-SIGN (clone 9E9A8, Biolegend), anti-human CD206 (clone 15-2, Biolegend), anti-human c-myc epitope tag (clone SH1-26E7.1.3, Miltenyi Biotech), Cells were washed and resuspended in PBS. For cell number quantification CountBright® absolute counting beads (Life Technologies) were added.

2.9 Chip Cytometry

5 µm tissue cryosections were stained on chipcytometry slides (Zellkraftwerk) with anti-human CD11c (clone EP1347Y, abcam), anti-human CD69 (clone D-3, Santa Cruz), anti-rabbit IgG (polyclonal, Jackson ImmunoResearch), and anti-mouse IgG (polyclonal, Jackson ImmunoResearch). The fluorescence was measured using the Zellscanner ONE (Zellkraftwerk).

3 Results

3.1 THP-1 models and proof-of-concept

THP-1 monocytic leukemia cells, like normal monocytes, retain the ability to be differentiated into macrophages and dendritic cells given proper stimuli [67, 69]. Addition of PMA to THP-1 cultures results in loss of proliferation as well as irreversible differentiation into macrophages with an inflammatory phenotype, while the addition of GM-CSF and IL-4 similarly induces differentiation into dendritic cells.

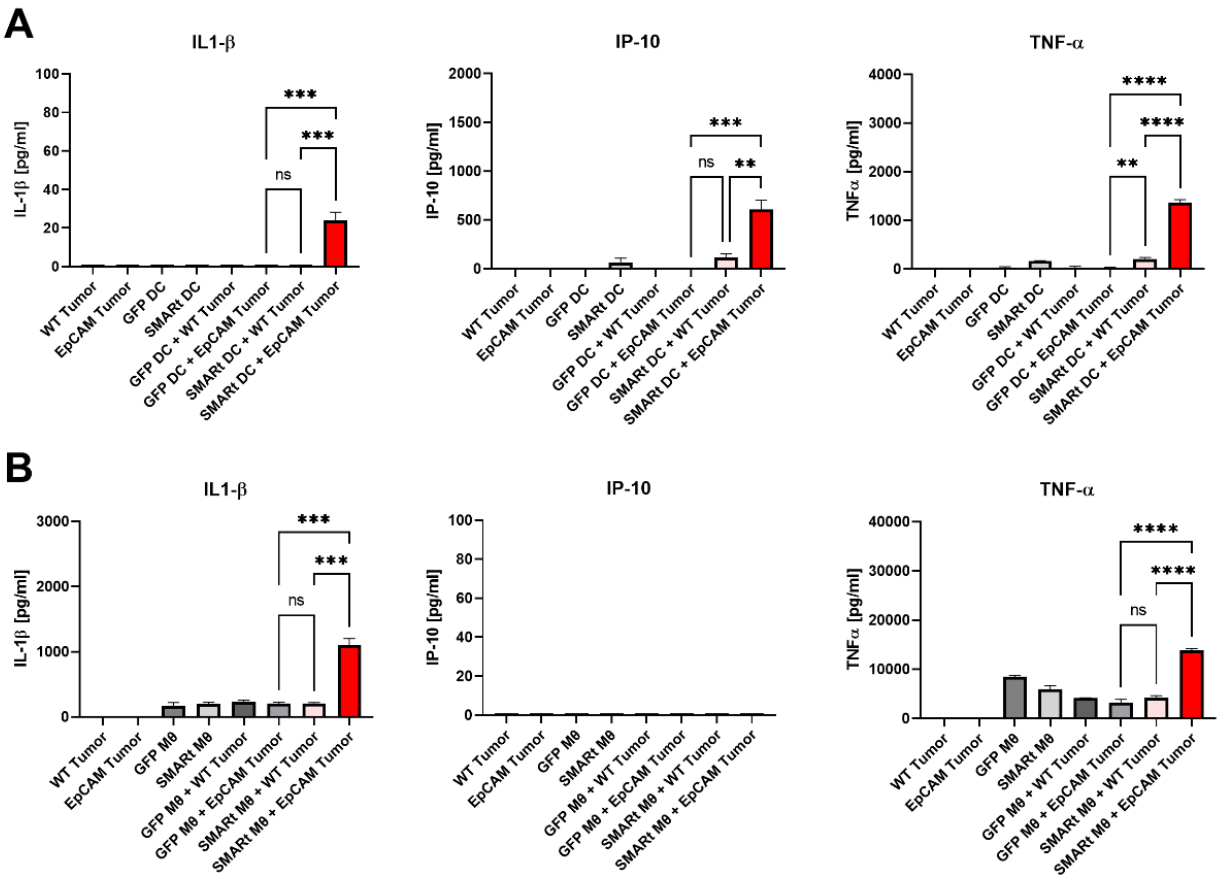

Figure 1: SMARt proof-of-concept in THP-1 derived dendritic cells and macrophages

Retrovirally transduced THP-1 monocytes that expressed an anti-EpCAM SMARt were differentiated for two days in either PMA (macrophages) or GM-CSF and IL-4 (dendritic cells) and cultured with EpCAM-overexpressing tumor cells. Release of IL-1β, IP-10, and TNF-α were measured from the supernatants and measured via ELISA for the THP-1-derived dendritic cells **(A)**, and for the THP-1-derived macrophages **(B)** as proxy markers for activation. Analyses of differences between groups were performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001. Cytokine release experiments show mean values ± SD and are representative of three independent experiments.

THP-1 cells were retrovirally transduced with a SMARt construct targeting epithelial cell adhesion molecule (EpCAM) and confirmed to be expressing it on the surface via staining with c-myc (Figure 1). Positive cells were sorted and expanded. SMARt-THP-1 cells were then differentiated into monocyte-derived dendritic cells and co-cultured with wild type T11 and T11-EpCAM cells (Figure 1). IL-1β, IP-10, and TNFα levels of the SMARt THP-1 cells co-cultured with wild type remained at similar levels to unstimulated cells, while levels in cocultures with antigen-expressing tumor cells dramatically increased, a result consistent with DC activation. These results proved that the SMARt concept could indeed work and lead to antigen-specific CD40-mediated activation of dendritic cells.

3.2 Design and establishment of an adenoviral vector for recombinant receptor expression on primary dendritic cells

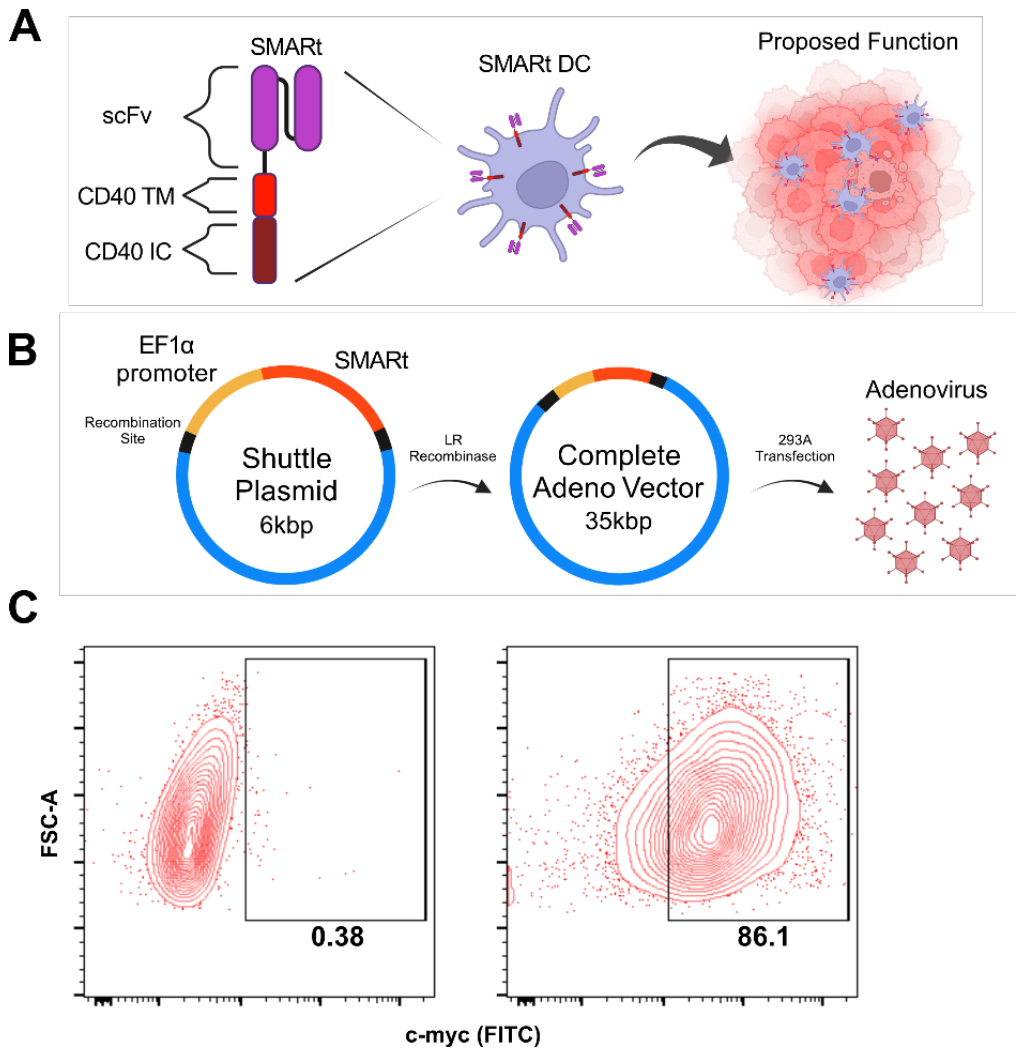

Figure 2: Expression of SMARt on primary dendritic cells through adenoviral transduction

Schematic of structure of the SMARt receptor, its inclusion in dendritic cells, and its intended inflammatory function within the tumor **(A)**. The SMARt transgene under the control of an EF1α promoter was first cloned into a shuttle plasmid that was then recombined into an adenoviral backbone plasmid, then transfected into 293A cells for virus packaging **(B)**. Expression of the SMARt receptor on primary dendritic cells after infection with SMARt-encoding adenovirus. A c-myc tag is included in the scFv extracellular domain and used for staining of the receptor on cell surface **(C)**.

Following successful proofing of the SMARt concept in the THP-1 model, a new expression vector was necessary as myeloid cells are not efficiently transduced by HIV-pseudotyped lentivirus due to the inhibitory effects of SAMDH1 [70]. It was described in literature that recombinant adenovirus type 5 can be utilized to transduce primary monocyte-derived macrophages [71, 72], and thus we utilized also an adenovirus type 5 system to express our receptor on dendritic cells. The construct was placed under the control of an EF1α promoter and integrated into the adenoviral vector via a recombination reaction (ViraPower, ThermoFischer). The resulting vector was used to generate adenovirus type 5 encoding the SMARt receptor. This virus would then be expanded and harvested. mdDC differentiated after five days were infected with the virus for two days and afterwards checked for expression via FACs staining with an anti-cmyc antibody (Figure 2). The adenovirus type 5 virus that was used incorporates a fiber knob protein that binds coxsackie and adenovirus receptor (CD46) for viral entry [73, 74]. Though CD46 is not highly expressed on mdDC [75-77], we have not encountered problems achieving sufficiently high levels of transduction efficiency which routinely exceed 70%.

3.3 SMARt-DC gain a novel antigen-specific cytotoxic capability mediated through CD40 activation

DC transduced with the SMARt receptor or control GFP were co-cultured with either MiaPaCa02 wild-type cells or MiaPaCa02-MSLN, both stained with proliferation dye, and then processed through flow cytometry. All control DC as well as SMARt DC co-cultured with wild-type MiaPaCa02 displayed little to no tumor cytotoxicity, while SMARt DC co-cultured with MiaPaCa02-MSLN displayed a dose-dependent cytotoxicity

that kinetically is much slower and less efficient than CAR-mediated killing (Figure 4). We further tested the killing effects on two other pancreatic cancer cell lines, Suit02 - Suit02-MSLN, and BxPC3 that demonstrated similar results (Figure 4). In further investigation of this killing effect, we also tested the system under the effects of the CD40-TRAF inhibitor 6877002, which revealed that inhibition of CD40 ligation to TRAF6, its downstream transcription factor, abrogates the observed cytotoxic effect, which in total confirms the predicted mechanistic dependency on CD40 (Figure 3).

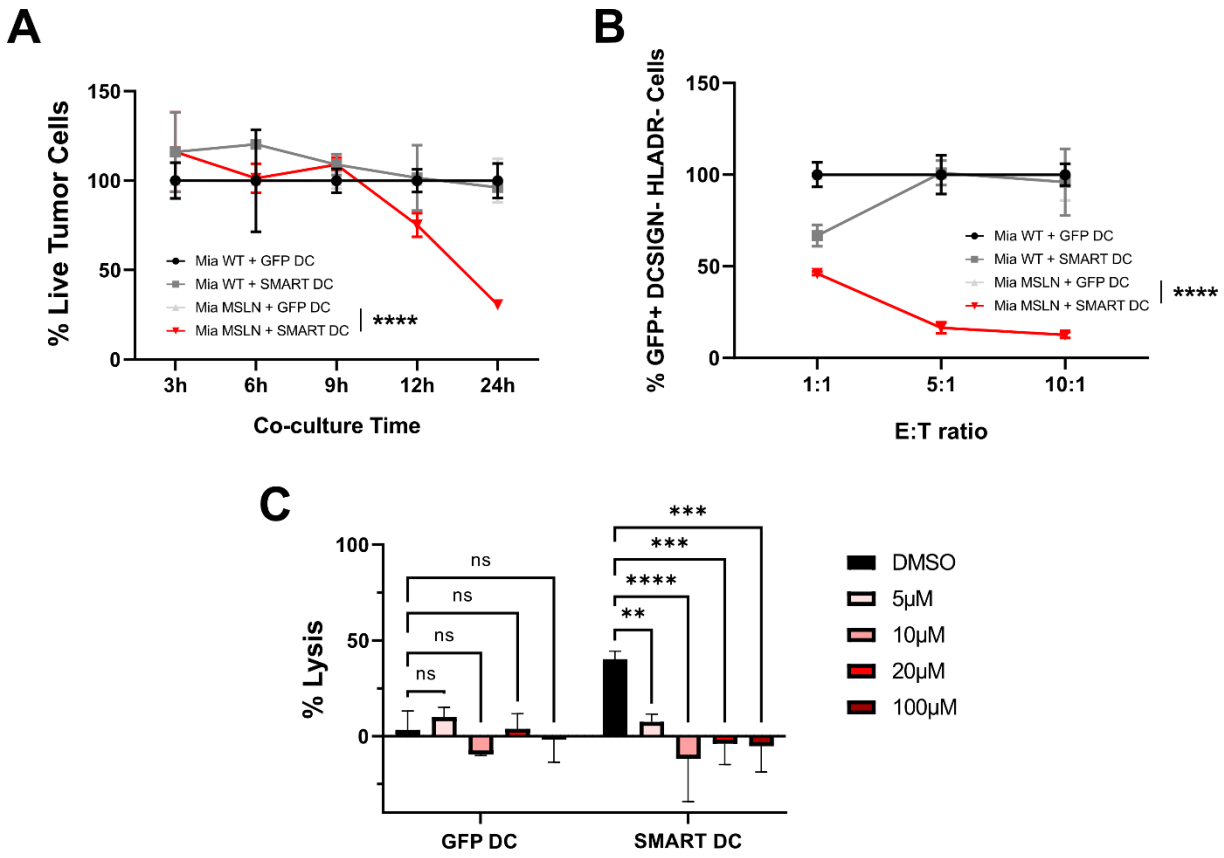

Figure 3: SMARt receptor activation in DC induces a novel, antigen-specific, CD40-mediated cytotoxic effect

MSLN-expressing and non-expressing MiaPaCa02 tumor cells were co-cultured with control GFP-expressing or SMARt-DC in a 1:1 effector:target (E:T) ratio and killing through many timepoints was measured through a flow-cytometry based assay **(A)**. The same co-culture was conducted for 24 hours using various E:T ratios and killing was measured through a flow-cytometry based assay **(B)**. Control and SMARt-DC were cultured with ffLuc+ MiaPaCa02-MSLN with DMSO or various concentrations of the CD40-TRAF inhibitor 6877002, and killing was measured through a luciferase-based assay (**C**). Analyses of differences between groups were performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001. All experiments show mean values ± SD and are representative of three independent experiments.

It is noted that with lower, more physiologically relevant effector-to-target ratios i.e., below 1:1, the cytotoxic effect observed from antigen-specific activation of SMARt-DC does indeed becomes less prominent and increasingly donor-specific, suggesting that this effect may not be expected to be a primary mediator of anti-tumor effect in actual tumors (Figure 4). It further hints that cytotoxicity is linked to the sufficient accumulation of cytotoxic factors, namely inflammatory cytokines, and also to slower killing processes such as phagocytosis. We will further explore both avenues of investigation in later results.

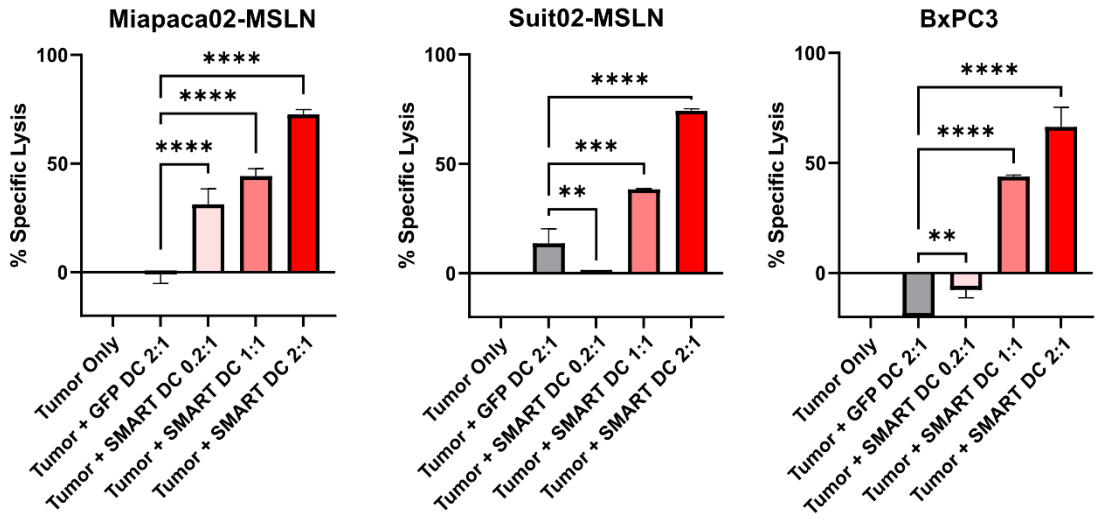

Figure 4: SMARt-receptor expressing DC induce a novel, antigen-specific, CD40-mediated cytotoxic effect against various pancreatic tumor cell lines

Three different human pancreatic cell lines expressing ffLuc, MiaPaCa02-MSLN, Suit02-MSLN, and BxPC3, and killing was measured through a luciferase-based assay. Analyses of differences between groups were performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001. All experiments show mean values ± SD and are representative of three independent experiments.

3.4 SMARt-DC maintain a semi-mature CD1c DC-like phenotype

The phenotype of SMARt-DC were examined in comparison to untransduced, immature mdDC, as well as LPS-matured mdDC. Adenoviral transduction of DC (and macrophages) is frequently described to impart enhanced inflammatory and

costimulatory capacity on the DC [78]. Thus, as we examined markers such as CD40, CD80, CD86, and HLA-DR that are associated with an inflammatory phenotype, SMARt-DC expression frequently present in between the mature LPS-stimulated control and the immature unstimulated control (Figure 5), indicating that the adenovirus-mediated expression of the SMARt receptor does confer a level of inflammation that induces a semi-mature status to the DC in line with literature. For mdDC archetypical markers such as CD11c and DC-SIGN, expression levels remain relatively the same as expected. When clustered on surface marker expression profiles, SMARt-DC present as a distinct cluster that groups close to but removed from LPS-matured dendritic cells, highlighting this distinct semi-mature phenotype (Figure 5). It is noted that both groups are far removed from the immature DC grouping, indicating the clear shift in phenotyping that either LPS or SMARt transduction confers. Both points of data point to a SMARt-DC presenting as a distinct phenotype of semi-mature mdDC that are inflammatory in nature.

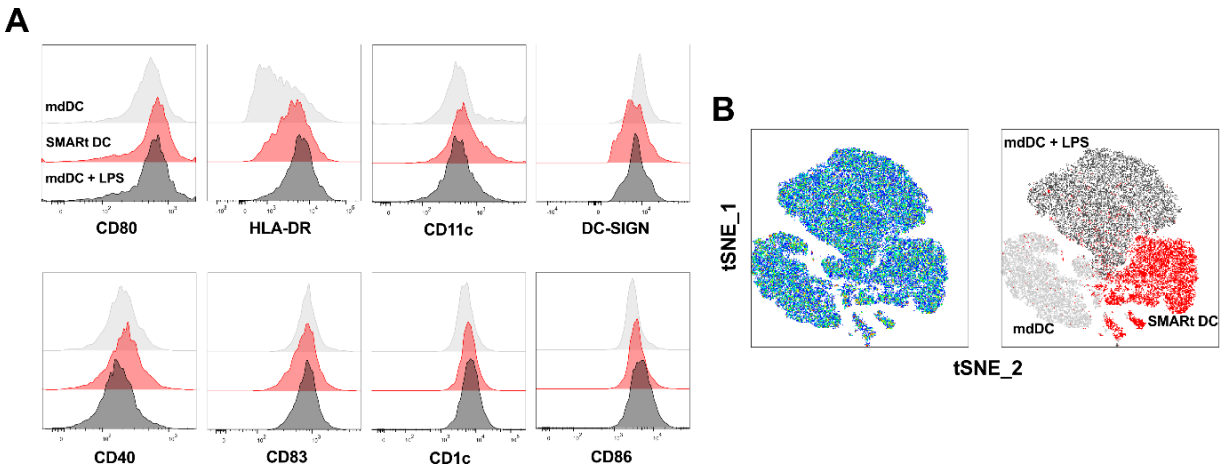

Figure 5: Phenotype of SMARt-DC

The expression (MFI) of various dendritic cell-related phenotypic, inflammatory, or maturation markers are depicted as histograms. Groups represented at immature monocyte-derived DC, SMARt-DC, and mature, LPS-stimulated monocyte-derived DC **(A)**. tSNE analysis of surface markers, size, and complexity of the three groups of cells and their clustering **(B)**. tSNE analysis was conducted within the FlowJo software.

3.5 SMARt-DC gain an antigen-specific phagocytosis function and maintain pinocytosis function

Co-culture of SMARt-DC with MSLN-bearing and non-bearing tumors revealed that the SMARt receptor confers a dose-dependent antigen-specific phagocytosis that contributes to the cytotoxicity effect previously observed (Figure 6). The percentage of SMARt-DC with intracellular (phagocytosed) GFP after 24 hours at initial glance was lower than control, but after normalizing to the number of alive tumor cells, it is observed that SMARt activation confers much greater phagocytotic potential that leads to tumor cell death (Figure 6). We then investigated another main avenue of antigen uptake: pinocytosis. For this, we assayed the pinocytotic potential of the SMARt-DC compared to untransduced mdDC as well as mature LPS-stimulated mdDC, measure via uptake of DQ-Ovalbumin. While mature, LPS-stimulated mdDC displayed lower pinocytotic capacity than immature mdDC, SMARt-DC displayed pinocytosis capacity equal to immature mdDC (Figure 6). It is known that maturation in DC causes reduction of antigen-uptake pathways, though here it is shown that the semi-mature phenotype of the SMARt-DC does not affect its ability to pinocytose and in fact increases its ability to phagocytose antigen. This does indeed raise the possibility that SMARt-induced phagocytosis and pinocytosis can result in acquired and presented tumor antigen.

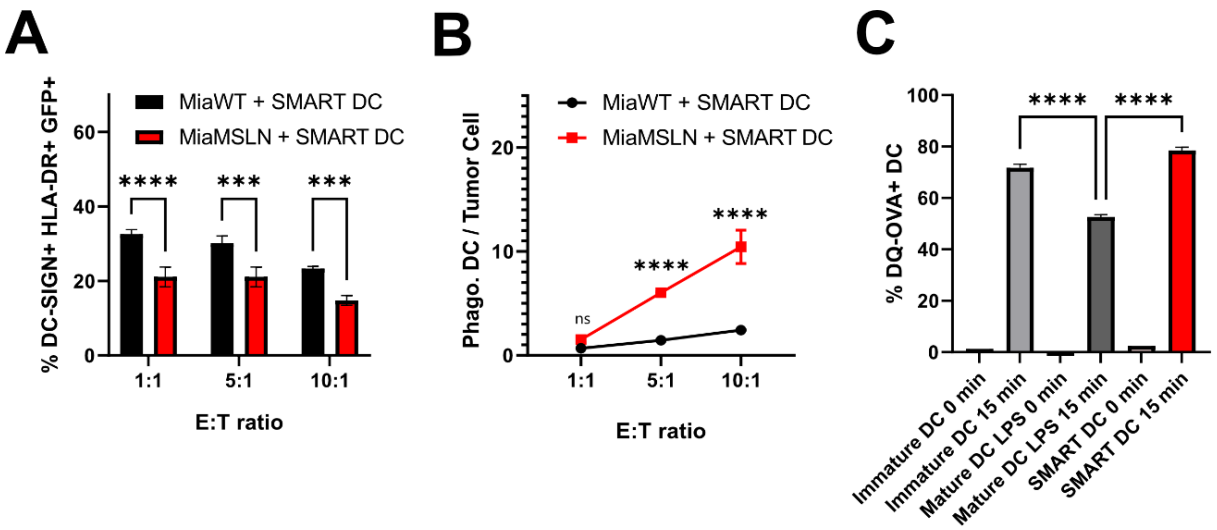

Figure 6: SMARt-DC induce stronger phagocytosis and maintain pinocytosis capacity

SMARt-DC were cultured with GFP+ MSLN-expressing or non-expressing MiaPaCa02 cells for 24 hours at various E:T ratio and phagocytosis was measured via a flow cytometry-based assay, with phagocytosing dendritic cells defined as DC-SIGN+, HLA-DR+, and GFP+ cells within the assay (**A**). The same co-culture was normalized to number of alive cells within the assay and represents the efficiency of cytotoxicity mediated by phagocytosis (**B**). Immature, LPS-stimulated, and SMARt-DC were incubated with DQ-Ovalbumin for 0 minutes (negative control) and 15 minutes via the manufacturer's recommended protocol and DC that pinocytosed the DQ-OVA are defined as DC-SIGN+, HLA-DR+, DQ-OVA+ (FITC) cells (**C**). Analyses of differences between groups were performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001. All experiments show mean values ± SD and are representative of three independent experiments with three donors.

3.6 Antigen-specific activation of SMARt receptors leads to inflammatory cytokine release

One major role of dendritic cells is to mobilize immune cells to localized inflammation sites via cytokine and chemokine secretion. While CD40 is not canonically known as a receptor that is involved in this innate immune function, the downstream transcription factor machinery in the CD40 pathway heavily overlaps with TLRs and RLRs, beginning with TRAF6 that binds directly on CD40 dimers [52]. We investigated the antigen-specific cytokine secretion induced by SMARt activation. First, we stimulated SMARt-DC with its cognate antigen, recombinant MSLN, coated on a plate. Supernatant from this assay displayed antigen-specific release of a representative cytokine TNF-α in only conditions with rMSLN and transduced DC (not shown). Next supernatants from co-cultures of SMARt-DC and control transduced DC with MiaPaCa-MSLN were collected and cytokine and chemokine levels were measured via the multiplexed proteomics approach Codeplex to ascertain the actual cytokine milieu released when all tumor-DC interactions are factored in (Figure 7). Supernatants from the SMARt-DC condition showed upregulated levels of GM-CSF, Granzyme B, IL-13, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, and TNF-β compared to controls. To verify the results of the Codeplex assay, we confirmed that ELISAs of the representative cytokine TNF-α corresponded closely with the results from the multiplex assay (not shown).

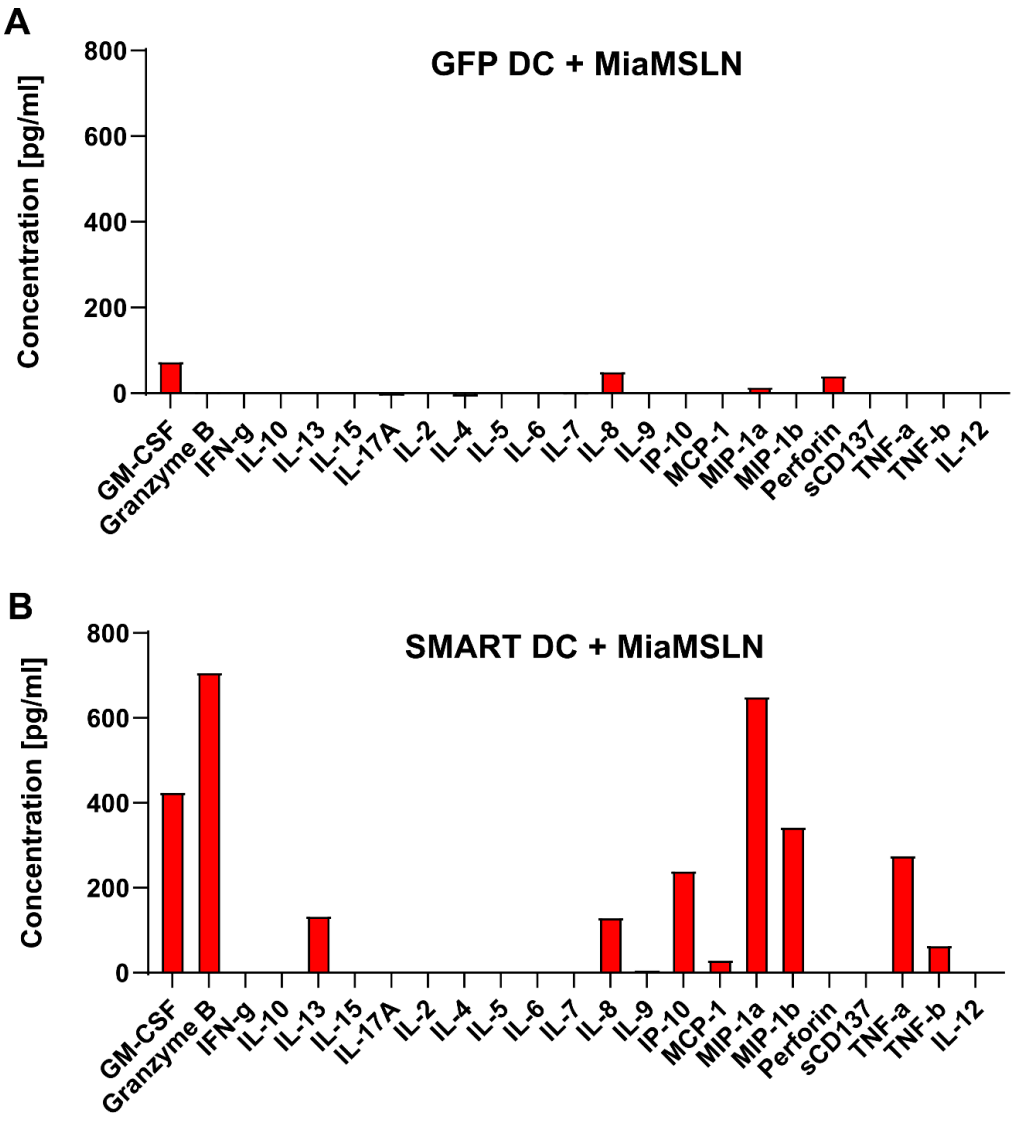

Figure 7: SMARt-DC activation induces inflammatory cytokine release

Control DC were cocultured for 24 hours with MSLN-expressing MiaPaCa02 pancreatic tumor cells at a 1:1 E:T ratio. Supernatants were collected and cytokine expression was assessed through the Isoplexis Codeplex multiplex cytokine assay (**A**). The same was done for SMARt-DC (**B**). Duplicates were used as recommended by the manufacturer and analysis of the data was processed internally using the IsoSpeak software 2.8.0.0. Only one donor was assayed.

3.7 SMARt DC are resistant to M2 macrophage-mediated suppression, as well as reprogram them to a more inflammatory phenotype

As a variety of immunosuppressive cells are expected to localize in the tumor microenvironments that we target the SMARt-DC to, we investigate the possible

interactions between such cells. We focus on M2-like macrophages here as they closely model the tumor-associated macrophages that are one of the most abundant immunosuppressive cell populations in pancreatic tumor stroma. M2-like macrophages were differentiated from monocytes and cultured with both tumor cells and also SMARt-DC or control DC and assayed for cytokine release and killing kinetics (Figure 8).

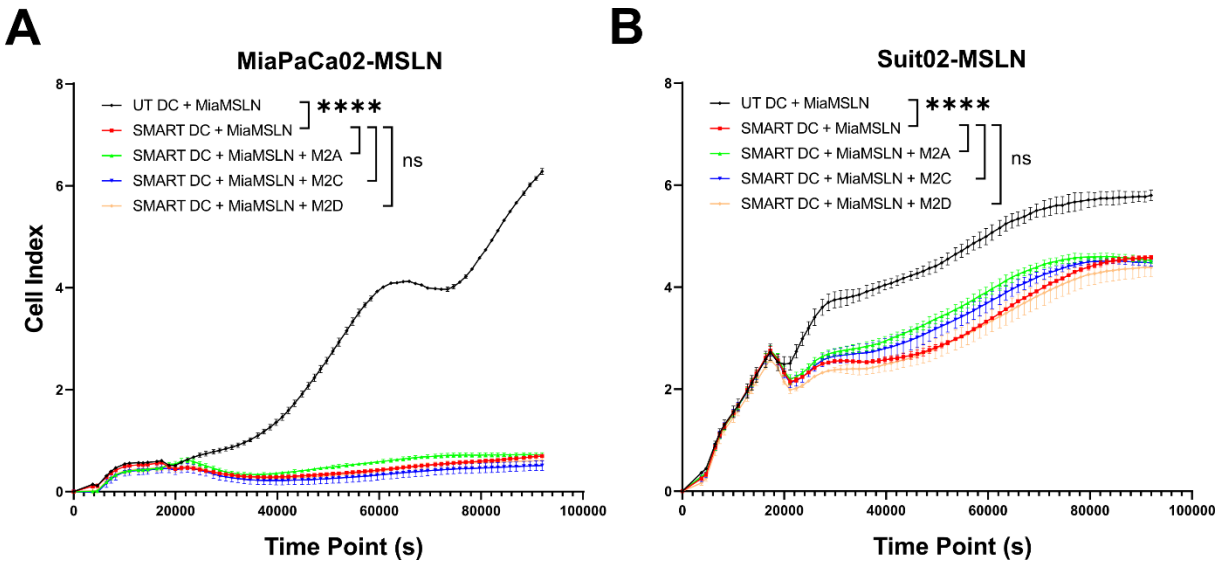

Figure 8: SMARt-DC-mediated anti-tumor functions are immune to M2-like macrophage suppression

Control or SMARt-DC, M2 macrophages, and either MiaPaCa02-MSLN (**A**) or Suit02-MSLN (**B**) cells were cultured at a 1:1:2 ratio, respectively, for 24 hours and killing was measured through the Xcelligence-based killing assay which measures impedance of attached cells. Analyses of differences between groups were assessed at the last timepoint and performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001. XCelligence experiment shows mean values ± SD and is representative of three independent experiments with three donors.

SMARt-DC were shown to maintain killing kinetics in the presence of the tolerogenic and immunosuppressive M2A, M2C, and M2D subtype macrophages. ELISA data of these assays reveal that in fact, the levels of the representative cytokine TNF-α were significantly boosted by the presence of the M2 macrophages in the SMARt-DC conditions, suggesting that either the activation of the dendritic cells caused inflammation in the M2 macrophages, or that the presence of the M2 macrophages

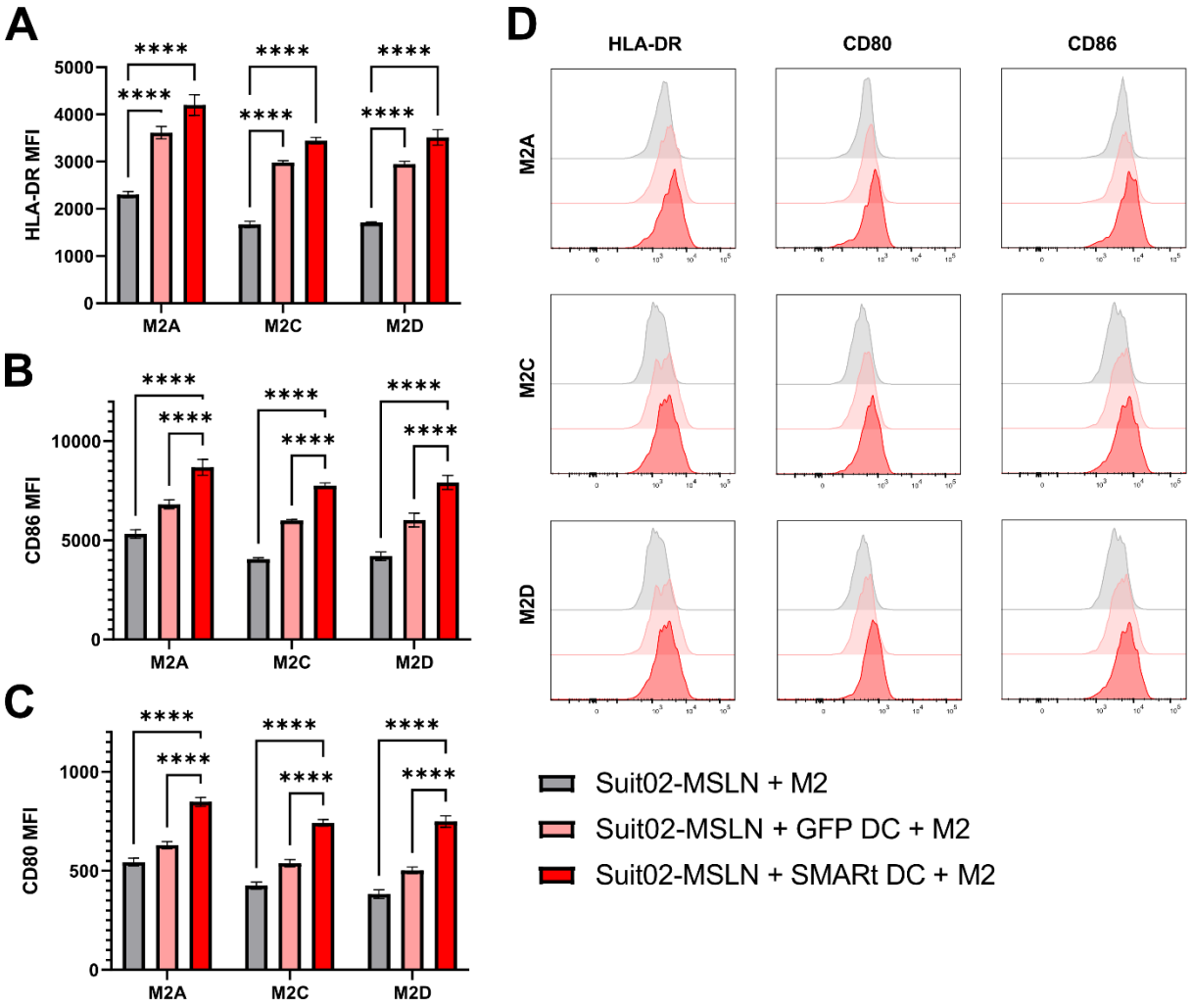

Figure 9: SMARt-DC reprogram immunosuppressive M2 macrophages to a more inflammatory phenotype

Control or SMARt-DC, M2 macrophages, and Suit02-MSLN cells were cultured at a 1:1:2 ratio, respectively for 24 hours. Macrophages were pre-stained with eFluor450 Proliferation dye and defined within the assay as eFluor450+ cells and their phenotype was assessed via flow cytometry. The MFI of the inflammation/activation-associated markers HLA-DR (**A**), CD86 (**B**), and CD80 (**C**) on the M2 macrophages were measured, as well as the total surface expression depicted in histograms displaying the phenotypic shift (**D**). Analyses of differences between groups were assessed at the last timepoint and performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001. All experiments show mean values ± SD and are representative of three independent experiments with three donors.

boosted the potency of SMARt activation (Figure 10). Assessment of phenotype of the M2 macrophages revealed that in all M2 subtypes, inflammatory surface markers were

elevated in conditions cultured with SMARt-DC, demonstrating a reprogramming of a relatively suppressive to a more inflammatory phenotype in the M2 macrophages

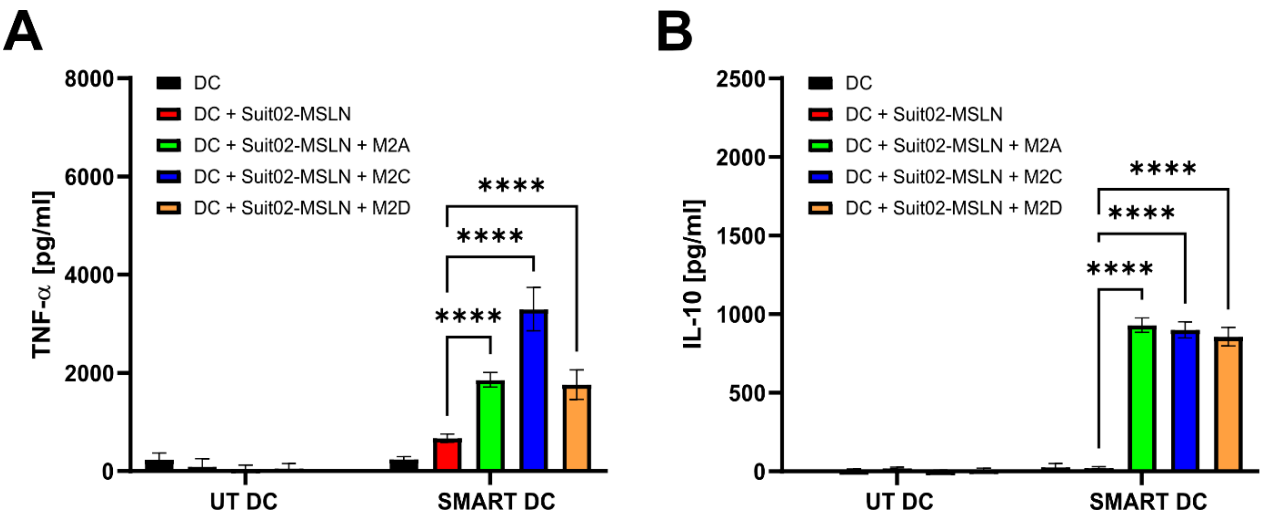

Figure 10: SMARt-DC reprogram immunosuppressive M2 macrophages to a more inflammatory phenotype

Control or SMARt-DC, M2 macrophages, and Suit02-MSLN cells were cultured at a 1:1:2 ratio, respectively for 24 hours. Supernatants were collected and measured via ELISA for the protein concentrations of TNF-α **(A)**, and for IL-10 **(B)**. Analyses of differences between groups were assessed at the last timepoint and performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001. All experiments show mean values ± SD and are representative of three independent experiments with three donors.

that was induced by SMARt-DC activation (Figure 9). Interestingly, the canonically immunosuppressive cytokine IL-10 that was recently shown to be able to restore intratumoral terminally exhausted T cells to a functional state and recover their anti-tumoral function [64], was highly upregulated in conditions with both M2-like macrophages and SMARt DC, but not in conditions with control DC (Figure 10).

3.8 SMARt DC synergize with CAR T cells

Because low E:T ratios are more physiologically relevant, and our data shows that SMARt-DC by themselves at these ratios do not lead to significant cytotoxicity (Figure 3), we investigated the effect of combining them with CAR T cell therapy.

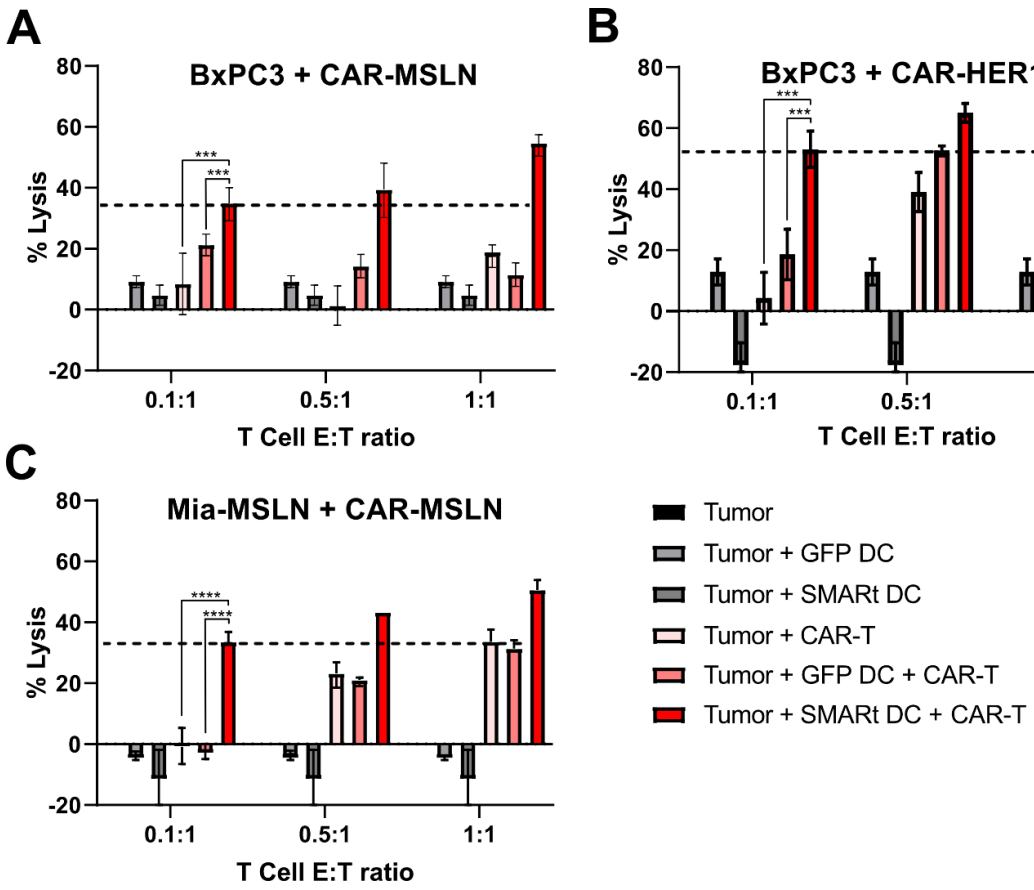

Figure 11: SMARt-DC exhibit strong synergistic killing with CAR-T cells

Control or SMARt-DC, anti-MSLN CAR-T cells, and ffLuc+ BxPC3 or Suit02-MSLN pancreatic cancer cells were cultured at 0.2:1 ratio between DC and tumor cells, and at the displayed ratios of T cells for 24 hours. Killing was measured via luciferase assay (**A**). The same assay was conducted with only BxPC3 cells using either anti-MSLN CAR-T cells, or anti-HER1 CAR-T cells and killing was measured via luciferase assay (**B**). Dashed lines are established at the level of tumor cells co-cultured with CAR-T cells and SMARt-DC at the 0.1:1 T-cell:Tumor ratio for comparison to tumor and CAR-T cells conditions at higher ratios. Analyses of differences between groups were performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001. Luciferase killing experiments show mean values ± SD and are representative of three independent experiments with three donors.

Not only are T cells and DC natural interaction partners in physiological immune responses [48], but the effects that are induced by the SMARt-DC target many of the issues that pose obstacles for CAR T cells in solid tumors. Our co-cultures with MSLN-expressing tumor cells, anti-MSLN CAR T cells, and anti-MSLN SMARt-DC or control DC reveal a synergistic effect between the two cell populations in inducing higher tumor cell cytotoxicity in multiple tumor models. It is frequently observed that the killing

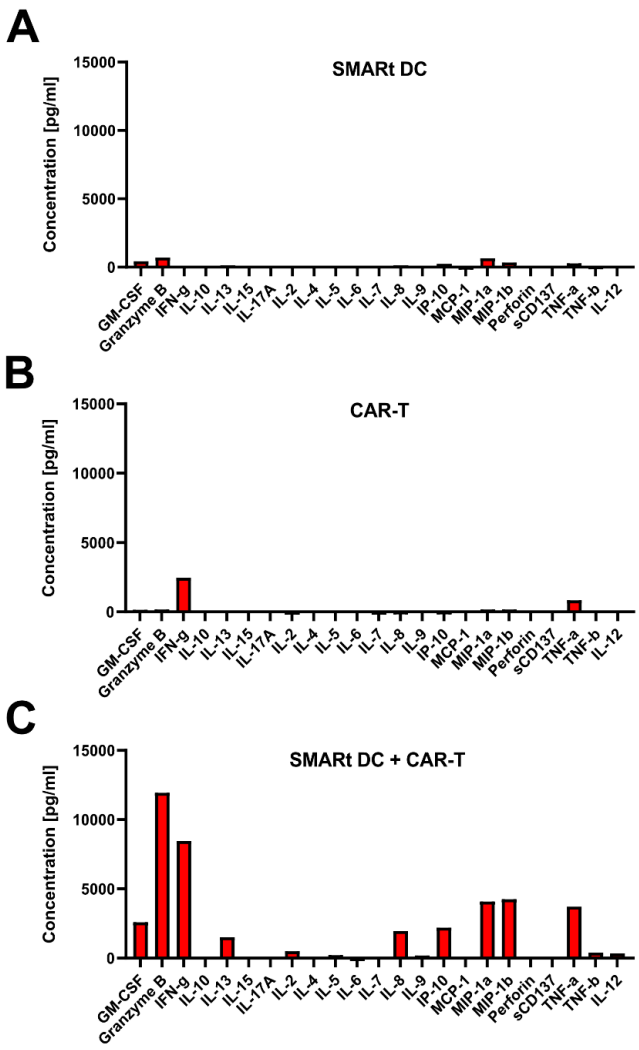

Figure 12: Cytokine release is increased by synergy between SMARt-DC and CAR-T cells

SMARt-DC, anti-MSLN CAR-T cells, and MiaPaCa02-MSLN pancreatic cancer cells were cultured at a 0.2:0.5:1 ratio, respectively, for 24 hours. Supernatants were collected and cytokine expression was assessed through the Isoplexis Codeplex multiplex cytokine assays. SMARt DC were co-cultured with MiaPaCa02-MSLN cells **(A)**. Anti-MSLN CAR-T cells and MiaPaCa02-MSLN cells were cocultured **(B)**. MiaPaCa02-MSLN cells, SMARt-DC, and anti-MSLN CAR-T cells were cocultured together **(C)**. Duplicates were used as recommended by the manufacturer and analysis of the data was processed internally using the IsoSpeak software 2.8.0.0. Only one donor was assayed.

capacity of the combined co-culture to be as potent as 10-fold the number of CAR T cells by themselves (Figure 11). We confirm that the synergy effect was not weakened using the same antigen target for both constructs in the BxPC3 tumor model which expresses physiological levels of MSLN (Figure 11), as antigen density and receptor competition could be a concern within tumors. We further investigated CAR T cell and

SMARt-DC synergy in relation to cytokine release. The multiplex Codeplex assay we conducted revealed significantly increased cytokine induction in the combined conditions compared to either CAR T cells or SMARt-DC condition alone, reinforcing the previous cytotoxicity data (Figure 12). Lastly, we observe that the phenotype of the SMARt-DC shifts markedly to a mature DC phenotype with high upregulation

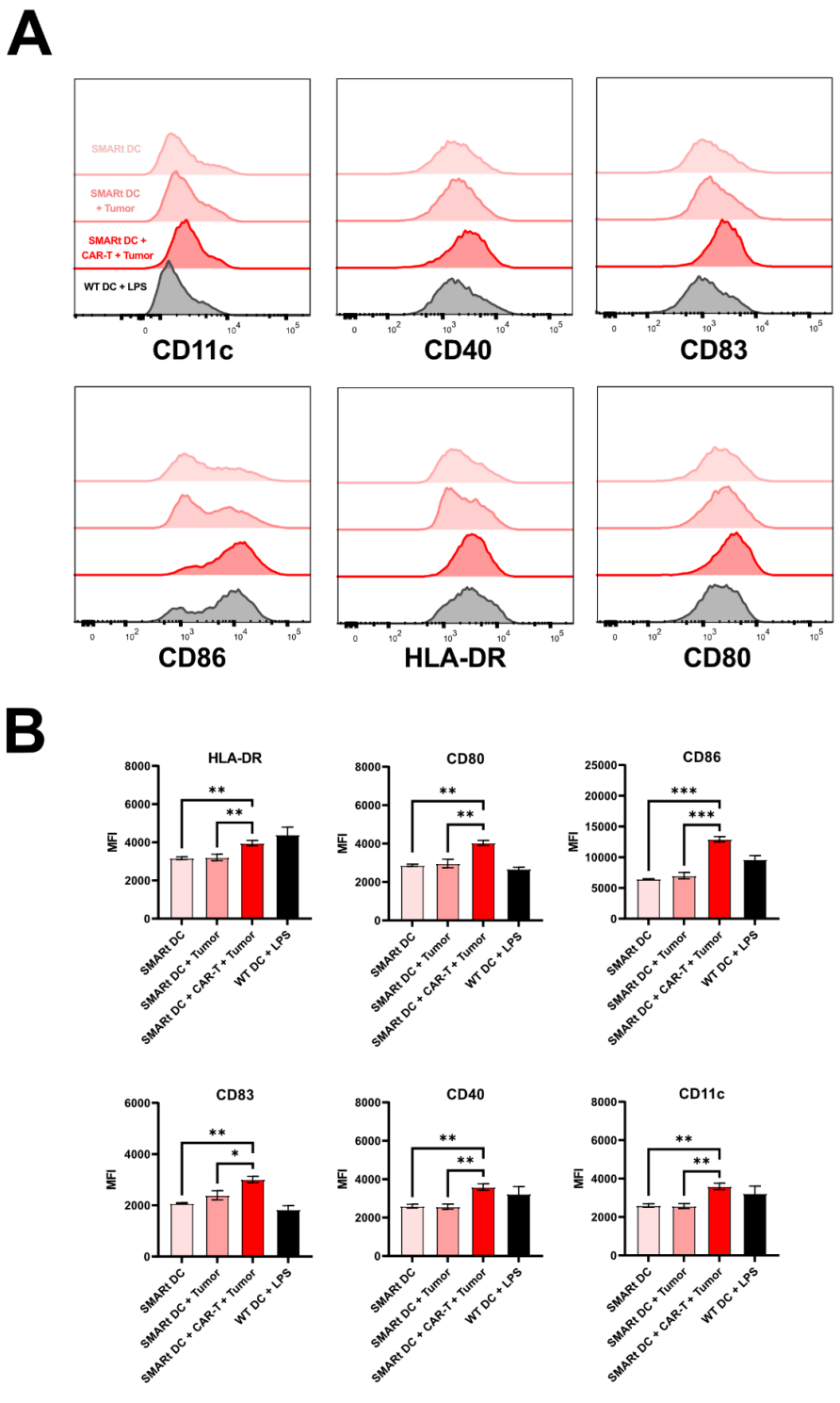

Figure 13: Synergy between SMARt-DC and CAR-T cells, and induces a stronger inflammatory phenotype on SMARt-DC

SMARt-DC, anti-MSLN CAR-T cells, and MiaPaCa02-MSLN pancreatic cancer cells were cultured at a 0.2:0.5:1 ratio, respectively, for 24 hours. Dendritic cells were pre-stained with eFluor450 proliferation dye and their phenotype was assessed via flow cytometry. SMARt-DC in this assay are defined as eFluor450+, c-myc+ cells. The total surface expression of the inflammation/activation-associated markers HLA-DR, CD86, CD80, CD40, CD83, and the phenotypic marker CD11c are depicted as histograms to display phenotypic shift (**A**) and quantified using the MFI of the markers (**B**). Analyses of differences between groups were performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001. All MFIs show mean values ± SD and are representative of three independent experiments with three donors.

of maturation markers as well as costimulatory markers (Figure 13). CAR T cell activation/exhaustion phenotypes (not shown) remain similar between tumor only, and tumor with control DC or SMARt-DC conditions. CAR T cell proliferation, however, was markedly affected by the presence of dendritic cells.

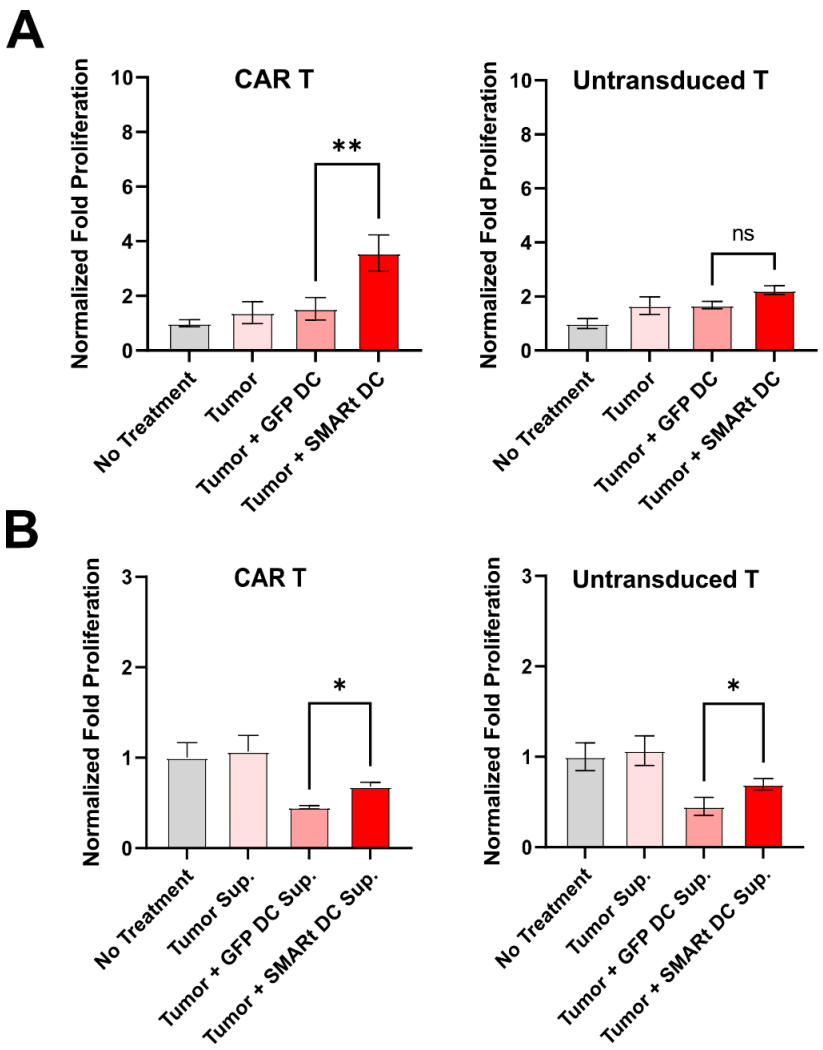

Figure 14: Activation of SMARt-DC induce greater proliferation of CAR-T cells

SMARt-DC, anti-MSLN CAR-T or untransduced T cells, and MiaPaCa02-MSLN pancreatic cancer cells were cultured at a 0.2:0.1:1 ratio, respectively, for 24 hours (**A**). Control or SMARt-DC were cultured with MiaPaCa02-MSLN pancreatic cancer cells at a 0.2:1 ratio for 24 hours, after which the supernatant was transferred and cultured with either anti-MSLN CAR-T or untransduced T cells alone for 24 hours (**B**). Human CD3/CD28 Dynabeads were used as positive controls in both assays. Proliferation of T cell populations was analyzed via flow cytometry, with UT cells defined as CD3+, c-myc-, and CAR-T cells as CD3+, c-myc+ within the assay. Analyses of differences between groups were performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001. All proliferation experiments show mean values ± SD and are representative of three independent experiments with three donors.

The effect on CAR T cell proliferation caused by direct co-culture with tumor cells and SMARt-DC or control DC, as well as culture with only the supernatant of tumor cells and DC were investigated. Direct co-culture, where contact interactions are enabled, displayed advantage in proliferation for CAR T cells that were cultured with SMARt-DC (Figure 14). The same result trend is found for supernatant co-culture, except that all conditions with dendritic cells have reduced CAR T cell proliferation below the control levels (Figure 14). While this unexpected reduction is reproducible, we currently have not investigated it more in depth and attribute it to unknown assay factors.

3.9 Combined SMARt DC and CAR T cell therapy synergize for increased tumor control in in vivo pancreatic xenograft models

SMARt-DC were used as monotherapy and in combination with anti-MSLN CAR T cells against the xenograft pancreatic tumor MiaPaCa-MSLN in NSG mice. As previously speculated, mice that received both control DC or SMARt-DC alone performed no better than the negative control (Figure 15). This falls in line with previous data reported on this tumor model, where anti-MSLN CAR T cell therapy alone also did not result in any tumor control [79]. However, in the mice that received both anti-MSLN SMARt-DC and anti-MSLN CAR T cells, there was delayed tumor outgrowth (Figure 14). Mice in this group experienced high rates of tumor ulceration and required removal due to prespecified endpoints. Despite this, survival approaching statistical significance was observed at the time of termination (Figure 15).

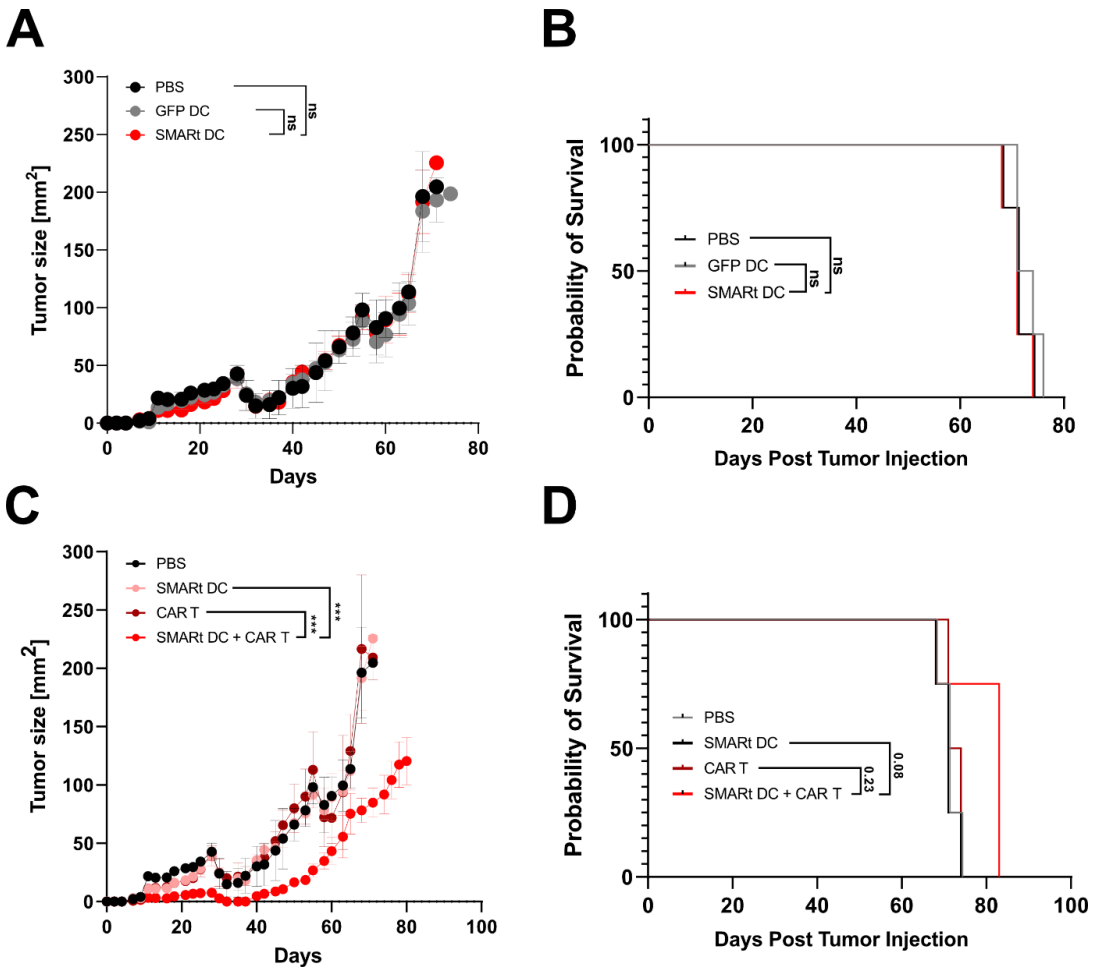

Figure 15: SMARt-DC function enables CAR-T anti-tumor efficacy in a human pancreatic xenograft model

NSG mice inoculated s.c. with 10^7 MiaPaCa-MSLN cells were treated with a single i.v. injection of PBS control or either $5x10^6$ control or SMARt-DC after tumors reached palpability. Tumor growth curves are depicted (**A**), as well as survival (**B**). NSG mice inoculated s.c. with $1x10^7$ MiaPaCa-MSLN cells were treated with a single i.v. injection of PBS control, $5x10^6$ SMARt-DC, $1x10^7$ CAR-T cells, or the combination of both cells after tumors reached palpability. Tumor growth curves are depicted (**C**), as well as survival (**D**). Analyses of differences in tumor growth between groups were performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. Analyses of survival differences between groups were performed using the log-rank test. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001.

3.10 SMARt DC induce greater localization of CAR T cells into the tumor

We initially surmised that one of the advantages of SMARt-DC-mediated release of inflammatory cytokines and chemokines would be the induced migration of cytotoxic T and NK cells that can respond to such gradients. To probe this question, we conducted trans-well migration assays with PBMC migrating towards supernatants from

conditions of co-culture with tumor cells, control or SMARt-DC, and CAR T cells. Supernatants from conditions of co-culture with SMARt-DC induce both increased migration of immune cells and enrichment of NK cells and T cells within the migrated cells compared to control DC (Figure 16). In addition, the combination of CAR T cells and SMARt-DC induced even greater migration and enrichment of cytotoxic cells, suggesting that the combination of these therapies in vivo may mobilize many more endogenous cells to the tumor microenvironment (Figure 16). We confirm intratumoral localizations in the MiaPaCa-MSLN in vivo xenograft model, where we observed that SMARt-DC do indeed localize intratumorally, though control DC do also at lower rates. Introduction of CAR T cells in combination induces greater SMARt-DC infiltration (Figure 16). Looking at the CAR T cell side of combinatorial therapy, we confirm basal level CAR T cell tumor infiltration in single therapy treated mice. In contrast, we observe a two-fold increase in CAR T cell tumor infiltration in mice treated with both CAR T cells and SMART-DC (Figure 16). This solidifies one of the major inferred mechanisms of SMARt-mediated anti-tumor immune mobilization.

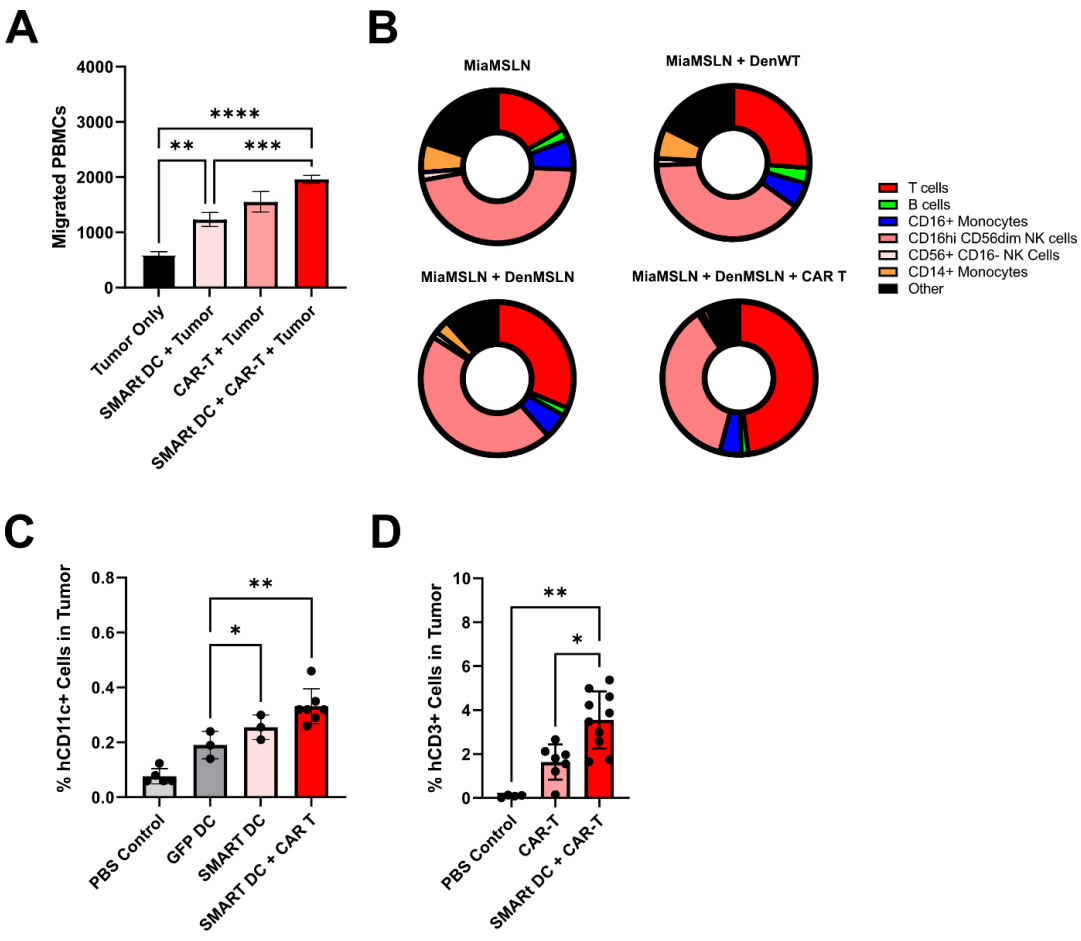

Figure 16: SMARt-DC activation induces migration of cytotoxic cells from blood as well as adoptive CAR-T cells towards the tumor

A Boyden chamber migration assay was conducted with a co-culture of MiaPaCa02-MSLN cells with SMARt-DC on the bottom portion, and fresh donor-matched PBMCs on the top portion. Migration is represented by the total number of cells migrated through 5µm pores to the bottom well after 6-hours incubation. Counts were normalized with counting beads and measured via flow cytometry (**A**). The migrated cell compartment was stained for major populations of blood lymphocytes and analyzed via flow cytometry (**B**). NSG mice inoculated s.c. with $1x10^7$ MiaPaCa-MSLN cells were treated with a single i.v. injection of PBS control, $5x10^6$ control DC, $5x10^6$ SMARt-DC, and $5x10^6$ SMARt-DC with $1x10^7$ CAR-T cells. Tumor infiltrating-DC are defined as human CD11c+ cells (**C**). Similarly inoculated mice were treated with a single i.v. injection of PBS control, $1x10^7$ CAR-T cells, or both $5x10^6$ SMARt-DC and $1x10^7$ CAR-T cells after tumors reached palpability. Mice were sacrificed one week after adoptive cell injection, with tumors harvested and examined as single cell suspensions via flow cytometry. Tumor infiltrating CAR-T cells are defined as human CD3+, c-myc+ cells (**D**). Analyses of differences between groups were performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001. All experiments show mean values ± SD.

3.11 SMARt DC persist for a period of weeks in vitro, and possibly longer in vivo

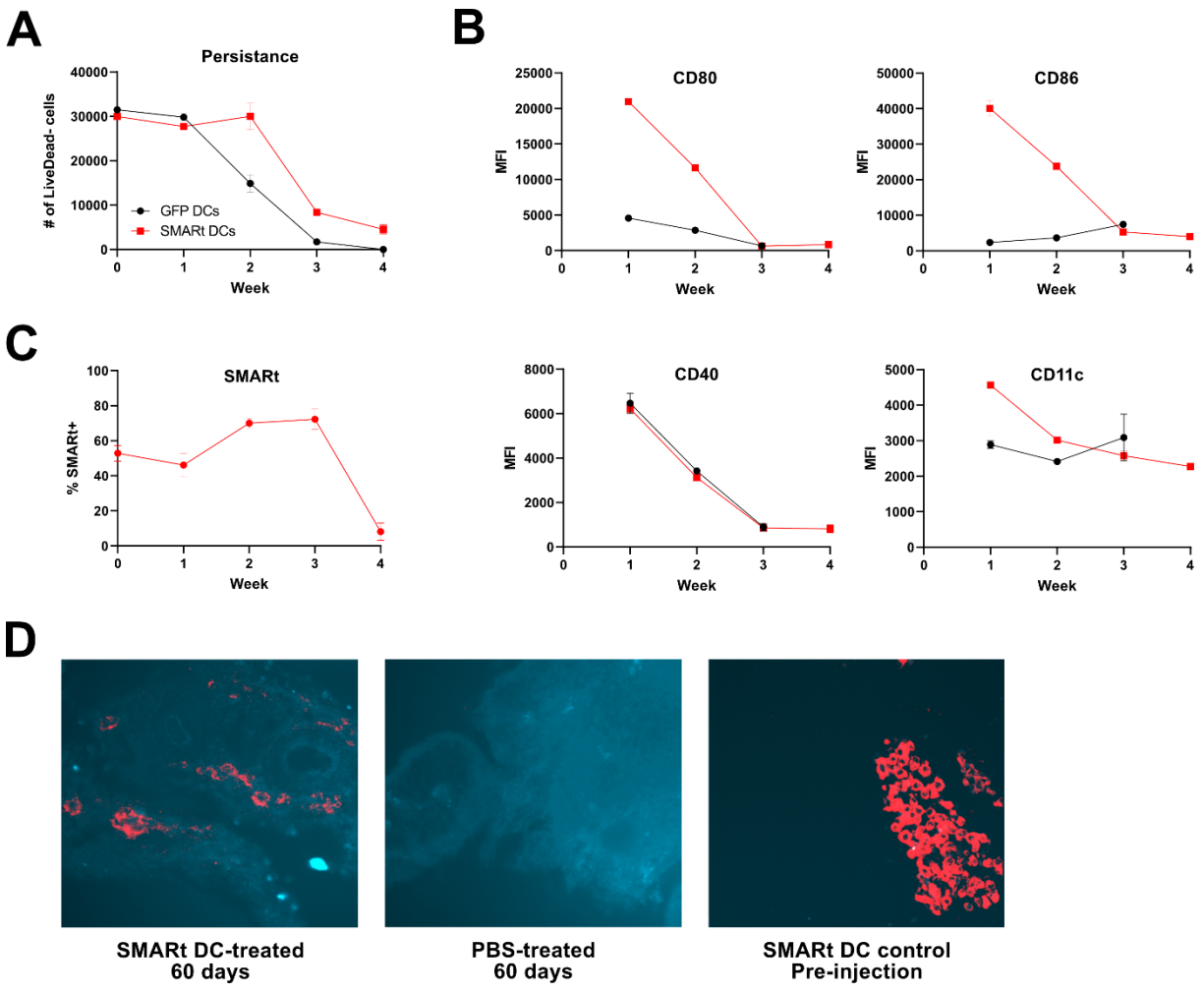

Figure 17: SMARt-DC persist both invitro and in vivo for long periods of time

After transduction, SMARt-DC and control DC were maintained in culture with GM-CSF and IL-4 replenished every 3 days. The DC, defined as CD11c+ cells, were analyzed at various timepoints for persistence (**A**), expression of inflammatory and phenotypic markers (**B**), and SMARt expression (**C**) via flow cytometry. NSG mice inoculated s.c. with $1x10^7$ MiaPaCa-MSLN cells were treated with a single i.v. injection of PBS control (n=4) or either $5x10^6$ control (n=4) or SMARt-DC after tumors reached palpability. Tumors were harvested as they reached prescribed endpoints, flash-frozen, and slices were analyzed via Zellscanner chip microscopy (**D**). Analyses of differences between groups were performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. $p < 0.05$ was considered statistically significant and represented as * < 0.05, ** < 0.01 and *** < 0.001. All flow cytometry experiments show mean values ± SD and are representative of three independent experiments with three donors.

Primary terminally differentiated dendritic cells isolated from human blood are known in literature to persist in culture only for a few days after isolation even under optimal conditions [80]. Thus, the persistence of monocyte-derived SMARt-DC is a point of primary concern. After transduction, we maintained cultures of SMARt and control DC in GM-CSF and IL-4 to test persistence. Control mdDC begin to lose viability in numbers after the first week of culture, while SMARt-DC maintain their viability for at least two weeks, while also demonstrating a slower rate of decline thereafter (Figure 14). Though this perhaps represents “optimal” conditions ex vivo, many potentially crucial factors that would be present in an in-vivo system remain missing. This is most evident in previously shown data of SMARt-DC infiltration into the in vivo xenograft model, which contained live cells even after 60 days of tumor growth (Figure 14).

4 Discussion

The scientific work outlined in this thesis dissertation aims to characterize the ideation, creation, and functional testing of a synthetic myeloid activating receptor (SMARt) engineered into monocyte-derived dendritic cells to be used as an adoptive cellular therapy for the treatment of pancreatic cancers. The experimental data here show that the SMARt expresses as intended on the dendritic cell and mediates an antigen-specific activation effect in the dendritic cells that has both cytotoxic and immune mobilizing capacities. SMARt-DC mediated cytotoxicity was shown to be correlated to both phagocytosis and cytokine signaling through cytokines such as TNF-α, while the capacity of the SMARt-DC to mobilize endogenous and adoptive immune cells is mediated through cell-cell interactions, release of immune-stimulating cytokines, as well as migration-inducing chemokines. SMARt-DC synergize in combination with CAR T cell therapy, with the use of both together inducing significant fold increases of tumor killing. The combination of CAR T cells and SMARt-DC also demonstrates reduction of tumor growth and significantly increased immune infiltration in an initial pancreatic xenograft model, which shows no response towards CAR-only treatment. While this data demonstrates preliminary efficacy as a cellular therapeutic treatment against pancreatic cancer, the underlying results and many unelucidated aspects pertaining to mechanism, efficacy, and safety remain will be discussed here.

4.1 SMARt-DC ideation and filling a niche

The origin of the SMARt begins with the antibody-derived scFv. scFv demonstrate straightforward customizability through successful combination with various transmembrane and intracellular domains in CAR, TRuCK, and other receptors [18, 21] and remains the optimal choice in adding antigen-specificity to recombinant receptors. Moving forward with construct design, the inclusion of the CD40 transmembrane and intracellular domains constitutes the defining feature of the SMARt that confers effector function in dendritic cells. CD40 signaling has been utilized in the cancer therapeutics setting mainly in agonist antibodies that activate CD40-expressing cells [81] and more recently as bispecific engagers that draw dendritic cells to interact with immunosuppressive cells in the TME [82]. A major drawback of the former was its

dose-limiting off-tumor cytotoxicity while the latter has not yet been well investigated clinically [81, 82]. We bypass these issues and harness the potential of anti-tumor CD40 signaling through combining it with the scFv. Conveniently, CD40 signaling in myeloid cells initiates through dimerization of CD40 intracellular domains upon extracellular binding of CD40L (CD154), similar to how CAR are thought to be activated by aggregation of CD3z domains upon binding of the scFv to its cognate antigen [18]. Thus, our recombinant receptor indeed resembles CAR in both structure and mechanism. However, its engineering onto DC allowed for fulfilling a unique, multirole niche. The full concept – SMARt-engineered DC – induce CD40-mediated inflammatory activation in antigen-expressing tumors, remodel the TME to become more immunopermissive, and to acquire antigen for further immune mobilization.

4.2 SMARt-DC-mediated direct anti-tumor function

We have extensively characterized the effector functions of the activation of the SMARt in dendritic cells. We rigorously examined antigen specificity using antigen-expressing and non-expressing cells, and with recombinant protein (data not shown), both demonstrating a high level of antigen specificity. Antigen-specific activation was linked to intracellular CD40-mediated downstream signaling through assays inhibiting the CD40-TRAF interaction, confirming the complete expected mechanism of the SMARt.

Activation of the receptor was shown to induce phagocytosis and a significant release of a variety of inflammatory cytokines. While increased phagocytotic killing can be plainly associated with anti-tumor function [44], the release of cytokines warrants a more nuanced examination in terms of overall effect on both tumor cells and the TME. While many of the released cytokines such as, IP-10, MIP-1α, MIP-1β, and MCP-1 have consensus anti-tumor function [55, 56], other released cytokines such as GM-CSF, IL-13, IL-8, and TNF-α are found to play both pro- and anti-tumorigenic roles through direct action on the tumor or in orchestrating signaling axes between tumor-associated cells within the TME [59, 62, 83, 84]. The cytokine-associated tumorigenesis mentioned above typically revolves around inducing low level tumor inflammation, which helps the tumor grow [62]. In contrast, we expect and show that SMARt-DC affects sudden and

immense release of cytokines. At least two pleiotropic inflammatory cytokines released by SMARt-DC, IL-1β and TNF-α, have been shown to be reliably anti-tumor in nature in that type of setting [58, 62]. Due to the complexity of the system, we opt to examine this cytokine question in a holistic sum-of-the-parts view, rather than an onerous examination of each individual cytokine function. The first and most important conclusion is that the SMARt-DC mediated release of cytokines on tumor binding does not increase the growth rates of the tumors in vitro at various E:T ratios, and in vivo at physiological levels. The cytokines and chemokines secreted attract a highly favorable influx of endogenous cytotoxic T cells and NK cells towards the tumor while not significantly increasing levels of other cell types. This chemotactic advantage is also conferred in the combinatorial therapy of SMARt-DC and CAR T cells, where the presence of SMARt-DC in the tumor correlates with higher CAR T cell infiltration. As a whole, the combination of phagocytosis and inflammatory cytokine release mediated by SMARt-DC constitute a decidedly anti-tumor function.

4.3 SMARt-DC and interactions with the M2 macrophages

SMARt-DC are able to maintain their inflammatory phenotype, and importantly, their antigen-specific cytotoxic capacity and cytokine release in the presence of tumor and M2-like TAM, an immunosuppressive population highly enriched within pancreatic tumors [85, 86]. Indeed SMARt-DC activation and the resulting inflammatory cytokines result in a distinct reprogramming of the macrophages to a more immunoinflammatory phenotype, marked by increased levels of costimulatory receptors CD80 and CD86, as well as the type II HLA-DR, all of which are canonically upregulated in inflammatory myeloid activation [65, 80]. Interestingly, we show higher cytokine levels released in conditions of SMARt-DC activation with M2 macrophages present, a result that when combined with the aforementioned phenotype data infers that the SMARt-DC are causing the M2 macrophages themselves to release additional inflammatory cytokines. Only in conditions with both SMARt-DC and M2 macrophages do we observed release of IL-10, a cytokine traditionally associated with immune suppression but recently shown to restore function in endogenous terminally exhausted intratumoral T cells [64]. Though IL-10 in our system is only one cytokine with merely postulated function, its

expression here illustrates the high possibility of interaction between SMARt-DC and tumor-associated cells resulting in novel anti-tumor functions that warrant further investigation. Altogether the data overview reflects a robust reshaping of the TME that is mediated through SMARt-DC activation.

4.4 SMARt-DC-mediated antigen presentation and the potential for endogenous immune mobilization

mdDC have been shown to possess robust antigen presentation capability of both HLA-I and HLA-II restricted antigens to a variety of other immune cells [87, 88]. This concept underlies the main biological mechanism of the concept of the dendritic cell vaccine, which currently lists one approved therapy [1]. mdDC are loaded with tumor antigen and reintroduced into the patient to elicit an endogenous immune response against that antigen [1]. In lieu of antigen pre-loading as with DC vaccines, we postulate SMARt-DC will instead autonomously acquire tumor antigen. We demonstrate both SMARt-DC localization into the tumor (access to antigen), and their unimpaired ability to acquire antigen from their surroundings through pinocytosis and phagocytosis. We thus infer that these cells will capably present these antigens while inside the tumor, but also if they migrate to lymph nodes as they mature. CD40 signaling incidentally signals a positive feedback loop for more CD40 expression which results in higher costimulation capacity [46, 54]. Accordingly, SMARt activation harnesses this aspect of CD40 signaling to boost costimulation of interacting cells in addition to presenting tumor antigen. Future work will be dedicated addressing this topic further, as SMARt-DC-mediated tumor antigen presentation on both MHC-I and MHC-II are both expected, but not yet proven. Furthermore, we've observed hopeful signs hinting at significant reinvigoration of exhausted intratumoral immune cells mediated through SMARt-DC activation. Tumor-specific TIL feature in varying degrees in many solid tumors, with the commonality being suppression and dysfunction mediated by the TME [89]. Many reports show that these intratumoral TIL are a potentially pivotal reservoir of tumor-specific cells that can be utilized against tumors if targeted for priming and reinvigoration [11, 90]. Thus, in addition to mobilizing new immune responses against

the tumor, there is significant importance in fully investigating the role that SMARt-DC may play in harnessing these TIL to repower previously suppressed immune responses.

4.5 SMARt-DC in the context of other related cellular therapies

CAR-M and CAR-P constitute the most similar therapeutic concepts comparable to SMARt-DC. Similar to SMARt-DC, they phagocytose tumor cells and elicit some degree of inflammation and TME remodelling [44, 45]. The selection of macrophages as the basal cell therapy infers a reliance on phagocytosis as the main effector function. Indeed, CAR-M demonstrated anti-tumor efficacy in both in vivo and inv vitro HER2+ breast cancer models as a monotherapy, while CAR-P efficacy was demonstrated in-vitro [44, 45]. This high degree of phagocytosis likely owes much to the selection of the intracellular domains on the CAR constructs themselves. Fcγ receptors used on CAR-P are the quintessential receptors mediating phagocytosis for macrophages, while CD3ζ used in CAR-M bears strong homology to it [44, 91]. Megf10, also used in CAR-P, mediates clearance of apoptotic cells by myeloid cells [92]. In addition to phagocytosis, CAR-M demonstrated in vitro cross-presentation of CD8 T cells, while CAR-P did not [44, 45]. Human mdM are however suggested in literature to have non-functional MHC-II presentation, thereby limiting the scope of a possible endogenous immune response to only CTL [89, 90]. In constrast to these approaches, we designed SMARt-DC to trade away the high phagocytosis-based direct killing potential of macrophages [93] for the possibility of increased inflammatory immune co-stimulation and antigen-presentation capability, both properties of CD40 signaling and DC [65, 93]. While some phagocytosis was expected [46, 81], we found that SMARt-DC could in many cases elicit phagocytosis-based cytotoxicity comparable even to CAR T cell cytotoxicity. In vivo anti-tumor cytotoxicity was insufficient to consider SMARt-DC for monotherapy. However, though this may be a result of the tumor entities and models targeted and not of the cytotoxic potential of the treatment itself, as CAR T cells in our models also proved similarly ineffective as monotherapy.

Another cellular therapy that SMARt-DC contrast against are dendritic cell vaccines. DC vaccines demonstrate that a DC-based therapy can prove efficacious against solid tumors and indeed even receive regulatory approval [1, 7, 8]. Peptide

loading on the MHC molecules of dendritic cells, which forms the basis of DC vaccines such as Sipuleucel-T [1], theoretically bypasses the need to acquire de-novo antigen for presentation. However, this also limits the subsequent immune mobilization to a small subset of T cells that are reactive to the presented peptides. Though there is some evidence that DC vaccines can lead to epitope spreading [94, 95], this is likely dwarfed by the repertoire that can be elicited by SMARt-DC designed to acquire tumor neoantigen de-novo. These approaches also do not elicit direct tumor cytotoxicity, and the T cell effectors that are generated encounter the same suppressive issues with solid tumors and the TME that T cell-based cellular therapies do [3, 96]. SMARt-DC thus represents an option within the spectrum of cellular therapies that balances direct cytotoxicity with high potential for endogenous immune mobilization.

4.6 Considerations for pancreatic cancer as the initial tumor entity target and MSLN as the antigen

A great deal of the morbidity of pancreatic cancer derives from the biology of the tumor, which is defined by a highly immunosuppressive TME, a well-developed stroma that physically excludes potential treatments, and aggressive metastasis [49, 50]. We initially chose this tumor entity as a target because successful function of SMARt-DC should address many of these issues and provide an avenue for treatment. However, we postulate that many immune-restricted tumor entities such as colorectal, ovarian, and some breast cancers [96, 97] would benefit highly from SMARt-DC therapy for similar reasons as pancreatic cancer. With different tumor indications, the use modalities of SMARt-DC may also shift. For instance, in vivo data for our pancreatic models precluded monotherapy. However, less aggressive or resistant tumors may enable that option. Tumor entities for which checkpoint inhibition is currently approved may maximize benefit with SMARt-DC combinatorial therapy, an avenue we are currently investigating.

With pancreatic cancer settled as the initial target, mesothelin (MSLN) was selected as the antigen target due to its near-universal expression in pancreatic cancer tissues [98, 99], as well as the demonstrated efficacy of MSLN-targeting immunotherapies against pancreatic tumors preclinically [100, 101]. MSLN is also the

most developed cellular therapy-targeted pancreatic cancer antigen clinically, with the most clinical trials involving cellular therapies ongoing and completed [102]. There is strong potential for other targets however, with the only true limitation being low levels of expression in healthy tissues. Due to toxicity concerns, targets such as HER2, Claudin 18.2, GPC3, CEA, and others [103, 104] which are more tightly regulated would fare much better than a target which is more broadly expressed, such as EGFR [105, 106]. Furthermore, as SMARt-DC by design targets the TME and acquires neoantigen from tumors, highly specific but heterogeneously expressed tumor antigen targets such as CanAg [107] can be the targets specified by the SMARt scFv, whereas normally these targets are precluded from traditional CAR T cell therapy. We believe strongly in the potential for SMARt-DC therapy to be a platform enabler in a variety of immune-restricted tumors using a multitude of targets. We have begun studies with SMARt-DC on other tumor indications with prominently expressed MSLN, as well as created SMARt directed against the appropriate antigen targets of other indications.

4.7 Efficacy of SMARt-DC as a standalone treatment, and the logical combination with CAR T cells and other cancer therapies

In vivo data from our pancreatic cancer models suggest that SMARt-DC (and also CAR T cells) by themselves as monotherapy do not mediate significant anti-tumor activity. We thus endeavored to combine SMARt-DC with CAR T cells, a natural combination in which the TME-remodeling capacities of myeloid cell therapies [44] can be exploited to allow greater CAR T cell infiltration and tumor clearance and where CAR T cell-mediated tumor killing generates large amounts of antigen [108, 109] to be up taken and presented by the SMARt-DC. A substantial synergy is demonstrated between the SMARt-DC and CAR T cells in killing, inflammatory cytokine release, and CAR T cell proliferation in vitro, translating to significant efficacy in vivo. These results in total echo many other studies where CAR T cell therapy is combined with immunomodulating agents that similarly inflame the TME or directly enhance T-cell activity for combinatorial anti-tumor synergy [110-112]. We’ve additionally demonstrated that directing the SMARt-DC and CAR T cells towards two different antigens of the same tumor entity does not affect the efficacy of the combination. This differential targeting may in fact be

more beneficial to generating epitope spreading as more tumor cells are targeted in total, thus lowering the chances of leaving unaffected cells for tumor escape [110]. While safety profiles must always be vigilantly assessed when dealing with cellular therapies, we believe that combining SMARt-DC and CAR T cells enables the targeting of a wide range of targets and tumor indications not yet conceived of for either as a monotherapy.

Though we have demonstrated SMARt-DC as a logical and successful combinatorial therapy with CAR T cells, there is clear potential in pairing it also with other classes of immunotherapy, such as checkpoint inhibition. Checkpoint inhibition remains the standard-of-care treatment for various types of solid tumor and achieved blanket approval for mismatch repair-deficient (dMMR) solid tumor entities [113-115]. The enabling factor for efficacy in these dMMR tumors, and for checkpoint inhibition in general centers on the immune proficiency of the target tumor, based on factors such as TIL and NK infiltration, PD-1/CTLA-4 expression on these cells and their receptor expression on tumor cells, and the degree of overall immunosuppression within the tumor [116, 117]. Indeed, pancreatic cancer as an entity typically does not respond well to checkpoint inhibition due to its high degree of immune restriction [49, 51, 118]. We thus posit that our results in utilizing SMARt-DC against pancreatic cancer demonstrates its capabilities to inflame the TME and provide an avenue to enable checkpoint inhibition for this tumor entity, a concept that is under investigation. We believe this principle will stand true for other highly immune-restricted tumor entities such as breast, and ovarian cancers [96, 97].

Combinatorial therapy with CAR T cells and checkpoint inhibition both rely on the onus of SMARt-DC enabling the function of those respective therapies. An equally suited view on combinatorial therapy may be the opposite, with a prospective combination therapy enabling the function of SMARt-DC. Therapeutic agents that produce high amounts of direct tumor cell death will naturally produce free tumor antigen for uptake by SMARt-DC, while also addressing its weakness in direct tumor cytotoxicity. Antibody drug conjugates (ADC), radiotherapy, and chemotherapy all fall under this category of tumor-direct acting therapies [119-121]. There is already substantial evidence that chemotherapies and immunotherapies can be mutually

beneficial in certain combinations [122, 123]. The same holds true for radiotherapy and immunotherapies [124, 125]. These direct acting therapies not only provide tumor killing, but in many cases will also elicit some degree of TME remodeling [124, 125], possibly enhancing SMARt-DC infiltration and function. While investigating these combinations have not been our primary interest, we believe that in fact they may be very viable as combinations for currently inaccessible tumor entities. SMARt-DC may indeed be a way to revamp the efficacy of these existing therapies by providing an element of epitope spreading that would complement their direct effect on tumors.

4.8 SMARt-DC persistence and longevity within the tumor

Mature, fully differentiated dendritic cells harvested from human blood are viable in culture for only a few days [80]. SMARt-DC when cultured with GM-CSF and IL-4 can maintain phenotype and viability for at least two weeks, after which both begin to deteriorate. Not surprisingly, untransduced mdDC deteriorate much quicker, which can likely be attributed to the lack of GM-CSF and other cytokines secreted due to the low-level inflammation caused by adenoviral transduction in SMARt-DC [44, 78]. This is consistent with literature, in which both LPS stimulation and adenoviral transduction led to increased DC survival [78, 80]. CAR-M, similarly transduced with constructs encoded in adenovirus, were able to maintain detectable levels in vivo for over 60 days, though there was decrease in viability [44]. In vivo, we can also observe live, confirmed SMARt-DC in the tumor tissue as long as 60 days post injection. The difference between in vitro and in vivo persistence can be attributed to the presence of a more complete in vivo environment which might provide cytokines missing in culture. This echoes results that are observed with CAR T cells, which in our hands become unviable after two to three weeks in culture but maintain themselves in mice tumors and organs for months. In the human system, generation of an adaptive immune response against antigen is known to take roughly a week [126]. SMARt-DC can thus maintain themselves long enough for their main task to be accomplished, though further studies in humanized mice with capable immune repertoires are required to answer whether this does indeed happen within a sufficient timeframe.

5 Conclusions and future outlook

In pancreatic cancer, the various components of the TME synergize to promote tumor growth, orchestrate a network of immunosuppression, and physically excludes cellular and traditional cancer therapies, ultimately leading to dismal relapse rates and therapeutic outcomes [49, 51]. Despite the consequential preclinical and clinical efforts to address these issues posed by the TME of pancreatic and other solid cancers, it remains by and large the unresolved main barrier to beat to enable efficacious therapeutic responses [3].

Here, we describe a novel cellular therapeutic approach purpose driven to address issues posed by the TME – SMARt DC – that simultaneously inflames an otherwise immune-restricted tumor, drives the infiltration of various beneficial immune populations, and hopefully mobilizes an endogenous immune response that complements the antitumor effects of adoptive therapy. We were able to characterize the processes and mechanisms from which these SMARt DC can be developed from mdDC, transduced with adenovirus to express a recombinant receptor that antigen-specifically activates CD40 signaling in the DC and drives both phagocytosis and cytokine inflammation. We further outline the efficacy this may have against pancreatic cancer with various in vitro models and illustrate the considerable potential that SMARt DC show when combined with traditional CAR T cell therapy. We finally demonstrate the promising first steps of testing the combinational concept in in vivo xenograft pancreatic tumor models. These results represent a further step in the evolution of cellular therapies to address solid tumors, though many unaddressed points remain in developing SMARt-DC into a viable trial-ready therapy.

The development of SMARt-DC outlined in this thesis reflects the ongoing efforts in improving other cellular therapies – in CAR T cells and many others – to address issues posed by the TME. Novel improvements in these therapies that target elements of TME-mediated immune restriction result in more efficacious anti-tumor therapy. Furthermore, concepts that target multiple TME elements or remodel the TME altogether induce the immune permissiveness that enables efficacy for previously ineffective immunotherapeutics, opening the potential for a wide range of new therapeutic combinations. This study and others like it move forward the state-of-the-art

in solid tumor-targeting immunotherapies, and the collective success of these studies signals a promising future for patients suffering from solid tumors.

6 Abstract

While the advent of CAR T cells changed the paradigm of last-line treatment for several hematological malignancies, they unfortunately have not achieved the same therapeutic successes in solid tumors. In the case of pancreatic cancer, many of the issues faced by CAR T cells and other cellular immunotherapies can be sourced to the tumor microenvironment that can physically restrict the access of these therapies as well as exert robust immunosuppressive forces, both leading to therapeutic inefficacy.

We present here the novel concept of synthetic myeloid activating receptor (SMARt) dendritic cells as an approach to targeting the tumor microenvironment through inflammation and promotion of immune permissiveness. SMARt DC bind specifically to cognate antigen and induce downstream CD40 signaling, a process which induces phagocytosis of the target tumor cells as well as strong cytokine-mediated immune inflammation. We show that these DC can maintain inflammatory phenotype in the presence of immunosuppressive cells and can actively reprogram those cells to a more inflammatory phenotype. The therapy synergizes with CAR T cell therapy, where SMARt-DC-mediated immune permissiveness allows space for CAR T cell-mediated tumor killing, resulting in abundant available tumor antigen for SMARt-DC uptake and presentation. This synergy induces several fold-increases in tumor killing *in vitro*, as well as enables anti-tumor efficacy in pancreatic xenograft cancer models that are resistant to CAR T cell monotherapy.

Our results outline a novel platform therapy that enables cellular therapy in pancreatic cancer but could be also repurposed for other solid tumor entities. Additionally, this approach reinforces the feasibility of cellular therapeutic approaches that stress TME targeting in enabling efficacy in solid cancer entities.

7 Bibliography

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8 List of abbreviations

A	
Acute lymphocytic leukemia	ALL
Adoptive T cell therapy	ACT
Antibody-dependent cellular phagocytosis	ADCP
Antibody-drug conjugate	ADC
C	
C-C chemokine ligand	CCL
C-C chemokine receptor	CCR
C-X-C chemokine ligand	CXCL
C-X-C chemokine receptor	CXCR
Chimeric antigen receptor	CAR
Chimeric antigen receptor macrophage	CAR-M
Chimeric antigen receptors that trigger phagocytosis	CAR-P
Cluster of Differentiation	CD
Cytokine release syndrome	CRS
Cytotoxic T lymphocyte-associated protein 4	CTLA-4
D	
Dendritic cells	DC
Damage-associated molecular pattern	DAMP
Deficient mismatch repair	dMMR
G	
Granulocyte-macrophage colony-stimulating factor	GM-CSF
G-protein coupled receptors	GPCR
H	
Human leukocyte antigen	HLA

I

Immune checkpoint blockade	ICB
Injected intratumorally	i.t.
Injected intravenously	i.v.
Interleukin	IL

M

Major histocompatibility complex	MHC
Mesothelin	MSLN
Monocyte-derived dendritic cell	mdDC
Monocyte-derived macrophage	mdM
Myeloid-derived suppressor cell	MDSC

N

Natural killer cells	NK cells

P

Pancreatic ductal adenocarcinoma	PDAC
Pathogen-associated molecular pattern	PAMP
Peripheral blood mononuclear cells	PBMC
Programmed cell death protein 1	PD-1
Programmed death-ligand 1	PDL-1

S

Single chain variable fragment	scFv
Subcutaneously	s.c.
Synthetic myeloid activating receptor	SMARt

T

T cell receptor	TCR

T cells redirected for antigen-unrestricted cytokine-initiated killing	TRUCK
TNF receptor-associated factor	TRAF
Transforming growth factor-β	TGF-β
Tumor infiltrating lymphocyte	TIL
Tumor-associated macrophage	TAM
Tumor microenvironment	TME
Tumor necrosis factor	TNF

U

Untransduced	UT

W

Wild-type	WT

9 Curriculum Vitae

Duc Huynh

Work & Relevant Experience

PhD Research – Kobold Lab, LMU Munich, Germany	Oct. 2018-Present
Master's Research – Fujita Lab, Kyoto University, Japan	Sept. 2016-Sept. 2018
Lab Tech, Children's Mercy Hospital, Kansas City, MO	May 2016-Sept. 2016
Lab Tech Volunteer, University of Missouri, Columbia, MO	Fall 2015
Lab Tech, University of Missouri, Columbia, MO	May 2014-Sept. 2014

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PhD in Medical Research — Oct. 2018-Present
Ludwig Maximilians Universität München (LMU Munich)
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Master–Kyoto University, Japan — Sept. 2016-Sept. 2018

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Oener, Ö. Umut, S. Joaquina, L. Vimeux, T. Tran, T. Hank, T. Baba, **D. Huynh**, R. T. A. Megens, K.-P. Janssen, M. Jastroch, D. Lamp, S. Ruehland, M. Di Pilato, J. N. Pruessmann, M. Thomas, C. Marr, S. Ormanns, A. Reischer, M. Hristov, E. Tartour, E. Donnadieu, S. Rothenfusser, P. Duewell, L. M. König, M. Schnurr, M. Subklewe, A. S. Liss, N. Halama, M. Reichert, T. R. Mempel, S. Endres and S. Kobold (2021).
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10 Acknowledgements

Where to start...perhaps like all the other acknowledgement speeches after presentations that I've done all these years. This only seems fitting. It normally goes something like "I'd first like to thank Stefan and Sebastian for having me in Klinpharm and this doctoral program". That line and its place before all other acknowledgements is of course well deserved.

Stefan Endres has been absolutely an absolutely phenomenal mentor, not only to me personally, but to each and every person that passes through Klinpharm, student, post-doc, professor alike. He's been the glue that keeps Klinpharm together during great and challenging times alike, and always has a knack for making someone feel good about themselves and their work, regardless of if it was deserved. He's been a shining example not only of great science, but also how to treat people and develop the people around you. In that aspect, his example will probably have a more outsized influence than any purely scientific advice he could've given.

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