

Exploiting the Potential of Vaccines and GGSK-1/30 mAb based ADC against MUC1 for Immunotherapy of Adenocarcinomas

DISSERTATION

Zur Erlangung des akademischen Grades

Doktor rerum naturalium

im Fachbereich Chemie

der Johannes Gutenberg-Universität Mainz

vorgelegt von

Isabelle Florence Silvestre

geboren am 21.05.1996 in Bad Urach

Mainz, 2025

Dekanin: Professor Dr. Eva Rentschler

1. Berichterstatter:

2. Berichterstatter:

Tag der mündl. Prüfung:

Eigenständigkeitserklärung

Dissertation im Fachbereich 09 Chemie an der Johannes Gutenberg-Universität Mainz

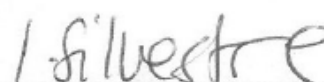
Ich, Isabelle Florence Silvestre, versichere, dass ich die vorliegende Doktorarbeit mit dem Titel „Exploiting the Potential of Vaccines and GGSK-1/30 mAb based ADC against MUC1 for Immunotherapy of Adenocarcinomas“ selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel (dazu zählen auch KI-basierte Anwendungen oder Werkzeuge*) benutzt habe. Sämtliche wörtlichen oder sinngemäßen Übernahmen und Zitate sind kenntlich gemacht und nachgewiesen (dies gilt auch für Texte, die durch generative KI, wie Chat GPT erzeugt wurden). Ich versichere, dass ich keine Hilfsmittel verwendet habe, deren Nutzung die Prüferin oder der Prüfer explizit ausgeschlossen hat.

Im Anhang „The Usage of AI“ habe ich die verwendeten KI-Tools dokumentiert. Mit Abgabe der vorliegenden Leistung übernehme ich die Verantwortung für das eingereichte Gesamtprodukt. Ich verantworte damit auch jegliche KI-generierten Inhalte, die ich in meine Arbeit übernommen habe. Die Richtigkeit übernommener (KI-generierter) Aussagen und Inhalte habe ich nach bestem Wissen und Gewissen geprüft.

Mir ist bekannt, dass ein Verstoß gegen die genannten Punkte prüfungsrechtliche Konsequenzen hat und insbesondere dazu führen kann, dass die Promotionsleistung als mit „nicht bestanden“ bewertet wird. Die Einschreibung kann für bis zu zwei Jahre widerrufen werden, wenn Studierende zweimal oder häufiger bei Prüfungsleistungen täuschen (§ 69 Abs. 4 und 5 HochSchG).

Mainz, 25.06.2025

(Ort, Datum)



(Unterschrift)

Table of Content

Table of Content	I
Abbreviations	IV
1 Introduction	1
1.1 Immunotherapy in cancer	1
1.2 Adenocarcinomas	2
1.2.1 Challenges and progress in BC and PDAC diagnosis and treatment	2
1.3 Tumor-associated MUC1	6
1.4 Vaccination strategies against BC and taMUC1	7
1.4.1 Identification of the MUC1(22)S ¹⁷ ST _N -p30 synthetic glycopeptide sequence and its usage as anti-MUC1 vaccination	9
1.4.2 The generation of the GGSK-1/30 mAb	12
1.5 Cancer therapy with mAbs and antibody drug conjugates	13
2 Aims	16
3 Materials and Methods	17
3.1 Devices, materials and chemicals	17
3.2 Prepared solutions and buffers	19
3.3 Cell culturing	21
3.3.1 Thawing and freezing the cells	21
3.3.2 Cell counting	22
3.3.3 Murine BC cell lines	22
3.3.4 Human cancer cell lines	23
3.4 Statistical analysis	24
3.5 In vivo experiments	24
3.5.1 Vaccination of mice	25
3.5.2 Blood collection and serum isolation	25
3.5.3 Ex vivo isolation of immune cells from spleen and mesenteric Lymphnode for subsequent FACS analysis	25
3.6 Western blot analysis of taMUC1 expression	25
3.7 ELISA of mice antisera	26
3.8 Fluorescence activated cell screening	27

3.8.1	Analyzing taMUC1 expression and MUC1 internalization <i>via</i> FACS -----	28
3.8.2	Analyzing cell viability and apoptosis and cell counting in FACS-----	29
3.8.3	Analysis of T cells in vaccinated mice-----	31
3.8.4	Analysis of B cells in vaccinated mice -----	33
3.9	ADC treatment assays -----	35
3.9.1	Synthesis of Ab drug conjugates-----	35
3.9.2	2D cultures for ADC treatments -----	36
3.9.3	Crystal violet viability staining (Figure 3-8 B) -----	38
3.9.4	3D culturing for spheroid formation -----	38
3.9.5	Surface measurement of spheroid microscopy images in image J -----	39
4	Results-----	41
4.1	The MUC1(22)S ¹⁷ ST _N -p30 supramolecular, synthetic and modular antitumor vaccination strategy-----	41
4.1.1	Humoral immune response of mice vaccinated with different ratios of the gel forming monomer within the new vaccine formulation -----	44
4.1.2	Can the optimized formulation containing the hydrogel-forming monomer match the former formulation with IFA in synthetic antitumor vaccines? ----	45
4.1.3	Optimized vaccine formulation shows high titer responses in wt mice as well as in humanized mice model-----	47
4.1.4	Comparison of immune responses in mice vaccinated with cleavable or uncleavable linkage and the addition of a STING agonist to the vaccine ----	48
4.2	Evaluating the (TA)-MUC1 specific mAb GGSK-1/30 as therapy in murine BC models -----	67
4.2.1	Internalization of taMUC1 receptor after mAb binding -----	68
4.2.2	Testing a new potent payload-linker technology for the use in GGSK-1/30 mAb based ADCs -----	70
4.3	Evaluating the (TA)-MUC1 specific mAb GGSK-1/30 as therapy in human BC models -----	73
4.3.1	In vitro treatment of 2D cultures from different human BC cell lines with GGSK-1/30 mAb coupled DM1 and SN38 -----	76
4.3.2	Treatment of 3D models for human BC with GGSK-1/30 based ADCs-----	80
4.4	Testing the GGSK-1/30 mAb and its ADCs on other adenocarcinomas-----	87

4.4.1	GGSK-1/30 binds specifically to many other human adenocarcinomas-----	88
4.4.2	Screening for taMUC1 expression in various human adenocarcinoma cell lines -----	90
4.4.3	In vitro treatment testing of GGSK-1/30-SN38 ADC on LNCAP prostate cancer cells, A549 lung cancer cells and PANC-1 PDAC cells -----	92
4.4.4	PANC-1 spheroid formation and treatment with GGSK-1/30-DM1 and GGSK-1/30-SN38 -----	94
4.4.5	GGSK-1/30 mAb in a preclinical tissue culturing model on PDAC samples from patients -----	97
5	Discussion and outlook-----	104
5.1	Hydrogel depot forming monomer as replacement for the usage of IFA in vaccine formulations in supramolecular vaccines -----	104
5.2	GGSK-1/30 based ADCs also show a curative effect in preclinical human BC model-----	106
5.3	GGSK-1/30 mAb should be considered as a new treatment on other tumor entities -----	108
5.4	Conclusion for the therapeutic use of anti-MUC1 vaccines and GGSK-1/30 mAb based therapies -----	110
6	Abstract -----	112
7	Kurzfassung -----	114
8	Appendix-----	VII
8.1	Supplementary figures-----	VII
8.2	Literature -----	XXIII
8.3	Index of figures-----	XXXV
8.4	Index of tables-----	XXXIX
8.5	The usage of AI Tools-----	XXXIX
8.6	Acknowledgements -----	XLI
8.7	Curriculum vitae -----	XLII

Abbreviations

%	percent	ELISA	Enzyme linked immune sorbent assay
% (v/v)	% by volume	EM	Effector memory
5-FU	5- Fluoro uracil	eq.	equivalents
Ab	Antibody, antibodies	FACS	Fluorescence activated cell scanning
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)	FDA	Food and drug administration
ADC	Ab drug conjugate	FBS	Fetal bovine serum
ADCC	Antigen-dependent cytotoxicity	FCS	Fetal calf serum
AF488	Alexa fluor 488	FITC	Fluorescein iso-thiocyanate
APC	Allophycocyanin	FOL B	follicular B cell
BB	Blocking buffer	Foxp3	Forkhead Box protein 3
BC	breast cancer	FSC	Forward scatter
BSA	Bovine serum albumin	g	gram
C	assigned bead count	Gata3	GATA-binding protein 3
C57Bl/6- {hMUC1}	human MUC1 transgenic mouse strain	GCB	germinal center B cell
CAR	chimeric antigen receptor	h	hour
CD	cluster of determination	hMUC1	human MUC1
CDC	Complement-dependent cytotoxicity	HR	Hormone receptor
CM	Central memory	HRP	Horse radish peroxidase
CSR B	class switch recombined B cell	Hu	human
CTL	Cytotoxic T cell	i.p.	intraperitoneal
Ctrl	control	IC	intracellular
Cy	Cyanine	IFA	Incomplete Freud adjuvant
d	Day, days	Ig	immunoglobulin
DAPI	4',6-Diamidin-2-phenylindol	IHC	immunohistochemistry
ddH ₂ O	double-distilled water	IL	Interleukin
DMEM	Dulbecco's modified Eagles Medium	LAG3	lymphocyte activation gene 3
DMSO	Dimethyl sulfoxide	M	Molar
dn	Double negative	mAb	Monoclonal Ab
DNA	Desoxyribonucleic acid	MACS	Magnetic activated cell sorting
DTPA	diethylenetriaminepentaacetic acid	MC	Maleimide caproyl linker
e.g.	Example given	MC- GGFG	MC linker plus GGFG-peptide spacer
MHC	major histocompatibility complex	SDS	sodium dodecyl sulfate
min	Minute	STING	stimulator of interferon genes

EDTA	Ethylenediaminetetraacetate	mL	Milliliter
mg	Milligram	taMUC1	Tumor-associated mucin-1
mLN	Mesenteric lymph node	t-SNE	t-distributed stochastic neighbor embedding
MUC1	Mucin-1	TACA	tumor-associated carbohydrate antigens
MUC1-C	C-terminal subunit	TCR β	T cell receptor beta chain
MUC1-N	N-terminal subunit	TECP	tris(2-carboxyethyl) phosphine hydrochloride
MZB	marginal zone B	tg	transgenic
NEJM	New England Journal of Medicine	Th	T helper
Neg ctrl	Negative control	TIGIT	T cell immunoreceptor with Ig and ITIM domains
NK	Natural killer	TLR	Toll-like receptor
nm	nanometer	TM	Test medium
ON	Over night	TNBC	Triple negative breast cancer
P/S	Penicillin/Streptomycin	Treg	Regulatory T cell
PBS	Phosphate buffered saline	TTox	Tetanus toxoid
PD-1	Programmed death 1	UMC	University Medical Center
PDAC	Pancreatic ductal adenocarcinoma	UV	ultraviolet
PE	Phycoerythrin	VE	Deionized (German, voll entionisiert)
PR	Progesterone receptor	vs	versus
RNA	ribonucleic acid	w	week
Roryt	retinoic acid-related orphan receptor gamma t	WB	Washing buffer
rpm	Rounds per minute	wt	Wild type
RPMI	Roswell Park Memorial Institute	wt%	Weight percent
s	seconds	x g	Times gravitational force
S	surface	α	anti
s.c.	subcutaneous	f	conversion factor
SA	Streptavidin	F_d	dilution factor
SA-HPO	Streptavidin-horseradish peroxidase	$\bar{n}$	mean of cells in counted squares
SSC	Side scatter	μg	microgram
SMCC	Succinimide 4-(N-maleimide methyl)-cyclohexane-1-carboxylate linker	μL	microliter

1 Introduction

1.1 Immunotherapy in cancer

In the late 19th century, Wilhelm Busch and Friedrich Fehleisen first discovered that acute inflammation caused a significant decrease in tumor size^{1,2}. This prompted American surgeon William B. Coley to discover that injecting streptococci into advanced inoperable tumors triggers an immune response and successfully treats the tumor, marking the inception of immunotherapy^{3,4}.

However, this pioneering immunotherapeutic strategy was not pursued further because surgical, chemotherapeutic, and radiotherapy-based cancer treatments have come to the fore for around a century. In the early 20th century, Paul Ehrlich suggested that the immune system might be able to suppress tumor growth by screening the body for abnormal cells. Driven by the first studies on immunosuppressed mice, the role of the immune system in tumor suppression was better understood. Immunotherapy returned to cancer treatment by the end of the 20th century and was inaugurated by cytokine-based treatments⁵. Another great milestone was the discovery of a method for the recombinant generation of monoclonal antibodies (mAb) by hybridomas through the fusion of plasma B and myeloma cells by Georges Köhler and César Milstein in 1975.^{6,7}

This discovery has paved the way for the design of selective mAbs directed against a wide variety of cellular target structures that are currently used in both cancer diagnostics and the treatment of malignant diseases. In addition to mAb therapies, checkpoint inhibitors, interferons, and interleukins, tumor vaccines are also used as part of immunotherapy against cancer^{8–10}. In the field of anti-cancer vaccines, a distinction is made between prophylactic vaccinations, designed to prevent triggers of cancer formation, such as HPV vaccination for cervical cancer or hepatitis B vaccine for liver cancer, and therapeutic vaccinations, which are used to treat patients who have already developed the disease. In the domain of therapeutic vaccines, a plethora of strategies are currently available, including DNA/RNA delivering vaccines, tumor antigen-presenting peptide vaccines, cell-based strategies, or vaccines utilizing a viral or bacterial vector. Peptide vaccines for example have shown the advantage of inducing highly antigen specific responses and are therefore associated with low adverse off-target effects.¹¹

Currently, many other creative and innovative biomolecular approaches are being implemented to offer increasingly modern immunotherapies. Therefore, the focus is increasingly on strategies enabling personalized medicine, for example, through chimeric antigen receptor (CAR)-T-cell therapy. In this method, cluster of determination (CD)4 and CD8 positive T cells are extracted from the blood of a patient, genetically modified to recognize tumor antigens, and reintroduced to enhance antitumor immunity¹². Another recent strategy involves personalized RNA mutanome vaccines¹³. Here a sample is taken from a patient's malignancy, then sequenced for neoantigen discovery and lastly an individualized mRNA vaccine is created matching precisely the patient's tumor expression profile.^{9,14,15}

1.2 Adenocarcinomas

Adenocarcinoma is a carcinoma subtype that typically develops in glandular epithelial tissues and can occur in numerous different tissues. The most commonly associated cancer types are lung, pancreatic, prostate, esophageal, colorectal, gastric, and mammary adenocarcinomas. As with most types of cancer, adenocarcinomas are typically associated with uncontrolled cell growth and invasion of the surrounding tissues.^{16,17} Other characteristics, such as prognostic markers and treatments, must be considered individually for each affected region and will subsequently be discussed for breast cancer (BC) and pancreatic adenocarcinoma (PDAC), as they are key subjects in this thesis.

1.2.1 Challenges and progress in BC and PDAC diagnosis and treatment

BC is globally the most frequent cancer and the leading cause of cancer-related deaths for women.^{18,19} In Germany BC represents around 30 % of all newly diagnosed cancer diseases among collected date for the year of 2018 (Figure 1-1).^{18,19}

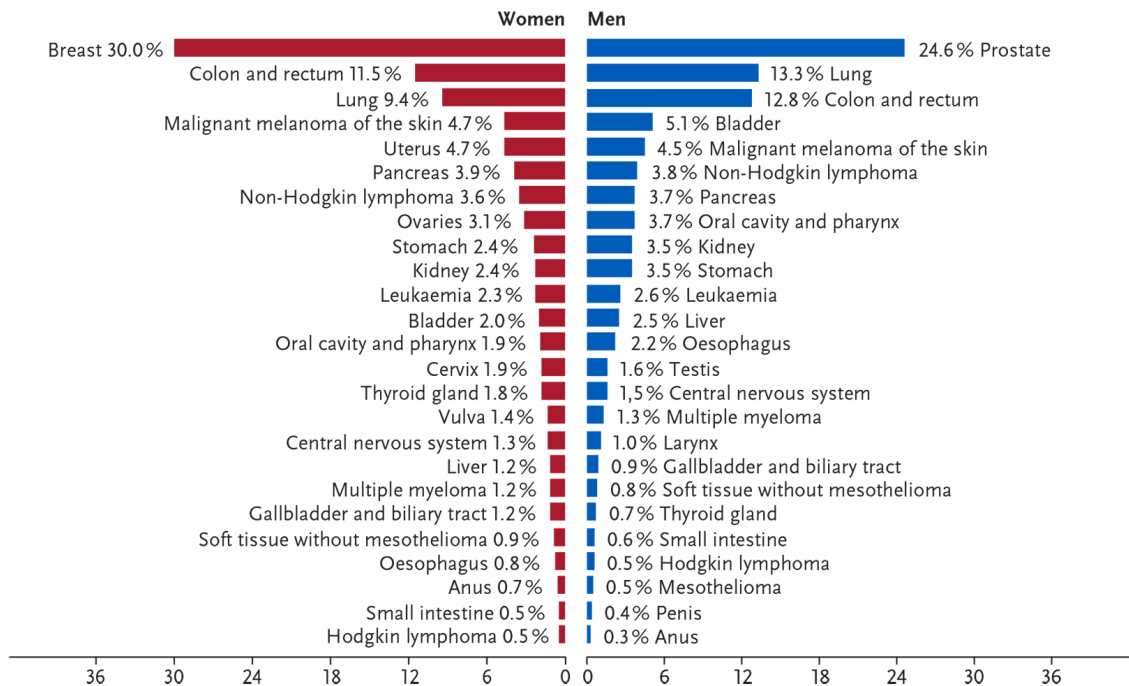


Figure 1-1: Frequency of cancer types in the percentage of newly diagnosed cancer cases in Germany in 2018.

Among all newly diagnosed female cancer patients 30,5 % patients suffered from BC representing the largest amount of cancer patients.¹⁹

Based on the expression of the prognostic markers estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2), human BC can be categorized into Luminal A (ER⁺, PR⁺, HER2⁻) or Luminal B (ER⁺, PR^{+/-}, HER2⁻) hormone-dependent BC, HER2 positive (HER2⁺) BC, or triple-negative BC (TNBC), expressing none of the mentioned markers²⁰.

Overall, early stage non-metastatic BC is related to a good prognosis, and significant improvements have been made in the therapy of this disease over the past few decades. Still, approximately 5–10 % of patients experience local recurrences after treatment, and 20–40 % of patients develop metastases before the cancer can be detected^{21–23}. Conventional BC therapy consists of surgical resection of the detected solid tumor tissue combined with radiation therapy, hormonal therapy, and chemotherapy to improve survival rates by reducing the risk of metastases^{22,24}. In addition to these conventional therapies, immunotherapy has gained traction, enabling the body's immune system to fight cancer. Targeted HER2-mAb treatments like pertuzumab (Perjeta®) and trastuzumab (Herceptin®) have shown success in treating HER2-positive BC^{25–27}. However, the receptor is expressed in only approximately 20 % of BCs. The TNBC subtype accounts for approximately 15 % of BC cases and cannot be effectively treated

with hormonal therapies or HER2-targeting treatments. Consequently, there is a necessity for the continuous investigation of novel markers that address this BC subtype. Furthermore, the general further development of better diagnostics and treatments for all BC subtypes is yet far from being completed.^{22,28}

Pancreatic cancer is not as frequent as BC among all cancer types in Germany (Figure 1-1)¹⁹; however, it is one of the deadliest cancers. Due to its late discovery, it is one of the most severe cancer diseases with a poor prognosis of only 5-10 % overall 5-year survival rate^{29,30}. Only 15–20 % of PDACs, the most common subtype of pancreatic cancer, are detected at a resectable stage. Classical treatment then consists of a combination of chemotherapy for example with gemcitabine, 5-Fluorouracil (5-FU), or Folfirinox, followed by surgery and then a second round of chemotherapeutic treatment. Late-stage metastatic PDAC usually cannot be removed surgically, and a standard treatment procedure does not really exist. In fact, a mixture of radiotherapies, check point inhibitors, and chemotherapy is given to these patients with the hope to slow down the progress.^{30–}

33

Currently, many new research approaches are under evaluation, and some of them are already in clinical trials. Newer concepts for immunoadjuvant PDAC treatment combine classical treatments with immune checkpoint inhibitors. These target molecules of a tumor's immune evasion that otherwise suppress the immune response against the tumor. Examples of their usage in PDAC are ipilimumab and tremelimumab, mAbs against cytotoxic T lymphocyte (CTL) antigen 4 or Pembrolizumab, an mAb against programmed death 1 (PD-1)³⁴. Both are regulatory molecules on the surface of CTLs that downregulate the proliferation and effector functions of cells when activated³⁵. Unfortunately, these are only beneficial for patients with responsive, highly immune-infiltrated tumors. As it is the most significant barrier to immunotherapy, the influence of the PDAC tumor microenvironment has also been the subject of several recent studies. Two other options that have been evaluated in clinical studies are the abovementioned CAR-T cell therapy (1.1) and adoptive cell transfer, in which tumor antigen-specific T cells are extracted from patients, expanded *ex vivo*, and subsequently reintroduced to enhance antitumor immunity.^{36–39} However, both strategies require specific tumor antigens that characterize the tumor^{12,40}.

Recent studies strongly suggest that Mucin-1 (MUC1) is a new tumor antigen and prognostic marker for PDAC, as it is expressed in approximately 80 % of tumors. MUC1 expression is present in the early stage of PDAC, and its expression levels seem to correlate with the severity stage and clinical outcome. This could have been shown by a study of correlation between expression and survival rate from TCGA data (Figure 1-2).^{36,41-44}

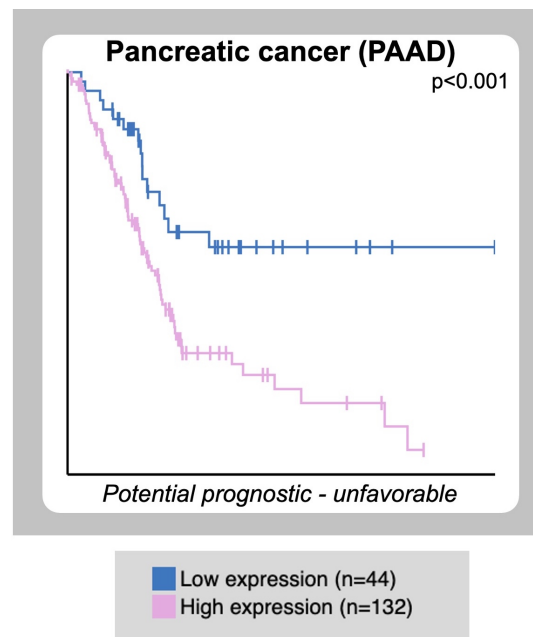


Figure 1-2: Correlation between MUC1 expression and patient survival.

Patients with pancreatic cancer seem to have a strongly unfavorable outcome when MUC1 is highly expressed. Data from different studies from TCGA summarized in the Human Protein Atlas.⁴⁴

Therefore, MUC1 is now increasingly considered in novel immunotherapies. In one study, adoptive cell transfer treatment with MUC1-CTLs was added as adjuvant therapy to gemcitabine after radical pancreatectomy and was able to significantly prolong the overall survival rate in postoperative pancreatic cancer patients⁴⁵. Also, there are already some Phase I studies investigating on MUC1 vaccines as a treatment for the PDAC example given (e.g). a vaccina virus targeting carcinoembryonic antigen and MUC1 to develop antigen-specific T cell responses.^{36,41}

For BC as well as for pancreatic cancer, there is an unmet medical need, and the development of further targeted therapies is required. Particularly, new treatments that focus on the molecular mechanisms of cancer-immune interactions are essential to improve outcomes and reduce side effects for patients. While progress has been made, particularly with immunotherapies and targeted agents for both diseases, much remains to be done to address the diversity and complexity of BC, especially for patients with

TNBC or those who develop resistance to current treatments. In addition, there should be a focus on discovering new target-specific tumor antigens, such as MUC1, to address the limitations of current PDAC therapies.

1.3 Tumor-associated MUC1

MUC1 is a transmembrane glycoprotein found in various epithelial tissues where it is associated with the mucus, which is giving it its name. Its function is to protect cells from environmental damage⁴⁶.

Tumor-associated MUC1 (taMUC1) is highly expressed in over 90 % of breast tumors, making it an excellent tumor-specific marker for both diagnosis and therapy. Overexpression of taMUC1 is associated with poor prognosis and increased metastatic potential. This additionally highlights its importance in cancer research.^{47–49} Unlike HER2, ER, and PR, it is characterized by aberrant glycosylation, strongly distinguishing it from MUC1 expressed in healthy epithelial cells (Figure 1-3)⁴⁷.

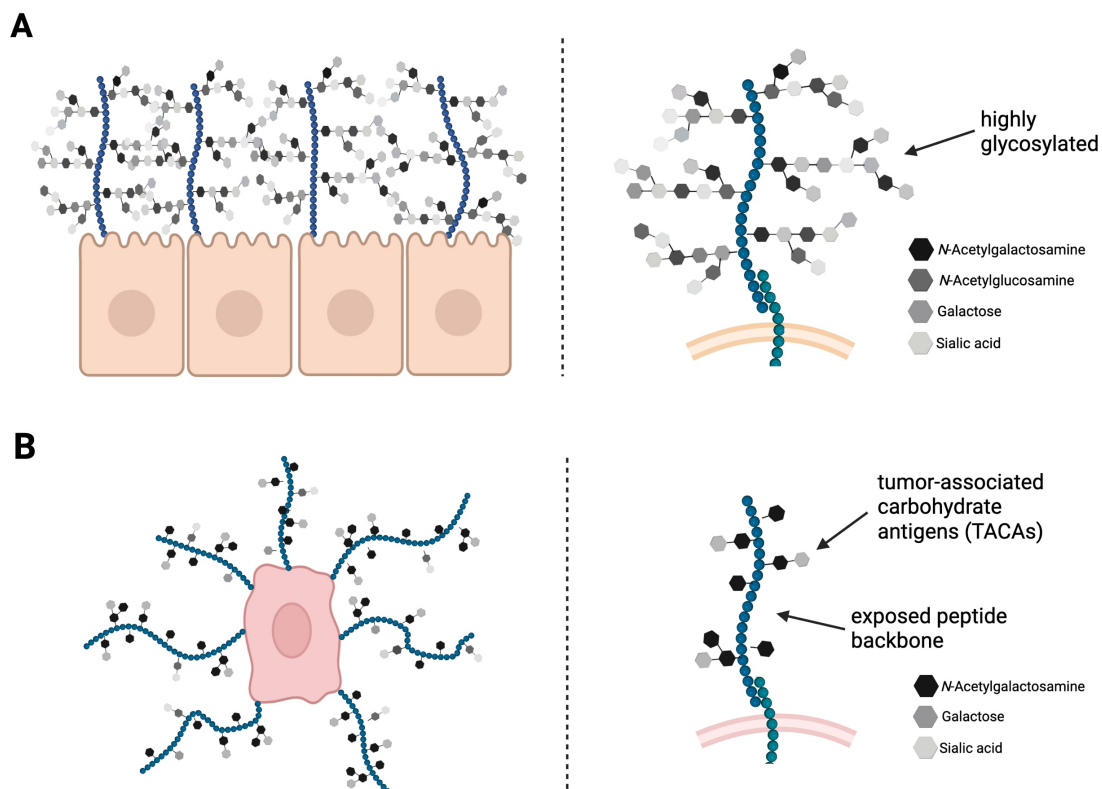


Figure 1-3: Structural changes between MUC1 in healthy tissue and taMUC1 in malignant tissue. Structure of MUC1 in normal tissue. B Structure of taMUC1 in tumorous tissue. This scheme was created using Biorender.com and adapted from the Dissertation of Riem Attariya⁵⁰.

Structurally, MUC1 consists of an extracellular N-terminal subunit (MUC1-N) and a C-terminal subunit (MUC1-C). MUC1-N contains a tandem repeat region rich in glycosylation sites, while MUC1-C contains a cytoplasmic domain involved in signal transduction. In normal tissues, MUC1 is extensively glycosylated and forms a protective mucosal barrier. However, in cancer, glycosylation is altered by decreased glycosyltransferase activity and increased sialyltransferase activity. This leads to the formation of tumor-associated carbohydrate antigens (TACAs) and loss of cell polarity, which promotes metastasis. Hypoglycosylation of taMUC1 exposes immunodominant epitopes that can trigger immune responses. Overexpression of taMUC1 promotes cancer cell proliferation and metastasis by modulating intrinsic signaling pathways and cell-cell interactions. taMUC1 does not seem to be the primary initiator of malignancy but plays a critical role in tumor progression by promoting adhesion, blocking apoptosis, and regulating growth signals^{51–53}.

Recent studies also suggest that taMUC1 is a key player in resistance to standard adjuvant therapies such as tamoxifen, trastuzumab, and systemic chemotherapy, particularly in hormone receptor-positive, HER2/neu-positive, and TNBC. The silencing of taMUC1 restores treatment sensitivity and reduces drug resistance by downregulating multiple drug resistance genes. Previous studies have also shown that targeting the taMUC1-C subunit can inhibit tumor growth in PDAC as well as in TNBC models. For example, the mAb TAB004, specific for hypoglycosylated taMUC1 epitopes, has shown significant antitumor activity in preclinical PDAC models when combined with immunotherapies^{54–56}.

These unique properties make anti-taMUC1 agents a promising therapeutic option. The consistent expression of taMUC1 throughout all stages of BC, including metastasis and recurrence, makes it a valuable target for diagnosis and targeted therapy.

1.4 Vaccination strategies against BC and taMUC1

Vaccinations are established strategies for preventing infectious diseases, but the use of tumor vaccines in therapy and cancer prevention is gaining increasing importance. Cancer vaccines are designed to specifically activate the immune system against tumor antigens, training it to recognize and eliminate cancer cells while sparing healthy tissue⁵⁷. Although research is still in its infancy, early clinical trials have shown promising results,

Introduction

offering opportunities for effective prevention and possible addition to existing BC treatments. In the long term, vaccines could lead to a turnaround in cancer treatment by preventing the disease rather than treating it.

Vaccination against BC is a particularly promising approach for preventing the development and progression of the most common cancer in women. Evidence is provided by the increasing number of vaccination strategies for BC treatment in clinical studies. An example of a preventive vaccination strategy against TNBC is the development of prophylactic vaccines targeting the oncogenic protein α -lactalbumin. This protein is normally expressed only in healthy breast cells during lactation but is upregulated in many cases of TNBC. A vaccine based on α -lactalbumin was able to induce a strong immune response and significantly inhibit tumor growth in mice in preclinical studies⁵⁸. An example of a therapeutic vaccine currently in use for the treatment of residual cancer cells in patients is the HER2 peptide vaccine E75 (nelipepimut-S), which has shown improved disease-free survival in clinical trials, particularly in patients with low HER2 expression^{59,60}.

One of the first clinical trials investigating a cancer vaccine against taMUC1 used an oxidized mannan MUC1 vaccine, a strategy where a specific taMUC1 fusion peptide is linked to oxidized variants of mannan to target macrophages or dendritic cells and induce a strong immune response against taMUC1. Follow-up over 12–15 years showed that the recurrence rate in vaccinated patients with early BC was significantly lower than that in the placebo group (12.5 % vs. 60 %).^{61,62} Another approach used a MUC1 DNA vaccine combined with immunomodulators IL-2, PD-1, and toll-like receptor (TLR) 9 agonists to target taMUC1. These vaccines showed significant tumor shrinkage in a murine lung cancer model and fast strong activation of CD8⁺ T cells 7–10 days after the last immunization⁶³. A new innovative approach uses mRNA nanoparticles encoding MUC1 in combination with CTLA-4 blockade. In preclinical studies, this strategy resulted in enhanced CTL responses and significant inhibition of tumor growth⁶⁴.

Vaccination against taMUC1 is a promising approach, especially for the immunotherapy of TNBC, as long-term clinical data and preclinical studies demonstrate the potential of cancer vaccinations. Most vaccine strategies use epitopes from the peptide backbone of taMUC1. However, literature has shown that this is associated with a higher risk of developing immune tolerance against the target antigen, whereas vaccines that present

glycopeptides of taMUC1 have a higher tendency to be recognized as foreign by the host immune system⁶⁵. Therefore, taMUC1 vaccine strategies that use taMUC1 glycopeptides should be considered for cancer treatment.

1.4.1 Identification of the MUC1(22)S¹⁷ST_N-p30 synthetic glycopeptide sequence and its usage as anti-MUC1 vaccination

Over the years of collaboration between the [REDACTED] and the [REDACTED] research group (Dpt. of Chemistry, JGU Mainz) and the Institute for Immunology of UMC Mainz several synthetic vaccines have been synthesized and tested in vivo to discover selective vaccine strategy directed against taMUC1.^{66–70}

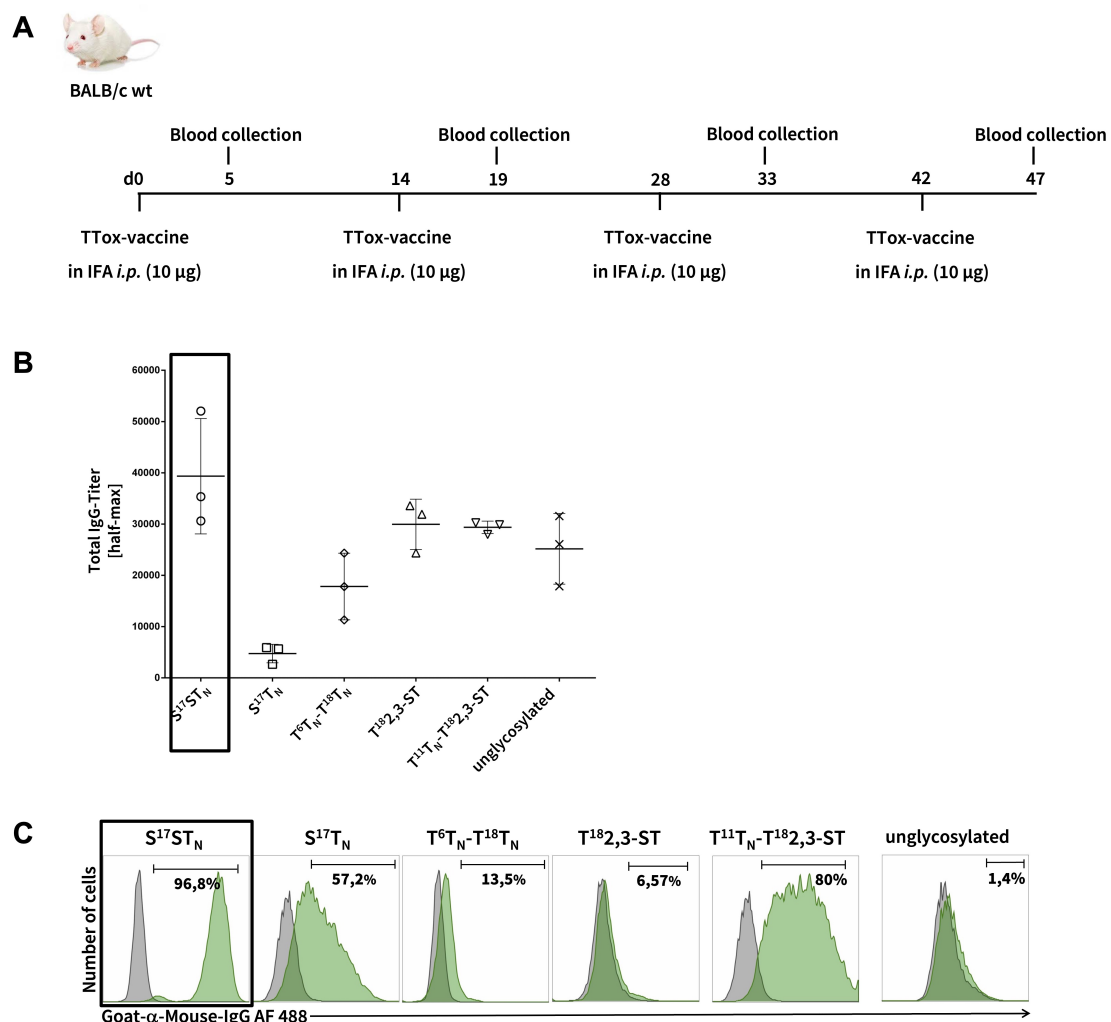


Figure 1-4: Comparison of humoral immune responses after vaccinating BALB/c mice with different synthetically prepared taMUC1 sequences in IFA.

A: Vaccination scheme, mice were vaccinated four times at intervals of two weeks with different vaccines. B: ELISA results show humoral response induction via IgG production by different vaccines. C: Antigen recognition on human T47D BC cells by serum Abs detected via FACS. In frame, the sequence subsequently used within the vaccine strategy is shown. Figure adapted from the Dissertation of Natascha Stergiou.⁷¹

Introduction

Figure 1-4 shows the data from former PhD student [REDACTED], who established a vaccination strategy for testing different taMUC1 sequences with altered glycosylation patterns, synthesized by former PhD student [REDACTED]⁷², adjuvanted with Tetanus Toxoid (TTTox), and Incomplete Freud' Adjuvant (IFA) in BALBc mice (A). Mice showed the best responses when vaccinated four times at intervals of two weeks. Blood was collected 5 days (d) after each injection to subsequently analyze the presence and amount of IgG in the serum via enzyme-linked immunosorbent assay (ELISA) (B) and the selective binding of the serum antibodies (Ab) to human T47D BC cells via flow cytometry (C).⁷¹ In comparison, one sequence emerged from all others with the highest IgG titer in the mice serum. The titer of this sequence, decorated with an ST_N at amino acid S¹⁷, was also significantly enhanced in comparison to the unglycosylated sequence and had at the same time the most distinct binding to BC cells in flow cytometry. The complete structure of the emerged vaccine is shown in Figure 1-5 A.

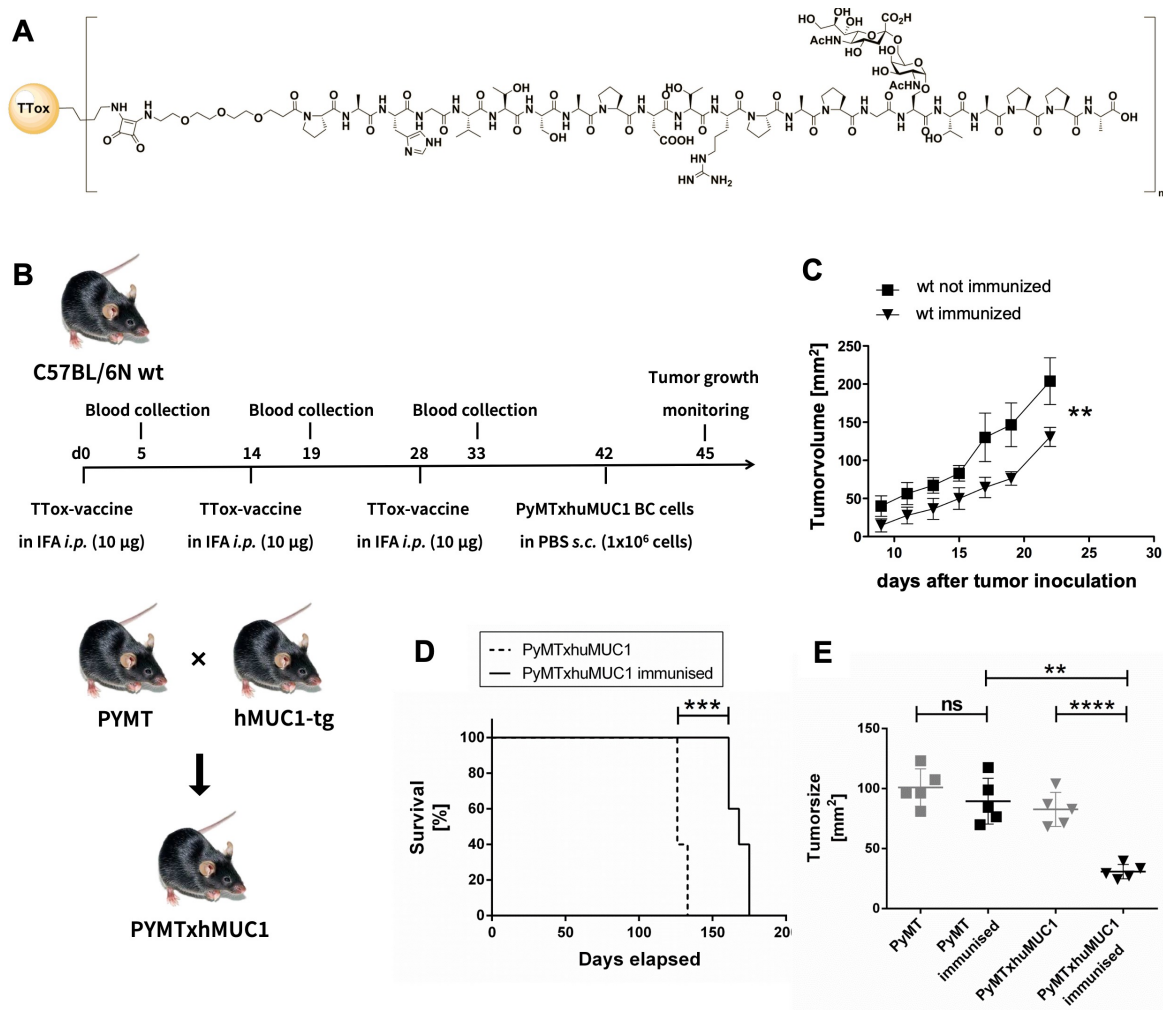


Figure 1-5: Preventive vaccination against taMUC1 with a synthetic glycopeptidic MUC1 sequence shows a significant reduction in tumor growth in a preclinical BC mouse model.

A: Sequence of MUC1(22)S¹⁷ST_N-TTTox used for immunization to subsequently generate the GGSK-1/30 mAb. B: Treatment scheme for preventive vaccinations of mice. C: Tumor growth monitoring of untreated vs. vaccinated C57BL/6 wt mice, adapted from the dissertation of Natascha Stergiou⁷¹. D: Survival of untreated vs. vaccinated PyMTxhMUC1 mice and E: Tumor growth monitoring of untreated vs. vaccinated PyMT and PyMTxhMUC1 mice adapted from Stergiou et al.⁷³

The applicability of this synthetic vaccine was assessed in two different preclinical mouse models. The first group consisted of C57BL/6N mice bearing tumors of the murine BC cell line PyMT, humanized to express human MUC1 (hMUC1). Tumor growth of mice immunized prior to tumor inoculation and of mice that did not receive any prior treatment was monitored, revealing that vaccination with the synthetic glycopeptidic taMUC1 sequence significantly reduced tumor growth (Figure 1-5 C). The second model consisted of PyMTxhMUC1 mice, a crossbreed of hMUC1 expressing hMUC1-transgenic (tg) mice and PyMT mice, that spontaneously develop several tumors with similar morphology to human BC in the mammary glands starting from 14 weeks of age⁷³. Both mouse strains were used to investigate spontaneous tumor formation after preventive vaccination with

the MUC1(22)S¹⁷ST_N-TTox sequence. Vaccination of PyMTxhMUC1 mice at 6 weeks of age before tumor formation led to a significant increase in their survival (Figure 1-5 D). By comparing tumor sizes of vaccinated PyMTxMUC1 and PyMT mice at 20 weeks of age, the antigen specificity of vaccine treatment was highlighted (Figure 1-5 E). In summary, these results demonstrate that this synthetic glycopeptide structure could be used as an effective preventive therapy for hMUC1 positive BCs in mice.

Further improvements in the vaccine were made, first through the replacement of TTox, which is associated with severe side effects on the injection side⁷⁴. p30, a peptide sequence of TTox consisting of 30 amino acids associated with fewer side effects while maintaining T-cell stimulatory effectiveness, was introduced into the vaccine. Another improvement consisted of adding immunostimulatory adjuvants to the vaccine, which further enhanced the immune responses⁷¹.

The development of self-assembling nanorods in collaboration with the [REDACTED] working group enabled a modular vaccine platform co-presenting antigens and stimulatory molecules.⁷⁵ Our collaboration partners showed that peptide secondary structures can be harnessed to design monomers capable of self-assembling into nano-scaled supramolecular structures in aqueous media. The resulting supramolecular polymers exhibit high dynamic adaptability and inherent responsiveness to external stimuli, similar to biological systems. By decorating the surface with immunogenic molecular patterns, pathogen-mimicking entities and potential vaccine candidates can be developed.

The nanorods could be particularly suitable for effective antigen presentation owing to their virus-like shape. Further evolution of this multifunctional, highly reactive, and immunologically effective vaccine, whose structure can be varied in a targeted manner, will be the next aim and is addressed in this thesis.

1.4.2 The generation of the GGSK-1/30 mAb

Synthetic hu(TA)MUC1 glycopeptides can successfully mimic the altered glycosylation pattern of BC cells and elicit strong and specific immune responses as B-cell epitopes in antitumor vaccines. The vaccine has been shown to be particularly effective, eliciting the strongest Ab responses in several mouse models. With the help of this vaccine, the GGSK-1/30 mAb, which specifically recognizes human taMUC1, was developed through hybridoma technology.

ELISA analyses confirmed that GGSK-1/30 is an IgG1 Ab and optimally recognizes its target antigen at 1 µg/ml. GGSK-1/30 was also shown to be particularly specific in distinguishing between taMUC1 and physiological MUC1 expressed by healthy cells. This was demonstrated using FACS and fluorescence microscopy of human epithelial and BC cells. In this study the mAb appeared significantly stronger and more specific than other commercially available Abs tested SM3 and HMFG1 against MUC1.⁷¹

1.5 Cancer therapy with mAbs and antibody drug conjugates

The use of antibody-drug conjugates (ADCs) signifies a revolutionary advancement in the field of oncology, offering a distinctive approach that integrates the precision of mAbs with the efficacy of cytotoxic agents. This innovative strategy enables selective targeting and elimination of cancerous cells while concurrently minimizing damage to healthy tissues⁷⁶.

ADCs are composed of three primary components: first, an mAb engineered to recognize and bind to specific antigens overexpressed on cancer cells and ensure targeted delivery; second, a cytotoxic payload consisting of highly potent drugs, such as microtubule inhibitors or DNA-damaging agents, designed to induce cell death upon internalization; and third, a chemical bridge as a linker that connects the mAb to the payload, designed to be stable in circulation but to release the drug quickly inside the target cell⁷⁷. Thus, linker design is critical. It must balance stability in the bloodstream with efficient release of the cytotoxic agent inside the tumor cell. Advances in linker technology have led to improved stability and efficacy of ADCs⁷⁸. Linkers can be designed as either cleavable or not. Cleavable linkers allow the release of the drug by hydrolysis at low pH through reduction of disulfide bonds or by proteolysis. Linkers designed for proteolysis contain sites specifically recognized by cleaving enzymes, for example, cysteine proteases. Non-cleavable ones rely on the degradation of the Ab itself to release their cytotoxic payload.^{50,79}

The assumed mechanism of action after drug administration is shown in Figure 1-6.

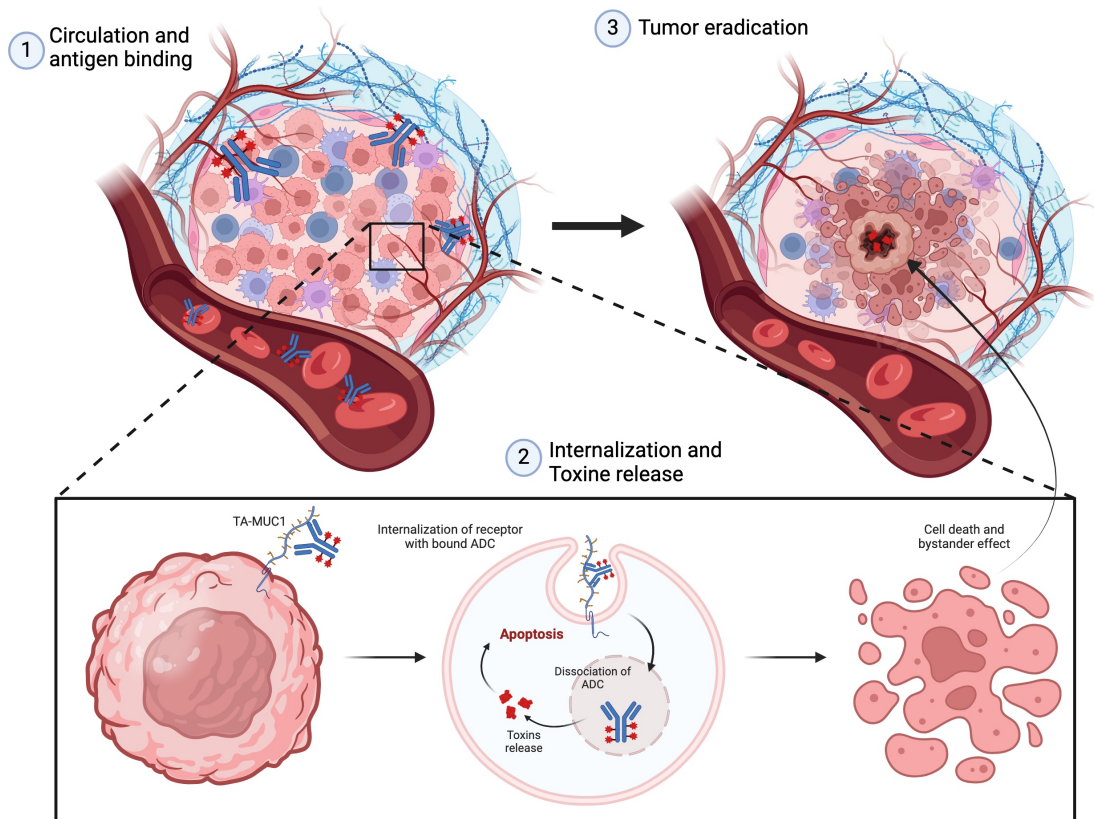


Figure 1-6: Assumed mechanism of tumor eradication under ADC treatment.

Step 1: ADC circulates in the bloodstream until it finds and binds to its antigen. Step 2: Endocytosis of antigen-bound ADC and release of toxin after lysis. Step 3: Tumor eradication through cytotoxin-induced apoptosis and bystander effect. This scheme was created using Biorender.com and adapted from Fu et al.⁸⁰.

ADC circulates through the body until the mAb component binds to its specific antigen on the surface of the cancer cell. This binding triggers internalization of the ADC-antigen complex into the cell via endocytosis. Once inside, the linker is cleaved, releasing the cytotoxic payload, which then exerts its lethal effect typically by disrupting critical cellular processes such as DNA replication or microtubule function.^{77,80}

Several ADCs have already been in clinical applications and are approved for the treatment of cancer⁷⁶; for example, gemtuzumab ozogamicin (Mylotarg®) for acute myeloid leukemia was the first ADC to be approved by the Food and Drug Administration (FDA) in the USA⁸¹. Trastuzumab emtansine (Kadcyla®) combines the HER2-mAb trastuzumab with a microtubule inhibitor and is used to treat HER2-positive BC⁸². The two ADCs have become the prevailing option for the treatment of HER2-positive BC, owing to a combination of enhanced efficacy and reduced systemic toxicity when contrasted with conventional chemotherapy.⁸³

ADC therapies also face some challenges, for example, the premature release of the payload or the so-called off-target effect, meaning the false detection of cells or tissues through the Ab, and therefore non-specific uptake of the toxin can lead to severe adverse events. Cancer cells can also develop resistance through various mechanisms, including antigen downregulation or drug efflux. The stability and distribution of ADCs can be influenced by the properties of the linker and the drug-to-Ab ratio, leading to off-target effects. Ongoing research focuses on the development of next-generation ADCs with novel payloads, improved linkers, and site-specific conjugation techniques to improve efficacy and safety profiles.

ADCs represent a significant advancement in targeted cancer therapy, offering the potential for more effective and less toxic treatments. Continued innovation in ADC design and a deeper understanding of tumor biology are essential to overcome current limitations and fully realize the therapeutic potential of ADCs in oncology.^{84–86}

2 Aims

The objective of this research project is to further develop new and innovative immunotherapy approaches, with a specific focus on targeting taMUC1 to enhance the treatment of BC and other adenocarcinomas.

Therefore, different vaccine formulations based on a synthetic modular vaccine platform that contains synthetic glycopeptides, varying adjuvants, and a new thermoresponsive hydrogel-forming element are tested *in vivo*. The improved formulation is then compared with the previous IFA-based formulation in the *in vivo* context.

In the second part the highly taMUC1 specific GGSK-1/30 mAb is evaluated for its potential to be integrated in combined therapeutic treatments for human BC. Then, ADCs are generated through coupling of the mAb with different toxins. Additionally, suitable 2D and 3D human BC *in vitro* models are established to evaluate ADCs.

The third focus of this project lays on the taMUC1 expression in other adenocarcinomas with emphasis on expanding its potential areas of application to the treatment of the highly prevalent PDAC. Consequently, further preclinical *in vitro* models of the human PDAC cell line PANC-1 and other adenocarcinoma cell lines are established, and the efficacy of GGSK-1/30 based treatments is subsequently evaluated for these tumor entities.

3 Materials and Methods

3.1 Devices, materials and chemicals

Table 3-1: Overview of all devices and materials used with their suppliers.

Device	Supplier
10 L oxygen concentrator	Devilbiss Healthcare, Mannheim, Germany
BD Aria II cytometer	BD Sciences, New Jersey, USA
BD Aria III cytometer	BD Sciences, New Jersey, USA
BD Canto cytometer	BD Sciences, New Jersey, USA
BD Fortessa LSRII cytometer	BD Sciences, New Jersey, USA
BD Fortessa Symphony A5/A5SE cytometer	BD Sciences, New Jersey, USA
BioRad Trans-Blot turbo plotter	BioRad, Hercules, USA
Bioruptor Plus	Diagenode, Seraing, Belgium
Centrifuge Megafuge 1.0, Biofuge fresco	Heraeus, Hanau, Germany
Centrifuge Megafuge 40R	Heraeus, Hanau, Germany
CO2 incubator	Thermo Fisher Scientific, Darmstadt, Germany
Counting chamber (Type: Neubauer)	Paul Marienfeld, Lauda-Königshofen, Germany
Electrophoresis power supply	Pharmacia Biotech, Amersham, UK
Heater for 1.5 mL reaction tubes	Hettich lab technologies, Tuttlingen, Germany
Isoflurane diffuser + Alda sorber	Rothacher-Medical GmbH, Heitenried, Switzerland
PCR thermocycler PeqStar	PeQLab Biotechnologie, Erlangen, Germany
Pipetboy/-girl comfort	INTREGRA Biosciences, Fernwald, Germany
Pipets (1 – 10 µl, 10 – 100 µl, 20 – 200 µl, 100 – 1000 µl)	Eppendorf, Hamburg, Germany
Practum scale	Satorius, Göttingen, Germany
QuickDrop photometer	Molecular Devices, München, Germany
Rebel microscope	ECHO, San Diego, USA
Safety cabinet (MSC-Advantage™)	Thermo Fisher Scientific, Darmstadt, Germany
SDS page chambers	BioRad, Hercules, USA
Small Centrifuge Z216MK	Hermle, Gosheim, Germany
Thermocycler T3	Biometra, Göttingen, Germany
Transmitted light cell counting microscope	Carl Zeiss, Jena, Germany
Transmitted light microscope	Carl Zeiss, Jena, Germany
Ultrasound bath USC-THD	VWR, Pennsylvania, USA
Vortex Genie 2	Mitlacher Laboratoriumstechnik, Mainz, Germany
Water bath	Memmert, Schwabach, Germany
ChemiDoc MP Western blot reader	BioRad, Hercules, USA

Table 3-2: Overview of all materials used with their suppliers.

Materials	Supplier
0.5-ml, 1.5-ml, 2-mL reaction tubes	Eppendorf, Hamburg, Germany
15-mL and 50-mL reaction tubes	Greiner, Frickenhausen, Germany
5 mL reaction tubes for cytometry	Greiner, Frickenhausen, Germany
Cell culture flasks (25 cm ² , 75 cm ² , 175 cm ² , coated)	Starlab, Hamburg, Germany
Cell culture plates (6-Well, 12-Well, 48-Well, coated)	Greiner, Frickenhausen, Germany
Cell scraper	Starlab, Hamburg, Germany
Cell strainer (40 and 70 µm)	Greiner, Frickenhausen, Germany
96-well Spheroid Microplates	Corning, Wiesbaden, Germany
Cryo conservation tubes (2 mL)	Sarstedt, Nümbrecht, Germany
Dissecting cutlery	Hammacher, Solingen, Germany
Flat Bottom 96-well plates	Greiner, Frickenhausen, Germany
PD-10 sephadex G25-M columns	Cytiva, Marlborough, Germany
Sterican injection needles, size 27Gx1/2"	Braun, Melsungen, Germany
Microtiter well plate (96-well)	Greiner, Frickenhausen, Germany
Omnifix-F syringes	Braun, Melsungen, Germany
V-Bottom 96-well plates	Greiner, Frickenhausen, Germany

Standard chemicals were supplied by Merck (Darmstadt, Germany) and Roth (Karlsruhe, Germany) in pro analysis quality. All chemicals from other suppliers and media supplements are listed in Table 3-3.

Table 3-3: Overview of used substances and their suppliers.

Substance	Supplier
Accutase®	Gibco, Darmstadt, Germany
Aminoacids for culture media	Sigma-Aldrich, Steinheim, Germany
Anti-rabbit IgG, HRP-linked Ab	Cell Signalling Technologies, Frankfurt, Deutschland
Biozym LE agarose	Biozym, Oldendorf, Germany
Blasticidin	InvivoGen, Toulouse, France
Bovine serum albumin (BSA)	Roth, Karlsruhe, Germany
DMEM powder	PAN Biotech, Aidenbach, Germany
DNAase I	Roche, Basel, Switzerland
Dulbecco's Modified Eagle-Medium (DMEM)	Gibco, Life Technologies, Carlsbad, USA
D-PBS	Sigma-Aldrich, Steinheim, Germany
Collagenase IV	Thermo Fisher Scientific, Darmstadt, Germany
Cytotoxicity Assay Kit (CFSE, 7-AAD)	Abcam, Cambridge, UK
Fetal calf serum (FCS)	Gibco, Life Technologies, Carlsbad, USA
Fixable viability dye eFluor 780	Thermo Fischer, Darmstadt, Germany

Fixation und Permeabilisation Puffer Set	Thermo Fisher (eBioscience), Darmstadt, Germany
Geneticin G418 sulfat	Gibco, Darmstadt, Germany
Minimal essential medium (MEM)	Gibco, Darmstadt, Germany
McCoy's 5A	Sigma-Aldrich, Steinheim, Germany
Mouse-IgA+IgM+IgG-HRP	Abcam, Cambridge, UK
Nitrocellulose	BioRad, Munic, Germany
Nuclease free water	Qiagen, Hilden, Germany
PageRuler Plus Prestained Protein Ladder	Thermo Fisher Scientific, Darmstadt, Germany
PCR mycoplasma test kit (Venor®GeM Classic)	Minerva Biolabs, Berlin, Germany
Penicillin/streptomycin (P/S)	Serva, Heidelberg, Deutschland
Roswell Park Memorial Institute (RPMI)-1640	Gibco, Life Technologies, Carlsbad, USA
Sodium pyruvat	Serva, Heidelberg, Germany
Streptavidin-horseradish peroxidase (SA-HPO)	Roche, Basel, Switzerland
Streptomycin	Serva, Heidelberg, Germany
Vitamin B12	Serva, Heidelberg, Germany
Water, nuclease-free	Qiagen, Hilden, Germany
WesternBright ECL (Western blotting detection kit)	Advansta, San Jose, USA

3.2 Prepared solutions and buffers

Table 3-4: List of in-house prepared solutions and their components.

ACK lysis buffer	In double distilled water (ddH ₂ O) + 40 mM NH ₄ Cl + 1 mM KHCO ₃ + 0.1 mM ethylenediaminetetraacetic acid (EDTA) pH 7.3 and filtered 0.2 µm
Amino acid solution for IMDM	Complete deionized water (VE)-water: + 32 mM alanine + 17 mM asparagine + 26 mM aspartic acid + 58 mM glutamine acid + 40 mM proline + 114 mM sodium pyruvate + 1:185 vitamin solution containing 1 mM biotin, 184 µM vitamin B12, 0.5 µM HCl
Block buffer (BB) for ELISA	In 1x PBS + 1 % BSA + 0.2 % (v/v) Tween

Materials and Methods

Citrate buffer	+ 40 mM citric acid + 60 mM $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ pH 4,4 – 4,5
Coating buffer	In VE-water + 0.1 M $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ pH 9.3
DMEM10	DMEM by Gibco + 1 % (v/v) penicillin/streptomycin + 1 % (v/v) sodium pyruvate + 4 mM glutamine + 10 % (v/v) FCS
Enzyme mix for tumor dissociation	Iscoves (2 mL per tumor) + 2 mg/mL collagenase IV + 20 μL DNase I
FACS buffer	In 1x PBS + 0.5 % BSA + 5 mM EDTA + 0.01 % NaN_3
IMDM	In ddH ₂ O + 17.67 g/L DMEM (dry powder) + 44 mM NaHCO_3 + 12.5 mM HEPES + 1 % (v/v) amino acid solution + 1:2500 cysteine solution (58 μM in 0.25 M HCl) + 128 μM $\text{Na}_2\text{Se}_2\text{O}_3$ + 0.1 % (v/v) penicillin/streptomycin + 50 μM β -mercaptoethanol pH = 6.9
LNCAP medium	RPMI-1640 by Gibco + 1 % (v/v) penicillin/streptomycin + 1 % (v/v) sodium pyruvate + 2 mM glutamine + 10 % (v/v) FCS
Minimal essential Medium (MEM)	MEM dry powder + 2.2 g/L NaHCO_3 + 4.7 g/L HEPES + 1 % (v/v) penicillin/streptomycin pH 7.2

MACS Buffer	D-PBS + 2 mM EDTA + 0.5 % BSA
MG63 culture medium	EMEM + 2 mM glutamine + 1 % non-essential amino acids + 10 % FBS
PBS	In VE-water + 1.4 M NaCl + 0.1 M NaH ₂ PO ₄ pH = 7.2
Test medium (TM) 10	In IMDM + 1 % (v/v) Na-Pyruvat + 1 % (v/v) glutamine + 10 % (v/v) FBS
TM 5	In IMDM + 1 % (v/v) Na-Pyruvat + 1 % (v/v) glutamine + 5 % (v/v) FBS
Trypan blue solution	1:4 Gibco™ trypan blue solution 0,4 % in PBS
U2 culture medium	McCoy's 5A + 2 mM glutamine + 10 % FBS
Wash buffer (WB) for ELISA	In 1x PBS + 1:100 Tween20

3.3 Cell culturing

Cell culture work was carried out in a safety cabinet under a filtered laminar air flow. All materials and chemicals used for cell culture were previously sterilized or sterile filtered. The storage and cultivation of the cells were carried out in a cell incubator at 37 °C, approximately 95 % humidity, and 5 % CO₂.

3.3.1 Thawing and freezing the cells

For cell cultivation, the cryogenic tube was removed from liquid nitrogen, rapidly thawed in a water bath (~37 °C), and immediately taken up in 3 – 5 milliliter (mL) of culture

Materials and Methods

medium. The cells were then centrifuged at $550 \times g$ at 4°C for 5 min, picked up in fresh medium, and cultivated in either a cell culture flask or plate.

For cryopreservation, cells were harvested by trypsinization or incubation with Accutase, the cell number was determined, and the medium was removed ($550 \times g$, 5 min, 4°C). The cell pellet was collected in ice-cooled FCS containing 10 % DMSO and 1 mL was transferred to 2 mL cryogenic tubes. The tubes were then frozen in an isopropanol box ON at -70°C and subsequently transferred to liquid nitrogen after 24 h.

3.3.2 Cell counting

To determine the number of living cells, the cells were harvested and resuspended in the appropriate medium. From this suspension, 10 microliter (μL) was taken and diluted in a 96-well plate with 90 μL trypan blue solution. This dye causes blue coloration of cells with damaged cell membranes. Living cells with intact cell membranes remain uncolored.

The solution was then transferred to a Neubauer counting chamber (depth 0.1 mm) using a pipette, and the living cells were counted under a transmitted light microscope. 4 squares were counted for each sample. The mean value of the cell numbers from the individual squares ($\bar{n}$) was used to calculate the number of living cells per mL, together with dilution factor ($F_d = 10$) and conversion factor of the counting chamber ($f = 10^4$), using the following equation:

$$\text{count} \left[\frac{\text{Cells}}{\text{ml}} \right] = \bar{n} \cdot F_d \cdot f$$

3.3.3 Murine BC cell lines

3.3.3.1 PyMT and PyMTxhMUC1

PyMT cells were previously isolated by our research group from female PyMT mice (C57BL/6-R10) that spontaneously developed BC tumors starting at approximately 13 weeks of age. The cells were encultured as described previously (3.3.1) and nourished with TM5. PyMTxMUC1 cells were isolated from PyMTxhMUC1 mice (C57BL/6-TG(MUC1)79.24GEND/JxR10), a crossbreed of hMUC1 expressing C57Bl/6-{hMUC1} mice, and PyMT mice by preparation of a tumor-bearing mouse. The isolated tumor was cut into small pieces ($<1,5 \text{ mm}$) and then lysed with the enzyme mix for tumor dissociation described before (3.2) by incubation at 37°C and 225 rpm for 1,5 h. The obtained cell

suspension was passed through a 70 μm cell strainer and then cultured with TM5. Both cell lines were diluted in a ratio between 1:5 and 1:10 two to three times a week.

3.3.4 Human cancer cell lines

3.3.4.1 MDA-MB-231 BC cells and A549 lung cancer cells

Both cell lines were provided by the Weil/Seah Ling Working Group at the MPI-P, Mainz. The cells were encultured into 25 cm^2 flasks as described previously (3.3.1), then cultured in 75 cm^2 flasks, nourished with DMEM10 medium, and subcultured twice a week in a ratio of 1:5 to 1:10.

3.3.4.2 MCF-7 and T47D BC cells

Both cell lines were already established in the working group of former PhD student Natasha Stergiou. They were encultured into 25 cm^2 flasks as described previously (3.3.1), then cultured in 75 cm^2 flasks, nourished with TM10 medium, and subcultured once a week to once every two weeks in a ratio of 1:2.

3.3.4.3 PANC-1 PDAC cells

This cell line was obtained from Cytion (CLS Cell Line Service GmbH, Eppelheim, Germany). They were encultured into 25 cm^2 flasks as described previously (3.3.1), then cultured in 75 cm^2 flasks, nourished with TM10 medium, and subcultured once or twice a week in a ratio from 1:2 to 1:5.

3.3.4.4 LNCaP prostate carcinoma cells

This cell line was obtained from Cytion (CLS Cell Line Service GmbH, Eppelheim, Germany). They were encultured into 48 or 12 well plates as described previously (3.3.1), then cultured in 6 well plates or 75 cm^2 flasks, nourished with RPMI10 medium, and subcultured once a week to once every two weeks in a ratio of 1:2.

3.3.4.5 MG63 osteosarcoma cells and U2 osteosarcoma cells

Both cell lines were provided in culture by my colleague, Keteryene Rodrigues da Silva. MG63 cells were cultured in MG63 culture medium, and U2 cells were cultured in U2 culture medium.

3.4 Statistical analysis

Statistical analyses were performed for all experiments containing at least three biological or technical replicates using GraphPad Prism (Versions 9 and 10), and the mean with standard variation is plotted in the figure. Depending on the experimental settings, multiple t-tests or a two-way ANOVA was performed. The specific test performed is indicated for each figure individually in the caption. If the statistical test resulted in a significant p-value according to the New England Journal of Medicine (NEJM) style: $p > 0.05$ (ns/nd), $p \leq 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***), the corresponding asterisks were added. To maintain clarity within the figures, $p > 0.05$ was not included in some of the figures. If so, they are marked accordingly in the caption.

3.5 In vivo experiments

C57Bl/6 wt mice were obtained from Charles River Laboratories (USA) or Janvier Laboratories (France). C57Bl/6-{hMUC1} mice are originally from The Jackson laboratories, USA listed under the strain 024631 C57BL/6-tg(MUC1)79.24Gend/J but were bred in house at the translational animal research center Mainz. Animals were kept, treated, and bred according to the German welfare law §1 TierSchVersV. Food and water were provided *ad libitum*, and mice were kept in a 12/12h d/night cycle. The described in vivo experiments were approved within the animal experiment application G19-1-099.

3.5.1 Vaccination of mice

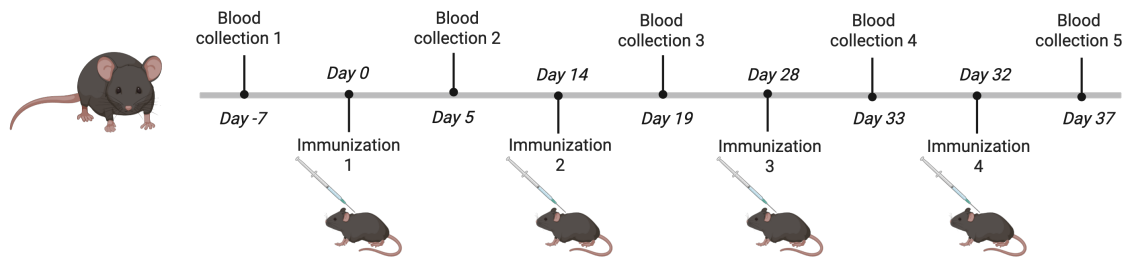


Figure 3-1: Vaccination scheme for C57Bl/6 and C57Bl/6-{hMUC1} mice.
Created using Biorender.com.

Mice were vaccinated using the scheme shown in Figure 3-1. Every mouse was injected *i.p.* or subcutaneously (s.c.) with the formulation to be tested four times at two-week intervals for 2 weeks. For C57Bl6 wt mice were taken for the experiment at 8 w old. C57Bl6-{hMUC1} mice were used between 6 and 16 w of age.

3.5.2 Blood collection and serum isolation

Blood was collected from the mice by puncturing the *vena facialis* 7 d before the first vaccination and 5 d after each vaccination (Figure 3-1). Serum was isolated from the blood through centrifugation at $12200 \times g$ for 15 min.^{87, 88}

3.5.3 Ex vivo isolation of immune cells from spleen and mesenteric Lymphnode for subsequent FACS analysis

Mice were sacrificed by CO₂ exposition and then opened with a ventral incision to prepare the spleen and mesenteric lymph node (mLN). Both organs were stored in PBS on ice. Subsequently, each organ was filtered through a 40 μ m cell strainer and taken up in MEM with 2 % FCS into a 50 mL-falcon. If necessary, red blood cells were lysed with ACK lysis buffer by incubating for 2 minutes (min) at RT, then removing the solution by centrifugation at $550 \times g$ for 5 min. Then, cells were resuspended in FACS buffer and transferred into V-bottom 96-well plates for FACS staining (3.8.3 and 3.8.4.).

3.6 Western blot analysis of taMUC1 expression

First, a separating gel and collecting gel were cast with the preparations listed in Table 3-5.

Table 3-5: Composition of SDS-PAGE gels.

Ingredients for 1 Gel	Separation gel (10 %)	Collecting gel (4 %)
ddH ₂ O	4,8 mL	3,2 mL
1,5 M Tris buffer (pH 3,6)	2,5 mL	--
0,5 M Tris buffer (pH 6,8)	--	1,2 mL
SDS (10 %)	100 µL	50 µL
Acrylamide (40 %, bis 29:1)	2,5 mL	0,5 mL
APS (10 %)	50 µL	5 µL
TEMED	10 µL	

Then, $1 - 2 \times 10^6$ cells per sample were taken up in 200 µL urea buffer and incubated at 95 °C for 6 min. They were then sonicated in BioRuptor for 15 cycles of 30 s each. Protein quantity determination was carried out from each sample against a BSA standard series. For this purpose, 5 µL of the samples were mixed with 75 µL Pierce 660 nm Protein Assay Reagent in an ELISA plate (NUNC 96-well ft), and the absorbance at 660 nm was determined after 5 min incubation on the Tecan spectrometer.

The samples were then mixed with SDS loading buffer (1:6) in an adjusted ratio and applied to the gel, which was run for approximately 15 min at 30 mA and approximately 40 min at 50 mA. After gel electrophoresis, the proteins contained in the gel were transferred to a cellulose membrane activated with methanol using the BioRad Trans-Blot-Turbo. They were then blocked with 5 % BSA solution in TBST (30 min on a shaker at RT). After blocking, the membrane was washed twice in TBS (5 min on shaker at RT), and then Ab staining was performed. The secondary Ab was incubated on a shaker for 1 hour (h) at RT. Subsequently, two washes with TBST and two washes with TBS were performed, and the protein bands were detected by incubating the membrane for 2 min with the chemiluminescence reagent WesternBright ECL. An exposure time of 300 s was selected, and an image of the membrane was taken at 10 s intervals.

3.7 ELISA of mice antisera

Flat-bottom 96-well plates were coated overnight with 1 mg/mL peptide-BSA conjugate in coating buffer (4 °C, 50 µL per well). Then, the plates were washed three times with WB and 150 µL BB per well was added and incubated for 30 min at 37 °C. After washing three more times with WP, the samples were titrated 1:2 with the first dilution representing 1:250 (2 µL serum in 500 µL BB) or 125 ng/mL for mouse IgG control. Samples were incubated

for 90 min at 37 °C, and then the plate was washed three times with WB. Next, the secondary Ab mouse-IgA +IgM+IgG-HRP was added at a concentration of 1:1000 in BB (50 µL per well) and incubated for 60 min at 37 °C. The plate was again washed three times with WP, and then a mixture of 1 milligrams (mg) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) per mL and 1:4000 H₂O₂ in citrate buffer (50 µL per well) was added. After the color change, the absorption at 410 nm was detected with a Tecan plate reader.

3.8 Fluorescence activated cell screening

In fluorescence-activated cell screening (FACS) analysis, cells are passed through a flow cytometer using a stream of liquid. Here, the cells pass through several laser beams, where they are detected and can be distinguished by granularity and size.

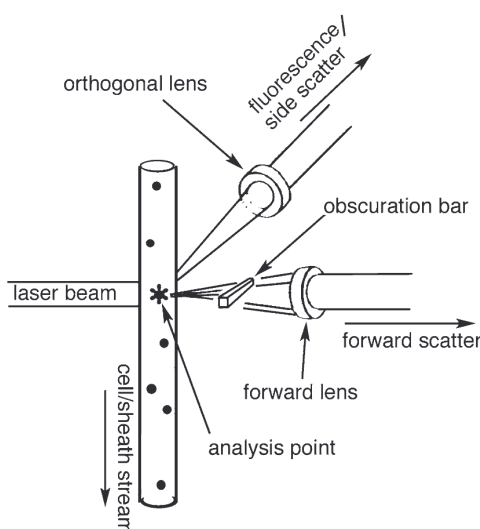


Figure 3-2: Schematic representation of cell detection by the laser beam using forward or side scatter.⁸⁹

The size of the cells is determined by a forward scatter (FSC) detector recording the light from the laser on the opposite side of the liquid flow. A second detector, side scatter (SSC), is used to distinguish the cells according to their granularity by recording the light scattering at a 90° angle to the laser beam directed at the liquid flow. Using fluorophore-coupled Ab's directed against surface proteins, FACS analysis enables the rapid analysis of large numbers of cells according to various factors and cell characteristics.^{89,90}

All FACS data were analyzed using FlowJo Software version 10 (BD Biosciences, Franklin Lakes, NJ, USA).

3.8.1 Analyzing taMUC1 expression and MUC1 internalization via FACS

For the internalization experiment, PyMT and PyMTxMUC1 cells were harvested with Accutase (5 min, 37°C) and then washed with PBS (650 × g, 5 min, 4 °C). After that 1–1.5×10⁶ cells per sample were transferred to a 15 mL falcon and mixed with GGSK-1/30-Ab in FACS buffer. The different samples were then incubated for 0, 1, 2, 4, or 8 h at 37 °C in an incubator and then kept on ice. One hundred microliters were taken from each sample and stained in a 96-well plate with secondary Ab (goat α-mouse-IgG, FITC-coupled) and fixable viability dye (1:1000). The cells were then incubated for 30 min at 4 °C in the dark. The cells were then washed twice with PBS (850 × g, 2 min, RT), and 1 × 10⁴ cells were recorded on the BD Canto flow cytometer.

Table 3-6: Abs used for taMUC1 internalization experiments and taMUC1 expression controls.

Name / target	Fluorescent dye	Dilution	Supplier
GGSK-1/30 / taMUC1	-	1:1000	Own production
GGSK-1/30 / taMUC1	AF488	1:1000	Own production
mouse IgG1 Isotype ctrl	AF488	1:500	BD Pharmingen, Frankfurt, Germany
Goat α-mouse-IgG	AF647 or AF488	1:2000	Invitrogen, Waltham, USA
fixable viability dye	eF780	1:1000	Thermo Fischer, Darmstadt, Germany

To analyze taMUC1 expression in different cell lines, cells were harvested with Accutase (5 min, 37 °C) and washed with FACS buffer (650 × g, 5 min, 4 °C). Between 1–2×10⁵ cells per sample were transferred into a V-Bottom 96-well plate and stained with GGSK-1/30 α-taMUC1-FITC and fixable viability dye (1:1000) for 20 min at 4 °C in the dark. The cells were then washed twice with PBS (850 × g, 2 min, RT), and 1×10⁴ cells were recorded on the BD Canto flow cytometer.

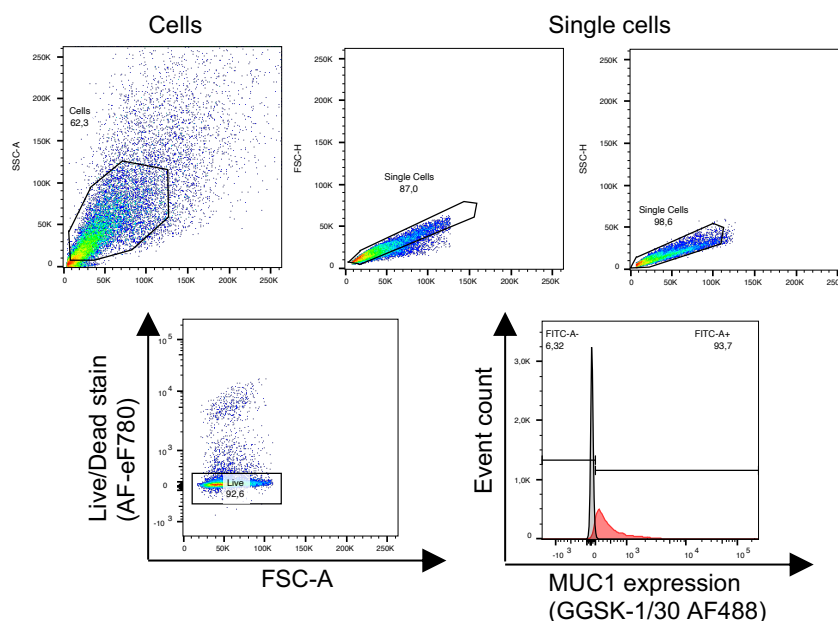


Figure 3-3 Gating procedure for identifying Ab- or antiserum-bound cells.

Between $1\text{--}2 \times 10^5$ cells were recorded from each sample. To gate the target cells, the first size (FSC) was plotted against granularity (SSC). This allowed the identification of the cell population and differentiation from cell fragments. In the next step, single cells were distinguished from cell clusters by plotting the area of cells against their height. By staining with a live-dead dye (Fixable Viability Dye, APC-eF780), the dead cells could be distinguished from living cells, since living cells did not take up the dye. To differentiate cells that were bound by the specific anti-taMUC1 mAb-GGSK1/30, negative controls (neg ctrl) were included in each FACS measurement. These were either unstained cells, stained PyMT (MUC1 neg. cells) or isotype mAb cells. The neg ctrl was used to define the basic intensity, with a maximum of 1 % false-positive events. Positive cells could then be distinguished by applying the gate of the neg ctrl to the samples to be tested. FACS measurements were performed on a BD Canto.

3.8.2 Analyzing cell viability and apoptosis and cell counting in FACS

To analyze the viability and apoptosis of the treated cells by FACS, prepared single-cell solutions of the samples were stained by adding 50 μL of the staining mixture listed in Table 3-7.

Table 3-7: Reagents and Abs contained in the staining mixture for cell viability and apoptosis analysis.

Antigen	Fluorescent dye	Dilution	Supplier
7-AAD	-	1:21	Abcam, Cambridge, UK
Annexin V	BV421	1:100	Biolegend, San Diego, USA
CFSE	AF488	1:10	Abcam, Cambridge, UK
GGSK-1/30 α -taMUC1 biotinylated	-	1:500	Own production
Streptavidin	PE	1:500	Thermo Fischer, Darmstadt, Germany
fixable viability dye	eF780	1:1000	Thermo Fischer, Darmstadt, Germany

The cells were incubated with this mixture for 20 min at 4 °C in the dark. Then, the samples were washed twice with 100 μ L FACS buffer per well (850 $\times$ g, 2 min, RT) and resuspended in FACS buffer to be measured at BD Fortessa LSRII. Between 1 and 2 $\times 10^5$ cells were recorded from each sample. The gating procedure for this analysis is illustrated in Figure 3-4.

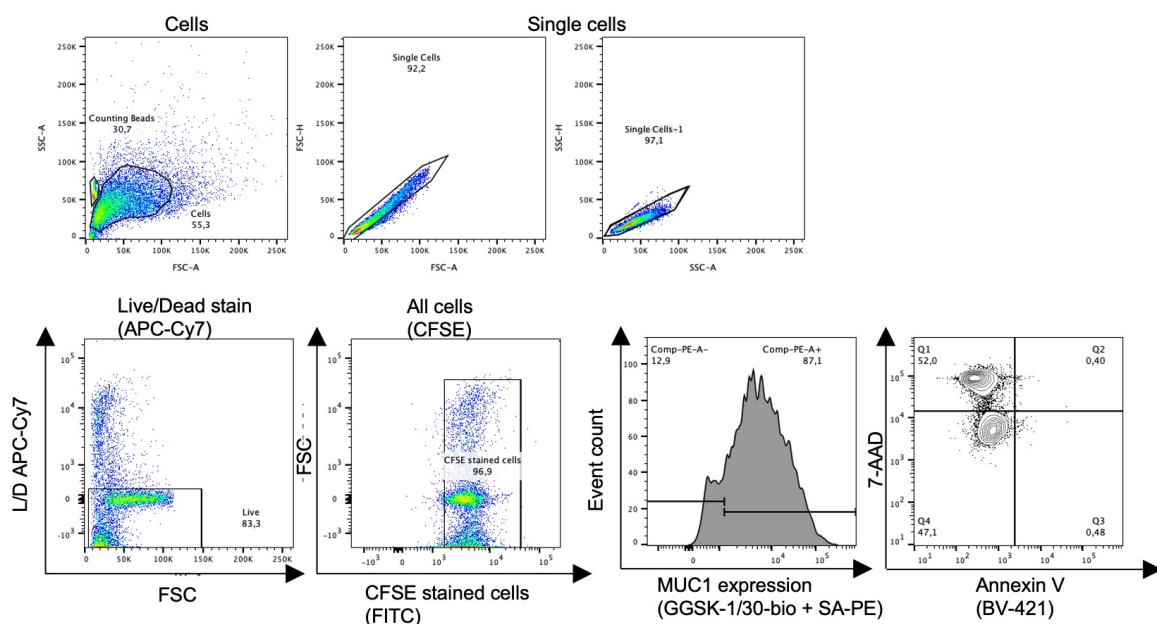


Figure 3-4: Gating procedure for viability and apoptosis analysis via FACS.

As described previously (3.8.1), target cells were identified with FSC and SSC und by distinguishing single cells. From this population, CFSE-positive cells were determined and viability was checked by staining with eF780 fixable viability dye. Subsequently, cell death and apoptosis were analyzed by examining 7-AAD and Annexin-V among CFSE-positive cells. taMUC1 expression was analyzed in living cells as described in 3.8.1, determined with fixable viability dye.

Determination of the cell number in the sample is performed by putting the event counts from the target cell population (A) and the event count of the bead population (B) from the FSC/SSC plot together with the assigned bead count of the lot ($C = 0,51 \cdot 10^5/50 \mu\text{L}$) into the following equation:

$$\frac{A}{B} \times \frac{C}{V_{\text{sample}}} = C_{\text{sample}} [\text{cells}/\mu\text{L}]$$

3.8.3 Analysis of T cells in vaccinated mice

To analyze immunoactivation in T-cells of treated mice by FACS, prepared single-cell solutions of the spleen and mLN samples were stained by adding 50 μL of the surface (S) staining mixture listed in Table 3-8.

Table 3-8: Reagents and Abs contained in the staining mixture for T-cell immune-activation analysis

Antigen/Name	Fluorescent dye	S / Intracellular (IC) staining mix	Dilution	Supplier
CD45	BUV805	S	1:500	BD OptiBuild, Frankfurt, Germany
CD3	BUV661	S	1:200	BD Horizon, Frankfurt, Germany
CD8	BUV563	S	1:200	BD Horizon, Frankfurt, Germany
CD4	BUV496	S + IC	1:500	BD Horizon, Frankfurt, Germany
Gata3	BUV395	IC	1:200	BD Horizon, Frankfurt, Germany
Roryt	BV786	IC	1:200	BD Horizon, Frankfurt, Germany
CD127	BV711	S	1:200	BD Horizon, Frankfurt, Germany
TCR β	BV605	S	1:200	BD Biosciences, Frankfurt, Germany
CD25	BV480	S	1:400	Biolegend, San Diego, USA
FOXP3	eF450	IC	1:200	Thermo Fischer, Darmstadt, Germany
LAG3	PerCP-eF710	S	1:200	Thermo Fischer, Darmstadt, Germany
KI67	FITC	S	1:200	Biolegend, San Diego, USA
CD62L	PE-Cy7	S	1:400	Biolegend, San Diego, USA
Klrg1	PE-Dazzle594	S	1:200	Biolegend, San Diego, USA
CD69	PE	S	1:400	Biolegend, San Diego, USA
Fixable viability dye	eF780	S	1:1000	Thermo Fischer, Darmstadt, Germany
TIGIT	APC-R700	S	1:200	BD Biosciences, Frankfurt, Germany
CD44	APC	S	1:250	Biolegend, San Diego, USA
FC block	-	S	1:100	BD Biosciences, Frankfurt, Germany

The cells were incubated with this mixture for 30 min at 4 °C in the dark. The samples were then washed ($850 \times g$, 2 min, RT) twice with 100 μL of FACS buffer per well. Freshly prepared fixation/permeabilization mix (50 μL per well) was added to the samples and

Materials and Methods

incubated ON. Then, samples were washed ($850 \times g$, 2 min, RT) two times in permeabilization buffer (prediluted 1:10 in ddH₂O). Intracellular staining was performed by incubating the intracellular staining mixture for 1 h at 4 °C in the dark. Samples were washed twice with permeabilization buffer ($850 \times g$, 2 min, RT), then once more with FACS buffer ($850 \times g$, 2 min, RT). Finally, cells were resuspended in FACS buffer and analyzed on a BD Fortessa Symphony A5/A5SE cytometer.

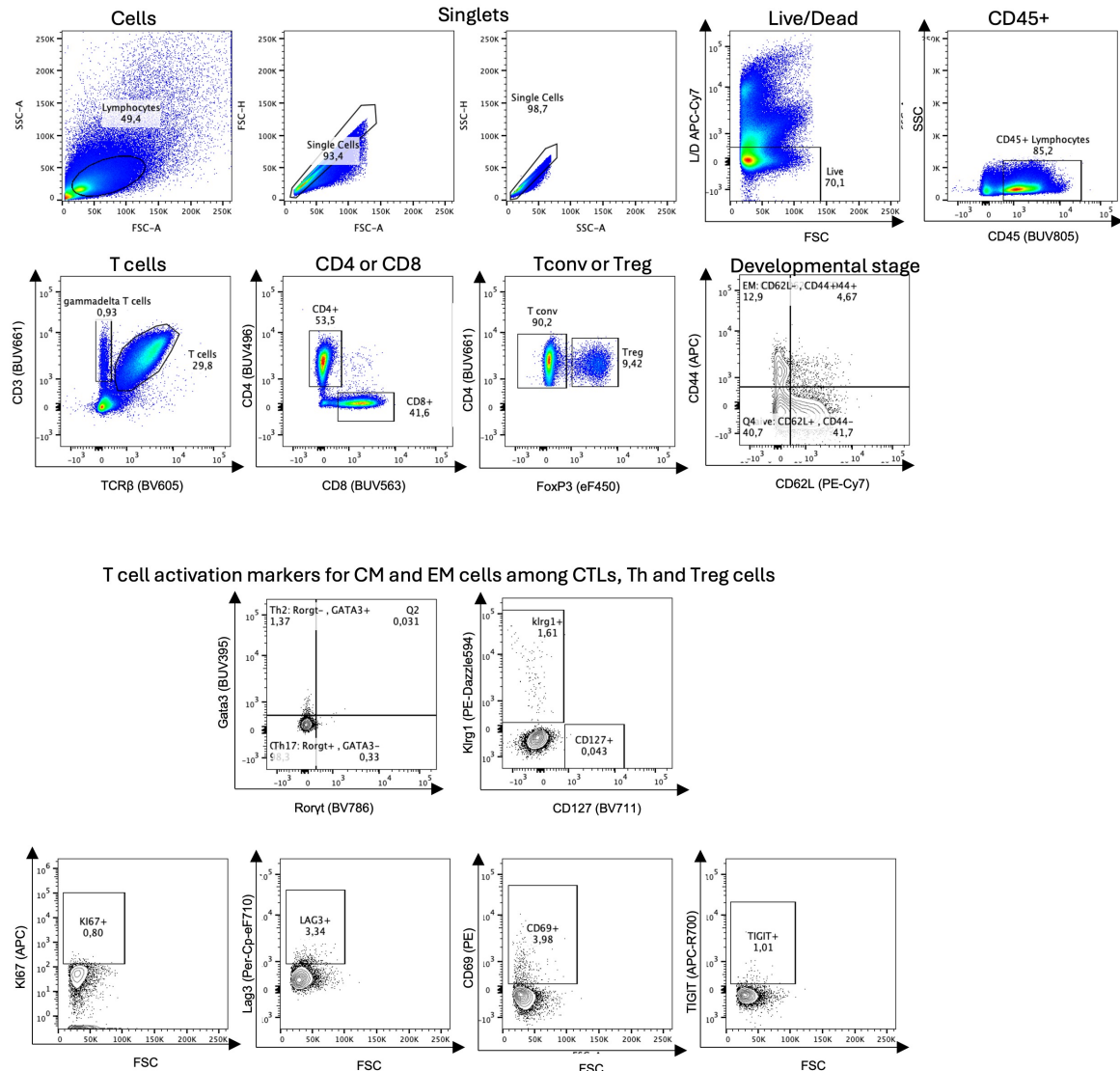


Figure 3-5: Gating procedure for analysis of T cell subsets and activation marker expression.

Figure 3-5 shows the gating strategy selected for the T cell analysis. The viable singlet target cell population was identified as described before (3.8.1). Lymphocytes were determined by gating CD45⁺ cells. $\alpha\beta$ -T cells (CD3⁺TCR β ⁺) and $\gamma\delta$ -T cells (CD3⁺TCR β ⁻) were identified by plotting CD3 against TCR β . From the double positive subset, CTL (CD8⁺) and CD4⁺ T cells were discriminated. Regulatory T (Treg) cells were identified from

the CD4⁺ subset by the additional expression of forkhead box protein 3 (FoxP3). In addition, among the CD4⁺ subsets, Th₁₇ (Roryt⁺) and Th₂ (GATA-binding protein 3 (Gata3)⁺) cells could be distinguished. Among CD8⁺ CTLs, CD4⁺FoxP3⁻ T-helper (Th) cells, and CD4⁺FoxP3⁺ Treg cells, the expression of the following activation states was categorized: CD44⁺CD62L⁺ naïve T cells, central memory T cells CD44⁺CD62L⁺, and effector memory T cells CD44⁺CD62L⁻. Subsequently, the expression of klrg1, CD127, KI67, lymphocyte activation gene 3 (LAG3), CD69, and T cell immunoreceptor with Ig and ITIM domains (TIGIT) was analyzed in the different states.

3.8.4 Analysis of B cells in vaccinated mice

To analyze immunoactivation in B cells of treated mice by FACS, prepared single-cell solutions of the spleen and mLN samples were stained by adding 50 µL of the surface staining mixture listed in Table 3-9.

Table 3-9 Reagents and Abs contained in the staining mixture for B-cell immune-activation analysis.

Antigen/Name	Fluorescent dye	S / IC staining mix	Dilution	Supplier
CD45	BUV805	S	1:500	Biolegend, San Diego, USA
CD23	BUV661	S	1:200	BD OptiBuild, Frankfurt, Germany
IgG1	BUV615	S	1:200	BD Biosciences, Frankfurt, Germany
CD38	BUV563	S	1:200	BD OptiBuild, Frankfurt, Germany
IgM	BUV496	S	1:200	BD OptiBuild, Frankfurt, Germany
B220	BV786	S	1:200	BD Biosciences, Frankfurt, Germany
CD95	BV650	S	1:200	BD OptiBuild, Frankfurt, Germany
IgD	BV605	S	1:200	BD Biosciences, Frankfurt, Germany
I-A/I-E (MHCII)	BV480	S	1:200	BD Horizon, Frankfurt, Germany
CD138	BB700	S	1:200	BD OptiBuild, Frankfurt, Germany
CD62L	FITC	S	1:400	Biolegend, San Diego, USA
CD21/35	PE-Cy7	S	1:200	Biolegend, San Diego, USA
CD93	PE-Dazzle594	S	1:200	Biolegend, San Diego, USA
Fixable viability dye	eF780	S	1:1000	Thermo Fischer, Darmstadt, Germany
CD19	APC	S	1:200	Biolegend, San Diego, USA

The cells were incubated with this mixture for 30 min at 4 °C in the dark. The samples were then washed twice with 100 µL FACS buffer per well (850 × g, 2 min, RT). Freshly prepared fixation/permeabilization mix (50 µL per well) was added to the samples and incubated ON. The samples were then washed twice more with FACS buffer (850 × g,

2 min, RT). Finally, cells were resuspended in FACS buffer and analyzed on a BD Fortessa Symphony A5/A5SE cytometer.

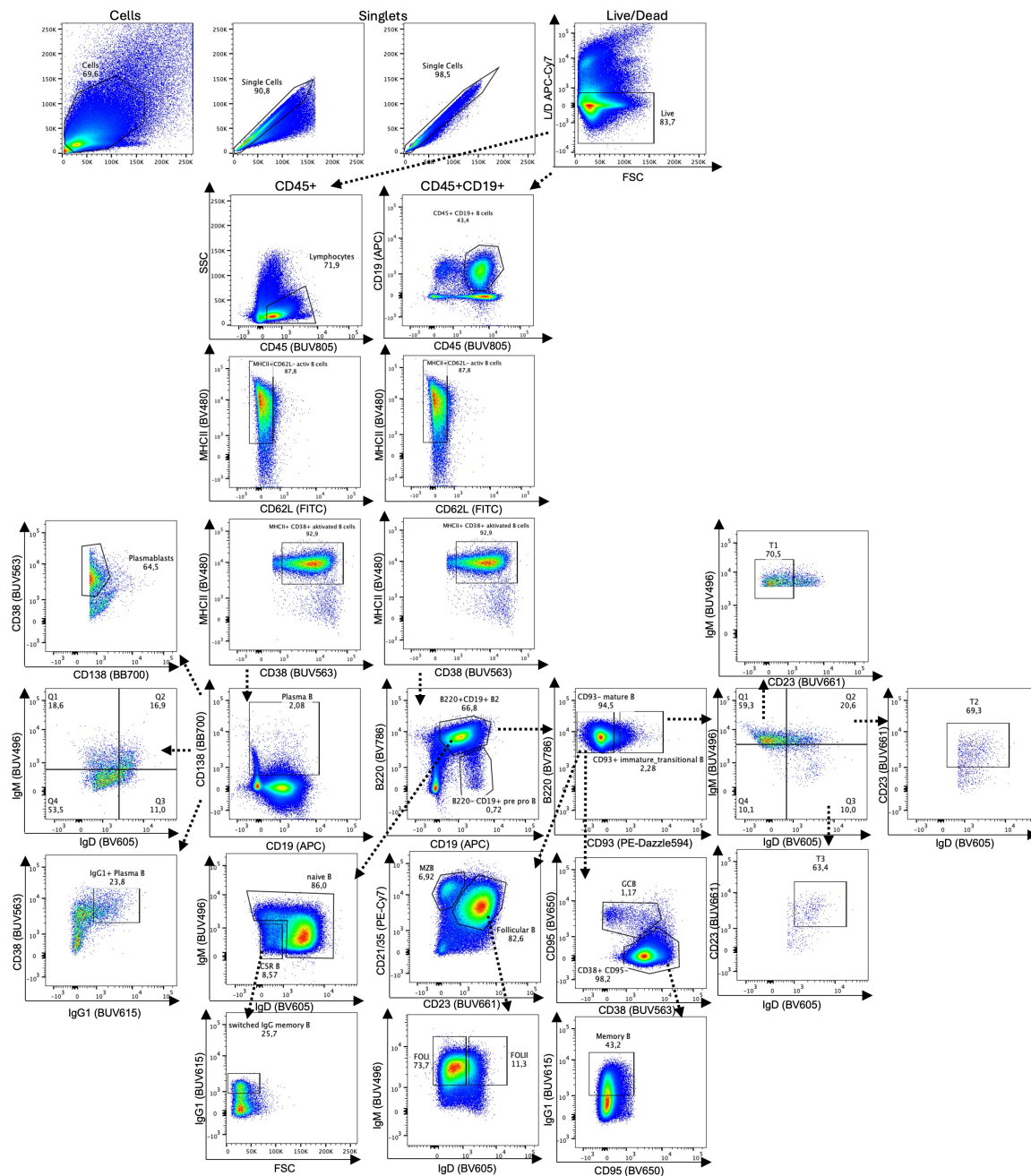


Figure 3-6: Gating procedure for analysis of B-cell subsets and activation marker expression.

Figure 3-6 shows the gating strategy selected for the B cell analysis. As described previously for T cell analysis (3.8.3), the CD45⁺ lymphocytes were gated. From CD45 positive cells activated plasma cells (CD19⁺/CD38⁺MHCII⁺CD62L⁻CD138⁺) were detected by plotting MHCII against CD62L, then against CD38, followed by plotting CD19 against CD138. Among activated plasma cells, CD138⁺CD38^{high} plasmablasts were detected, and the surface presentation of IgM, IgD, and IgG was analyzed.

Activated CD19⁺CD38⁺MHCII⁺CD62 L⁻ B cells could be differentiated into a B220⁻ pre-pro B cell population and conventional CD19⁺B220⁺ B. cells, which were then classified as mature (CD93⁻) and immature or transitional (CD93⁺) B cells based on their CD93 expression. Among CD93 positive B cells, the 3 transitional states T1(IgM⁺IgD⁻CD23⁻), T2 (IgM⁺IgD⁺CD23⁺), and T3 (IgM⁻IgD⁺CD23⁺) were determined by first plotting IgM against IgD and then analyzing the CD23 expression in each sector Q1-Q3. Within mature B cells, the CD95⁺CD38^{low} germinal center (GCB), CD21/35^{high}CD23^{low} marginal zone B (MZB), and CD21/35^{med}CD23⁺ follicular B (FOL B) cell subtypes can be further classified. B cells with a memory function (CD38⁺CD95^{low}IgG⁺) could be determined by plotting CD38 against CD95 and then looking at IgG1 expression. Follicular B cells could further be classified as FOLI (IgD⁺IgM⁻) and FOLII (IgD⁺IgM⁺) subtypes by plotting IgM against IgD. Among conventional B cells, IgD⁺IgM⁺ cells were classified as naïve B cells and differentiated from IgD⁻IgM⁻ class switch recombined B cells (CSR B) and subsequently from IgG⁺ switched memory cells.

3.9 ADC treatment assays

3.9.1 Synthesis of Ab drug conjugates

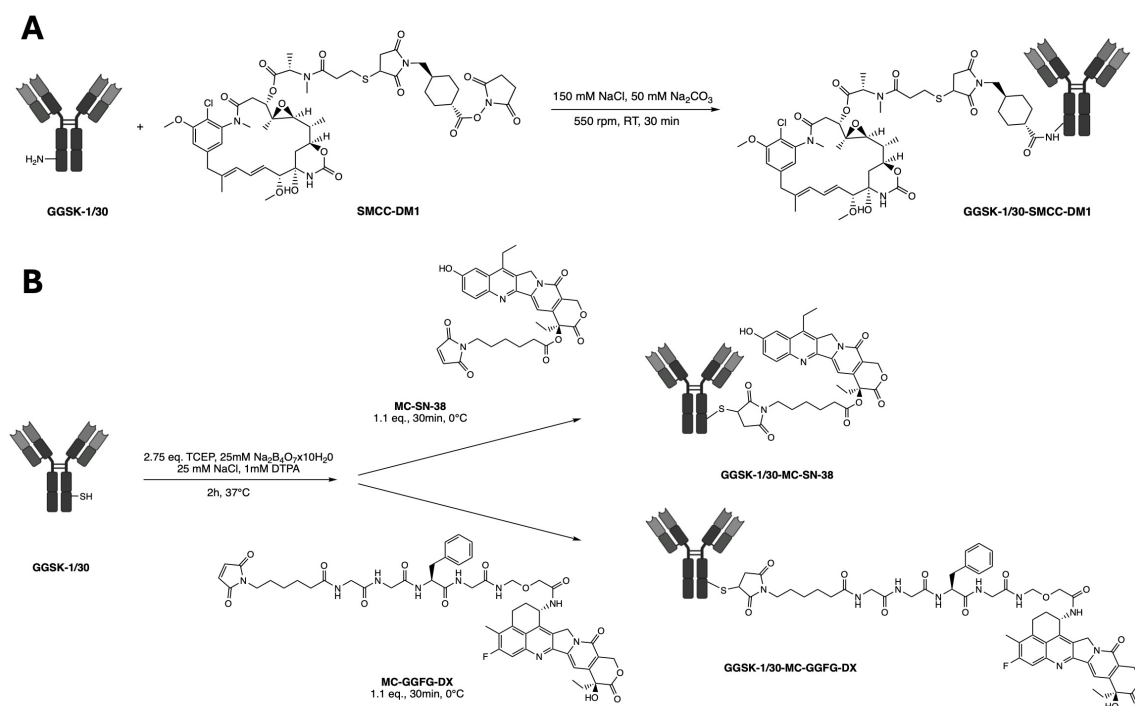


Figure 3-7: Reaction schemes for the synthesis of the coupling of GGSK-1/30 and the three linker-toxin conjugates SMCC-DM1 (A), MC-GGFG-DX, and MC-SN38 (B).

The scheme was thankfully provided by our collaboration partner Professor Pol Besenius.

Materials and Methods

For procedure A (Figure 3-7 A) 1– 5 mg of GGSK-1/30-Ab was added to 900 μL of 0.9 % NaCl solution. Subsequently, 7 μL of Na_2CO_3 solution (50 mM) was added. 4 and 8 eq. of succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC)-DM1 toxin were dissolved in 10 and 20 μL of acetonitrile, respectively, and then added. The reaction solution was incubated for 30 min at 550 rpm at RT. In procedure B (Figure 3-7 B), 1 – 5 mg was mixed with 2.75 molar equivalent of tris(2-carboxyethyl) phosphine hydrochloride (TCEP) in 0.025 molar (M) sodium borate, 0.025 M NaCl, and 1 mM diethylenetriaminepentaacetic acid (DTPA) for 2 h at 37 °C. Without purification, the mixture was cooled to 0 °C and 1.1 eq. of maleimide caproyl linker (MC)-SN-38 or MC linker plus GGFG-peptide spacer (MC-GGFG)-DX was added slowly, and the mixture was incubated for another 30 min at 0 °C. The toxin-coupled ADCs were all purified and isolated using a PD-10 column and the concentration was determined using a quick-drop photometer.⁵⁰

3.9.2 2D cultures for ADC treatments

To investigate the effect of ADCs in vitro, a 2D treatment assay procedure was established. The steps of this procedure are schematically shown in the following figure (Figure 3-8).

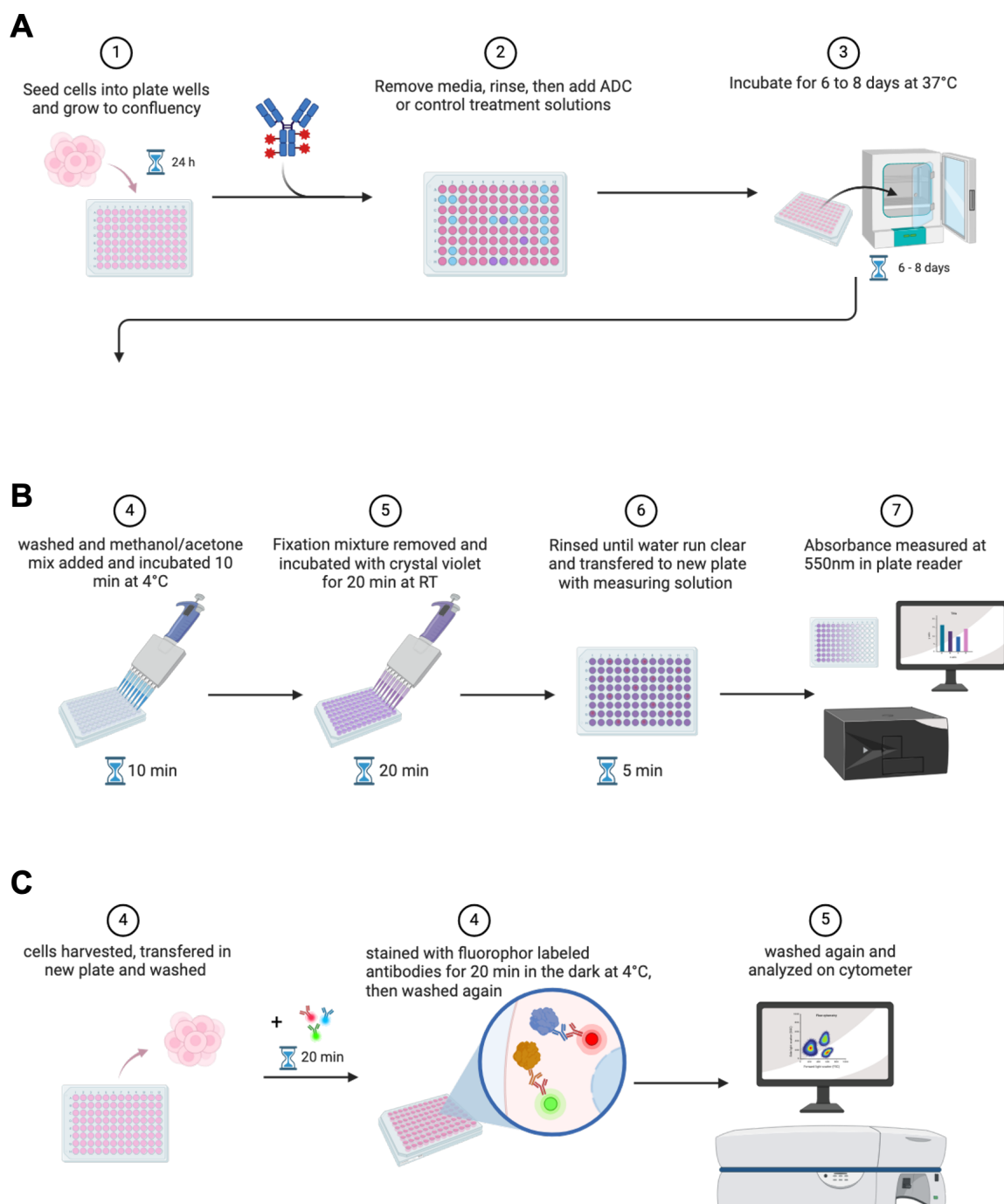


Figure 3-8: Scheme of the 2D treatment assay procedure.

A. Culture and treatment of the cells. B. Analysis via crystal violet viability staining. C. Analysis by FACS viability staining. Created using Biorender.com.

In the first step (1), cells were plated into a flat-bottom 96-well plate. The seeding densities of the different cell lines are listed in the following table (Table 3-10).

Table 3-10: Cell density seeded out for the 2D treatment assay.

Cell line	Density (cells per well)
PyMT	500
PyMTxMUC1	500
MCF-7	1000
T47D	2500
MDA-MB-231	750
PANC-1	4000
A549	1000

After 24 h in the incubator, the culture medium was replaced with the different treatment conditions (2). Depending on the confluency of the neg ctrl, cells were kept in the incubator between d6 and d8 after beginning the treatment (3) and then analyzed with crystal violet viability staining (B, 4-7) described in 3.9.3, or in FACS (C, 4-6) described in 3.8.2.

3.9.3 Crystal violet viability staining (Figure 3-8 B)

The medium of the cells from the prepared 96-well plates was removed, and 100 μ L fixation solution (100 mL PBS + 10 mL formaldehyde 37%) per well was added and incubated for 5 min at RT. This was then removed and 50 μ L crystal violet solution (0,5 gram (g) crystal violet + 80 mL millipore H₂O + 20 mL MeOH) was added per well and incubated for 20 min at RT. The solution was then removed, and the plate was rinsed with water. 125 μ L of measuring solution (50 % ethanol + 0,1 % glacial acetic acid) per well was added and resuspended. Subsequently, 100 μ L from each well was transferred to an ELISA plate (NUNC 96 well ft). The samples were imaged at 570 nm on a Tecan plate reader.

3.9.4 3D culturing for spheroid formation

To investigate the effect of ADCs on tumor spheroids, the procedure schematically shown in Figure 3-9 was followed.

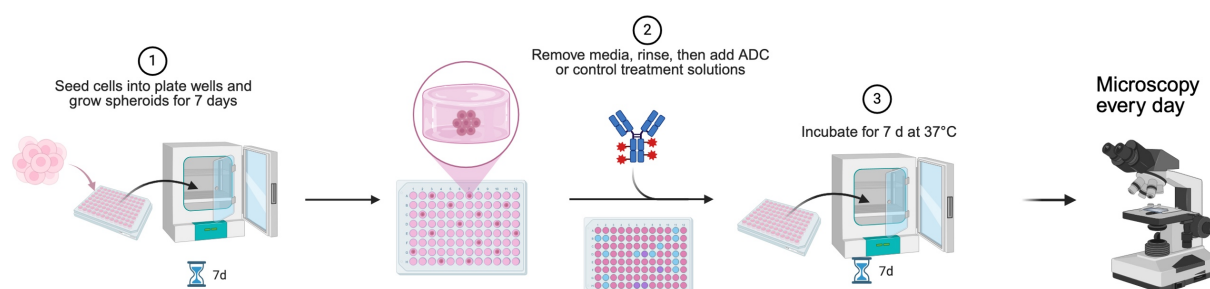


Figure 3-9: Procedure of 3D treatment assays.

Created using Biorender.com.

In the first step (1), cells were plated into low-attachment 96-well spheroid microplates. The seeding densities of the different cell lines are listed in the following table (Table 3-10).

Table 3-11: Cell densities seeded for the 2D treatment assay.

Cell line	Density (cells per well)
MCF-7	11000
T47D	11000
MDA-MB-231	10000
PANC-1	10000

Then, the cells were placed into the incubator for 7 d to allow spheroid formation, which was controlled every 2nd d under the microscope. Then, the culture medium was replaced with the different treatment conditions (2), and the spheroids were kept in the incubator for another 7 d (3). To follow the effects of the treatments, the spheroids were watched under the microscope every day.

3.9.5 Surface measurement of spheroid microscopy images in image J

For better visualization of the treatment effects on the spheroids, growth curves were generated from the cross-sectional area of the spheroids from the microscopy images. Therefore, the images were loaded into the Fiji Image J software and a scale bar was adjusted (Figure 3-10, 1) according to the scale settings obtained from the microscope (ratio: 0,8783 $\mu\text{m}/\text{pixel}$). Then, the image was modified using the following steps to allow the software to obtain the shape of the of the pictured spheroid.

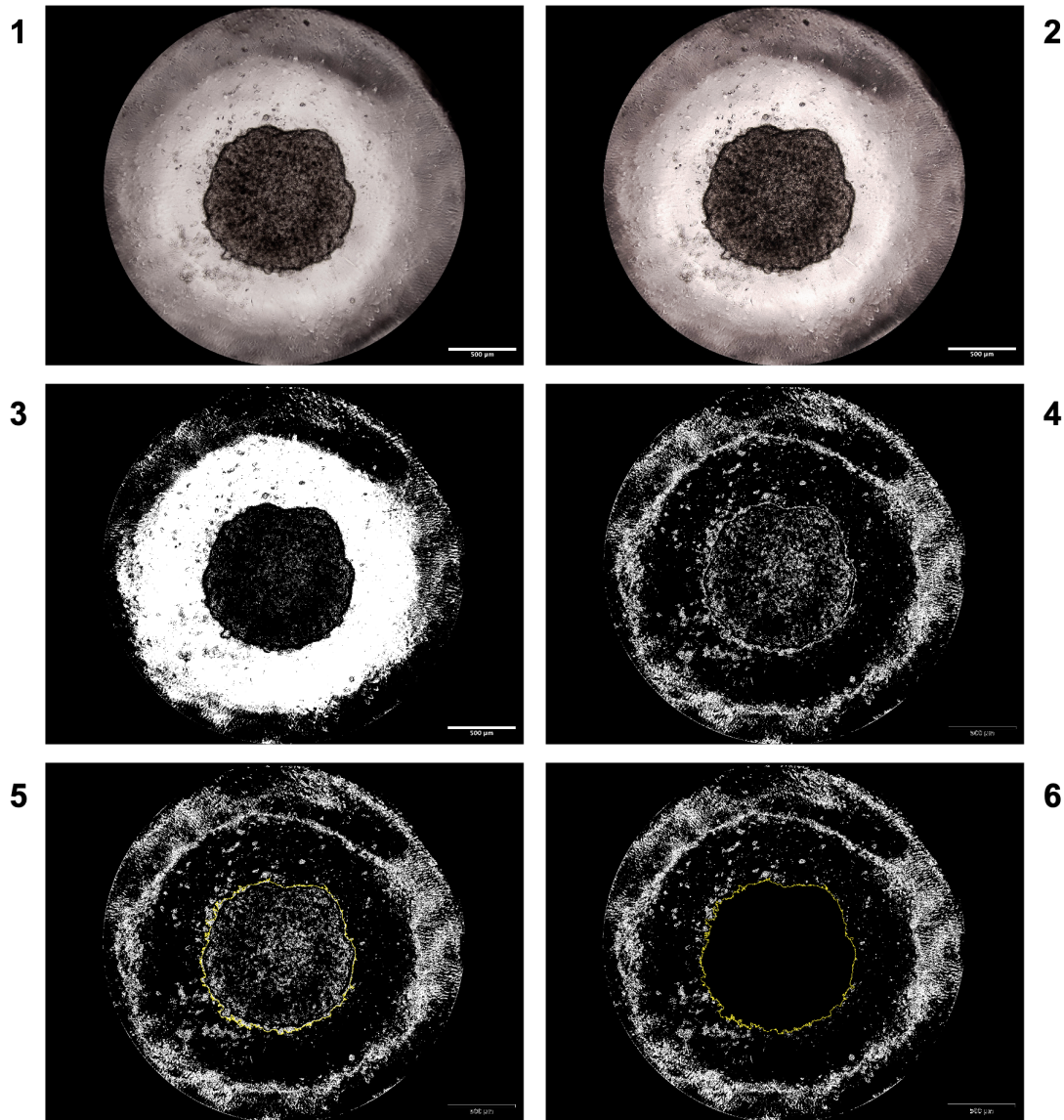


Figure 3-10: Transformations performed on the microscopy images with Fiji Image J to measure the cross-sectional area of the spheroids.

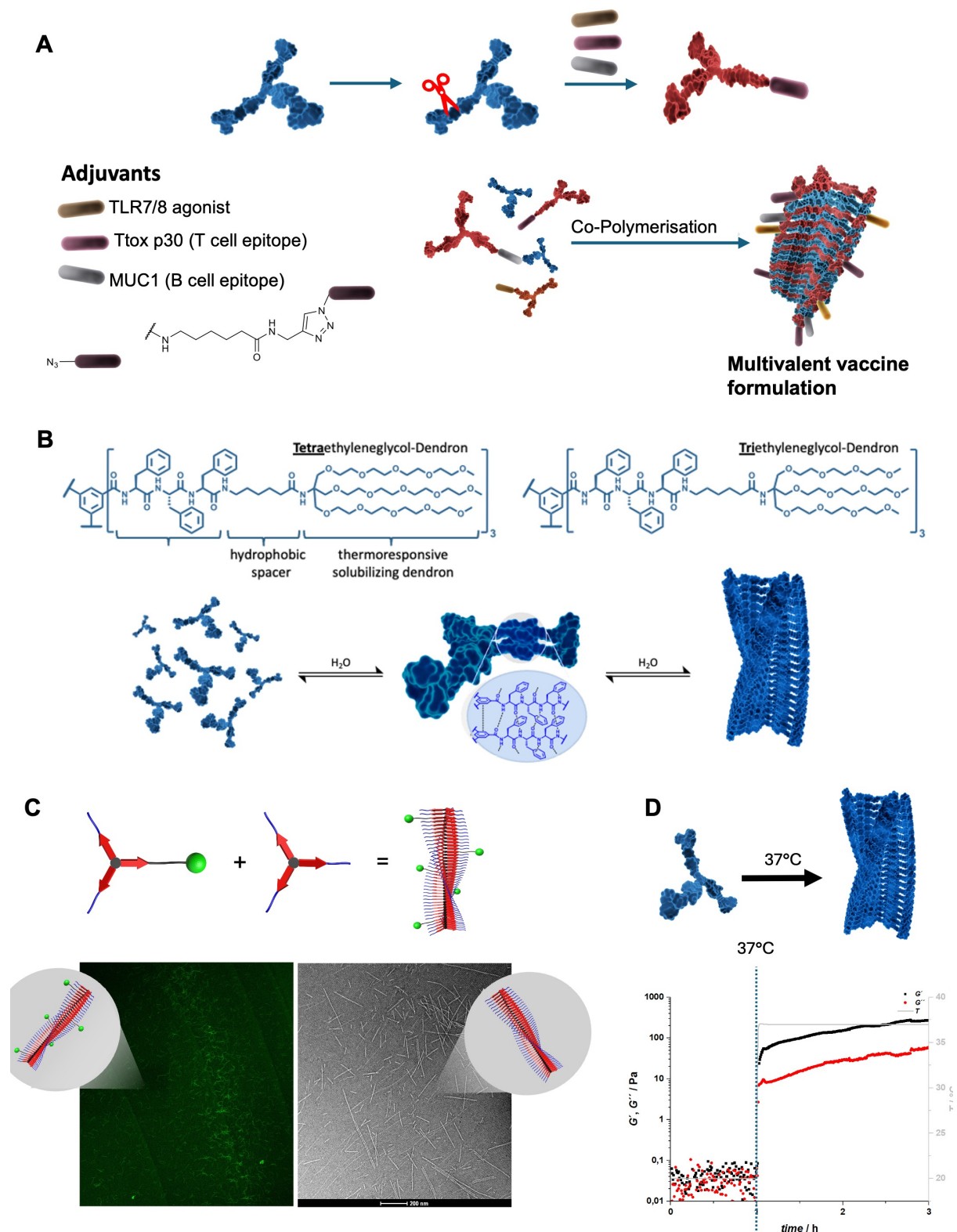
If necessary, the image contrast was sharpened and enhanced (Figure 3-10, 2). To make the shape detection more accurate, the image was then converted into a binary resolution (Figure 3-10, 3). The “find edges” processing tool was applied to the image (Figure 3-10 and 4). The shape of the spheroid was selected with the wand tracing tool, resulting in a yellow highlighting of the spheroid outline (Figure 3-10, 5). The area within the selected shape was calculated using the “Analyze measure” command (Figure 3-10, 6).

4 Results

4.1 The MUC1(22)S¹⁷ST_N-p30 supramolecular, synthetic and modular antitumor vaccination strategy

Preliminary studies showed that the synthetic glycopeptide MUC1(22)S¹⁷ST_N led to high Ig titers, and FACS analysis confirmed the strong binding affinities of the generated Abs to T47D tumor cells. Inserting this glycopeptide sequence as a B-cell epitope into monomers of self-assembling nanorods allows the efficient generation of multifunctional and multivalent structures without the need for extensive purification steps of protein conjugates. This leads to further improvement in the vaccine capacity to trigger the antitumoral immune response.^{69,75,91}

In this approach, antigens are conjugated to star-shaped peptide amphiphiles, with each monomer serving a defined function. In water, these monomers self-assemble via noncovalent interactions into rod-shaped nanostructures that display different immune epitopes. B-cell epitopes are synthetic 22-amino acid glycopeptides bearing tumor-associated sialylated ST_N antigens from taMUC1 that are altered during tumorigenesis. T-cell activation is triggered by a fragment of TTox (p30), whereas immune stimulation is enhanced by an imidazoquinoline-based TLR7/8 agonist. Mannose moieties can also be incorporated for the recruitment of macrophages. All epitopes were conjugated to monomers via Click Chemistry. Upon mixing in water, the hydrogen bonding and hydrophobic interactions of poly L-phenylalanine drive the formation of rod-like nanostructures. These virus-mimicking TEG-based nanorods are biocompatible and optimized for effective antigen presentation and immune activation. Achieving this requires the co-stimulation of B cells with T helper cells, which can be facilitated by the co-presentation of different epitopes and immunostimulatory agents on the surface of multicomponent supramolecular polymers. These formulations adjuvanted by IFA were previously tested in mice and could already demonstrate the immunogenic potential of modular supramolecular vaccine platforms as a novel approach for the development of synthetic antitumor vaccines.^{75,92,93} All experiments from this section are part of a collaboration with [REDACTED] and [REDACTED] from the group of [REDACTED] (Dpt. of Chemistry, JGU Mainz). Figure 4-1 A schematic recapitulates the described system and the vaccine formulations tested within the project.



The right side of Figure 4-1 C shows the nanorod shape analyzed via transmission electron microscopy, in which the rod-shaped structures are approximately 200 nm in size. To prepare the fluorescence microscopy image on the left side of Figure 4-1 C, monomers of the system were linked to the fluorescence dye Cy-3.

With the idea of replacing IFA within the formulation with thermoset supramolecular hydrogels to form the necessary vaccine depot, a hydrogel forming was introduced into the vaccine building set. Figure 4-1 B shows the schematic structure of this hydrogel-forming monomer, in which a tetra- and triethylenglycol-dendron are attached via a spacer to poly-L-phenylalanine. Mixing in water creates thermoresponsive nanorods. Figure 4-1 D shows how a solution containing the hydrogel-forming monomer behaves at different temperatures. At approximately 37 °C, the solution forms a gel. This enables its application as a depot forming agent for vaccine injections, offering a promising alternative to conventional adjuvant-based vaccination strategies.

For cancer vaccine formulations, an appropriate adjuvant is mandatory to address the right immune cell population to fight cancer. To determine the optimal amount of p30 and TLR7/8 for the new formulation, different adjuvants were previously tested in vivo in our working group (Figure 4-2).

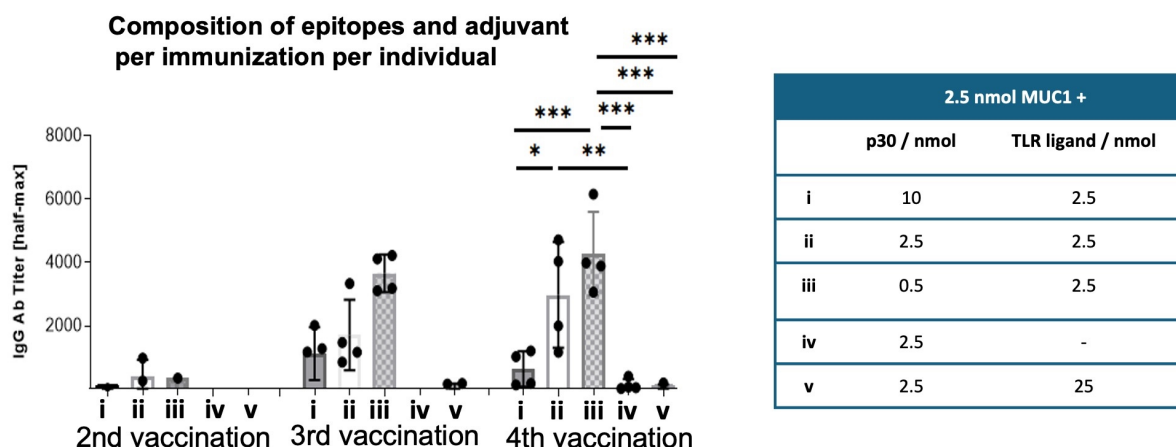


Figure 4-2: Optimization of MUC1(22)S¹⁷ST_N-p30 supramolecular antitumor vaccine (II).
Effects of variation in the concentration of p30 and TLR7/8 Ligand on humoral immune response.

The experiment highlights that the ratios of the adjuvant-holding monomers within the formulation have an impact on the humoral response in mice (Figure 4-2Figure 4-6 A). The five tested formulations all contained 2,5 nmol of MUC1(22)S¹⁷ST_N but differed in the amount of T cell epitope p30 and TLR7/8 agonist. The first three had a constant amount of TLR7/8 agonist (2,5 nmol) with decreasing ratios of 10 (i), 2,5 (ii) and 0,5 nmol (iii) p30.

Results

The results show that decreasing the amount of p30 had a significant effect on the humoral immune response, making 0,5 nmol the best option for this vaccine strategy. In formulation (iv), the TLR7/8 agonist was removed, and in formulation (v), its ratio was increased by 10 times (25 nmol). Both lead to a massive decrease in humoral responses, suggesting that a TLR7/8 adjuvant is required. However, increasing the ratio even had detrimental effects. Therefore, the original quantity of 2,5 nmol was retained.

4.1.1 Humoral immune response of mice vaccinated with different ratios of the gel forming monomer within the new vaccine formulation

To integrate the structural hydrogel forming monomer into the existent formulation in the right ratio without diminishing its efficacy, different formulations containing 0,5 wt%, 1 wt% or 1,5 wt% of the structural hydrogel forming monomer were tested *in vivo*. Mice were treated as shown in the following vaccination scheme (Figure 4-3 A). C57Bl/6 wt mice were injected *i.p.* four times at intervals of 2 weeks. A serum sample was isolated from the blood of mice before the first and after each round of immunization. The humoral response to the different formulations was determined by analyzing the presence of immunoglobulins (Ig) in serum samples. Figure 4-3 B shows a comparison of the half-maximal Ig titers determined from serum samples from each group.

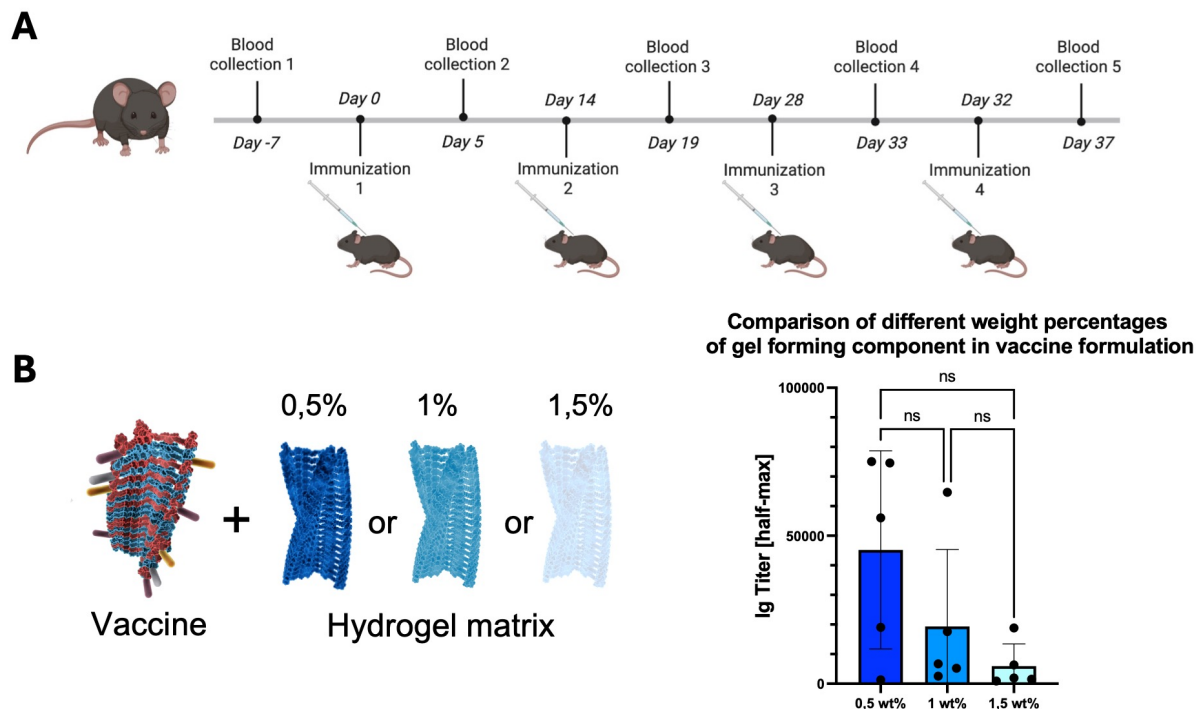


Figure 4-3: Optimization of the MUC1(22)S¹⁷ST_N-p30 supramolecular antitumor vaccine (I): Testing the effect of different ratios of the hydrogel forming monomer on the humoral response.

In the Vaccination scheme, pre-immune serum of the mice was collected 7 d before the first vaccination, and then the mice were vaccinated four times at intervals of 2 weeks. Blood was collected 5 d after each vaccination. B Testing the effect of different wt% of hydrogel-forming component in the vaccine formulation on humoral response in Ig ELISA. Parts of this figure were created using Biorender.com. Statistical analysis: Brown-Forsythe and Welch one-way ANOVA with Dunnett's T3 multiple comparisons, $p > 0.05$ (ns), $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

The figure shows that raising the amount of hydrogel forming monomer does not lead to a better response. In fact, mice vaccinated with 1.5 wt% of the monomer within the vaccine showed almost no reaction. Both lower dose formulations triggered a higher humoral response, but due to high standard variations, the results were not significant. Qualitatively, the formulation with 0,5 wt% seems to induce the highest Ig production; therefore, it was selected for subsequent analysis.

4.1.2 Can the optimized formulation containing the hydrogel-forming monomer match the former formulation with IFA in synthetic antitumor vaccines?

To determine the future applicability of the optimized vaccine strategy, it is necessary to ascertain that the resulting immune response is as effective as the former formulation. This was demonstrated by the comparison of both formulations in vivo in the next experiment. Groups of five C57Bl/6 wt mice obtained either the IFA vaccine containing 2,5 nmol MUC1-ST_N, 0,5 nmol p30 and 2,5 nmol TLR7/8 or the hydrogel vaccine with

Results

additionally 0,25 wt% TetraEG-Monomer and 0,25 wt% TriEG-Monomer. Vaccination and blood collection was performed as described before (4.1.1). The humoral response was checked in each blood collection after each immunization round. Additional blood collections at 2, 4, and 6 weeks after the last round of immunization were taken to evaluate the two vaccines based on their ability to produce a longer-lasting immune response. Figure 4-4 shows the results of the ELISA from serum samples displayed as half-maximal titers.

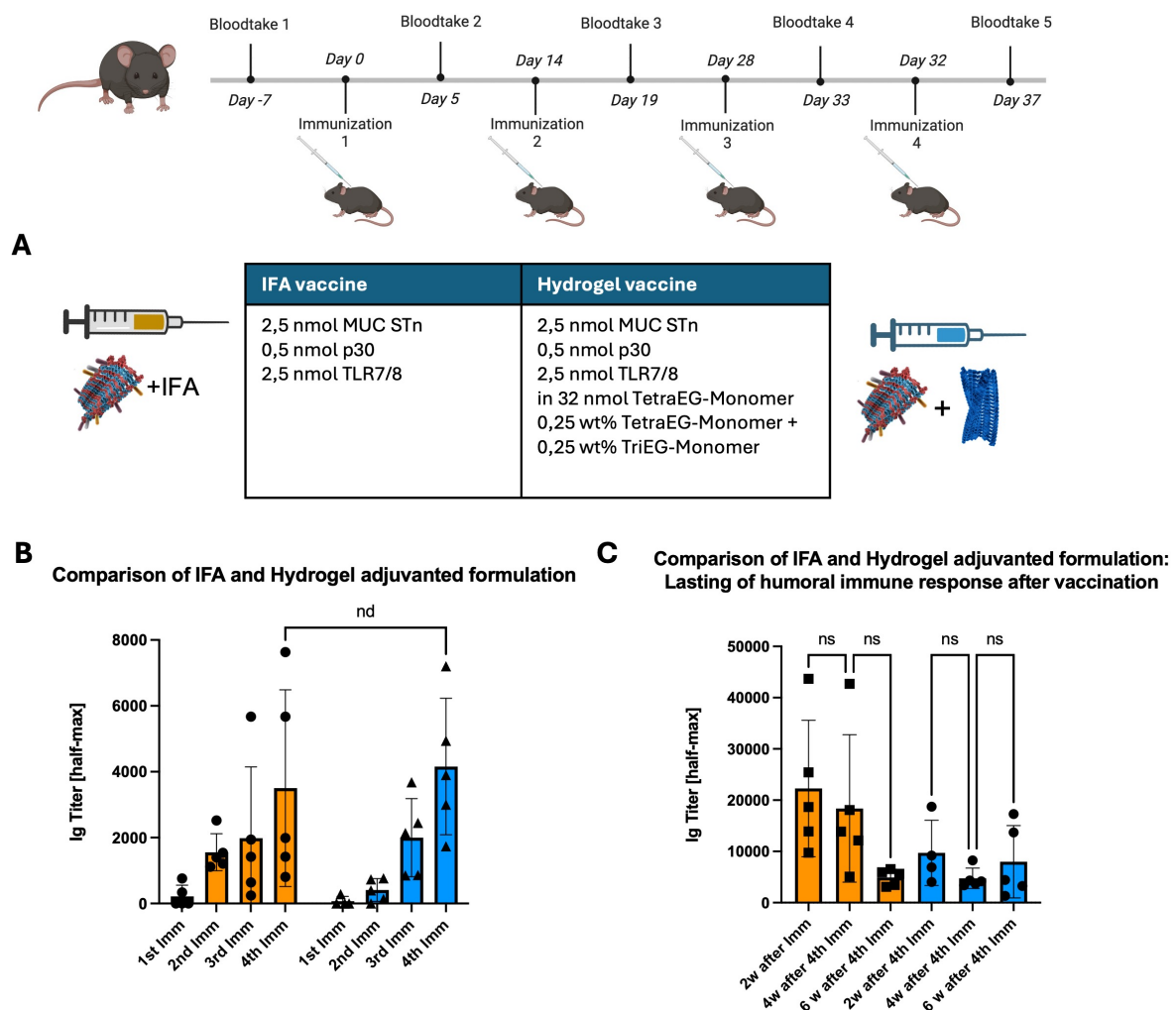


Figure 4-4: Optimization of the MUC1(22)S¹⁷ST_N-p30 supramolecular antitumor vaccine (III): Comparison of IFA and hydrogel formulations.

A: Ingredients of IFA and hydrogel formulations. B: Comparison of humoral immune responses after each immunization with IFA (orange) or hydrogel-based (blue) formulations. C: Comparison of remaining Ig titers at 2, 4, and 6 weeks after immunization. Parts of this figure were created using Biorender.com. Statistical analysis: B. Welch unpaired t-test from 4th Imm., C: one-way ANOVA with Tukey's multiple comparison, $p > 0.05$ (ns), $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

The results show that both strategies lead to strong humoral responses. Each round of immunization led to a boosting effect on the humoral response in both cases. After the second injection the IFA formulation has a slightly stronger inducing effect, but this levels off with the third and fourth round. Looking at the Ig present in the samples taken 2, 4, and 6 weeks after the last immunization, there seems to be a decrease over time for the IFA formulation, but the differences are not significant. For the hydrogel formulation, no such tendency was observed. This could indicate that the new formulation might have a better depot effect, leading to a more gradual presentation of immune cell epitopes. Consequently, this then induces a more constant and therefore longer-lasting induction of the humoral immune response.⁹⁶

4.1.3 Optimized vaccine formulation shows high titer responses in wt mice as well as in humanized mice model

In the context of antitumor vaccines, one major challenge for vaccine candidates is to overcome self-tolerance mechanisms against self-derived tumor antigens to elicit an immune response against the tumor. To demonstrate that the new formulation could do so, it was tested in the mouse strain C57Bl6-{hMUC1}, which was genetically modified to carry hMUC1 instead of the murine variant. Therefore, mice from this strain should better mimic the situation in humans by presumably being tolerant to healthy hMUC1.

Five C57Bl6-{hMUC1} mice were vaccinated along with five C57Bl6-wt mice according to the vaccination scheme described before (4.1.1). Serum from both groups was analyzed for humoral immune response by determining Ig levels using ELISA (Figure 4-5).

Results

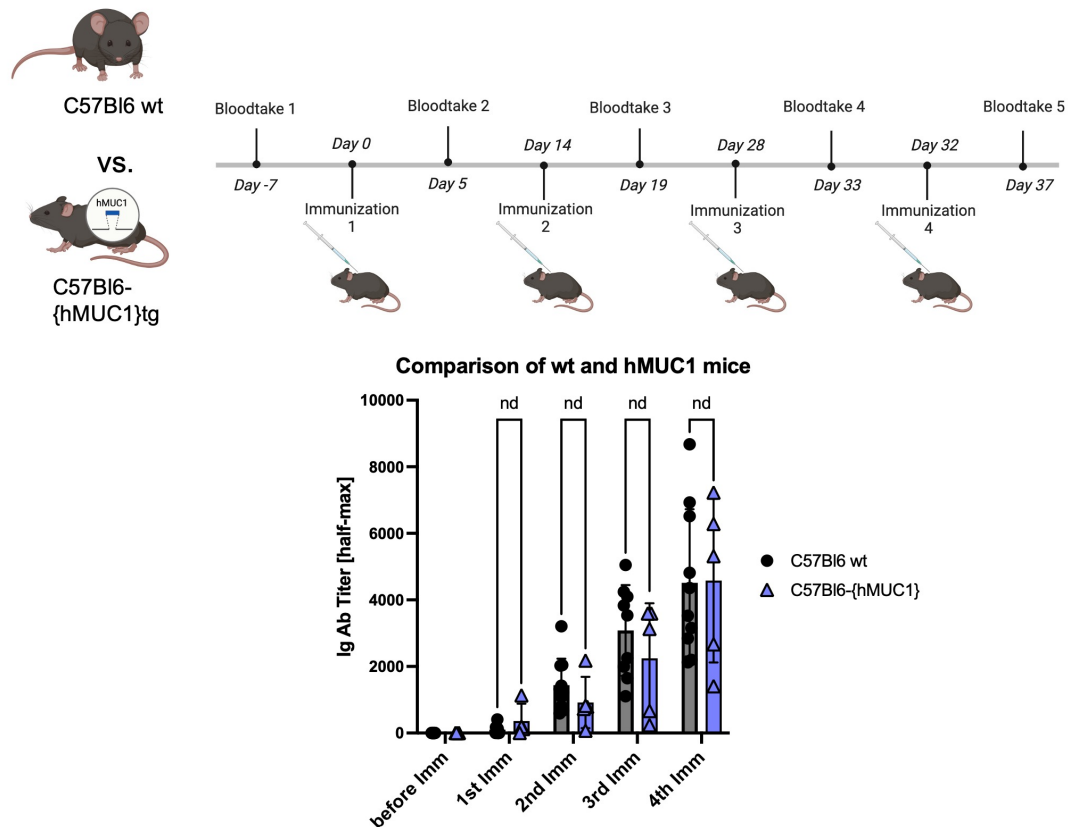


Figure 4-5: Comparison of humoral responses in C57Bl6 wt and hMUC1 transgenic mice after vaccination with the new hydrogel adjuvant formulation.

Parts of this figure were created using Biorender. com. Statistical analysis: Welch unpaired t-test, $p > 0.05$ (nd), $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

Half-max Ig titers showed no significant differences between wt and transgenic (tg) mice at all stages of vaccination, demonstrating that taMUC1-Abs formed equally in the serum of hMUC1-bearing mice and mice without hMUC1. This suggests that the vaccine formulation was able to easily overcome hMUC1-self-tolerance, as the immune response against taMUC1 was as strong as that in mice that do not have the hMUC1 antigen.

4.1.4 Comparison of immune responses in mice vaccinated with cleavable or uncleavable linkage and the addition of a STING agonist to the vaccine

Thus far, it has been shown that the right amount of adjuvant is important to generate a stable humoral response, that the introduction of a hydrogel-forming monomer can replace the use of IFA, and that the optimized vaccine can overcome self-tolerance. In the next step, a more potential immunostimulatory molecule was introduced. This synthetic structure acts as a stimulator of interferon genes (STING) agonist. Literature has demonstrated that STING agonists tend to have an improved synergistic effect in nearly every anti-cancer therapy. They target the cyclic GMP-AMP synthase-STING pathway.

Activation of this pathway leads to enhanced type-I-interferon and inflammatory cytokine production. This recruits antitumoral immune cells to the site turning the tumor microenvironment into a tumor repressive phenotype.^{97–99}

In the next experiment, a synthetic STING agonist structure was compared to the TLR7/8 agonist, and the impact of the introduction of a cleavable linking system for adjuvants was tested in vivo. Cleavable linkage should enable the release of ligands directly after uptake into cells and avoid the necessity for prior molecular degradation of the vaccine. Groups of five C57Bl/6-wt mice were vaccinated with the four different combinations according to the vaccination scheme previously described (4.1.1) and are shown in Figure 4-6 A.

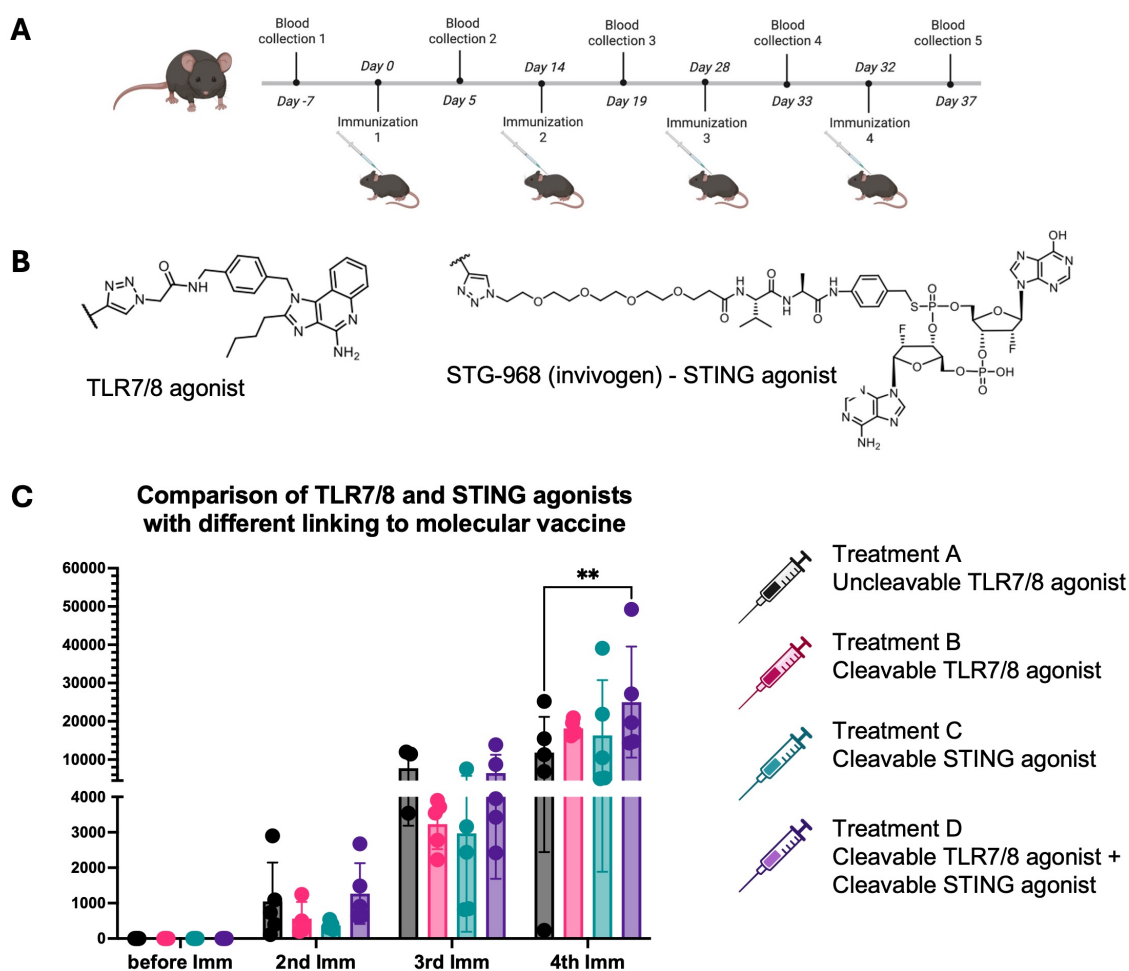


Figure 4-6: Testing different adjuvant variations within the MUC1(22)S¹⁷ST_N-p30 supramolecular antitumor vaccine.

A Vaccination scheme: Pre-immune serum was taken 7 d before first vaccination. Mice were vaccinated 4x at intervals of 2 weeks. Blood was taken 5 d after each vaccination. B Structures of TLR7/8 (left) and STING agonist (right). C Humoral response detected via ELISA after the different treatments. Parts of this figure were created using Biorender.com. Statistical analysis of treatments against each other in 4th Imm.: Tukey's corrected two-way ANOVA, $p > 0.05$ not shown, $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

Results

Treatment A represents the previously optimized vaccine formulation and was compared to three different formulations. In the first variation (Treatment B), the TLR7/8 agonist was connected to the monomer via a cleavable linker. The next variation (Treatment C) contained the STING agonist, which was also connected through the cleavable linker. The last variation (Treatment D) combined with treatments B and C. Figure 4-6 B shows the structure of the two synthetic structures, TLR7/8 agonist on the left and STING agonist on the right side, that were compared within the formulations. Figure 4-6 C shows the results of the ELISA analysis of Ig formation in the serum of the mice after each vaccination.

All four treatments induced a humoral response. The results show that each round of vaccination has a boosting effect on the response. Interestingly, the change of the agonist or the introduction of the cleavable linker alone did not have a boosting effect on Ig formation. Only the combination of both showed a significant increase in Ig detectable in the serum.

To further characterize differences in the immune response induced by the four different treatments, secondary lymphatic organs were collected 3 w after the last vaccination. They were then analyzed in comparison to an untreated ctrl group of mice for immunoactivation marker expression in B and T cells of the spleen and the mesenteric lymph node (mLN) via FACS.

To gain a general overview of the T and B cell subsets present in the spleen and mLN and to better visualize differences between untreated and treated groups, t-distributed stochastic neighbor embedding (t-SNE) of the data was computed. Therefore, pre-gated living CD45- and CD3-positive T cells or CD19-positive B cells were analyzed using the unsupervised t-SNE clustering plug-in tool of FlowJo¹⁰⁰ to create the tSNE plots. The subsequent FlowSOM plug-in¹⁰¹ analysis created a heatmap displaying marker expression in each cluster and was used to annotate the cell types in the different clusters. Heatmaps for each tSNE analysis are shown in the supplementary section (Figure 8-1).

FlowSOM cluster analysis was then applied on the tSNE plots created before resulting in the plots shown in Figure 4-7 for B cells of the spleen and in Figure 4-8 for B cells of the mLNs.

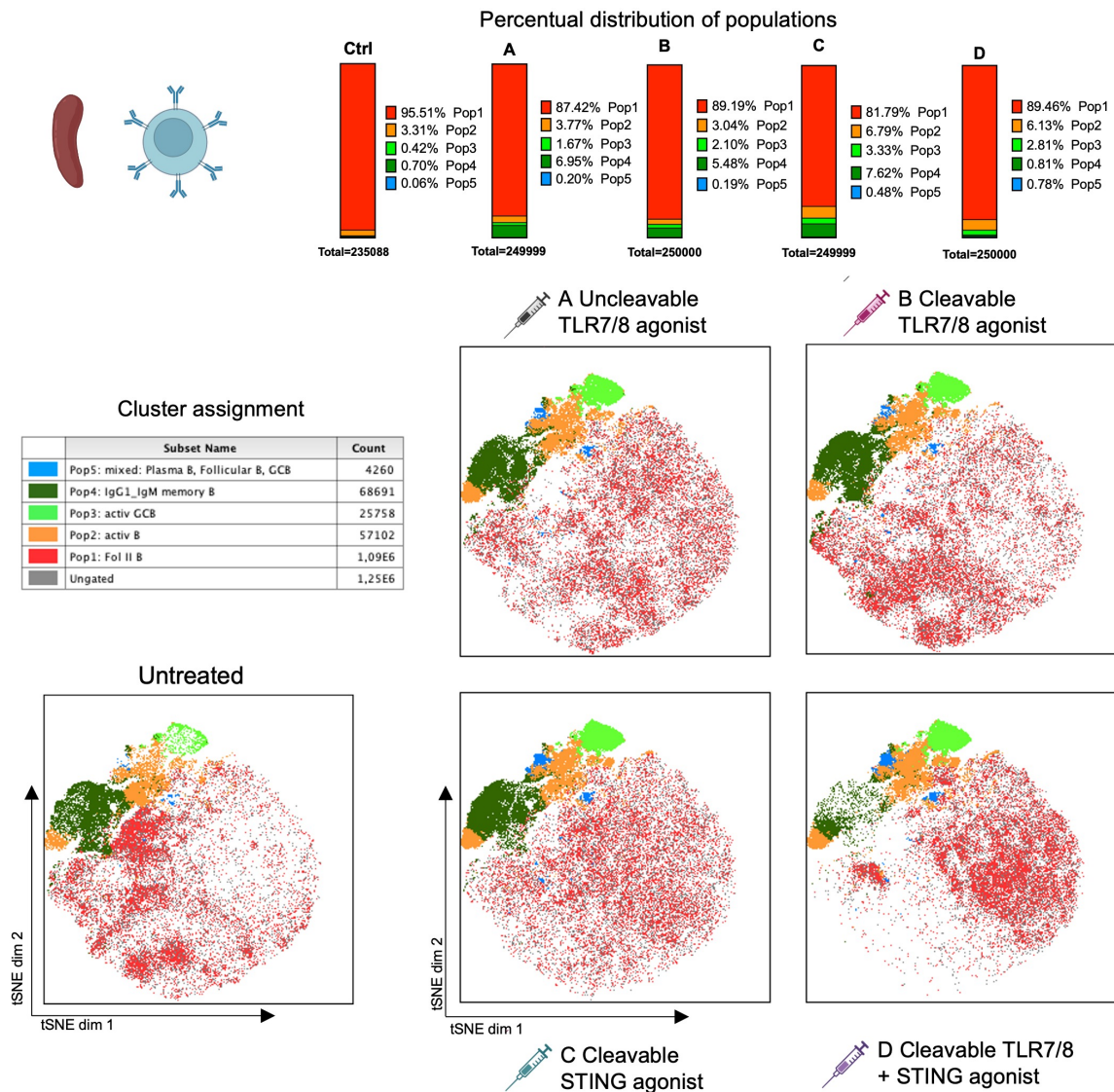


Figure 4-7: tSNE cluster analysis of B cells from the spleen of mice after vaccination with differently adjuvated vaccine formulations as measured by FACS.

CD45⁺CD19⁺ cells were pre-gated and 50000 cells per sample were computed as tSNE. Marker expression in FlowSOM clusters was checked with the Heat Maps, and subtypes were accordingly defined. Computation was performed in FlowJo v10.10.0 with DownSample, tSNE¹⁰⁰, and FlowSOM¹⁰¹ R-based Plug-ins. Parts of this figure were created using Biorender. com.

The tSNE and FlowSOM analyses of the data allowed the determination of the following clusters in B cells from the spleen: CD21/35⁺CD23⁺IgD⁺ type II FOL B cells (Population 1), CD38⁺ activated B cells (Population 2), CD95⁺CD38⁺MHCII⁺ activated germinal center (GCB) cells (Population 3), IgG1⁺ and IgD⁺ CD95⁺CD38⁺ memory B cells (Population 4), and a mixed CD38^{high}CD138⁺CD95⁺IgM⁺IgG1⁺IgD⁺CD21/35⁺ cluster presumably containing CD21/35⁺CD23⁺ follicular B, CD95⁺ GCB, and CD138⁺ plasma cells (Population 5).

Results

Cluster analysis of B cells in the spleen showed that a major proportion of B cells belong to the type II Fol B phenotype. This cell type is known to participate in inducing T-cell dependent antigen-responses¹⁰². Comparing the cluster between the different treatments, it seems that all treatments led to an increase in active GCB cells in comparison to the untreated sample, meaning that humoral response had taken place in the germinal center zone of lymphatic organs. The strongest increase, with a doubling amount of approximately 6 % of active GCBs, was observed in treatments C and D, which contained the STING agonist. Additionally, treatment A, B, and C induced the formation of IgG or IgM memory B cells, whereas treatment D did not. In addition, the follicular II B cell population decreased in all treatments to 80-90 % compared to the ctrl (95 %), and shifts in the population were observed on the tSNE. The shifts in this population could be explained by the fact that this cell type has a high variety in their Ig repertoire¹⁰², leading to the assumption that each treatment might have induced different compositions of Ig repertoire.

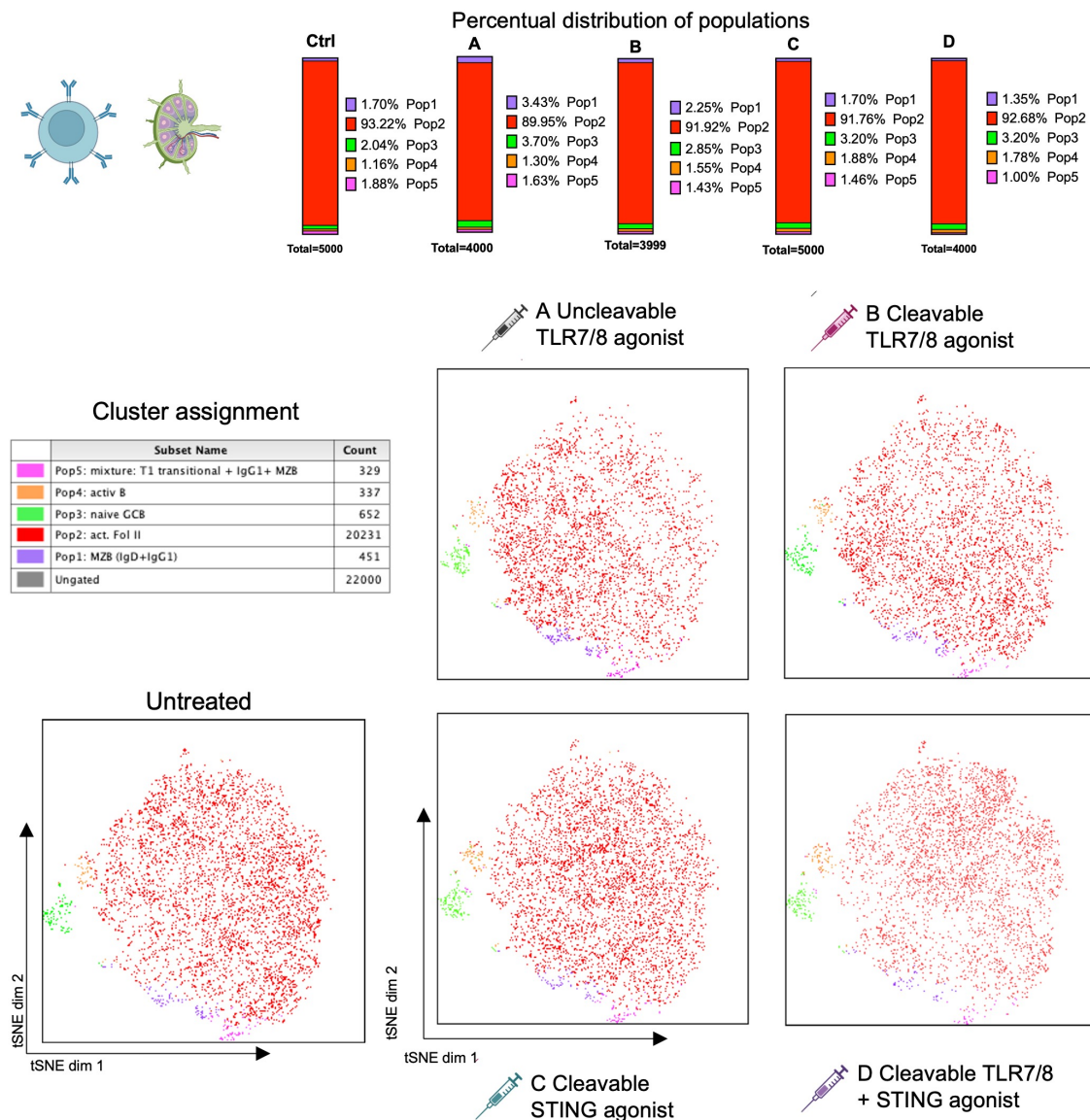


Figure 4-8: Figure 4-9: tSNE cluster analysis of B cells from the mLN of mice after vaccination with differently adjuvated vaccine formulations as measured by FACS.

CD45⁺CD19⁺ cells were pre-gated and 4000-5000 cells were computed as tSNE. Marker expression in FlowSOM clusters was checked with the Heat Maps, and subtypes were accordingly defined. Computation was performed in FlowJo v10.10.0 with DownSample, tSNE¹⁰⁰ and FlowSOM¹⁰¹ R-based Plug-ins. Parts of this figure were created using Biorender. com.

Analogous to data processing of the spleen, the following clusters could be differentiated for the mLN: IgG1-pos. CD21/35⁺CD38⁺IgM^{med}IgD^{high} MZB cells (Population 1), CD21/35⁺CD23⁺IgD⁺MHCII⁺ activated Fol II B cells (Population 2), CD95⁺ IgM⁺ naïve GCB cells (Population 3), CD38⁺MHCII⁺ activated B cells (Population 4), and a mixed CD93⁺MHCII^{med}IgG1⁺IgM⁺CD38⁺ cluster presumably containing T1 transitional B cells and IgG1 expressing MZB (Population 5). Again, the most frequently represented population is activated type II follicular B cells. However, this time, no shift in this population can be observed in the different conditions. Notable differences can be observed in the amount

Results

of population 1. Treatment A showed a doubled amount of MZB cells (3,43 %) in comparison to untreated samples (1,70 %). For treatment B, this cell type also increased to 2,70 % while in treatment C no difference in comparison to untreated samples can be seen, and treatment D even shows a decrease (1,35 %) in this population. In addition, all treatments led to an increased number of GCBs present in the mLN, while the mixed cluster (population 5) showed a small decrease in all treated cells (1,0-1,6 %) compared to the ctrl (around 1,9%).

Next, the selection of activated MHCII⁺CD38⁺CD62L⁻ B cells was subsequently analyzed in more detail for the expression of lineage markers and their Ig presentation. The resulting figures are shown in Figure 4-10 for the spleen and Figure 4-11 for the mLN.

Figure 4-10 A and Figure 4-11 A show the distribution of B cell subsets among activated B cells. As expected, in the context of vaccination in both the spleen and the mLN, the most frequent subset represents CD93⁻ mature B cells. Comparing the four treatments, the results show that treatment with the uncleavable TLR-7/8 agonist formulation (A) or combinatory treatment with cleavable attached TLR-7/8 and STING agonist (D) led to a significant increase in mature B cells in the spleen. The same effect is observed for the mLN. Accordingly, a significant decrease in naïve B cells is found for treatment D (Figure 4-10 C). The same tendency can be found for treatment A, but without significance.

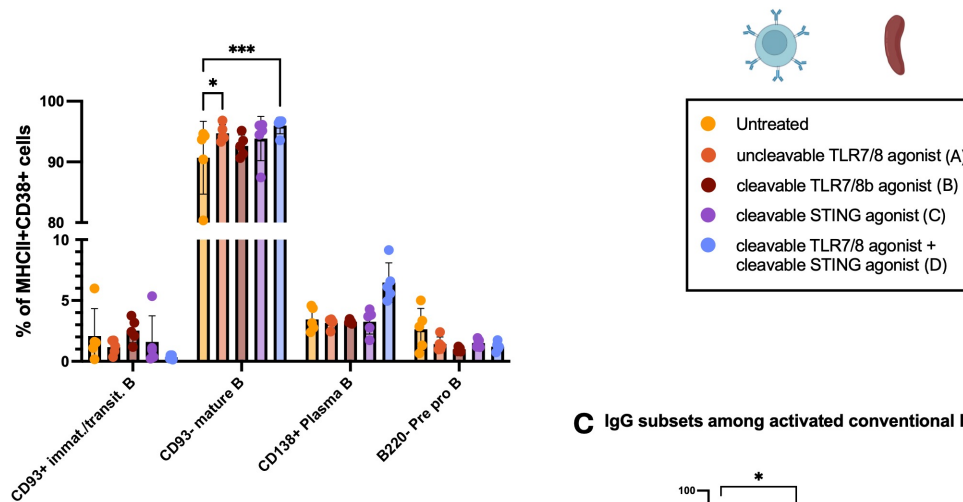
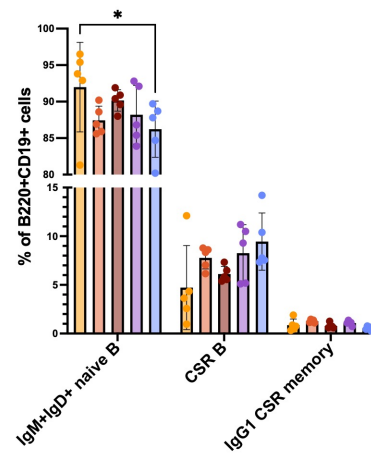
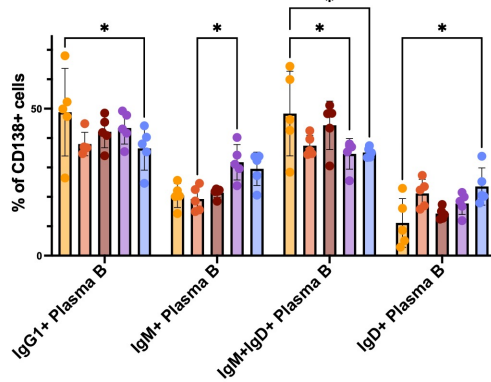
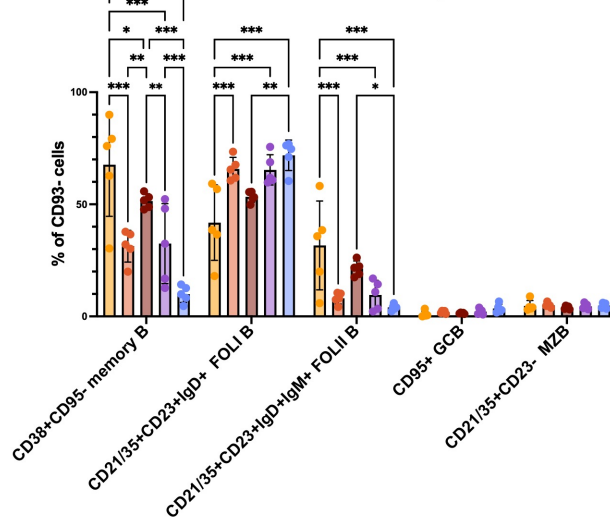
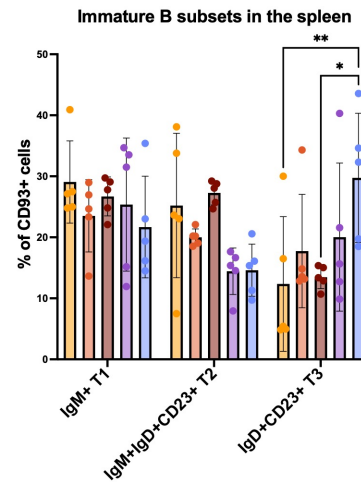
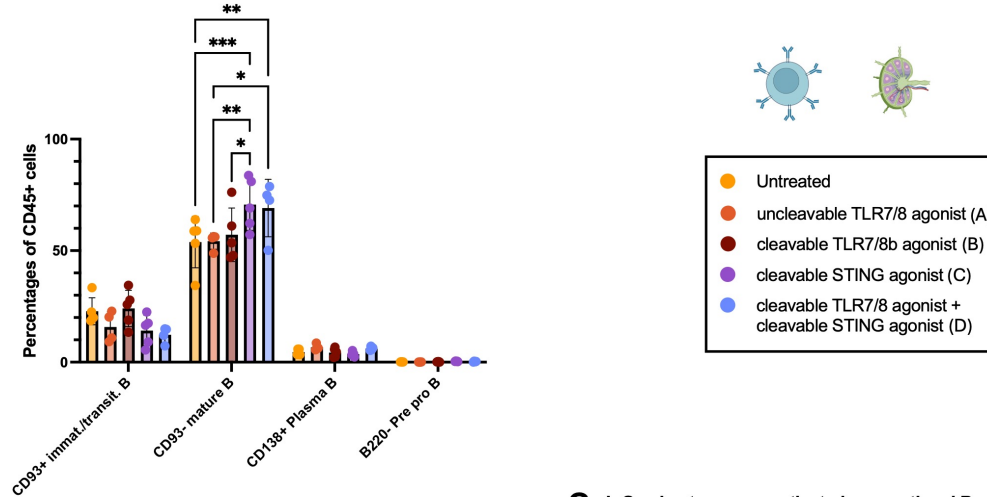
A B cell subtypes among activated B cells in the spleen**C IgG subsets among activated conventional B cells of the spleen****B IgG subsets among activated plasma B cells in the spleen****D Mature B subsets in the spleen****E Immature B subsets in the spleen**

Figure 4-10: Analysis of differences in B cell immunoactivation in the spleen after vaccination with adjuvant through TLR7/8 agonist and STING agonist coupled with different linkers.

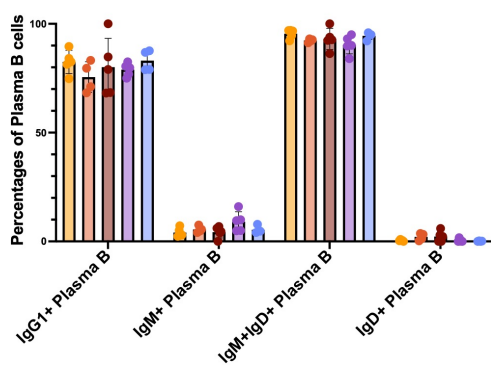
A Main types of B cells in the spleen. General activation of B cells and activation in plasma and germinal center B cells. C Plasma cell subsets. D Mature B-cell subsets. E Immature B-cell subsets. Parts of this figure were created using Biorender. com. Statistical analysis: Tukey's corrected two-way ANOVA, $p > 0.05$ not shown, $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Results

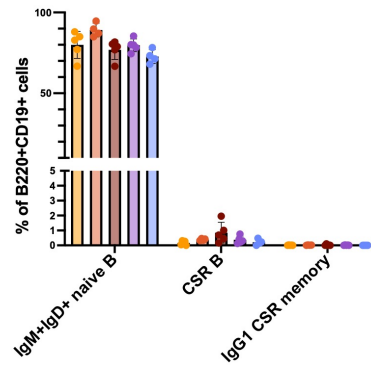
A B cell subtypes among activated B cells in the mLN



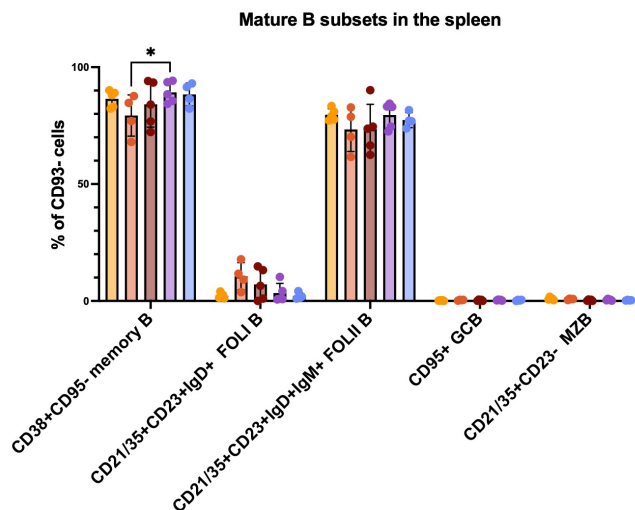
B IgG subsets among activated plasma B cells in the mLN



C IgG subsets among activated conventional B cells of the spleen



D



E

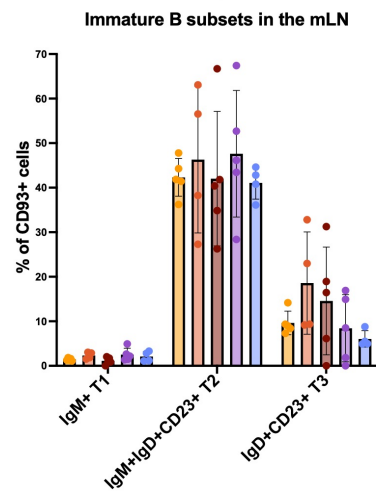


Figure 4-11: Analysis of differences in B cell immunoactivation in the mLN after vaccination with adjuvant through TLR7/8 agonist and STING agonist coupled with different linkers.

(A) The Main types of B cells in the mLN. General activation of B cells and activation in plasma and germinal center B cells. C Plasma cell subsets. D Mature B-cell subsets. E Immature B-cell subsets. Parts of this figure were created using Biorender. com. Statistical analysis: Tukey's corrected two-way ANOVA, $p > 0.05$ not shown, $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

As shown in Figure 4-10 B and Figure 4-11 B, plasma cells were divided into subsets based on their Ig presentation. Among the activated plasma cells in the spleen, approximately 50 % express IgG1. Contrary to expectations, the combined formulation containing cleavable attached TLR7/8 and STING agonists resulted in a lower amount of IgG1 positive cells. The high standard variation in the untreated group could explain this observed effect. For treatments C and D, a decrease in IgM/IgD-positive plasma cells was observed, matching expectations, as the expression of these markers is associated with a more naïve phenotype. Consequently, IgM and IgD single-positive plasma cells increased under this treatment. In the mLN, IgG1 presenting and IgD/IgM double-positive plasma cells can be found, and no differences between the different conditions are observed.

Observations in activated conventional B cells (Figure 4-10 C and Figure 4-11 C) are similar to those in plasma cells. Again, naïve B cells decreased in the spleen after all treatments. This decrease can be found to be significant for the combined treatment D. On the same side, the amount of CSR B cells is found to be slightly enhanced by all treatments, but here the findings are not significant. In IgG1 CSR memory B cells of the spleen, as well as in all assessed conventional B cell subtypes of the mLN, no changes were observed.

Most differences between the treated groups were found in mature B cell subsets (Figure 4-10 D and Figure 4-11 D). Memory B cells of the spleen were significantly reduced in all treated groups, whereas the strongest decrease was induced by treatment D and the weakest by treatment B. Contrarily, in the mLN, no such effects were observed; only treatment A showed a significantly reduced number of activated memory B cells compared to treatment C. Vaccinating the mice regardless of the formulation leads to a shift from type II follicular to type I follicular B cells in the spleen. In the mLN, no changes were found. For activated mature B cells, findings in the spleen again match expectations, as the increase in the late transitional T1 subset can be seen for all treatments but is significant only for treatment D in comparison to the untreated ctrl. No significant changes were observed in the mLN.

In summary, tSNE evaluation of B cells from the spleen and mLN showed that all four tested formulations led to the enhancement of clusters containing B cell subtypes more associated with activation or a more differentiated phenotype, such as GCBs, whereas

Results

clusters containing more naïve phenotypes, such as transitional B cells, became less frequent. The presence of more FOL II B cells and GCB cells than MZB cells indicates that the mice reacted to an exogenous antigen¹⁰³. Single marker expression analysis of MHCII⁺CD38⁺CD62L⁻ B cells analysis supports the results of tSNE analysis by showing an increase in B cells of a mature phenotype and a decrease in naïve B cells. Although in total there are more FOL II B cells in the spleen, among the activated B cells, there seems to be an induction of FOL I B cells, which are usually associated with a more naïve phenotype but also with a high production of IgG1¹⁰³. Comparing all four treatments, it appears that treatment D, combining both TLR7/8 and STING agonist attached via a cleavable linker, differed the most from the untreated control, indicating that this treatment, as already shown in ELISA, led to the strongest humoral response formation.

Having shown the effects of the treatments on the cellular humoral response, the effects on T cells in the spleen and mLN were analyzed, as they play an important role in adaptive, long-lasting immune-response, cellular toxicity against the tumor cells delivered through CD8 positive cytotoxic T cells (CTLs), as well as for Ab formation through CD4 positive T helper cells (T_H). The next figures show the results of the tSNE and FlowSOM computational analyses for the spleen (Figure 4-12) and mLN (Figure 4-13).

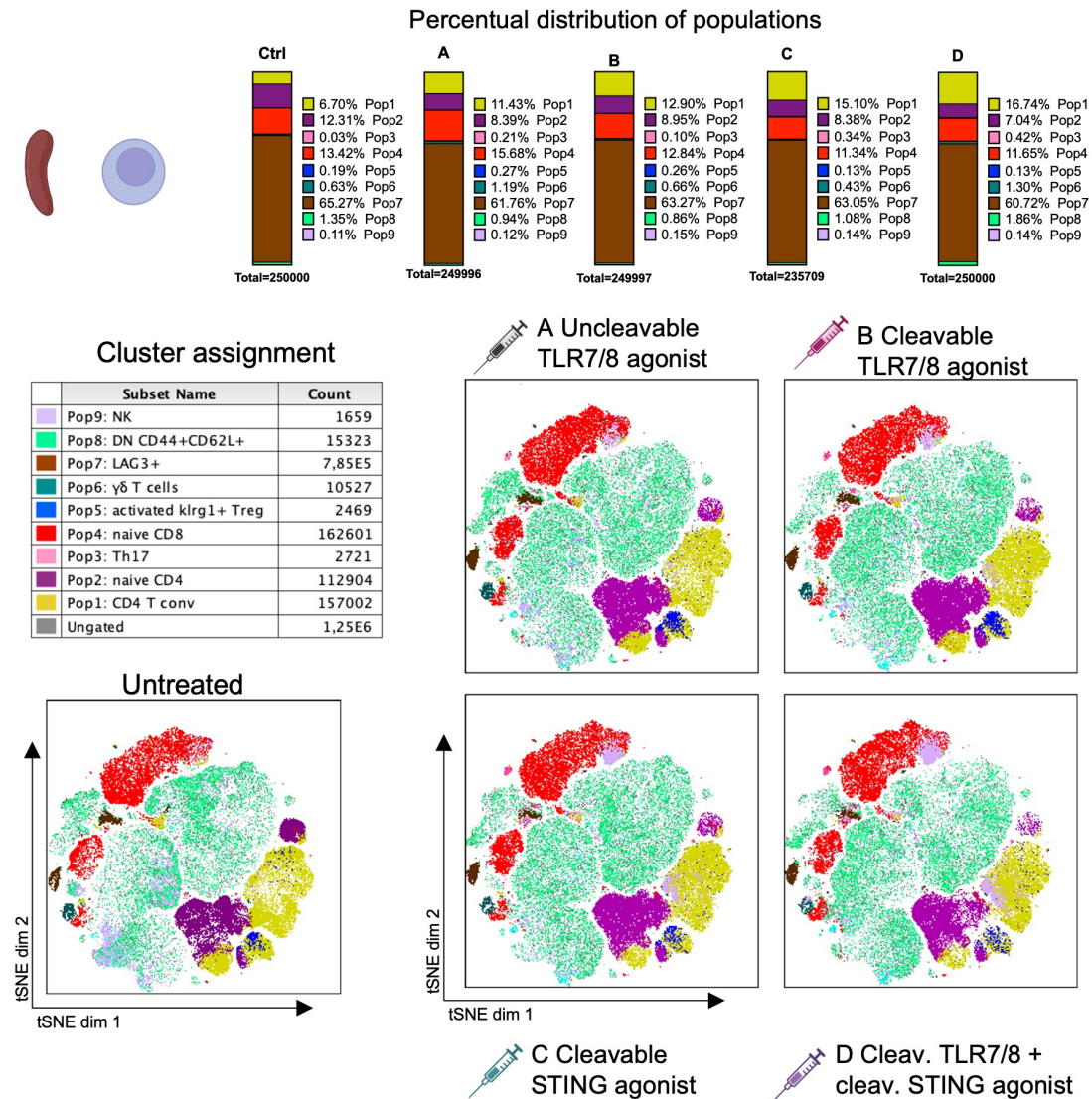


Figure 4-12: tSNE cluster analysis of T cells from the spleen of mice after vaccination with differently adjuvated vaccine formulations measured by FACS.

CD45 positive cells were pre-gated and 50000 cells per sample were computed as tSNE. Marker expression in FlowSOM clusters was checked with the Heat Maps, and subtypes were accordingly defined. Computation was performed in FlowJo v10.10.0 with DownSample, tSNE, and FlowSOM R-based Plug-ins. Parts of this figure were created using Biorender.com.

The tSNE and FlowSOM analysis of the data allowed the determination of the following cluster for the spleen: TCR β ⁺CD4⁺ Tconv (Population1), TCR β ⁺CD4⁺CD62L⁺ naive Tconv (Population 2), TCR β ⁺CD127⁺CD44⁺Roryt⁺ activated T helper cells (T_H)17 (Population 3), TCR β ⁺CD8⁺CD62L⁺ naive CTLs (Population 4), TCR β ⁺CD4⁺Foxp3⁺klrg1⁺Lag3^{med}CD44⁺ Treg (Population 5), TCR β ⁻ $\gamma\delta$ -T cells (Population 6), a CD3^{low}TCR β ^{low}Lag3⁺ subset (Population 7), a CD4/CD8 double negative (dn) CD44^{low}CD62L^{low} presumably naïve natural killer (NK) cells (Population 8) and a CD4/CD8 dn klr1+CD44⁺ subset presumably activated NK cells (Population 9). A comparison of tSNE plots and frequencies of populations between the treatments showed a strong gradual increase in Tconv between

Results

the different treated groups to A: 11,43%, B: 12,90%, C: 15,10 % and D: 16,74 % compared to the untreated ctrl (6,70 %). Consequently, a decrease in naïve CD4 T cells from 12 % to 7-9 % can be observed. Although a rather minor population among all T cells, T_H17 cells were also found to be enhanced in treatments A, C, and D and klrp1 positive Tregs were enhanced after treatment B.

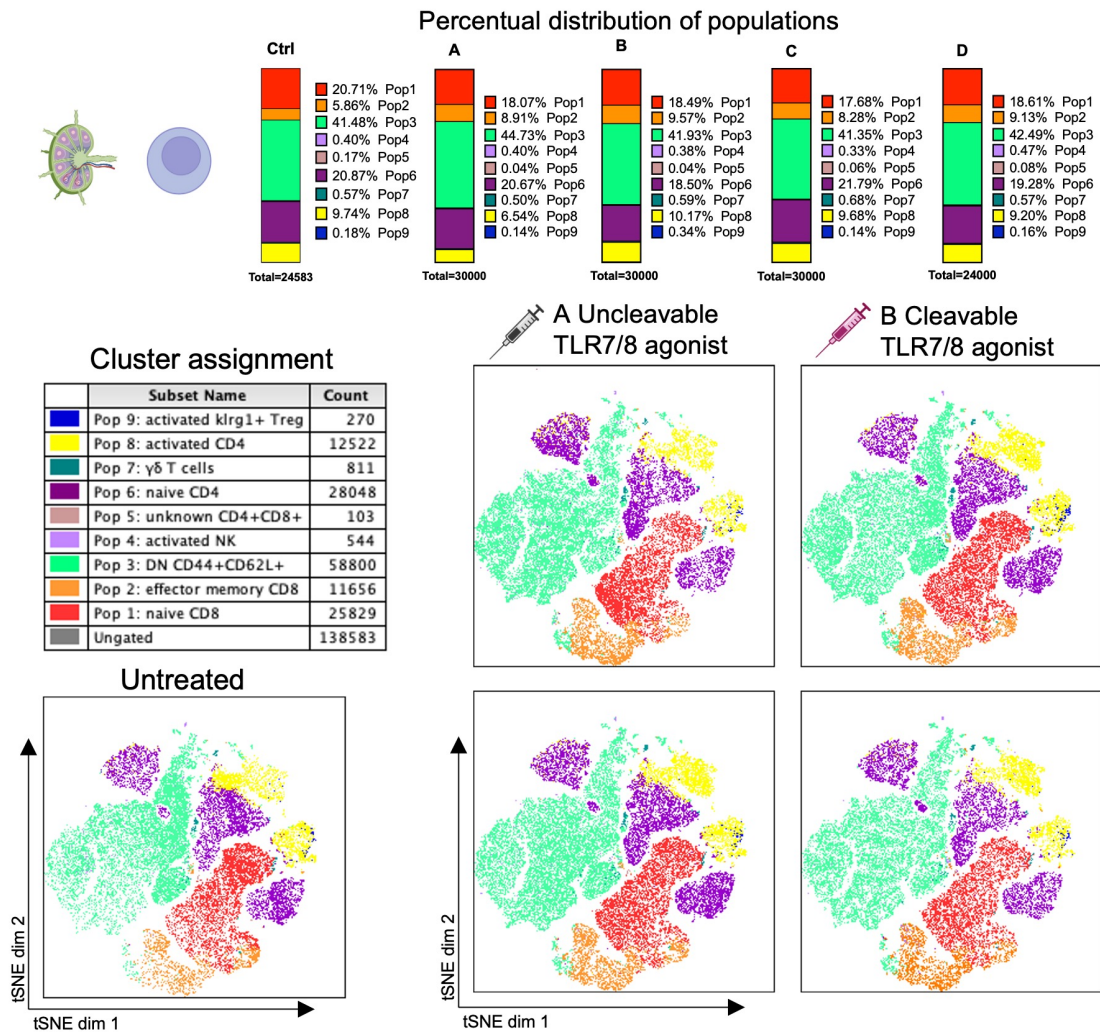


Figure 4-13: tSNE cluster analysis of T cells from the spleens of mice after vaccination with differently adjuvated vaccine formulations as measured by FACS.

CD45 positive cells were pre-gated and 50000 cells per sample were computed as tSNE. Marker expression in FlowSOM clusters was checked with the Heat Maps, and subtypes were accordingly defined. Computation was performed in FlowJo v10.10.0 with DownSample, tSNE, and FlowSOM R-based Plug-ins. Parts of this figure were created using Biorender.com.

Analogous analysis of the mLN revealed the determination of the following populations: TCRβ⁺CD8⁺CD62L⁺ naïve CTLs (Population1), TCRβ⁺CD8⁺CD44⁺CD62L⁺ central memory (CM) CTLs (Population 2), a CD4/CD8 dn CD44+CD62L+ subset (Population 3), a CD4/CD8 dn klrp1+CD44⁺ subset presumably activated NK cells (Population 4), a TCRβ⁺CD4⁺CD8⁺CD62L⁺CD25⁺CD127⁺Lag3⁺ TIGIT⁺ subset (Population 5), TCRβ⁻ γδT

cells (Population 6), $\text{TCR}\beta^+\text{CD4}^+\text{CD62L}^+$ naive Tconv (Population 7), $\text{TCR}\beta^+\text{CD4}^+\text{CD44}^+$ activated Tconv (Population 8), and $\text{TCR}\beta^+\text{CD4}^+\text{Foxp3}^+\text{klrg1}^+\text{CD44}^+$ CD69^+ Treg (Population 9). When comparing the effects of the different treatments on T cells in the mLN, an increase from around 6 to 9 % of the effector memory CD8 T cell cluster was found after all treatments, along with a reduction in naïve CD8 T cells from 20,71 % to around 18 %. In treatment A, a reduction in the population of activated CD4 T cells to approximately 7 % was observed, whereas in all other conditions, the amount was approximately 10 %. Some shifts in the tSNEs between the untreated group and all treated groups were observed in the major population, with a frequency of approximately 40 % containing cells from a CD45^{low} CD4/CD8 double-negative $\text{CD44}^+\text{CD62L}^+$ central memory phenotype.

Like for the B cells, T cells were subsequently analyzed for their activation marker expression. Next figure (Figure 4-14) shows the distribution of the main T cell subtypes, Tconv, Treg, and CTL cells in the spleen (A) and mLN (B).

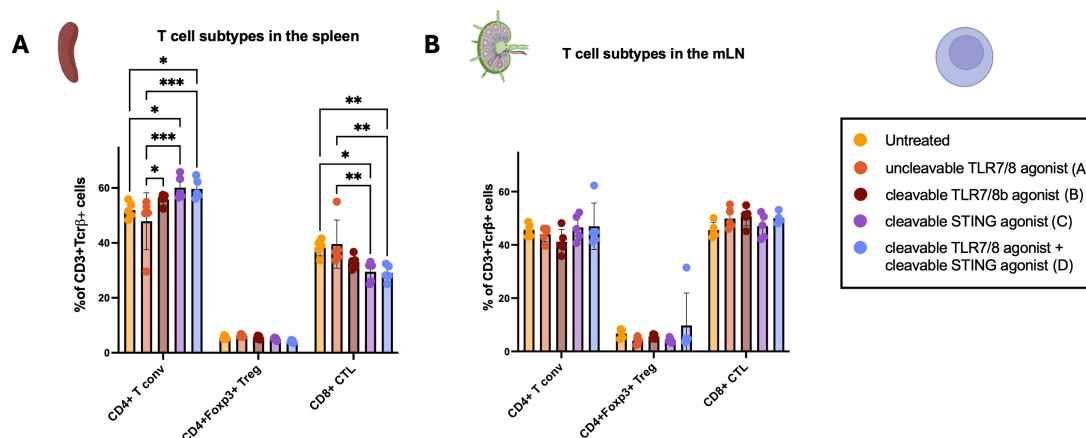


Figure 4-14: Expression of T cell subsets in the spleen (A) and mLN (B) after different treatments. A part of this image was created using Biorender.com. Statistical analysis: Tukey's corrected two-way ANOVA, $p > 0.05$ not shown, $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

The spleen results showed that both treatments containing the STING agonist elicited a rise in the proportion of CD4+ Tconv cells and, consequently, a lowering in the proportion of CD8+ CTLs. For CD4+FoxP3 Tregs as well as in all subtypes of the mLN, no significant changes were observed.

Subsequently, a more detailed analysis of the central memory (CM, $\text{CD44}^+\text{CD62L}^+$) and effector memory (EM, $\text{CD44}^+\text{CD62L}^-$) state was evaluated among Tconv (A), CTL (B), and Tregs (C). To focus only on the markers that showed differences between the

Results

individual treatments, heatmaps were created for EM and CM T cells from each subset shown in the supplementary section (Figure 8-2) that show the expression of each analyzed activation marker on each subset. Only the markers showing variations among the treatment conditions were included in the following graphs for the spleen (Figure 4-15) and the mLN (Figure 4-16).

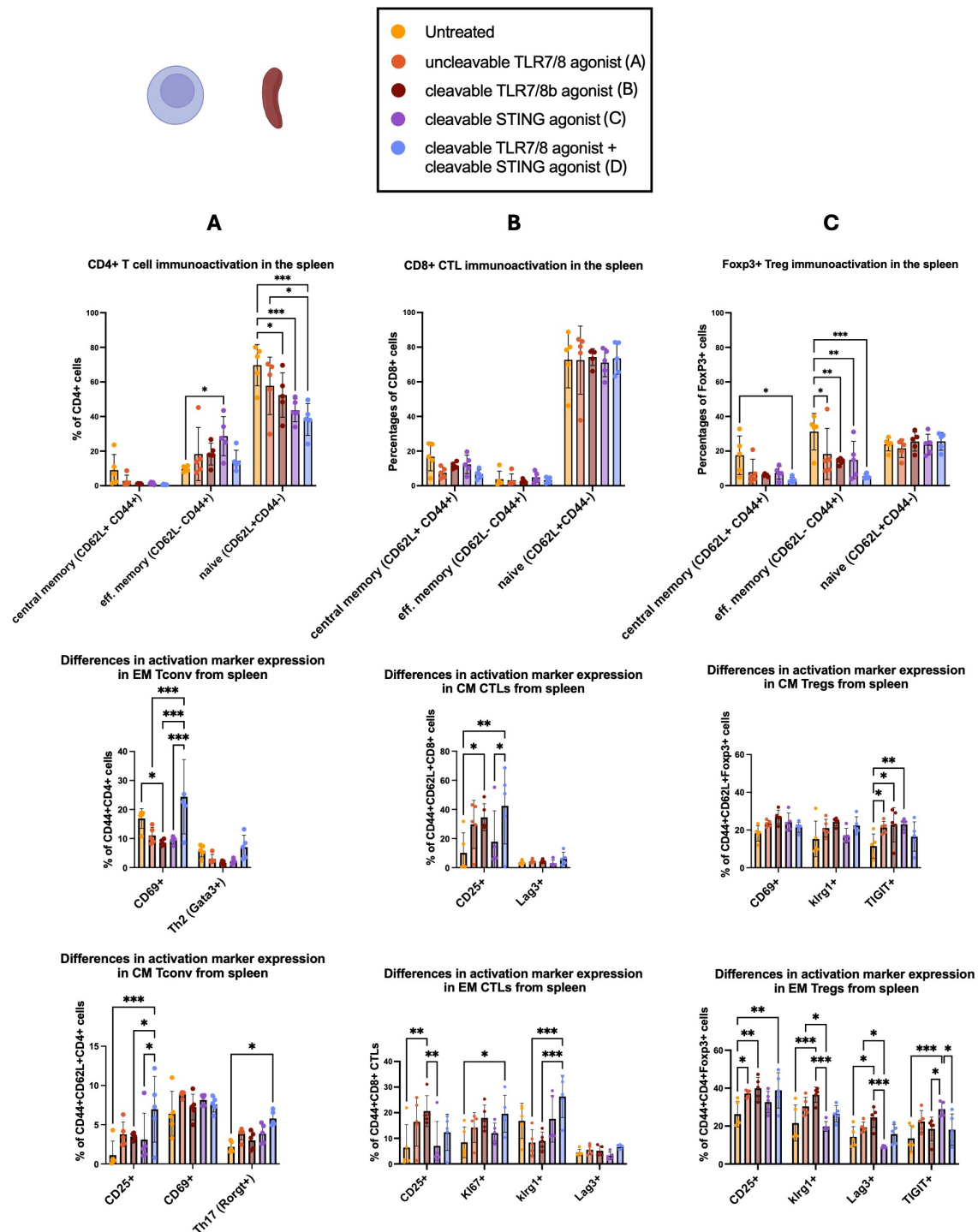


Figure 4-15: Activation state and expression of activation markers of T_H cell (A), CTLs (B), and Treg (C) subsets in spleens from untreated and vaccinated mice.

of this image was created using Biorender.com. Statistical analysis: Tukey's corrected two-way or Brown-Forsythe and Welch ANOVA test, $p > 0.05$ not shown, $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

In the spleen, Tconv (Figure 4-15 A) CM cells showed a gradual decrease in all treatment groups from treatments A to D. Accordingly, the number of EM cells increased, with the exception of treatment D. Treatment D significantly induced the expression of CD25 and Rorγt in CM cells. In EM cells, treatment with B and C led to a significant reduction in

Results

CD69 expression, whereas D significantly increased the expression of this marker. In addition, treatment D elicited a slightly insignificant increase in Gata3 expression.

For CTLs of the spleen (Figure 4-15 B), no changes in the effector status were observed. Looking at the changes in the expression of single markers between the untreated and treated groups, treatments B and D were able to significantly induce CD25 expression in CM CTLs. Treatment B showed the same effect for EM CTLs, while treatment D elicited increased Kl67 expression in this subset. In addition, a significant variation in klrg1 expression was observed between treatments A and B compared to treatment C, but not to the untreated ctrl.

For Tregs (Figure 4-15 C), a decrease in ones with a CM or EM can be observed for all treated groups. For CM Tregs a significant in TIGIT expression can be found for all treated groups except for treatment D. A similar trend was observed for CD69 expression, but the differences were not significant. In EM Tregs CD25 expression is enhanced by treatment A, B and C. Treatment B also elicited the expression of klrg1 and Lag3 in this subset. Treatment C also induced TIGIT expression.

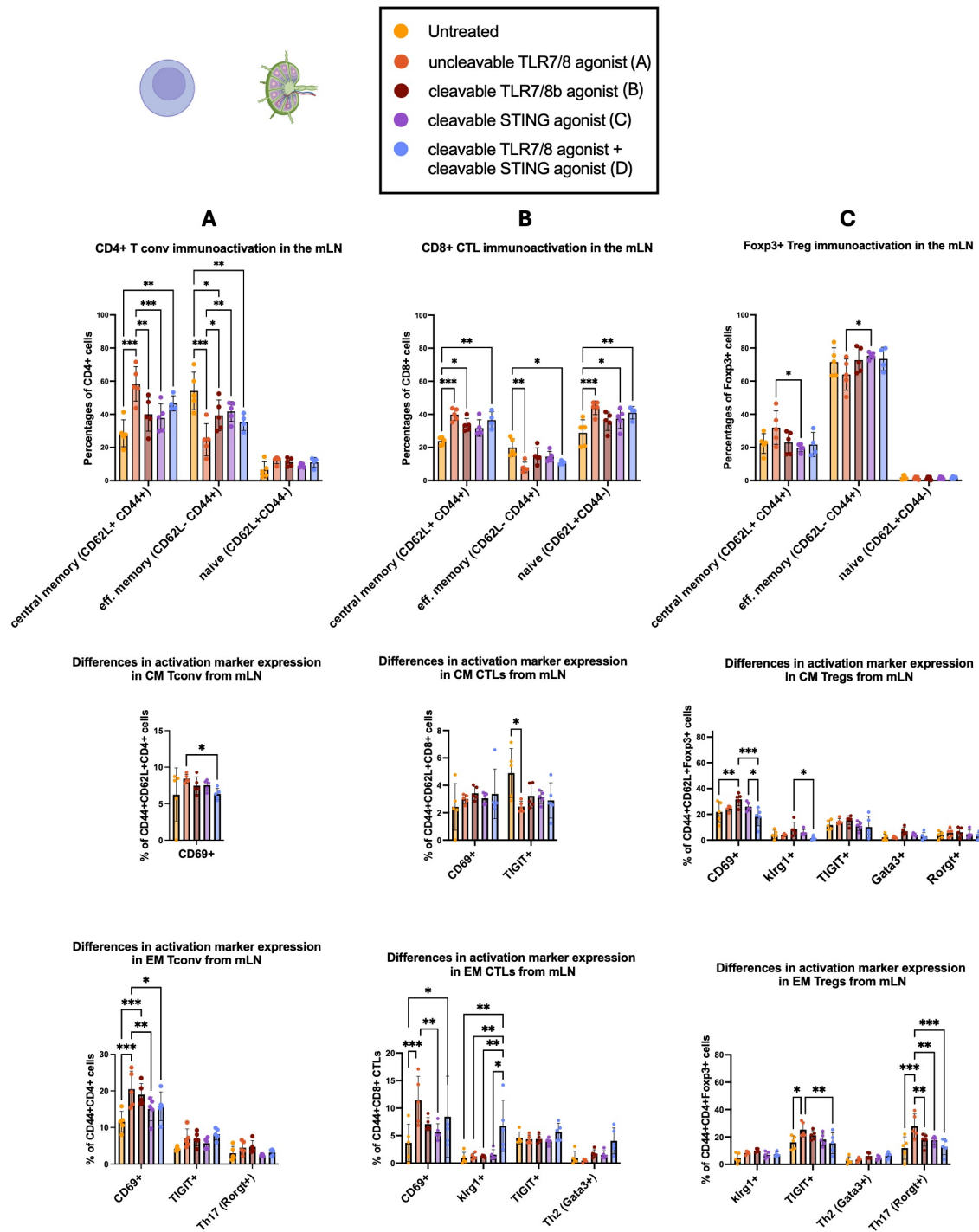


Figure 4-16: Activation state and expression of activation markers of T_H cells (A), CTLs (B), and Tregs (C) subsets in spleens from untreated and vaccinated mice.

A part of this image was created using Biorender.com. Statistical analysis: Tukey's corrected two-way or Brown-Forsythe and Welch ANOVA test, $p > 0.05$ not shown, $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

In the mLN, different expression patterns for the T cell subsets are also found. Generally, more EM and CM cells and fewer naïve cells are present in this organ. For Tconv cells (Figure 4-16 A), a significant shift was observed upon all treatments from the EM to CM phenotype. Within the CM phenotype, the only notable difference was that treatment A

Results

induced significantly higher CD69 expression than treatment D. In EM T conv, treatments A and B also led to increased CD69 expression. Along with Tconv, CTLs also shifted from CM towards the EM phenotype (Figure 4-16 B). Again, only one difference in the activation patterns can be noted for CM CTLs. Treatment A induced a significant reduction in TIGIT expression. In EM CTLs, increased CD69 expression was observed in all treated groups but appeared to be significant only in mice treated with formulations A and D. Also, *klrg1* was significantly induced by treatment D. Looking at the Treg subset, the opposite frequency of fewer CM and more EM phenotypic cells can be found regardless of the treatment, which shows no changes here. Nevertheless, differences were found in CD69 expression in CM Tregs and TIGIT or Ror γ t expression in EM cells that were all induced by treatment B.

Summarized tSNE and FlowSOM analysis of T cells in the spleen and mLN revealed that the treatments did influence cellular immunity as naïve phenotypes were reduced, while the number of activated effector phenotypes was increased. In addition, the individual analysis of activation marker expression on the different T cell subsets showed enhancement of T_H cell activity and suppression of Treg activity after treatment, indicating the presence of T cells. Immunity was induced. Looking at the spleen treatment, D, similar to B cells, seemed to perform the best in inducing peripheral T cell immunity, but when looking at the mLN, local T cell immunity was more heterogeneous among the different treatments. One possible explanation for this could be the late time point of organ isolation, 3 w after the last immunization.

Overall, FACS analysis of B and T cells mainly supported the observations from ELISA that the combination of TLR7/8 agonist and STING agonist attached via a cleavable linker would be beneficial for cancer treatment. The tendencies and variances between the four treatments showed a higher complexity, and the tendency for treatment D appears lower in FACS analysis as they are monitoring the global situation in the host body, whereas the ELISA selectively displays the taMUC1-specific response. For future FACS analyses of the response to vaccines, it would therefore be beneficial to find a method that enables the tracing of immune cell populations specifically involved in the process that led to the observed Ig response.

4.2 Evaluating the (TA)-MUC1 specific mAb GGSK-1/30 as therapy in murine BC models

mAbs have become indispensable tools in modern cancer therapy, particularly for the targeted modulation of specific tumor antigens. GGSK-1/30 is a promising mAb that specifically targets taMUC1 and should therefore be a new approach for BC treatment. To validate the principle, the mAb was first tested in different murine settings.

The HER2-Ab trastuzumab has been approved or is currently in several clinical trials as an ADC conjugated with the toxins emtansine (DM-1), deruxtecan (DX), and irinotecan (SN-38) for the treatment of HER2-positive cancers^{25–27,82,104–109}. Based on these three ADCs, analog conjugates were produced with GGSK-1/30 mAb for testing the mAb as a treatment for BC. Preliminary experiments, not shown here, demonstrated that all three elevated dosages in the range of 100–200 µg/mL were necessary to elicit effective eradication of PyMTxMUC1 cells in vitro. However, in her dissertation, former PhD student Riem Attariya demonstrated that the administration of GGSK-1/30-ADCs at much lower dosages of approximately 10 µg/g body weight to mice implanted with PyMTxhMUC1 tumors resulted in a substantial inhibitory effect on tumor growth (Figure 4-17 A–C) compared to untreated ctrls (Figure 4-17 D). In mice implanted with PyMT tumors lacking target marker expression (Figure 4-17 E and F) the treatment showed no effect, highlighting the specificity of the treatment.⁵⁰

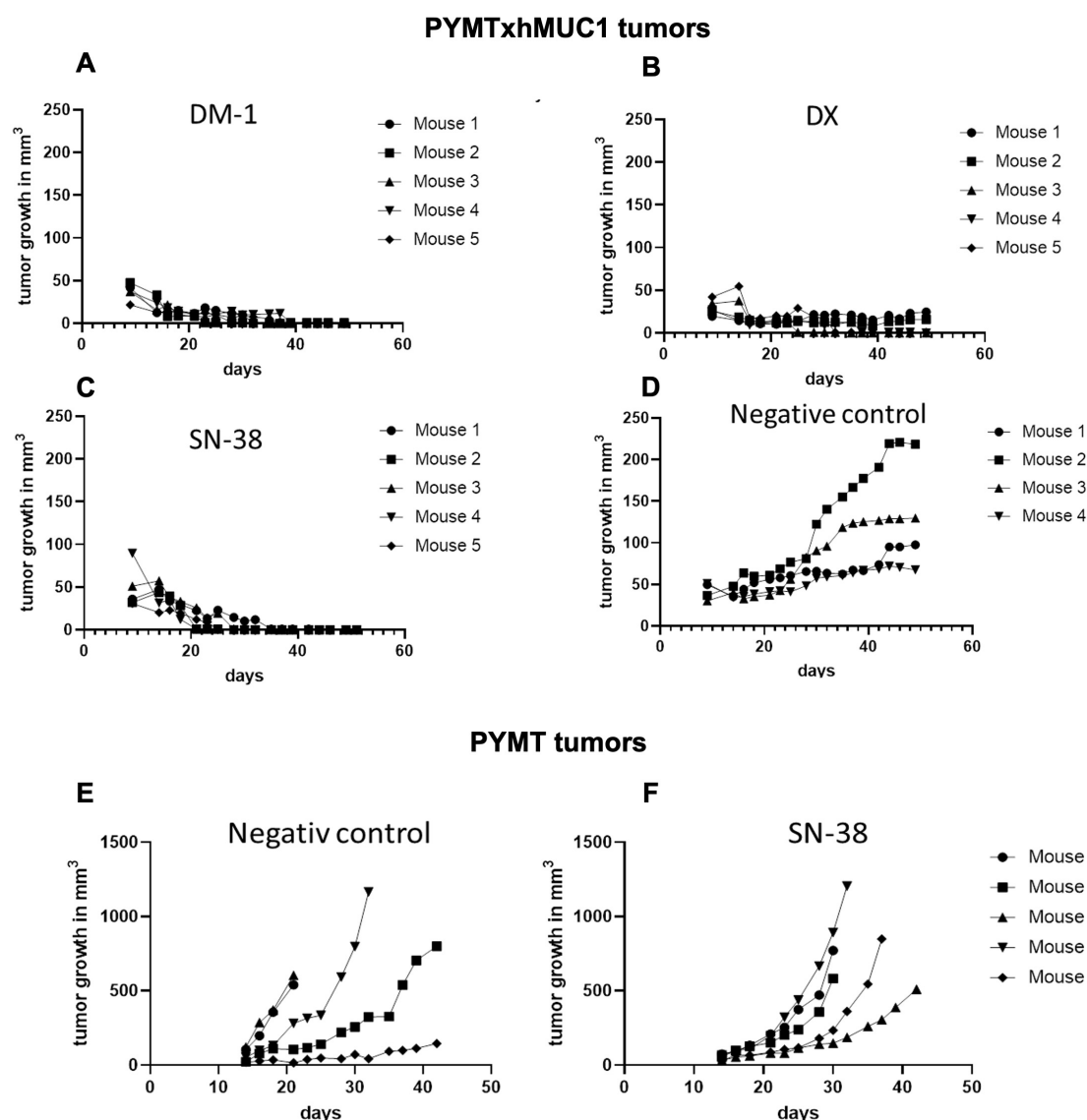


Figure 4-17: Tumor growth monitoring of mice implanted with humanized PyMTxhMUC1 or PyMT tumors and then treated with different GSK-1/30 based ADCs.

PyMTxhMUC1 tumors treated with A GSK-1/30-DM-1, B GSK-1/30-DX, or C GSK-1/30-SN-38 and D untreated. E: Untreated PyMT tumors, F: PyMT tumors treated with GSK-1/30-SN38. Figure adapted from the Dissertation of Riem Attariya⁵⁰.

These promising studies in mice demonstrate the strong efficacy of GSK-1/30 based ADCs. The next step is to test their effects in human in vitro models to allow a differentiated evaluation of the compounds under human-like conditions and to close the bridge between animal data and potential future clinical applications.

4.2.1 Internalization of taMUC1 receptor after mAb binding

The clathrin-mediated endocytosis of MUC1 in healthy tissue based on its O-glycosylation state could already be showed in literature¹¹⁰. This leaves the question of

whether internalization can also be triggered upon Ab binding, consequently giving evidence to the assumed mechanism of action of the GGSK-1/30-ADCs.

To determine if the mAb would be internalized, it was applied to samples of the murine BC cell line PyMT and the PyMTxhMUC1 variant genetically modified to express hMUC1. The first sample was taken and analyzed directly after staining and the others were first incubated for 0, 1, 2, 4, and 8 h at 37 °C and then stored on ice to inhibit further cellular activity. Subsequently, each sample was either analyzed in a western blot or stained with a secondary Ab for FACS analysis. Figure 4-18 shows the results of FACS analysis after different incubation times.

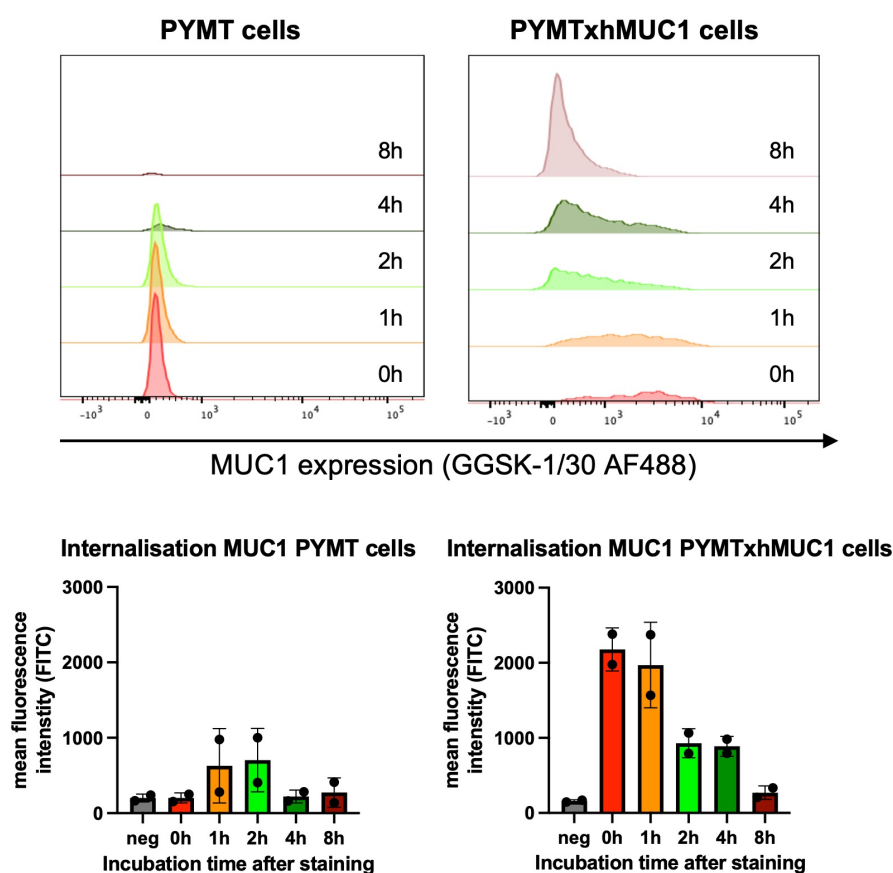


Figure 4-18: FACS analysis of MUC1 internalization upon GGSK-1/30 binding.

PyMT and PyMTxhMUC1 cells were stained with GGSK-1/30, FITC Goat- α -mouse-IgG, and APC-eF780 live/dead stain and then incubated for 0, 1, 2, 4, and 8 h at 37 °C. The mean fluorescence intensity of the FITC channel of pre-gated living cells was recorded using FACS (n=2).

The left side shows the stacked histograms (top) and the changes in MFI (bottom) for PyMT cells and the right side for PyMTxMUC1 cells. As expected, the GGSK-1/30 mAb specifically targeting hMUC1 could not bind the PyMT cells. In the 2 and 4 h samples, there was a slight increase in MFI, but by looking at the stack of histograms, there seemed to be no shift, which would indicate bound cells. Instead, the peak of the negative cells

Results

appeared to be larger. For PyMTxhMUC1 cells, a clear shift to higher fluorescence intensities can be observed in the sample taken directly after staining, indicating the Ab binding of taMUC1 on the cell surfaces. Subsequently, a reduction in the signal can be observed with increasing incubation time after staining, leading to the conclusion that the tumor marker was internalized upon Ab binding. Western blot analysis of the samples eliminated the possibility that the observations recorded were the result of inconsistent MUC1-protein amounts between the cell samples.

The results revealed through FACS analysis accompanied by a specific diminishing of MUC1-expressing tumors observed in vivo strongly suggested that the internalization of taMUC1 occurs upon binding to GGSK-1/30.

4.2.2 Testing a new potent payload-linker technology for the use in GGSK-1/30 mAb based ADCs

The Vincerx company (Palo Alto, USA) has developed a customizable ADC platform¹¹¹ and has already shown promising results with the development of novel small molecules, for example, VIP236, a 7-ethyl camptothecin, or the kinesin spindle protein KSP/Eg5, for use in ADCs against solid cancers^{112,113}. The GGSK-1/30 mAb already showed very promising results in mice as a targeting structure within ADC therapy with the tested toxins related to trastuzumab. In the course of a collaboration with Vincerx, the objective was to assess the feasibility of integrating our lead structure into their novel ADC platform. For the optimization of GGSK-1/30 ADCs, the use of their cleavable legumain linker, which should enhance selectivity for cancer cells, was of interest, as it has been claimed in the literature that the toxic payload will only be released once delivered through the Ab into the cell¹¹⁴. This would highly increase the specificity and efficacy of the anti-MUC1 ADC.

To evaluate the obtained ADC in vitro, it was compared with an ADC based on an IgG1 isotype Ab, a treatment with the equivalent amount of free toxin, and so far, the best option GGSK-1/30-SN38. PyMTxhMUC1 cells were used for the crystal violet viability assay. This assay represents an easy, fast, and cheap way to visualize the number of cells that survive treatment. In addition, many samples can be processed simultaneously, making it easily applicable for comparison. Limitations are that it can only be used for adherent cells and that no further differentiation or cell death can be induced. The results are shown in the following figure (Figure 4-19).

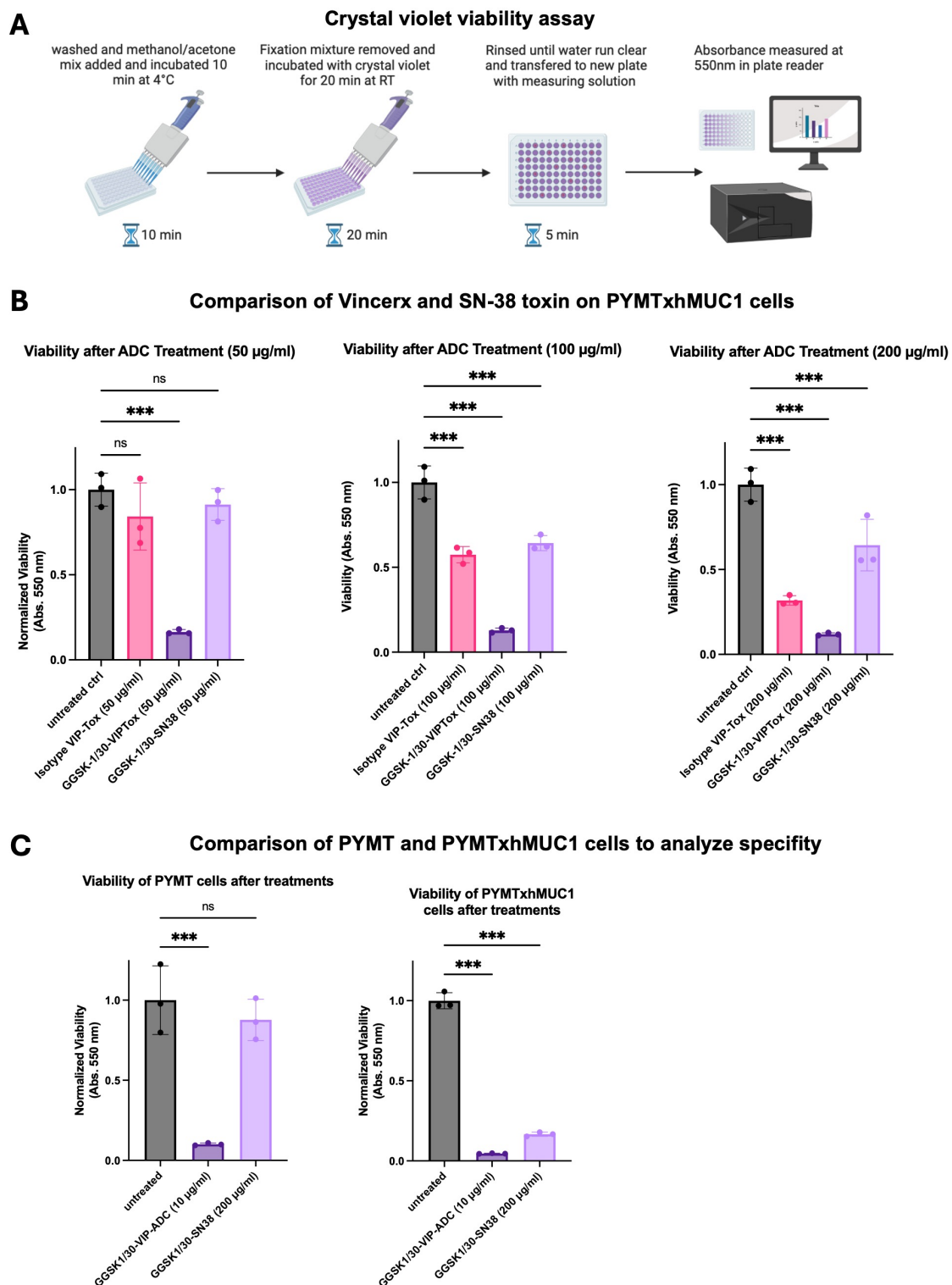


Figure 4-19: Comparison of VIP331 toxin from Vincerx company and SN-38 coupled on GSK-1/30 in a 2D treatment assay of PyMT and PyMTxMUC1 cells.

A Crystal violet viability assay: Cells were washed, fixed, stained with crystal violet for 20 min at RT, transferred to a measuring solution, and absorbance at 550 nm was measured in the supernatant. B Testing different toxin concentrations in PyMTxhMUC1 cells. C Treatment of PyMT and PyMTxhMUC1 cells with both ADCs. Statistical analysis: one-way ANOVA. $p > 0.05$ not shown, $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Results

Figure 4-19 A shows schematically the functioning of the crystal violet viability assay. Treated cells were washed with fixation buffer and stained with crystal violet solution. After 20 min of staining and thoroughly washing several times with water, only adherent cells, and therefore cells that were still alive, remained in the plate. By adding the measuring solution, the coloring was removed from the remaining cells and transferred into a new 96-well plate. The number of living cells was then assessed by measuring the absorbance of the solution in the new plate. High intensity is thereby associated with a high number of cells and vice versa.

In an initial assay, the efficacy of killing PyMTxhMUC1 cells was assessed for GGSK-1/30-VIP-Tox in comparison to GGKS-1/30-SN-38. Concentrations of 50, 100, and 200 $\mu\text{g/mL}$ used in the previous assays for both ADCs were tested. Untreated cells were used as a reference and treatments of the VIP-Tox payload coupled to the MUC1 mAb (GGSK-1/30-VIP-Tox), an IgG1 isotype Ab (Isotype VIP-Tox), or the SN38-ADC (GGSK-1/30-SN38) were tested. Cells were measured after 7 d of treatment. Figure 4-19 B shows the results of this first assay. For all three tested concentrations, the ADC with the VIP-payload showed a significantly stronger efficacy in cell diminishing than the one linked to SN-38. Decreased viability was also observed for Isotype-ADC treated cells at concentrations of 100 and 200 $\mu\text{g/mL}$, indicating that this concentration is too high for VIP-Tox-ADC. SN-38-ADC could not reduce cell growth at 50 $\mu\text{g/mL}$, but significant effects were visible at 100 and 200 $\mu\text{g/mL}$.

In a second experiment conducted within the working group, VIP-Tox-ADC was titrated down, thereby demonstrating that a concentration of 10 $\mu\text{g/mL}$ was sufficient to induce a significant effect on the cells. Consequently, a subsequent experiment investigated whether enhancing the specificity of treatment by reducing the concentration of GGSK-1/30-VIP-Tox to this level would be a viable option.

Figure 4-19 C shows the treatment of PyMT cells that do not carry the target antigen and taMUC1 expressing PyMTxMUC1 cells. Both cell lines were treated with reduced concentrations of 10 $\mu\text{g/mL}$ VIP-ADC and 200 $\mu\text{g/mL}$ SN-38-ADC. Even at this 20 times lower concentration, VIP-ADC showed significantly stronger cell killing than SN38-ADC of PyMTxMUC1 cells, again highlighting its potency. However, SN-38-ADC selectively killed only PyMTxhMUC1 cells bearing the target marker, whereas VIP-Tox-ADC seemed to eliminate both cell types regardless of target marker expression, indicating that even in

this low concentration the toxin was too potent and therefore selective targeting of the treatment was lost.

For integration of this payload into GGSK-1/30 targeted therapies more follow-up dose response experiments would need to be performed in order to determine if the introduction of the new toxin really led to decrease in the high specificity of the ADC. Generally, the Vincerox toxin could be beneficial for the GGSK-1/30 treatment as it shows to strongly increase its ability to eliminate tumor cells. Meanwhile downstream experiments were therefore carried out with previous ADC payloads.

4.3 Evaluating the (TA)-MUC1 specific mAb GGSK-1/30 as therapy in human BC models

After the evaluation of GGSK-1/30 based ADCs in murine models, the next envisaged step was to transfer the observed results to the human context. Therefore, different approaches were investigated. First, the mAb was tested by immunohistochemistry (IHC) staining of human BC patient samples. Through collaboration with [REDACTED] (Institute for Pathology, UMC Mainz), various samples from BC patients were collected and stained with the GGSK-1/30 mAb to check for taMUC1 expression. Figure 4-20 shows the selection of different BC subtype samples with varying receptor statuses.

Results

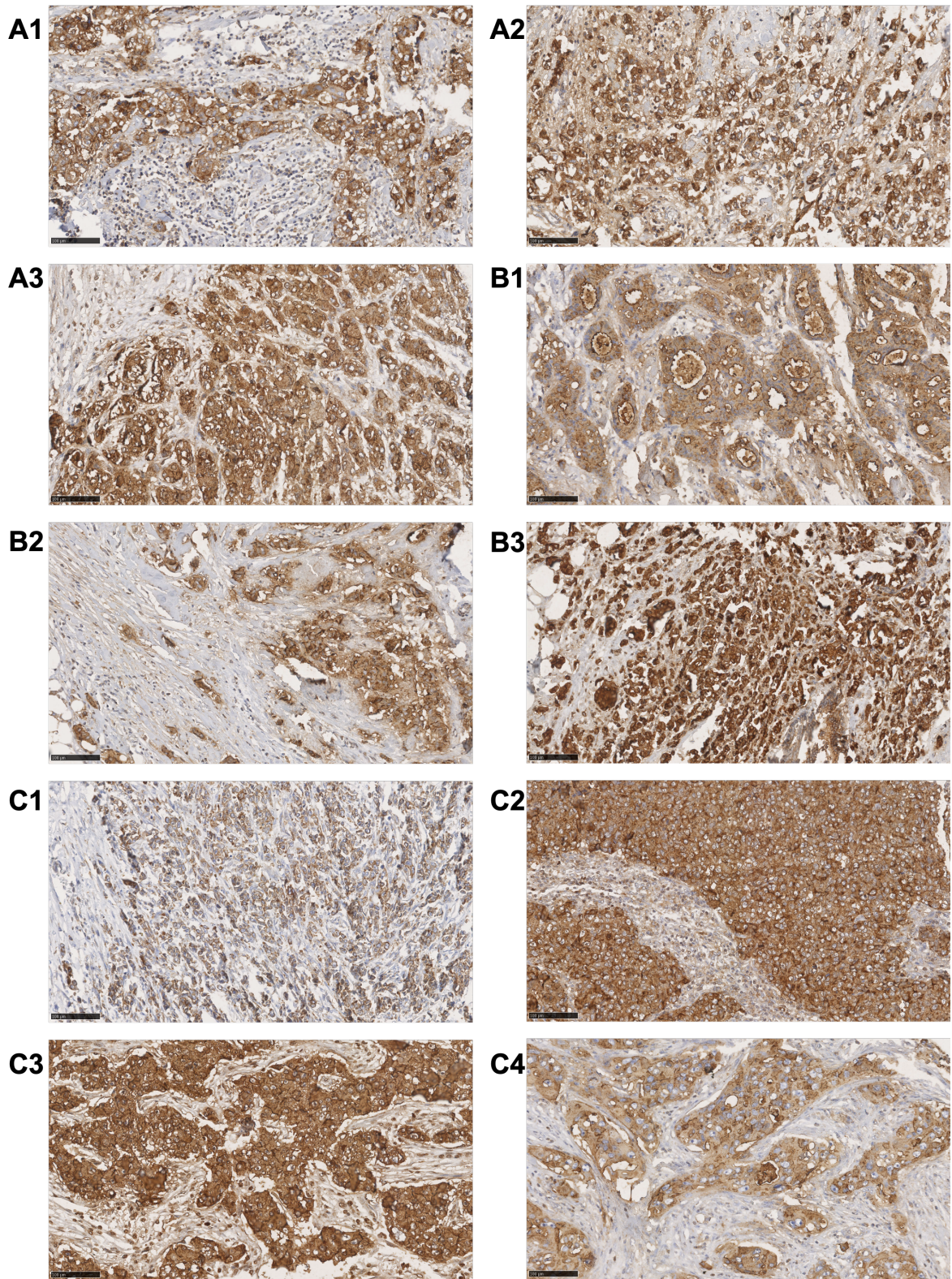


Figure 4-20: Histochemical screening for MUC1 expression in human BC samples collected in the pathological institute.

The cells were stained with GGSK-1/30 mAb. Scale 20x, scale bar = 100µm. A1-A3: Her2/neu pos. mamma CA B1-B3: Pr/Er pos mamma CA, C1-C4 TNBC.

Figure 4-20 A shows three samples from BC histologically classified as HER2/neu positive. In Figure 4-20 B, three PR- and ER-positive Luminal A representatives are shown,

whereas Figure 4-20 C shows four samples from the TNBC subtype. In all samples, taMUC1 expression was detected with the GSK-1/30 Ab. Thereby, the Ab seems to selectively only address tumor cells, leaving the surrounding tissue cells unstained, which highlights its strong potential as a diagnostic tool for the clinic. Especially promising is that among the TNBC samples, three of the four samples show strong taMUC1 expression and the last one also shows moderate expression. GSK-1/30 could therefore be able to fill a gap in current therapeutic approaches, as TNBC lacks the expression of the characteristic IHC markers HER2/neu and ER/PR.

GSK-1/30 mAb was additionally able to prove its utility in detecting taMUC1 expression in patient samples in a collaborative study between our institute and the clinic for obstetrics, gynecology, and women's health at UMC Mainz. 142 samples from patients with BC were collected and analyzed with GSK-1/30 mAb for taMUC1 expression to verify that taMUC1 is a prognostic marker for BC. Based on this, a correlation between taMUC1 expression and the clinical outcome of these patients was determined.

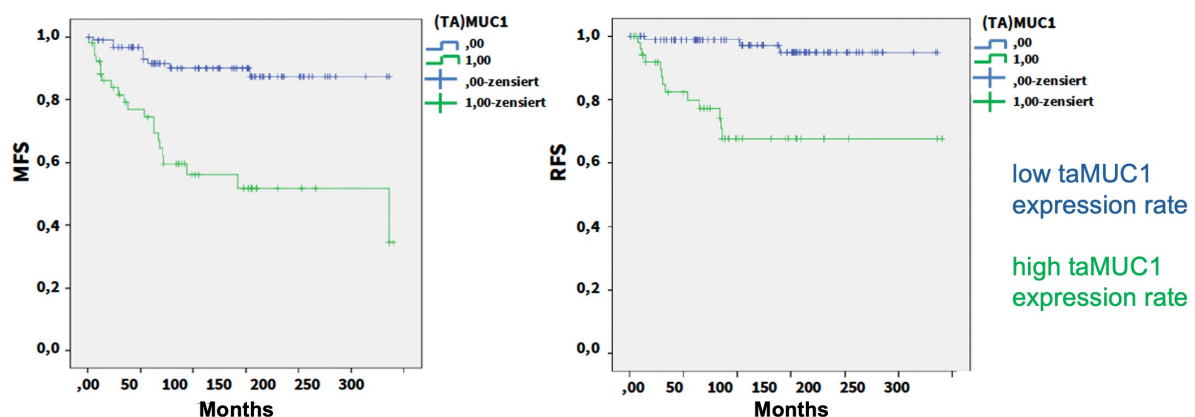


Figure 4-21: Correlation of (TA)MUC1 expression with the survival rate of metastasis-free and recurrence-free BC patients.

A total of 142 samples from BC patients were stained with the mAb GSK-1/30 (1 µg/ml). Correlation of cumulative survival to metastasis-free (MFS) and recurrence-free (RFS) scoring: Staining intensity (0 = negative, 1 = weak, 2 = medium, 3 = strong) multiplied by the % proportion of human (TA)MUC1-positive tumor cells (0 = 0%, 1 = 1–10%, 2 = 11–50%, 3 = 51–80%, 4 = 81–100%). Blue: low taMUC1 expression rate. Green: high taMUC1 expression rate. Figure adapted from the Dissertation of Natascha Stergiou⁷¹.

Figure 4-21 shows the metastasis-free (left plot) and recurrence-free (right plot) survival rates of patients correlated with a low taMUC1 expression rate in blue and a high taMUC1 expression rate in green. The data validates that taMUC1 expression is associated with strongly unfavorable outcome for BC patients.⁷¹

4.3.1 In vitro treatment of 2D cultures from different human BC cell lines with GGSK-1/30 mAb coupled DM1 and SN38

Knowing that GGSK-1/30 binds taMUC1 specifically and that taMUC1-expression is relevant for BC prognosis, suitable cellular models for ADC testing were investigated. Therefore, the following cell lines were tested:

MDA-MB-231 is a mamma adenocarcinoma cell line derived from a 51-year-old woman's cancer and is classified as a TNBC cell line^{115,116}. MCF-7 and T47D are both classified as Luminal A hormone-dependent BC, which means they are ER/PR positive and HER2 negative. Both were originally derived from a pleural effusion metastasis of a women's breast adenocarcinomas^{117,118}.

To test the suitability of the cell lines for treatment testing, taMUC1 expression was determined by FACS by staining with the GGSK-1/30 mAb coupled to the fluorophore AF488. Then, the first 2D treatment assay was performed on these cell lines with the GGSK-1/30-DM1 ADC, where different analytical methods for viability determination were assessed. To perform the assay, cells were incubated for 7 d either with 100 µg/mL GGSK-1/30-DM-1 or GGSK-1/30 without payload. An untreated ctrl was used as a reference. After the 7 d remaining viable cells were determined via crystal violet viability staining in FACS after staining the samples with eF780 fixable viability dye. Additionally, cell numbers in the samples were determined by cell counting with a lunar cell counter.

The next figure shows the MUC1 expression rates (Figure 4-22 A) and results from the 2D treatment assay (Figure 4-22 B-D).

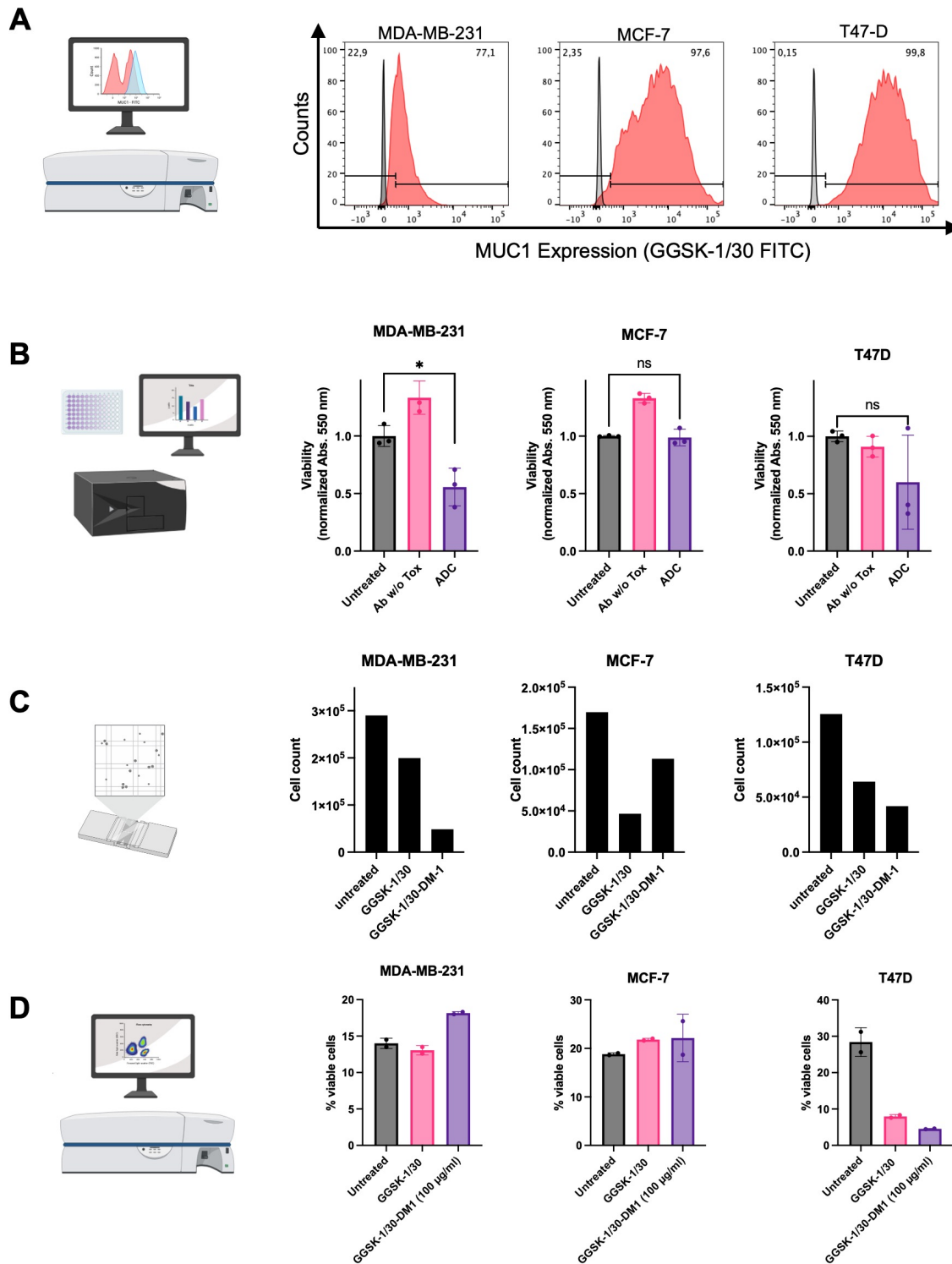


Figure 4-22: Treatment assay on different human BC cell lines in 2D culture with DM1 coupled GGSK-1/30 ADC.

A: FACS of hMUC1 expression levels before treatment (GGSK-1/30-AF488 1:1000). B: Results of crystal violet absorbance measurement after treatment with GGSK-1/30-DM-1 (100 µg/ml) for 7d. Statistical analysis (n=3): Welch-t-test, $p > 0.05$ not shown, $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). C: Total cell number in each sample determined with a Lunar cell counter (n=1). D: Analysis of cell viability by FACS after staining with eF780 fixable viability dye for 20 min at 4°C (n=2). Parts of this figure were created using Biorender. com.

Results

In all three human BC cell lines taMUC1 expression can be found. Both Luminal A class cell lines show expression rates of over 95 %. The TNBC cell line MDA-MB-231 also exhibits a high rate of 77 %. Therefore, all three were good choices for the subsequent ADC testing.

GGSK-1/30-DM-1 treatment of MDA-MB-231 leads to a significant reduction in the viability determined by crystal violet staining compared to the untreated ctrl. In contrast, treatment with GGSK-1/30 stimulated cell proliferation. When comparing the cell counts between the conditions, a reduction in total cell number can be seen after mAb treatment compared to the untreated ctrl, and in ADC treatment compared to mAb treatment alone. The results from the FACS analysis don't match the results from the crystal violet staining. The survival rates of untreated and mAb-treated cells appear to be almost the same, while ADC-treated cells show a higher survival rate. Generally, the number of viable cells is low, with 10 % and 20 % of cells being viable in all FACS samples.

For MCF-7 cells, the ADC treatment did not show a reduction in cell viability in either analytical method, but a decrease in total cell number was found in the ADC treated sample. Again, mAb treatment led to an increase in viability, as determined by crystal violet staining, whereas the total cell number decreased. Viability determined through FACS staining doesn't show any significant differences between the treatments, with all viabilities at approximately 20 %.

In T47D cells, treatment with GGSK-1/30 without payload results in a slight reduction in cell viability. The reduction after ADC treatment is more pronounced, but still not significant. The same viability pattern between the three treatments appeared in FACS analysis, which also showed a reduction in viability with Ab treatment and a slightly greater reduction with ADC treatment. The total cell number decreases in all three treatment groups, from the untreated sample to the mAb-treated and ADC-treated samples.

In summary, the tested methods for viability determination showed a high variation in the presented results for MDA-MB-231 and MCF-7. For T47D, all three methods supported each other, but the observed effect is not as strong as expected based on the previous murine in vivo results.

Subsequently, a second ADC combining GGSK-1/30 with the topoisomerase inhibitor SN38 was tested on 2D cultured BC cells. Instead of viability staining, apoptosis was

determined by FACS through Annexin-V-staining of the cells. Meanwhile, cell counting was also integrated in the FACS analysis using counting beads to achieve a clearer alignment of results. Cells were treated with the new SN-38 ADC, as described previously for DM1. Figure 4-23 shows the effects of treatment with GGSK-1/30-SN38 analyzed with crystal violet viability staining (A) and through the new via FACS analysis (B).

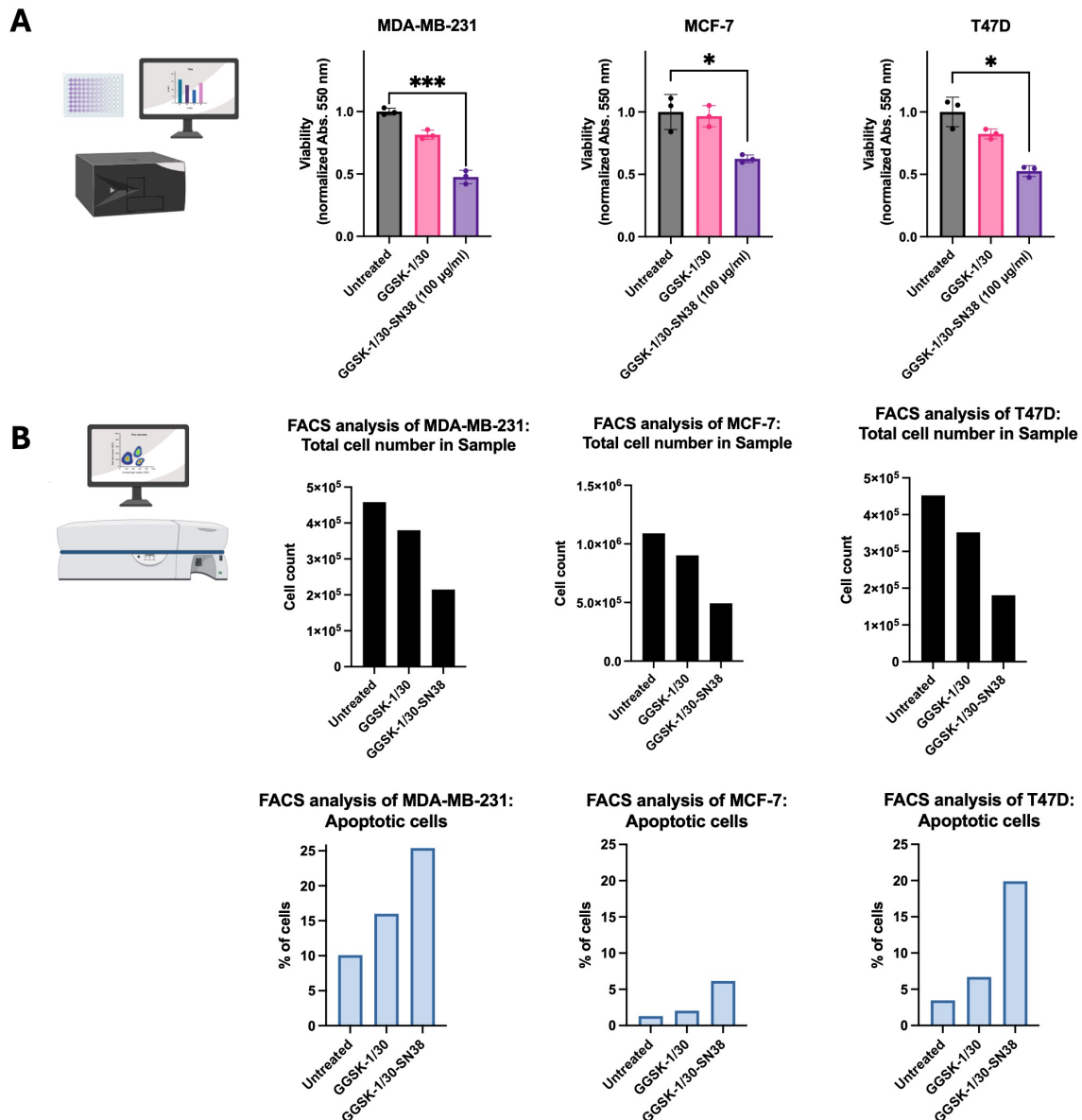


Figure 4-23: Treatment assay on different human BC cell lines in 2D culture with SN38 coupled GGSK-1/30 ADC.

A: Results of crystal violet viability assay after treatment with GGSK-1/30-SN38 (100 µg/ml) for 7 d. Statistical analysis (n=3): Welch-t-test, $p > 0.05$ not shown, $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). B: FACS analysis of cells using Invitrogen CountBright™ Plus Absolute Counting Beads and analysis of apoptosis by staining for 20 min at 4°C with AnnexinV-BV421 (n=2). Parts of this figure were created using Biorender. com.

Results

The results of the crystal violet assay show a significant reduction in ADC-treated cells compared to the untreated ctrl for all three cell lines. The treatment of the cell lines with mAb without payload don't show an enhancement in viability. The total count of cells within the FACS samples of each condition underlines the data from crystal violet viability staining. The cell counts decrease from for Ab treated and for ADC treated in all three cell lines compared to untreated cells. Consequently, the rate of apoptosis increases for Ab-treated and ADC-treated compared to untreated cells, also supporting the fact that GGSK-1/30-SN38 had a killing effect on the cells. Validated through the optimized analysis methods, which now all support each other, the 2D assay with MDA-MB-231, MCF-7, and T47D cells highlights the efficacy of SN-38 ADC, making it a suitable option for further evaluation as a treatment for human BC.

4.3.2 Treatment of 3D models for human BC with GGSK-1/30 based ADCs

The treatments of 2D cell cultures could provide promising results on the capacity of GGSK-1/30 based therapies to fight BC. While 2D culture models are an important tool for simple and cost-efficient or high-throughput screening of cancer drugs, they often fail to reproduce the complexity of an actual tumor. 3D models help provide results that are more likely to correlate with in vivo data and clinical outcomes. To address this, 3D culture models of BC cell lines were established.

To form spheroids, the selected cells were seeded into a specifically coated 96-well plate, which inhibits the adhesion of the cells on the borders. This leads to the formation of a cluster within 1–2 d that evolves into a sphere within 5–7 d. For each cell line, different cell numbers were tested to identify the best seeding number for the formation of well-sized and well-shaped spheres. Images were taken under the microscope at the beginning (d2), middle (d4), and late (d6) phases of spheroid formation shown in the supplementary section in Figure 8-3 for MDA-MB-231, Figure 8-4 for MCF-7, and Figure 8-5 for T47D.

Loose spheroids formed after 2–4 d in all three cell lines. While MDA-MB-231 and MCF-7 cells formed small and very densely shaped spheroids, T47D cells formed large spheroids that appears to have three layers. The middle layer appeared brighter and less dense under the microscope. In the middle, a dense and dark core is formed by d 6. In solid tumors, this phenomenon is referred to as necrotic core formation and occurs through the lack of nutrients in the center of the tumor or spheroid¹¹⁹.

A small number of seeded cells leads to the formation of a small spheroid, which makes it harder to see the differences in subsequent treatment testing. An excessively large number of seeded cells results in the formation of smaller spheres or spheroids without spherical shapes and arm-like extensions. Therefore, the largest possible number of cells without adverse effects on the spheroid shape was determined. The results show that 1×10^4 cells per well for MDA-MD-231 and 1.1×10^4 cells per well for MCF-7 and T47D as ideal seeding densities for 3D culturing.

After observing that the selected cell lines were able to form spheroids, the first ADC GSK-1/30-DM1 was tested. The 3D cultures were treated and analyzed according to the following scheme (Figure 4-24).

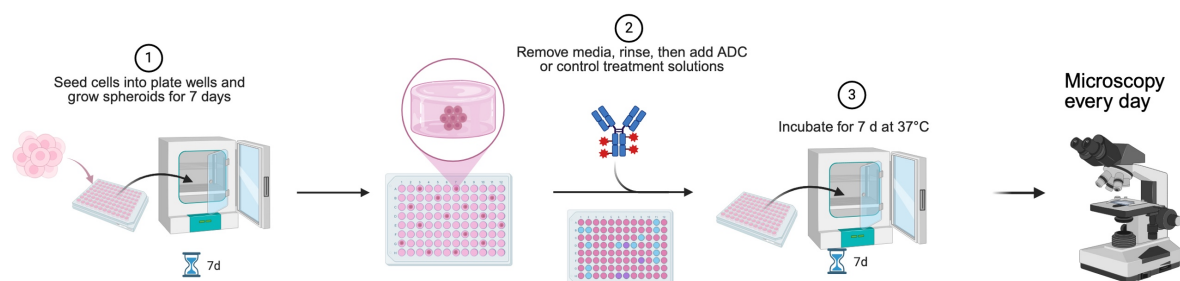


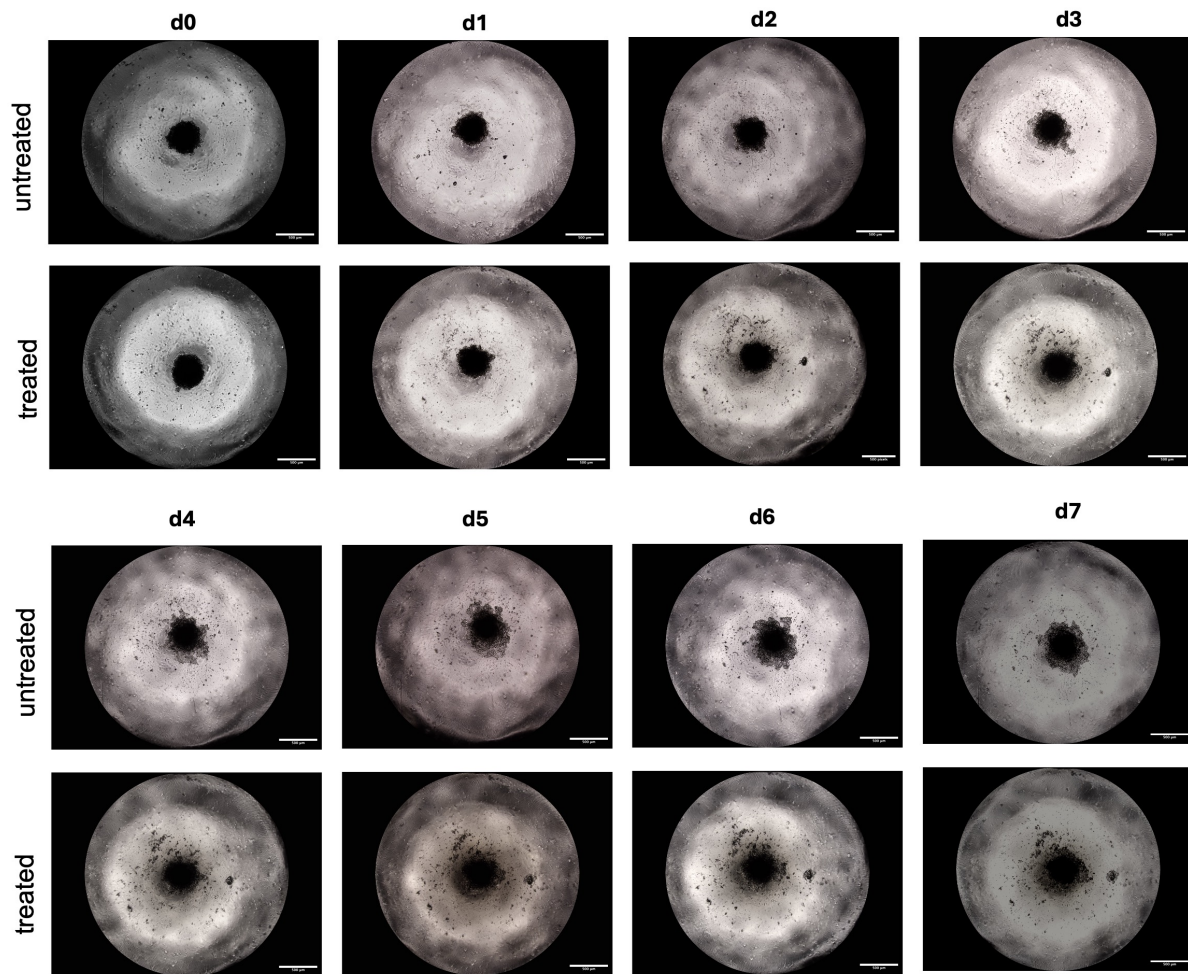
Figure 4-24: Procedure for spheroid formation, treatment, and analysis.

1: Cells were seeded in spheroid culture plates and incubated for 7 d. 2: The medium was replaced by the different treatments. 3: Cells were treated for 7 d and microscopy images were taken each d. Figure created using Biorender.com.

When proper spheroid formation could be observed under the microscope, treatments were added on d7 after seeding the cells. Groups of 5 spheroids were either kept as untreated ctrl or treated with GSK-1/30 alone or the GSK-1/30-DM1 ADC for another 7 d in the incubator. Every day, a picture of each spheroid was taken under the microscope to track changes.

Figure 4-25 shows a selection of untreated ADC ADC-treated spheroids and the size changes of all treatments for MDA-MB-231 spheroids. All other images taken from each spheroid on each timepoint are shown in the supplementary section in Figure 8-6 and Figure 8-7 for MDA-MB-231, in Figure 8-8 and Figure 8-9 for MCF-7, and in Figure 8-10 and Figure 8-11 for T47D. These images were then processed to determine the cross-section area of each spheroid to compare size changes between the different treatment groups.

Results



Size changes after treatment of MDA-MB-231 spheroids

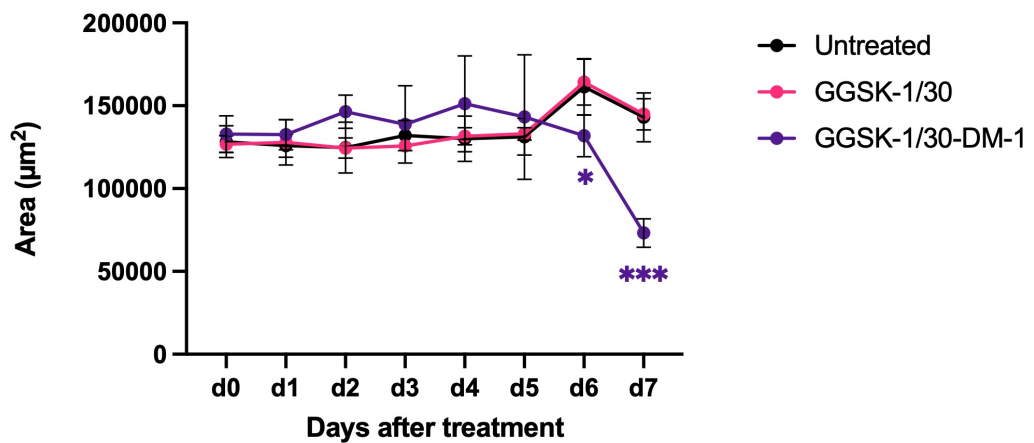


Figure 4-25: Microscopic imaging of MDA-MB-231 human BC cell spheroids and induced size changes of spheroids during the 7 d of treatment with DM1 coupled GGSK-1/30 ADC.

Scale 16x, scale bar = 500 μm . Size of spheroids was determined by measuring the cross-section area in Image J. Statistical analysis of untreated against ADC-treatments: Mixed-effects model with Geisser-Greenhouse correction, $p > 0.05$ not shown, $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

For the first 2 d, no changes are observed. From d3 onward, an increasing number of smaller cell clusters, presumably fragments, start to form around the main spheroidic

structure. In addition, the center of the spheroid started to appear increasingly blurred each day. Looking at the differences in sizes during the treatments, all three treatments were at the same level until d6, when ADC-treated spheroids started to shrink significantly. Spheroids treated with GSK-1/30 without toxin showed no changes in shape and size in comparison to the untreated group.

Results

Figure 4-26 shows a selection of untreated ADC ADC-treated spheroids and the size changes of all treatments for MCF-7 spheroids.

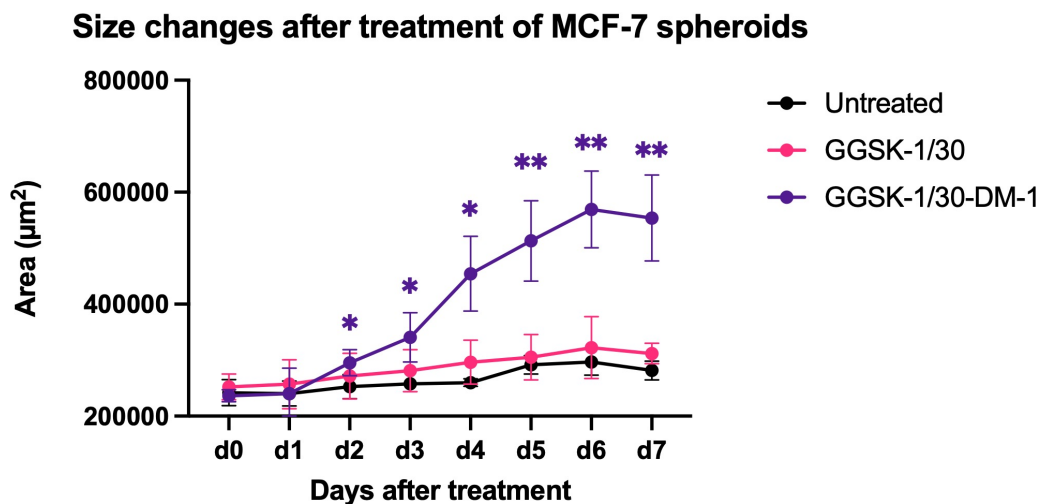
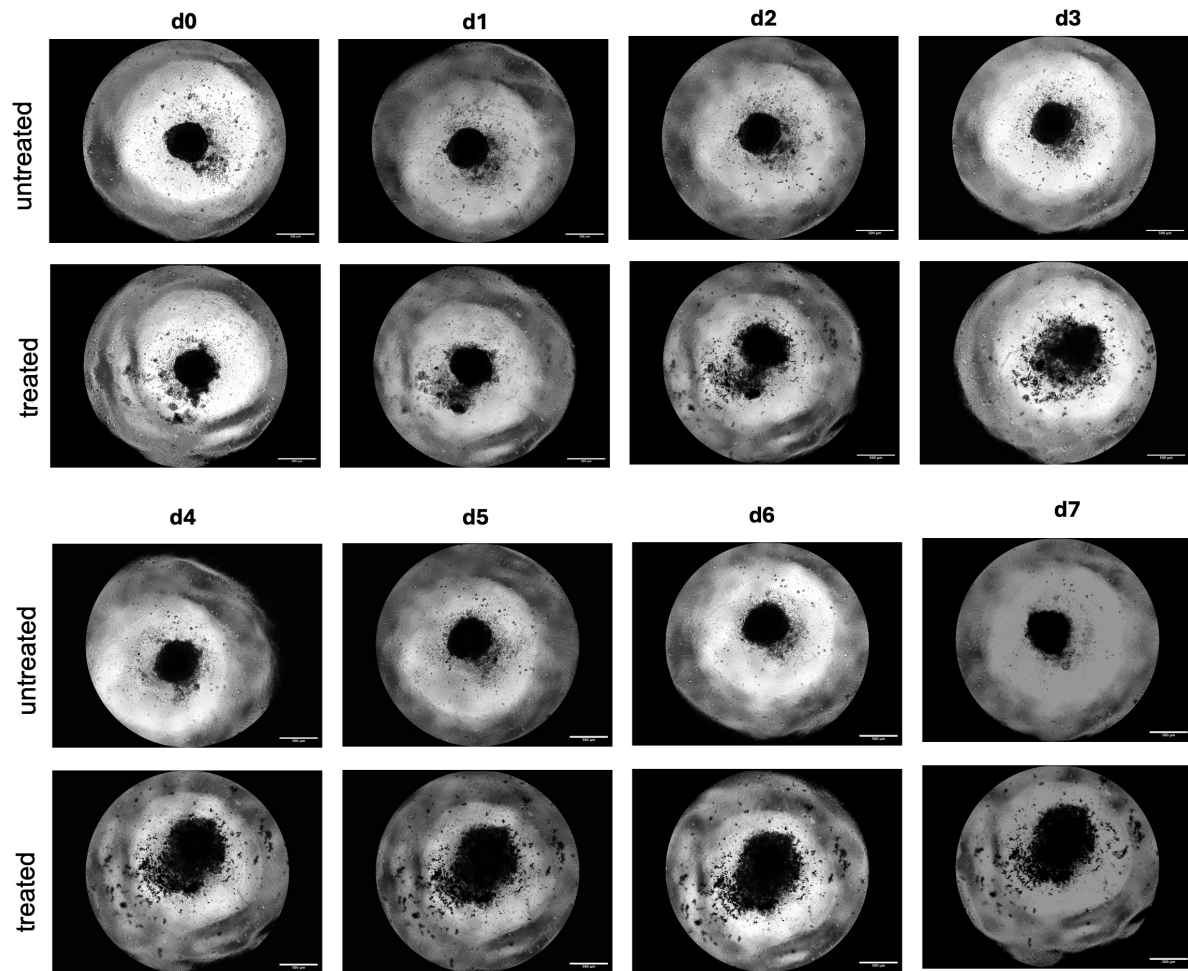


Figure 4-26: Microscopy imaging of MCF-7 human BC cell spheroids and induced size changes of the spheroids during 7 d of treatment with DM1 coupled GGSK-1/30 ADC.

Scale 16x, scale bar = 500 μm . Size of spheroids was determined by measuring the cross-section area in Image J. Statistical analysis of untreated against ADC-treatments: Mixed-effects model with Geisser-Greenhouse correction, $p > 0.05$ not shown, $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

From d 2, ADC-treated MCF-7 spheroids started to change in shape compared to their untreated reference. Their densely packed shape started to loosen, resulting in a significant enlargement of size. This effect intensified from d3 to d7 until the structures nearly doubled in size. On d7 of treatment, spheroids completely lost their spherical structure, and fragmented pieces could be observed around the former spheroid. Meanwhile, untreated spheroids maintained their shape and looked the same as on d0 of treated ones. Spheroids treated with the naked mAb do not differ in shape or size in comparison to the untreated group.

The next figure shows a selection of untreated and ADC-treated spheroids for each day of treatment and the resulting size changes of all treatments for T47D cells (Figure 4-27).

Results

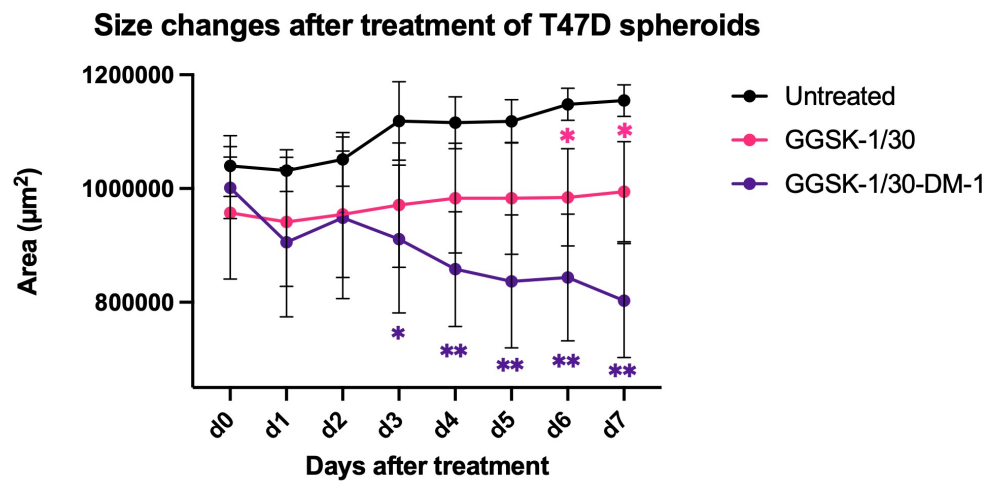
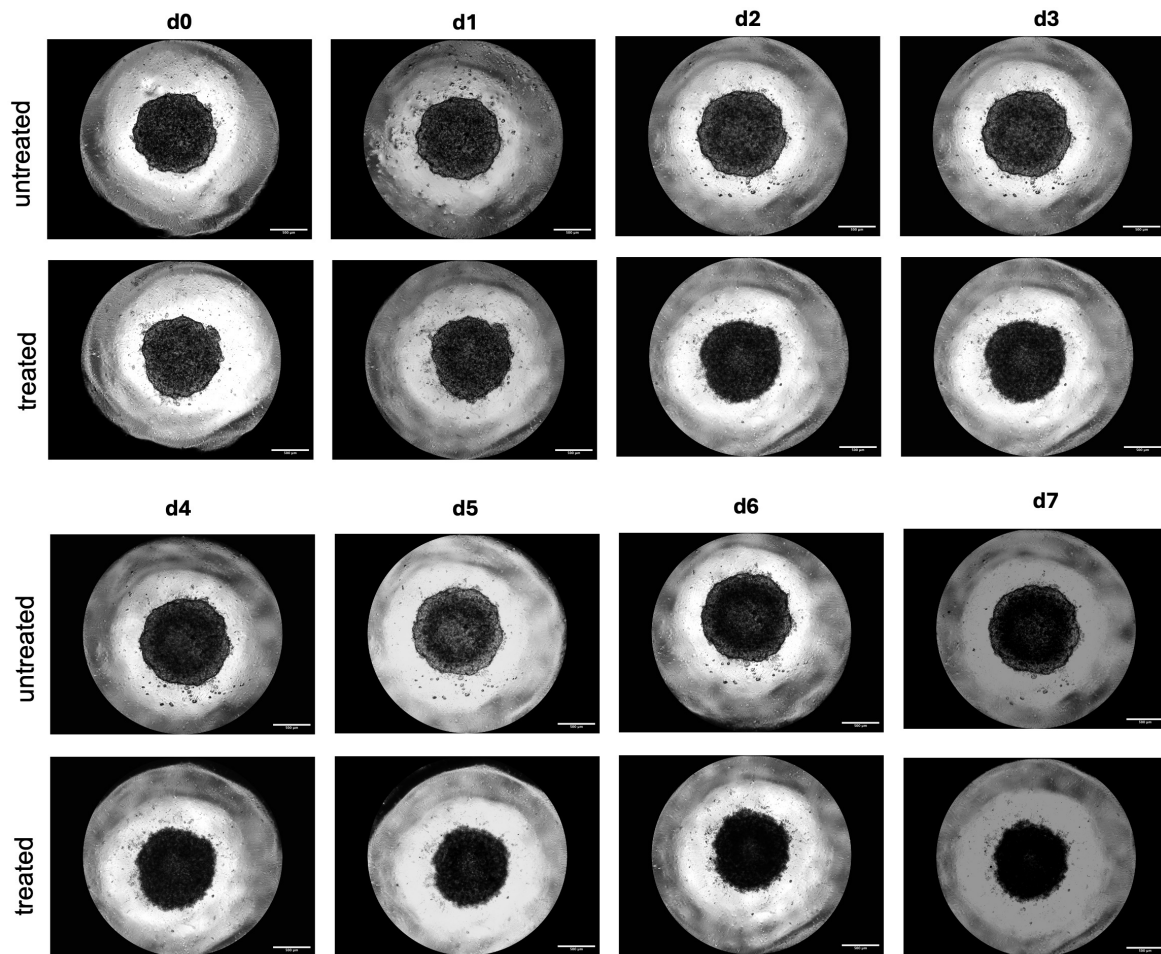


Figure 4-27: Microscopic imaging of T47D human BC cell spheroids and induced size changes of spheroids during 7 d of treatment with DM1 coupled GGSK-1/30 ADC.

Scale 16x, scale bar = 500 μm . Size of spheroids was determined by measuring the cross-section area in Image J. Statistical analysis of untreated against ADC-treatments: Mixed-effects model with Geisser-Greenhouse correction, $p > 0.05$ not shown, $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

For T47D spheroids, changes in shape between untreated and ADC-treated spheroids were observed starting from d2. First, the outer layer of the treated spheroids disappeared. The remaining layers increasingly shrank from day to day, leaving a dense

and dark-looking structure of presumably necrotic cells. The comparison of the spheroid sizes within the differently treated groups shows that the size of ADC-treated spheroids significantly decreases from d3, whereas the untreated and Ab only treated groups show a steady and slow increase in size day by day.

In comparison, the three types of spheroids reacted differently to the treatment. While spheroids built from MDA-MB-231 and T47D cells were reduced in size under the influence of ADC treatment, MCF-7 spheroids reacted by fragmentating and losing their density, resulting in an increase in size. MCF-7 and T47D spheroids reacted 5 d faster than those built from MDA-MB-231 cells.

To summarize, these results showed that GSK-1/30 treatments could be applied in tested human in vitro models. 2D cultures of the three BC cell lines were successfully converted into a 2D assay suitable for preliminary ADC testing. In addition, these cell lines can be cultured as spheroids, forming a 3D model for preclinical treatment testing. While data from the 2D assay pointed out GSK-1/30-DM1 as the less potent option in comparison to GSK-1/30-SN38, the data from 3D treatment assays could nicely demonstrate that the treatment with GSK-1/30-DM1 also has a strong cytotoxic effect on all three human BC cell lines. Interestingly, spheroid shapes differed between the three tested cell lines along with the way they reacted to the treatment.

4.4 Testing the GSK-1/30 mAb and its ADCs on other adenocarcinomas

As mentioned before, taMUC1 is suggested to be expressed in many other adenocarcinomas next to BC. Figure 4-28 shows the 15 tumor entities with the highest frequency of high MUC1 mRNA expression, extrapolated from different studies of the Cancer Genome Atlas (TCGA) Research Network by accessing MUC1 on the TCGA PAN Cancer Atlas database¹²⁰.

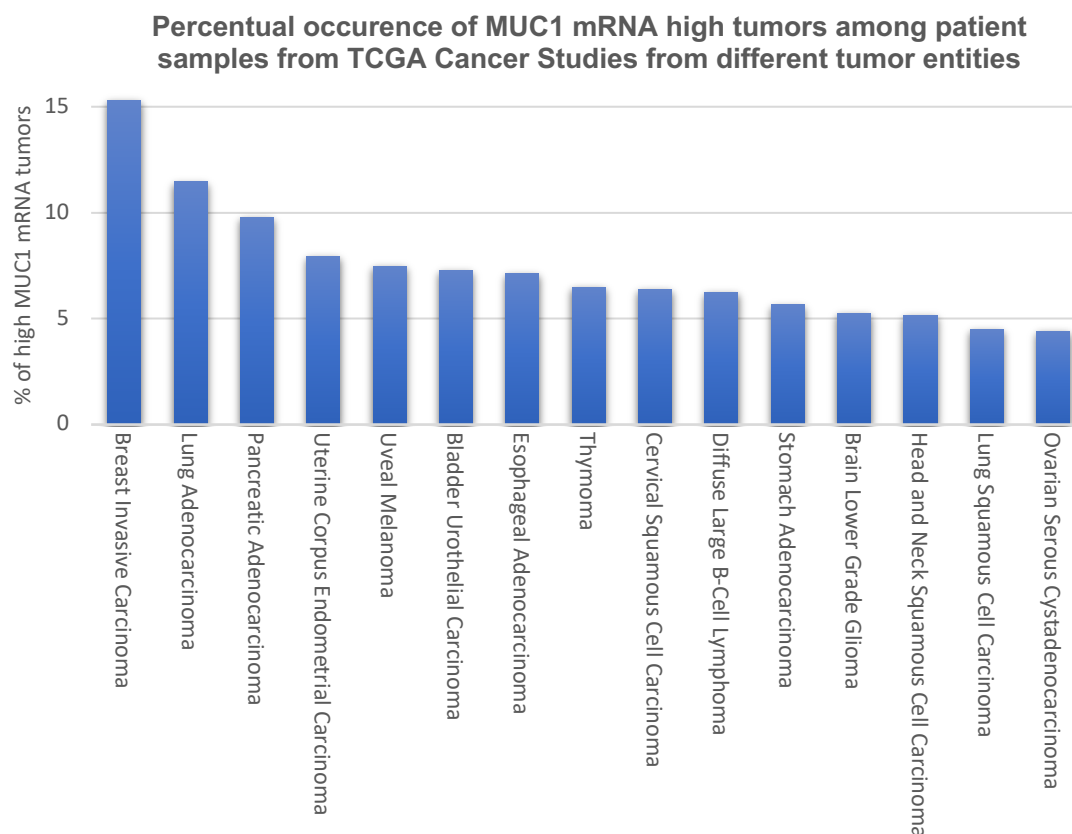


Figure 4-28: Percentual occurrence of tumors with high MUC1 expression among all tumors analyzed in studies for different types of cancer data sets extrapolated from PAN Cancer Atlas TCGA database¹²⁰.

Next to breast invasive carcinoma that showed the highest frequency of 15.31 % MUC1 high expressing tumors, other cancer types of the female reproductive organs also showed high MUC1 for example uterine corpus endometrial carcinoma (7.94 %), cervical squamous cell carcinoma (6.4 %), or ovarian serous cystadenocarcinoma (4.44 %). Interestingly, the database also shows that entities from completely different organs have a high occurrence of MUC1 positive tumors, including lung adenocarcinoma (11.48 %), PDAC (9.78 %), uveal melanoma (7.5 %), and brain low-grade glioma (5.25 %). This makes clear that many other cancers could be suitable for anti-MUC1 cancer treatment and suggests the need for further testing of highly taMUC1 specific GSK-1/30 mAb on other tumor entities.

4.4.1 GSK-1/30 binds specifically to many other human adenocarcinomas

Broad screening of biopsies from adenocarcinoma patients at the UMC of Mainz was performed in collaboration with Matthias Gaida from the Institute of Pathology at UMC Mainz to determine which cancer tissues would be recognized by GSK-1/30.

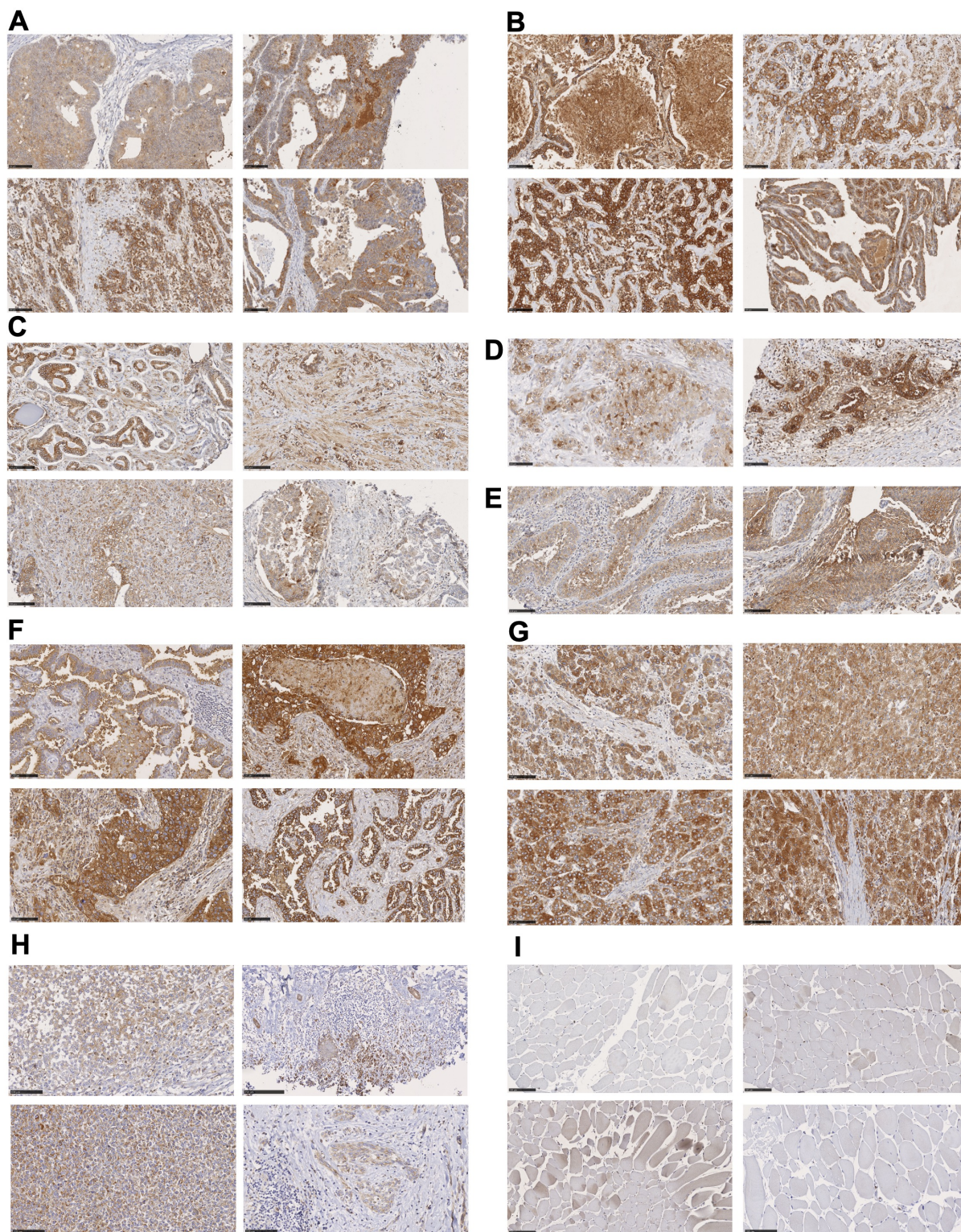


Figure 4-29: IHC screening for taMUC1 expression in human cancer samples collected in the pathological institute of UMC Mainz.

The cells were stained with GGSK-1/30. Scale 20x, scale bar = 100µm. Collaboration with Professor Matthias Gaida. A Colon adenocarcinoma, B Cholangial adenocarcinoma, C prostate adenocarcinoma, D pancreas adeno-CA, E cervical adenocarcinoma, F lung adenocarcinoma, G hepatocellular carcinoma, H malignant melanoma, and I surrounding healthy muscle tissue (MUC1 neg).

Results

Figure 4-29 shows the results of IHC screening of different biopsies after histochemistry staining for taMUC1. Within this selection, very strong taMUC1 expression can be observed for colon adenocarcinoma (A), cholangial adenocarcinoma (B), lung adenocarcinoma (F), and hepatocellular adenocarcinoma (G). Still, a relatively high expression is found in prostate adenocarcinoma (C), PDAC (D), and cervix adenocarcinoma (E). Malign melanoma (H) shows low taMUC1 expression in 3 of 4 tested samples. Healthy surrounding muscle tissues (I) were also stained and served as a neg ctrl for staining and this classification.

Screening revealed that GGSK-1/30 can selectively identify taMUC1 expressing malignant cells in many different tumors, highlighting its potential as a diagnostic marker. Consequently, this also enables the utilization of specific mAbs and derived ADCs for therapeutic purposes in various cancer types. Subsequent experiments were performed in order to develop models for further testing adenocarcinoma that can potentially be addressed by the GGSK-1/30 mAb based therapies.

4.4.2 Screening for taMUC1 expression in various human adenocarcinoma cell lines

With the promising results of the IHC screening, commercially available, suitable cell lines were searched from a selection of tumor entities. Their taMUC1 expression was confirmed via FACS by staining them with FITC-conjugated GGSK-1/30 mAb (Figure 4-30).

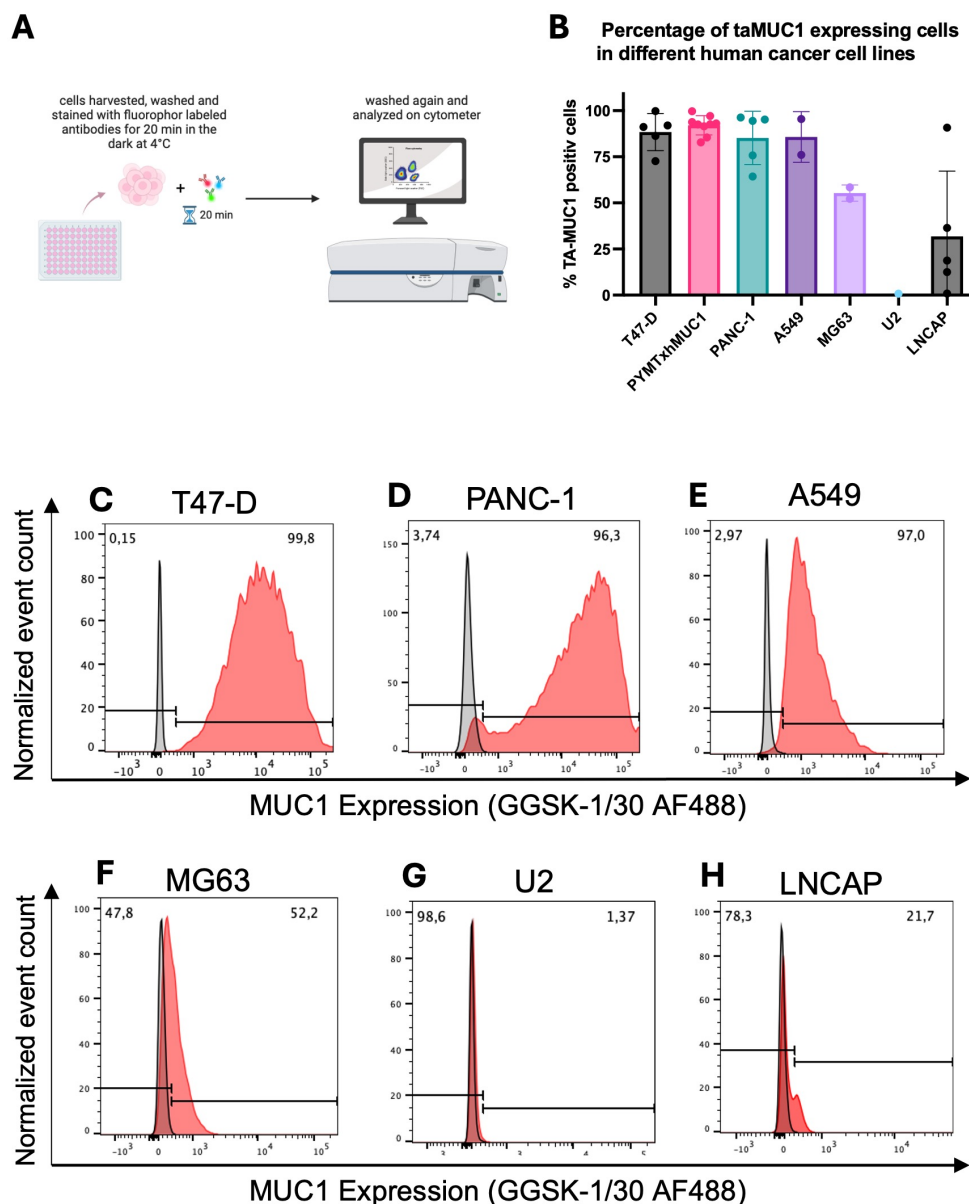


Figure 4-30: FACS screening for MUC1 expression in common commercially available human cancer cell lines from different tumor entities.

A Scheme of the staining procedure. The cells were stained with AF488-labeled GGSK-1/30 anti-MUC1 Ab (1:1000). B Mean taMUC1 expression in different cell lines. Histograms of taMUC1 expression in C positive ctrl T47D (Luminal A mamma adeno CA, taMUC1 high), D PANC-1 (pancreas CA, taMUC1 high), E A549 (lung CA, taMUC1 med.-high), F MG63 (osteosarcoma, taMUC1 med.), G U2 (osteosarcoma, taMUC1 neg.) and H LNCAP (prostate CA, taMUC1 low).

Figure 4-30 A briefly shows the staining procedure. The cells were stained with a mixture of FITC-GGSK-1/30 and APC-eF780 fixable viability dye for 20 min in the dark at 4 °C. After two washing steps, cells were analyzed on the BD Canto cytometer. Expression rates represented by percentages of taMUC1 positive of cells detected by the GGSK-1/30 mAb are summed in Figure 4-30 B. Samples of PyMTxhMUC1 (average of 92 % positive cells) and T47D (average of 88 % positive cells) were included in the screening as references for high taMUC1 expression. Throughout the screening, the Ab detected an

Results

average of 85 % taMUC1 positive cells in PANC-1, a human PDAC cell line, and A549 lung adenocarcinoma cells. LNCAP human prostate carcinoma cells showed an average of 32 % taMUC1 expression, and the human osteosarcoma cell line MG63 had an average expression rate of 52 %. U2, another osteosarcoma cell line, was included as an example cell line in which no taMUC1 expression could be detected. Figure 4-30 C–J shows single histograms of the selected samples within the different tested cell lines. Through this screening, taMUC1 positive adenocarcinoma cell lines could be identified, enabling new in vitro adenocarcinoma models to test GSK-1/30 based ADC treatments.

4.4.3 In vitro treatment testing of GSK-1/30-SN38 ADC on LNCAP prostate cancer cells, A549 lung cancer cells and PANC-1 PDAC cells

In the subsequent ADC testing, adenocarcinoma cell lines LNCAP with low taMUC1 expression, A549 with moderate to high taMUC1 expression, and PANC-1 with high taMUC1 expression were selected. As SN38-ADC previously showed stronger effects in the 2D cultures at eliminating BC cells than DM-1-ADC, it was selected to first be tested on these cells. An untreated ctrl and treatment with mAb alone were used as references. Cells were analyzed by crystal violet viability staining and for total cell number, viability, and apoptosis rate by cytometry. The results of this assay are shown in Figure 4-31.

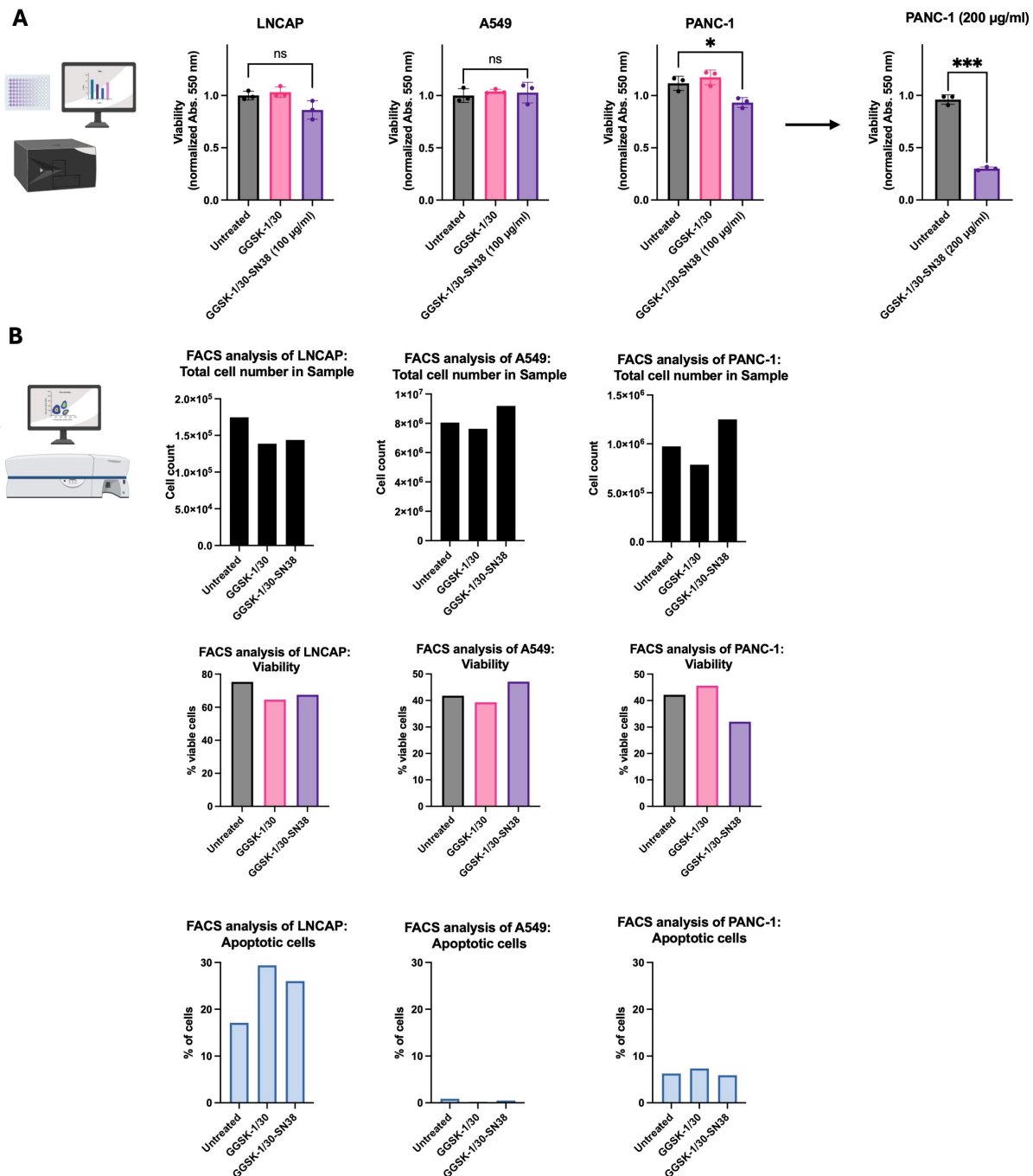


Figure 4-31: Analysis of treatment assay on prostate carcinoma cell line LNCAP, lung carcinoma cell line A549, and PDAC cell line PANC-1 in 2D culture with SN-38 coupled with GSK-1/30 ADC. A. Results of crystal violet staining. Statistical analysis (n=3): Welsch-t-test, $p > 0.05$ not shown, $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). B: Results from FACS analysis: cell number, viability, and percentage of apoptotic cells in the samples (n=2).

Figure 4-31 A shows the viability of untreated and GSK-1/30 or GSK-1/30-SN38 treated cells determined through crystal violet staining for the three cell lines. In Figure 4-31, B cell counts, viability, and apoptotic cell rates from samples of each condition determined via FACS are shown. For LNCAP cells with low taMUC1 expression rates, treatment had a minor effect. For this cell line, crystal violet staining and viability

Results

determined by FACS showed a small, insignificant reduction in viability for both mAb- and ADC-treated cells. Total cell number stayed within similar ranges between untreated cells (1.7×10^5 cells) and both treated cell groups (1.4×10^5 cells). Looking at the number of apoptotic cells, an increase of approximately 10 % was observed in mAb- and ADC-treated cells. Although having showed a high MUC1 expression rate, A549 cells were unaffected by ADC treatment. No reduction in viability through either determination method, accompanied by no major changes in total cell numbers and nearly 0 % of apoptotic cells in each sample from the different treatment conditions, can be found in the results.

PANC-1 cells seemed to respond to SN38-ADC as they showed a significant reduction in viability compared to the untreated cells in the crystal violet staining. This change could also be observed when defining viability via FACS; however, despite this, the percentage of apoptotic cells detected was low and consistent among all conditions. Cell counting via FACS resulted in 9.7×10^5 cells in the untreated sample, 7.9×10^5 cells for the mAb treated sample and 1.25×10^6 cells in the ADC treated sample. Thus, the total cell number decreased when treated with Ab alone but increased against expectation after ADC treatment. A higher concentration of ADC was tested on this cell line to verify the observed viability reduction and its dependency on the amount of ADC within the treatment. PANC-1 cells treated with the double dose of 200 $\mu\text{g/mL}$ SN-38 ADC showed a much greater reduction in viability with only around 30 % of the remaining cells in comparison to the untreated ctrl.

In summary, the screening of different adenocarcinoma cells in a 2D treatment assay could show that the GSK-1/30 based ADCs can eliminate PANC-1 cells and could therefore be used to treat PDAC. PANC-1 cells were subsequently used to develop a 3D in vitro model for PDAC to further evaluate the potency of GSK-1/30 based therapy for this tumor entity.

4.4.4 PANC-1 spheroid formation and treatment with GSK-1/30-DM1 and GSK-1/30-SN38

Spheroid formation and the right seeding number for the 3D treatment assay were tested as described for human BC cell lines (4.3.2). Seeding densities of 8×10^3 , 9×10^3 , 1×10^4 and 1.1×10^4 were tested. Microscopy images were taken of each well in the beginning

(d2), middle (d4), and late phase (d6) of spheroid formation. A selection from each density tested is shown in Figure 8-12 for PANC-1 cells. On d2, loose clustering of the PANC-1 cells could be seen from each tested density, and spheroid formation occurred in each of them from d4 after seeding the cells. On d6, the shape of the spheroids appeared denser. Similar to spheroids formed from T47D BC cells, PANC-1 cells form spheroids with different layers. The outer layer resembles a shell and covers a thin middle layer of less densely packed cells. In the center, the above-mentioned necrotic core, which appears dark and densely packed, can be found. A seeding density of 1×10^4 cells was selected for subsequent experiments as the resulting spheroids are larger than those made from lower cell numbers but still hold a clear spheric shape. On the other hand, the spheroids derived from this density are more spheroidal than those made from 1.1×10^4 cells. Consequently, they are the optimal choice for subsequent 3D treatment assays.

For treatment testing, cells were cultured for 7 d before adding the different treatments to the culture media. Groups of five spheroids were either left untreated or treated with GGSK-1/30 Ab alone or coupled to DM-1 or SN-38. Then, microscopy images were taken from each spheroid each day to observe the effects of the different treatments on their shape and structure. The cross-sectional area was measured from the captured images. Figure 4-32 shows a selection of microscopic images from untreated spheroids in comparison to spheroids treated with GGSK-1/30-DM1 and the size changes during the 7 d of treatment for all treatments. Images from all other spheroids are shown in Figure 8-13 and Figure 8-14.

Results

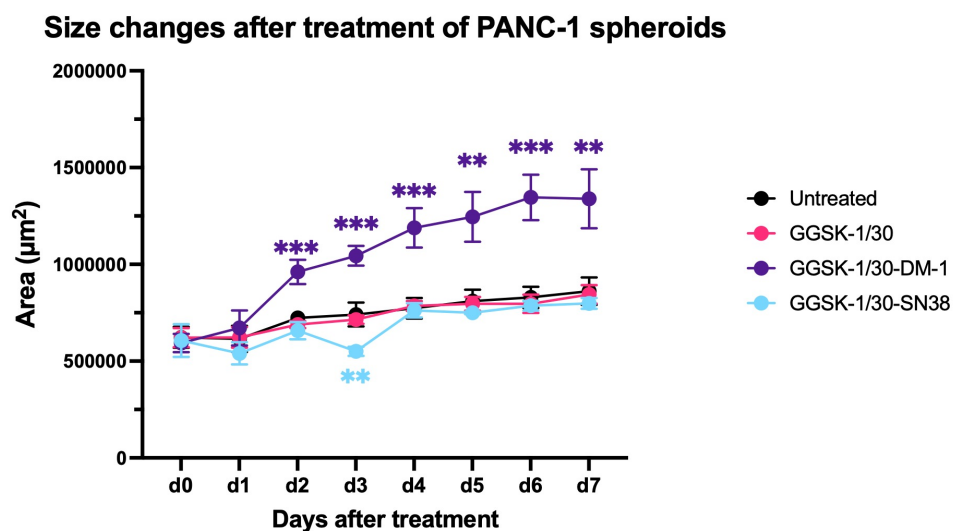
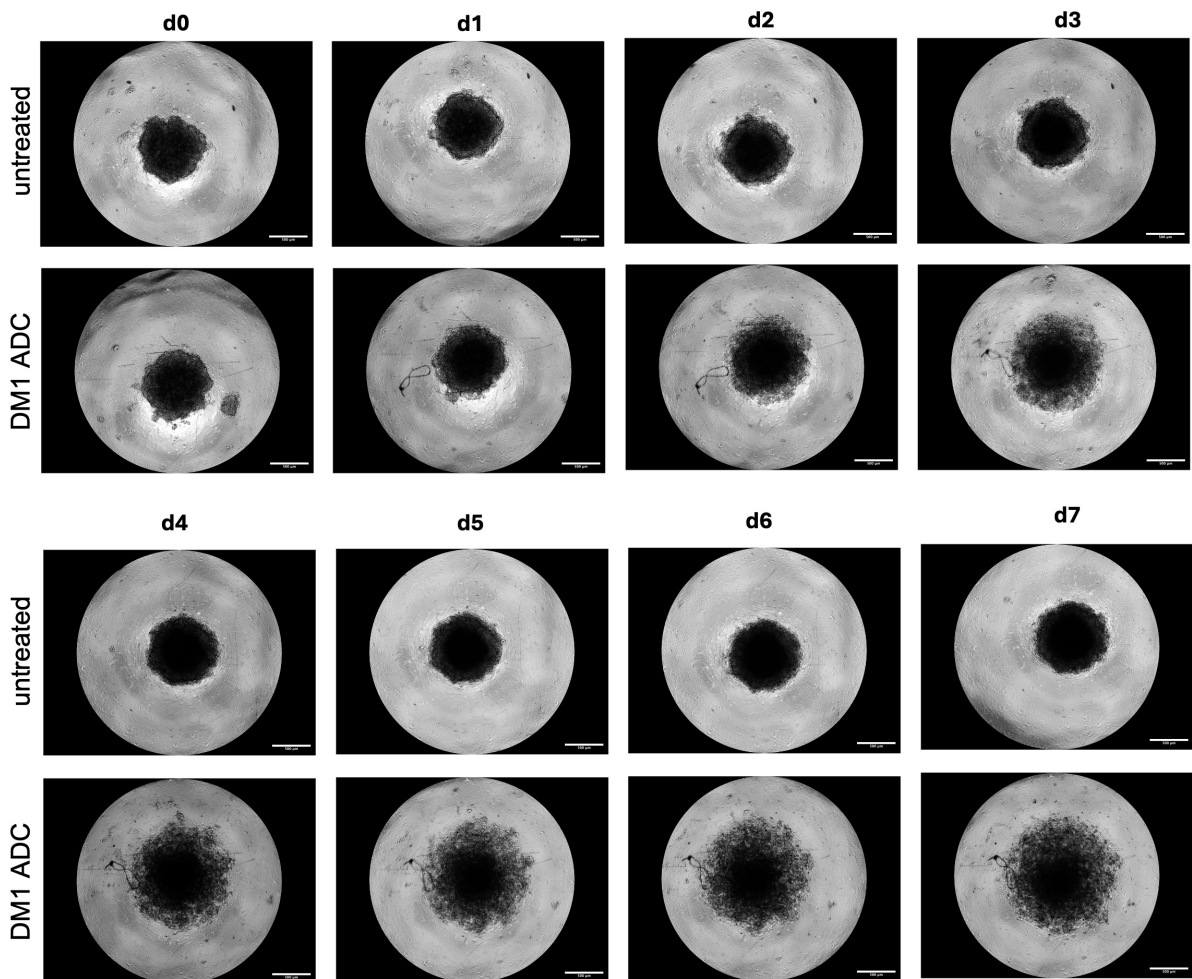


Figure 4-32: Microscopic imaging of PANC-1 human PDAC cell spheroids and induced size changes of the spheroids during 7 d of treatment with DM1 and SN-38 coupled ADCs.

Scale 16x, scale bar = 500 μm . Size of spheroids was determined by measuring the cross-section area in Image J. Statistical analysis of untreated against ADC-treatments: Mixed-effects model with Geisser-Greenhouse correction and Dunnett's multiple comparison, $p > 0.05$ not shown, $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Regarding the shapes of spheroids treated with DM-1, ADC changes can be observed at an early stage of treatment. By d2, the outer layer, which acts like a shell, disappears. After that, the gradual disintegration of the spheroid can be observed by the separation of an increasing number of constituent cells day by day. Consequently, the measured size of SN-38 ADC-treated spheroids increases significantly in comparison to the untreated ctrl as of d2. On d7 DM1-ADC treated spheroids have nearly doubled in size and their shape mainly consists in loose semi-detached cell clusters with presumably only the necrotic core remaining¹¹⁹.

For spheroids either treated with GGSK-1/30 alone or coupled with the SN-38 toxin, no such strong effect is observed. Only a slow onset of effect can be assumed starting from d5 of treatment but no changes in size can be found. GGSK-1/30 treated spheroids seem to behave exactly like untreated ones regarding shape and size within all 7d of treatment.

4.4.5 GGSK-1/30 mAb in a preclinical tissue culturing model on PDAC samples from patients

Within a collaboration with [REDACTED] and [REDACTED] (Institute for Pathology, UMC Mainz), the GGSK-1/30 mAb could be evaluated on a second 3D model. In this model, tissue slices generated by cutting thin slices from PDAC biopsies are processed directly after surgery. In this way, these slices maintained viability for several days in culture, which enabled the testing of treatments.

Before the ADCs could be tested on the tissue culture model, several preliminary experiments had to be conducted to predict the success of treatment. The first step was to establish whether the Ab could reach and sufficiently penetrate the tissue in culture, which is crucial, as treatment will only be successful if this occurs. Therefore, a PDAC sample was first tested by classical IHC for the expression of taMUC1 with 1 µg/mL of GGSK-1/30. The slices of this tissue were cultured and stained with GGSK-1/30. The tissue sample was then formalin-fixed and paraffin-embedded (FFPE) and separated into thin ICH stain sections. These were then either stained with a secondary detection Ab directly or first stained again for taMUC1, which was then also detected via the secondary Ab. In this way, the directly stained samples monitor taMUC1 reached by the Ab in culture, while restained samples act as a ctrl for how much taMUC1 was present in this area.

Results

The following figure shows the procedure just described (Figure 4-33 B) and the results of IHC imaging before (Figure 4-33 A) and after (Figure 4-33 C) tissue culture.

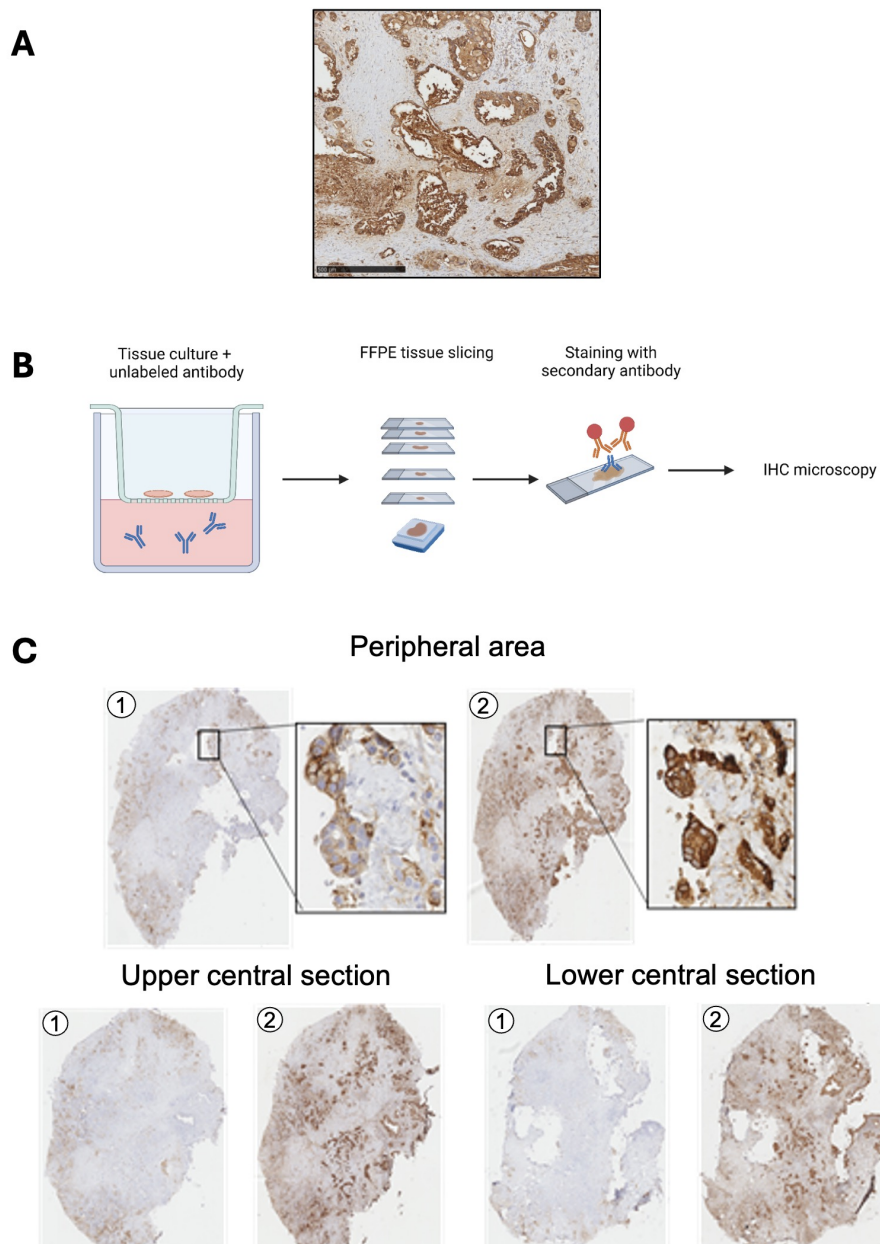


Figure 4-33: Establishment of PDAC tissue slice culture and Ab penetration assay testing if GSK-1/30 can reach the center of the tissue slice.

Results of collaboration with Laura Klein from the Gaidas working group. A IHC staining of PDAC samples with 1 μ g/mL GSK-1/30 mAb. Scale bar = 500 μ m. B Scheme of the tissue culture penetration assay: First, a tissue slice is cultured with unlabeled GSK-1/30, then fixed with the FFPE method and cut into several IHC cuts. Ab binding was then detected through secondary Ab staining. C IHC images from cuts of the peripheral and central areas of PDAC tissue slices. 1. taMUC1 was detected in stained cells in culture. 2. taMUC1 detected by IHC restaining after culture.

Figure 4-33 A shows the IHC staining against taMUC1 with the GSK-1/30 mAb and ensures that before starting, taMUC1 is also highly expressed in the selected patient tissue sample. Within Figure 4-33 A three pairs of cuts are shown. Each pair consisted of

an image from a cut processed by secondary staining after the culture (1) and the second image with the cut originating directly from the neighboring area within the slice that was restained after culturing (2). The first pairs of cuts shown belonged to the peripheral, the second to the upper central, and the third to the lower central section of the tissue slice. Respective isotype controls for each image shown in Figure 8-16 ensure that taMUC1 detection was specific. The detection of taMUC1 by the Ab in culture was visible in the peripheral area of the tissue slice. Every region where taMUC1 was expressed within the tissue was also reached and stained by the Ab in culture, but with a lower intensity compared to the restained cut. In addition, in both central sections, cultured GGSK-1/30 reached most of the taMUC1 expressing areas found in restaining, but the intensity decreased gradually from the border to the center of these cuts. Still, these results show that a potential treatment based on GGSK-1/30 should be able to address human PDAC as shown in the stained slices that the mAb was able to sufficiently penetrate the tissue cultures.

The next step involved testing the ability of GGSK-1/30 coupled to AF488 to image PDAC tissues using fluorescence microscopy. This method, as a complementary tool to existing detection methods such as cell cytometry and HRP-based IHC staining, will be important for the subsequent evaluation of the treated tissue slices.

Figure 4-34 shows the resulting fluorescence microscopy imaging of a PDAC sample stained with GGSK-1/30-AF488, DAPI, and cytokeratin.

Results

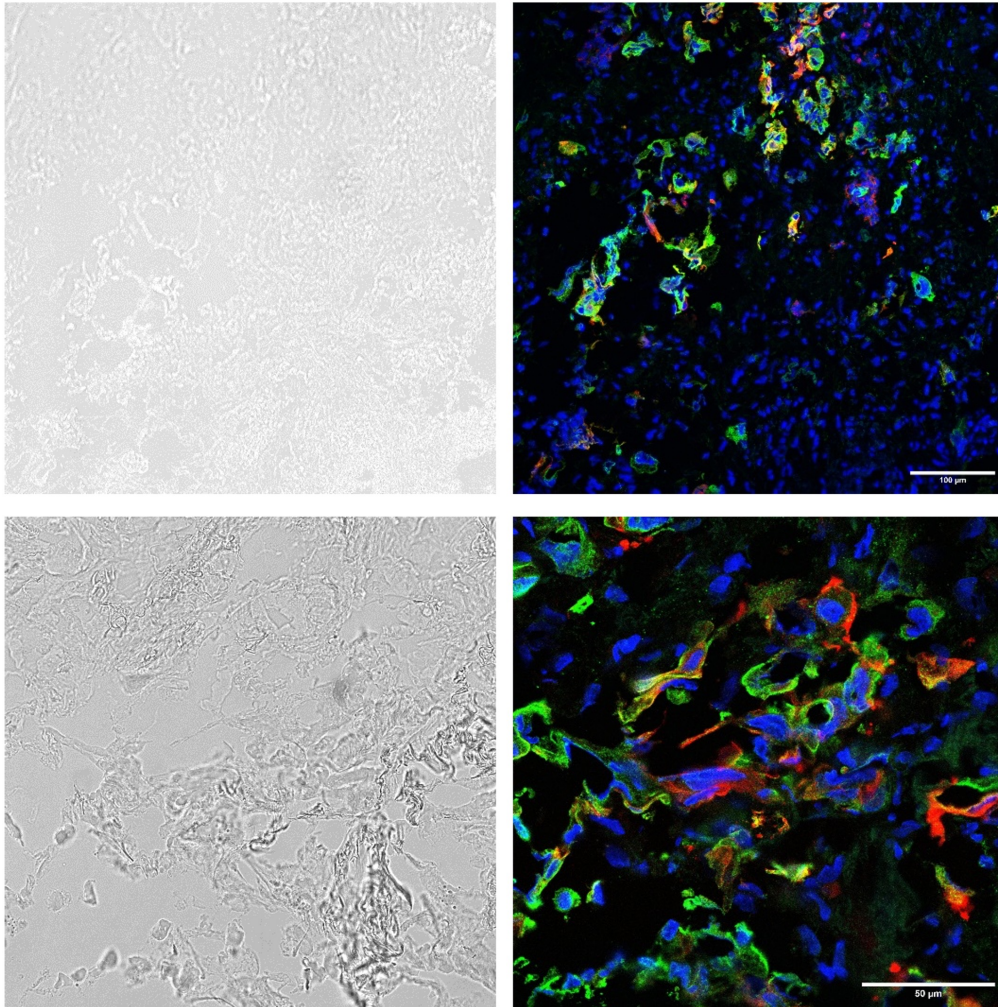


Figure 4-34: Testing AF488-GGSK-1/30 for fluorescence microscopy of a PDAC sample.

Fluorescence microscopy after staining with GGSK-1/30-AF488, DAPI, and cytokeratin. Left: transmission microscopy, right: merge of DAPI (blue), GGSK-1/30-AF488 for MUC1 (green), pan-cytokeratin (red) fluorescence microscopy images, Top: scale 20x (Scale bar = 100 µm); bottom: scale 63x (scale bar = 50 µm). Results from collaboration with [Laura Klein](#) from the Institute for Pathology, UMC Mainz.

The left side shows the transmission microscopy image of the PDAC sample at 20x magnification (top left) or 63x magnification (bottom left). In both images, the structure of the tissue can be seen. Higher magnification additionally allows the detection of single cells within the tissue structure. The images on the right side show a merge of images taken from different fluorescence stainings. taMUC1 was detected with AF488 labeled GGSK-1/30 (green), nuclei of single cells were stained with DAPI (blue), and cytokeratin of the cells was stained with a PAN-cytokeratin Ab (red). A second staining performed with isotype controls for each Ab (Figure 8-16) showed that the detection of the respective markers was specific. In the 30x magnified merge image (top right), many individual cells can be identified by the blue labelling of their nuclei. taMUC1 expression was only present in some of the cells, presumably tumor cells, all located in the same area of the imaged

part of the tissue. In the same area, more cytokeratin can also be found. Looking at the higher magnification (bottom, right), GGSK-1/30 can be found on the cell surface as well as inside the cell, which conclusively answers the question of whether taMUC1 is internalized upon Ab binding. At this magnification, cytokeratin is now also better recognizable inside some cells, and it is clearly visible how some cells use it to stabilize themselves or to move around. In conclusion, GGSK-1/30 labeled with AF488 is very suitable to track taMUC1 using fluorescence microscopy.

Having tested the last of the three envisaged analysis techniques, the next step was to analyze and select suitable candidates among the prepared ADCs of GGSK-1/30 with either emtansine (DM1), deruxtecan (DX), or irinotecan (SN-38) for the preclinical tissue culturing PDAC model.

Stable viability of the tissues could be assured in previous experiments for a culture duration of up to 4d. In previous trials, a treatment period of 7d was always investigated. Therefore, the three different GGSK-1/30 ADCs were subsequently tested in 2D culture on PANC-1 cells to predict which ADC will be the most potent, meaning the fastest acting and most effective treatment. Cultured cells were treated with 50, 100, and 200 $\mu\text{g/mL}$ of each ADC, and viability was analyzed on d2 and d4, in addition to the previously envisaged treatment duration of 7d. The determined viabilities are shown in Figure 4-35.

Results

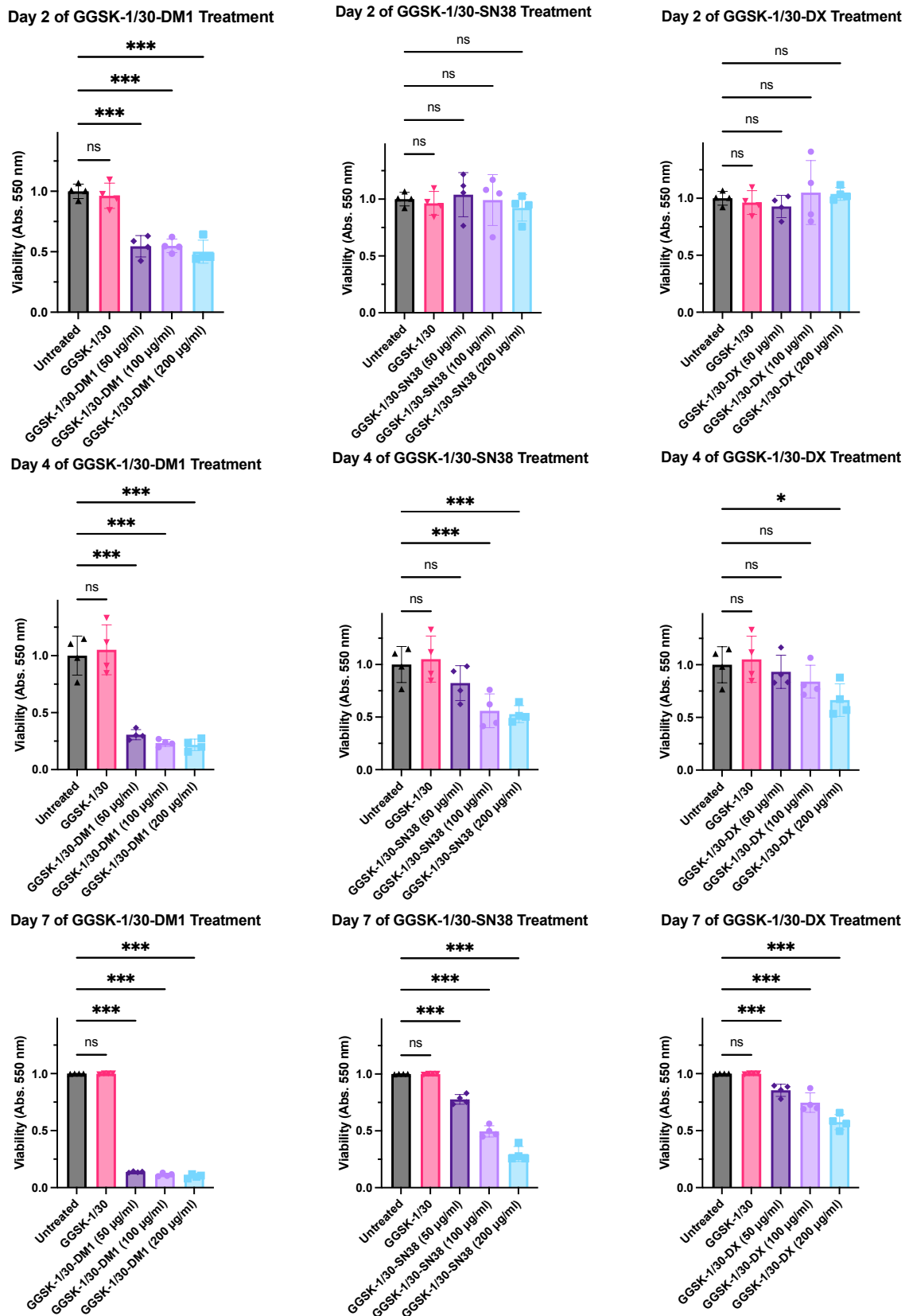


Figure 4-35: Screening for the most efficient ADC for PDAC tissue slice culture treatment.

PANC-1 cells treated with GGSK-1/30 ADCs coupled to payloads DM-1, DX, and SN-38 from left to right. The first row was treated for 2 d, the second row for 4 d, and the last row for 7 d. N=4, Statistical analysis: one-way ANOVA with Dunnett's multiple comparison, $p > 0.05$ not shown, $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

The results show that GGSK-1/30 coupled to the DM1 toxin has the strongest effect on PDAC cells and works the fastest. After just 2 d of treatment, viability was reduced to almost 50 % compared to the level of the untreated ctrl for all three tested concentrations of DM1-ADC. Elimination increased with longer treatment duration, leaving around 20 to 30 % of cells after 4d and only slightly more than 10 % of cells after 7 d of GGSK-1/30-DM1 treatment compared to the ctrl level. SN-38 and DX ADC showed no effect on viability at d2 of treatment. After 2 more days, the cells treated with 100 and 200 µg/mL GGSK-1/30 SN-38 showed a significant reduction in viability. After 7d a concentration of 50 µg/mL also induced a significant effect on cell viability. For GGSK-1/30-DX, only the concentration of 200 µg/mL showed a significant reduction on d4. On d7 all three tested concentrations of GGSK-1/30-DX showed a significant reduction in PANC-1 cells as for the two ADCs.

In total, the analysis showed that all three ADCs reduced viability after 7d of treatment. Classified by the lowest concentration required to achieve an effect and based on the strength of the observed effect, the ADCs can be categorized by effectiveness for the treatment of human PDAC cells as follows: GGSK-1/30-DX is considered the least potent, GGSK-1/30-SN38 is the intermediate, and GGKS-1/30-DM1 is the most potent combination. Additionally, the ADC coupled to DM1 acts the fastest, showing a already a strong effect on viability already 2-4 d before the others.

In summary, the experiments show that the GGSK-1/30 mAb can successfully be used in a 3D tissue slice culture model for human PDAC as it can reach taMUC1 within the tissue. It can also be used in IHC, fluorescence microscopy, and flow cytometry to detect taMUC1, covering all envisaged methods for subsequent analysis of treated tissue slices. Lastly, the comparison of the three ADCs of GGSK-1/30 with various payloads revealed that GGSK-1/30-DM1 is a highly potent and suitable candidate for further investigations in tissue slice treatments. Experiments with the use of this system for the actual testing of selected ADCs could not be carried out within the scope of this work, as the frequency and timing of the receipt of human tissue relies on the number of patients from surgery and therefore could not be planned. However, a continuation of this cooperation and the testing of GGSK-1/30-DM-1 as well as GGSK-1/30-SN38 and -DX will be the subject of future investigations.

5 Discussion and outlook

5.1 Hydrogel depot forming monomer as replacement for the usage of IFA in vaccine formulations in supramolecular vaccines

The first section of this work consisted of the investigation of different vaccine formulations based on a synthetic modular vaccine platform *in vivo*. All vaccine formulations elicited robust activation of humoral immunity, highlighting the strong potential of supramolecular vaccine platforms.

A few noteworthy observations were made when comparing different formulations. Although additional stimulation through TTox peptide p30 and TLR7/8 was shown to be important to stimulate humoral immune responses, increasing their ratios within the formulations showed a detrimental effect. According to literature, this could be explained by a phenomenon called peripheral tolerance in which T cells shut down to anergy or suppression after repeated high exposure of a specific antigen^{121,122}. Comparing the three varying ratios of hydrogel within the formulation, an inverse correlation between the amount of hydrogel forming monomer in the vaccine and its capability to induce humoral immune response was observed. This likely is due to the fact that the more hydrogel forming monomer the formulation contains the resulting vaccine forms a stronger crosslinked gel, making the presented epitopes less accessible for immune cells. Similar effects are described in literature pointing out that the evaluation of each individual formulation is mandatory^{123,124}.

Nevertheless, the new formulation performed as well as the previous IFA version, showing no loss of immunogenicity. The hydrogel formulation even offered certain advantages, such as its longer-lasting immune response or its integrated hydrogel forming monomer, which could be proven to be a functional alternative to IFA. Owing to the limitations of IFA, such as its local reactogenicity and restricted translational potential, the hydrogel system provides a more biocompatible and adjustable platform for antigen delivery and immune activation. This further supports its potential as a plausible alternative and positions the vaccine platform as a promising vehicle for future immunotherapeutic applications.

Many recent studies also emphasize the importance of formulations that are adaptable to each patient's needs^{125,126}. The modular vaccine platform presented in this study

represents a customizable strategy because structural hydrogel forming monomers can be easily integrated into the vaccine structure. This allows the vaccine to adapt to individual therapeutic requirements.

For further evaluation, suitable preclinical in vivo tumor models must be established to determine their prophylactic and therapeutic potential in oncological settings. It will also provide valuable insights into its mechanism of action and translational relevance.

In the context of BC, two models have already been established and are in use within our working group. One model consists of implanted PyMTxhMUC1 cells that express hMUC1 in C57Bl/6-wt mice, resulting in transgenic tumor-bearing mice. This model has been used in the context of ADC testing, as shown before (4.2), and could deliver valuable insights for ADC treatment testing and vaccination studies. The second model is a mouse strain (C57BL/6-TG(MUC1)79.24GEND/JxR10), which develops autochthonous tumors in the mammary glands that express human taMUC1. This strain was generated by crossbreeding hMUC1-tg mice with PyMT (R10) mice that bring in the spontaneous development of tumors in the mammary gland (Figure 5-1). This mouse strain was used for the evaluation of previous formulations. Due to stricter animal welfare regulations, this aspect could not be further investigated here.

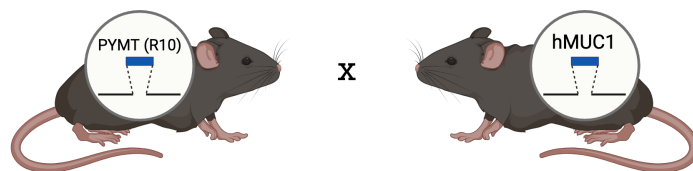


Figure 5-1: Generation of a murine BC in vivo model for MUC1 therapy testing.

To generate the PyMTxhMUC1 mouse model, mice from the PyMT strain, which spontaneously develop tumors in the mammary gland, were crossed with hMUC1-tg mice. The resulting strain spontaneously forms tumors that express hMUC1.

Subsequently, this strategy should be evaluated for the prevention and treatment of PDAC. A suitable model for this could consist of mice inoculated with Panc-02 cells that are transgenic for hMUC1 or genetically modified mouse models such as the KC model. In this model, mice are genetically modified with a pancreas progenitor cell-specific depletion of the K-ras gene, so that they developed small lesions selectively in the pancreas starting at 8 weeks of age. These lesions further developed to PDAC over the next two years mimicking the step-wise development of cancer seen in humans¹²⁷.

5.2 GGSK-1/30 based ADCs also show a curative effect in preclinical human BC model

For ADC testing, suitable robust cell lines were selected and harnessed to establish 2D and 3D treatments. Both models enabled the comparative assessment of two distinct anti-MUC1 ADCs, GGSK-1/30-DM1 and GGSK-1/30-SN38, consisting of the GGSK-1/30 mAb as the target structure and the cytotoxic payloads derived from irinotecan (SN38) and emtansine (DM1). This provided insight into their relative antitumor potential and therapeutic efficacy.

The 2D monolayer assays with both ADCs demonstrated that SN38, a potent topoisomerase I inhibitor, exerted a stronger cytotoxic effect on human BC cell lines than DM1, a microtubule inhibitor. The enhanced potency of SN38 in 2D cultures may be attributed to its mechanism of action, which is particularly effective during DNA replication in rapidly dividing cells. In contrast, DM1, a highly potent microtubule-disrupting agent, may be less effective in the flat monolayer configuration due to differences in drug uptake, distribution, or cell cycle dependency. Also to be mentioned is that the antibody-drug ratio of the synthesized ADCs could not yet be determined. Therefore, differences in each ADCs potential might also have occurred due to different amounts of toxin molecules bound to the mAbs leading to variances in the total toxin concentration in each treatment.

In comparison to the results of 2D culture, DM1 interestingly demonstrated pronounced antitumor activity in a 3D assay performed in parallel to the monolayer culture. ADC treatment led to significant changes in the structure of all three tested cell lines, but each reacted differently to treatment. While MDA-MB-231- and MCF-7-derived spheroids decreased in size upon treatment, T47D spheroids enlarged in size upon treatment and finally reacted in an alluvial destruction of the spheroid.

The disparity between the results of 2D and 3D assays underscores the limitations of monolayer models for evaluating ADCs and highlights the importance of incorporating 3D systems that better recapitulate in vivo tumor physiology. The lack of an effect in 2D cultures may stem from oversimplified growth conditions that fail to model crucial resistance mechanisms such as poor drug penetration, heterogeneous cell populations, or stromal influences. 3D culture conditions more accurately mimic the tumor microenvironment, including factors such as extracellular matrix deposition, cell-cell interactions, hypoxia, and limited drug penetration. These conditions may influence drug

sensitivity and more closely reflect the in vivo scenario. The observed higher efficacy of GGSK-1/30-DM1 on the spheroids could be explained by better accumulation or retention of the ADC within the tumor-like spheroids or by the microtubule inhibitor's ability to target non-proliferating or slowly proliferating cells present in the inner layers of the spheroids. An increasing number of studies, including the one presented in this work, demonstrate that 3D models correlate better with in vivo responses and clinical outcomes, emphasizing their importance in translational research. Within the next years the integration of 3D tumor in vitro models into the preclinical testing pipeline to improve the prediction of therapeutic success will become mandatory.^{128,129}

In conclusion, the findings of this study emphasize the need for advanced in vitro models for the preclinical evaluation of ADCs. They also highlight the need for further investigation in the GGSK-1/30 based ADCs, as they were shown to be highly potent in the treatment of BC.

The next step should consist of testing the GGSK-1/30-SN38 ADC within the 3D model as well to get a better insight on the comparison of both toxic payloads. To further characterize the therapeutic potential of GGSK-1/30 mAb based ADCs, functional assays should be performed to evaluate the capacity of the mAb to mediate antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). These mechanisms may significantly contribute to the overall antitumor activity, particularly in immunocompetent models or in combination with immunomodulatory agents. To further refine preclinical testing of anti-MUC1 ADCs and improve the prediction of clinical outcomes, future studies should incorporate more complex and physiologically relevant model systems. These could include: Co-culture models that combine tumor cells with stromal or immune cells, in order to simulate the cellular heterogeneity of the tumor microenvironment¹³⁰; organ-on-a-chip systems, which incorporate microfluidics and mechanical forces to mimic tissue architecture and dynamic drug perfusion¹³¹, which preserve the genetic and phenotypic characteristics of individual tumors and offer a personalized platform for ADC evaluation¹³²; or the in vivo xenograft or syngeneic mouse models described before can be used to validate in vitro findings under systemic physiological conditions, including immune response and metabolism.

5.3 GGSK-1/30 mAb should be considered as a new treatment on other tumor entities

taMUC1 expression could be determined through the GGSK-1/30 mAb on several additional tumor tissues to BC, strongly suggesting the expansion of GGSK-1/30 based treatments on other tumor entities. On the same side, initial functional 2D in vitro studies using GGSK-1/30-SN38 revealed that among the tested human adenocarcinoma cell lines, PANC-1 cells emerged as a particularly promising target, suggesting that PDAC tumors can be addressed by taMUC1-targeting treatments. This assumption was further underlined by 3D assays with the cell line, where a particularly strong effect was achieved by treatment of the spheroids with GGSK-1/30-DM1.

Considering the observed fact that the human lung adenocarcinoma cell line A549 in contrast to PANC-1 was not sensitive to the ADC up to a concentration of 100 µg/mL despite clearly detectable levels of taMUC1 on the tumor cell surface. This discrepancy raises important questions regarding the factors that determine ADC efficacy beyond mere target expression. One potential explanation for this phenomenon lies in the intracellular trafficking and processing of ADC following target binding. It has been hypothesized that discrepancies in internalization rates, lysosomal activity, or the expression of drug resistance transporters may have a substantial impact on therapeutic outcomes. Furthermore, the inherent properties of the tumors, including its proliferation rate, metabolic state and microenvironmental factors (e.g. hypoxia and stromal density), have the capacity to modulate the cytotoxic potential of the delivered payload.¹³³ These findings suggest that the expression of taMUC1 alone is not sufficient to guarantee treatment success, highlighting the importance of multifactorial evaluation when selecting tumors for targeted ADC therapy.

Nevertheless, this work identified PDAC as a valuable target for GGSK-1/30 based therapy in both 2D and 3D in vitro models. To achieve a further step in clinical translation, a tissue slice culture model for testing ADC treatments directly on patient-derived tissue samples was developed. The advantages of this model are the preservation of native tumor architecture, cellular heterogeneity, and tumor microenvironment. It also maintains critical interactions between tumor, stromal, and immune cells, providing a robust platform for evaluating drug efficacy, predicting patient-specific responses, and identifying biomarkers of therapeutic sensitivity or resistance^{134,135}. Preliminary results in the context

of this establishment were able to further highlight the extraordinary efficacy of GGSK-1/30-DM1 by rapidly killing PANC-1 in only 2d of culture. Another crucial question for future treatment of derived ADCs was answered by showing the ability of GGSK-1/30 mAbs to penetrate the cultured tissue.

In summary, the consistent expression of taMUC1 and the observed efficacies in 2D and 3D in vitro models and preliminary results from tissue slice culture establishment of PDAC provide a strong basis for the establishment of ADC therapy. However, PDAC presents specific challenges for drug delivery due to its highly desmoplastic stroma and inadequate vascularization, which could restrict accessibility and efficacy in vivo^{33,136}. Therefore, future studies must address how these barriers can be overcome, for example, through formulation engineering, combination with stroma-modulating agents, or novel delivery systems.

In order to exploit the full potential of anti-MUC1-based therapies, it is essential to establish a robust in vivo PDAC model. Like the KC-model mentioned before (5.1), these models must replicate key features of the human disease, such as immune evasion, stromal desmoplasia, and resistance to therapy. Future experiments will involve transfection of murine Panc-02 pancreatic cells with hMUC1 to form a humanized tumor implantation model. This model could be a valuable tool for testing monotherapies and combination regimens involving vaccines and ADCs. Regarding the usage of anti-MUC1 ADCs for PDAC, future investigations should focus on optimizing payload selection, the drug-to-antibody ratio (DAR), and tumor-specific penetration, particularly given the heterogeneous and fibrotic nature of PDAC tumors. In-depth evaluation of off-target toxicity and resistance mechanisms is also critical for translating these agents into clinical practice. Another valuable extension of this work is the application of ADCs to established PDAC tissue slices. This ex vivo model offers a high degree of physiological relevance and enables direct testing of drug efficacy within the native tumor architecture, including the stromal components and extracellular matrix barriers characteristic of PDAC. The positive results in these models would strongly justify further translational development.

5.4 Conclusion for the therapeutic use of anti-MUC1 vaccines and GGSK-1/30 mAb based therapies

These findings are encouraging for the development of anti-MUC1-based immunotherapeutic strategies in the form of vaccines and ADCs. The optimized vaccine formulation is a significant advancement, as it enables enhanced immunogenicity, efficient antigen presentation, and compatibility with additional immunomodulatory elements. This vaccine could be used as a monotherapy to elicit tumor-specific immunity or in combination with ADCs or immune checkpoint inhibitors to enhance efficacy through a multimodal approach. Combining vaccines and targeted therapies is a promising way to overcome tumor immune evasion and improve the long-term control of MUC1-expressing cancers.

In addition to optimizing the MUC1-targeting vaccine component, significant progress was made in developing 2D and 3D treatment models for ADC evaluation. Expanding the range of models used in ADC development enables researchers to gain a more comprehensive understanding of drug behavior, resistance mechanisms, and therapeutic windows. These advanced systems will also be critical for optimizing ADC design, including linker stability, payload selection, and antigen-targeting specificity. In the context of taMUC1-targeting strategies, such platforms will provide more accurate predictions of clinical efficacy and safety, ultimately accelerating the development of effective cancer therapies for promising ADCs.

Developing a functional ADC therapy that targets PDAC would be a significant advancement in the management of this aggressive and difficult-to-treat malignancy. PDAC is characterized by late diagnosis, dense desmoplastic stroma, poor drug penetration, and resistance to conventional chemotherapeutics and targeted agents. Current standard-of-care regimens, such as FOLFIRINOX or gemcitabine/nab-paclitaxel, offer limited survival benefits and are often associated with significant toxicity³².

An ADC that effectively targets a tumor-specific antigen such as taMUC1 could provide a highly selective and potent therapeutic option with an improved therapeutic index.

Improving response rates and survival outcomes in both first-line and refractory PDAC patients; enabling personalized treatment approaches, particularly if ADC sensitivity can be predicted based on tumor antigen expression levels; complementing or replacing

systemic chemotherapy in selected patient groups, improving quality of life and treatment tolerability; serving as a foundation for combination therapies with immune checkpoint inhibitors or stromal-modulating agents to overcome the immunosuppressive microenvironment of PDAC or expanding therapeutic options for patients with limited or no surgical options, as well as for those with metastatic disease. A successful ADC approach for PDAC would ultimately fill a critical unmet need in oncology by offering hope for a cancer type with one of the lowest survival rates and few effective therapeutic options.

In summary, the optimized anti-taMUC1 vaccine and ADCs show great promise as part of a multifaceted strategy to target taMUC1-expressing tumors. Future studies will determine the most effective ways to integrate these tools into clinically meaningful immunotherapeutic regimens, particularly for hard-to-treat cancers such as PDAC.

6 Abstract

BC remains one of the most common causes of cancer-related deaths in women. An established marker is the HER2/neu receptor, which is used for prognosis and therapy decisions.¹³⁷ The Ab trastuzumab has proven itself in HER2-positive tumors, but suitable diagnostic and therapeutic options are often lacking in HER2-negative tumors.^{25,73} An alternative marker is MUC1, whose tumor-associated form taMUC1 is overexpressed in over 90 % of BC tissues, particularly in TNBC. taMUC1 is also highly expressed in PDAC, one of the most aggressive tumor types with poor prognosis⁴¹. The strong biochemical differences between taMUC1 and normal MUC1 and its association with a worse clinical outcome make it an essential diagnostic or prognostic marker and highly valuable therapeutic target for these diseases^{47–49}. A highly specific mAb (GGSK-1/30) against taMUC1 and a vaccination strategy against the tumor antigen have been developed within the [REDACTED] research group^{67,70,91,138}.

To further develop the anti-MUC1 vaccination strategy, different formulations presenting synthetic glycopeptides, different adjuvants, and a new thermoresponsive hydrogel forming monomer on a synthetic modular vaccine platform were tested in vivo. All formulations induced a robust taMUC1 specific humoral immune response. The use of hydrogel-forming components proved to be an equivalent alternative to IFA, and thus represents a further development regarding the clinical use of the vaccination strategy.

The mAb GGSK-1/30 was developed to selectively bind taMUC1 without affecting normal epithelial cells. Previous studies have demonstrated its specificity for human BC tissue and its utility as a diagnostic and potentially therapeutic agent, including its use as a radiolabeled tracer for PET imaging.^{71,139} Based on this knowledge, various ADCs of GGSK-1/30 and trastuzumab-emtansine were prepared, and appropriate in vitro models were established for the testing of ADCs. The GGSK-1/30-SN38 conjugate was found to be the most effective against BC cells, whereas GGSK-1/30-DM1 was particularly effective against PANC-1 cells.

This work demonstrated the immense potential of immunotherapies targeting taMUC1 for the treatment of BC, pancreatic carcinoma, and other adenocarcinomas. Combining vaccination strategies with mAbs such as GGSK-1/30 can open completely new treatment possibilities for TNBC and PDAC by increasing therapeutic efficacy and delaying

resistance mechanisms. Continued research in this area is crucial to translate these findings into clinical success and improve patient outcomes.

7 Kurzfassung

Brustkrebs zählt weiterhin zu den häufigsten Krebstodesursachen bei Frauen. Ein etablierter Marker ist der HER2/neu-Rezeptor, der zur Prognose und Therapieentscheidung dient.¹³⁷ Der Antikörper Trastuzumab hat sich bei HER2-positiven Tumoren bewährt, jedoch fehlen bei HER2-negativen Tumoren oft geeignete Diagnose- und Therapiemöglichkeiten.^{25,73} Ein alternativer Marker ist MUC1, dessen tumorassoziierte Form taMUC1 in über 90 % des Brustkrebsgewebes und insbesondere beim TNBC überexprimiert wird. Auch beim PDAC, einer der aggressivsten Tumorarten mit schlechter Prognose, wird taMUC1 stark exprimiert⁴¹. Die großen biochemischen Unterschiede von taMUC1 zu normalem MUC1 und seine Assoziation zu einem schlechteren klinischen Ergebnis machen es zu einem wertvollen diagnostischen oder prognostischen Marker und zu einem guten therapeutischen Angriffspunkt für diese Erkrankungen^{47–49}. Ein hochspezifischer Antikörper (GGSK-1/30) gegen taMUC1 sowie eine Vakzinierungsstrategie gegen das Tumorantigen wurden innerhalb der Arbeitsgruppe [REDACTED] entwickelt.^{67,70,91,138}

Zur Weiterentwicklung und Optimierung der anti-MUC1-Impfstrategie, wurden in dieser Arbeit verschiedene Formulierungen einer synthetischen modularen Vakzinplattform, mit synthetischen Glykopeptiden, verschiedenen Adjuvantien und einem neuen thermoreaktiv hydrogelisierenden Monomer in Mäusen untersucht. Anhand dieser in vivo Experimente konnte gezeigt werden, dass alle Formulierungen eine robuste, taMUC1 spezifische humorale Immunantwort induzierten. Die Verwendung der Hydrogelbildenden Komponente erwies sich dabei als Alternative für zuvor verwendetes Inkompletes Freund'sches Adjuvanz (IFA) und stellte damit eine Weiterentwicklung hinsichtlich der klinischen Verwendung dieser Vakzinierungsstrategie dar.

Der mAb GGSK-1/30 wurde entwickelt, um taMUC1 selektiv zu binden, ohne normale Epithelzellen zu beeinträchtigen. Frühere Untersuchungen haben die Spezifität des Antikörpers für menschliches BC-Gewebe und seinen Nutzen als diagnostisches und potenziell therapeutisches Mittel gezeigt, einschließlich seiner Verwendung als radioaktiv markierter Tracer für die PET-Bildgebung.¹³⁹ Darauf aufbauend wurden in dieser Arbeit ADCs aus GGSK-1/30 und verschiedenen, aus Trastuzumab Anwendungen bekannten Toxinen hergestellt. Um diese für eine taMUC1 gerichtete Therapie zu etablieren und zu

validieren wurden passende in vitro Modelle etabliert. Dabei zeigte sich, dass das GGSK-1/30-SN38-Konjugat am wirksamsten gegen Brustkrebszellen war, während GGSK-1/30-DM1 besonders effektiv bei PANC-1-Zellen agierte.

Gegen taMUC1 gerichtete Immuntherapien stellen einen vielversprechenden Weg für die Behandlung von Brustkrebs, dem Pankreaskarzinom und anderen Adenokarzinomen dar. Durch die Kombination von Impfstrategien, wie beispielsweise der hiesig untersuchten, mit GGSK-1/30 eröffnen neue immuntherapeutische Behandlungsmöglichkeiten vor allem für TNBC und PDAC. Weitere Forschung in diesem Bereich ist von entscheidender Bedeutung, um die therapeutische Wirksamkeit weiter zu verbessern und Resistenzmechanismen zu verzögern und somit letztendlich Patienten in der Klinik verbesserte Behandlungsoptionen zu ermöglichen.

8 Appendix

8.1 Supplementary figures

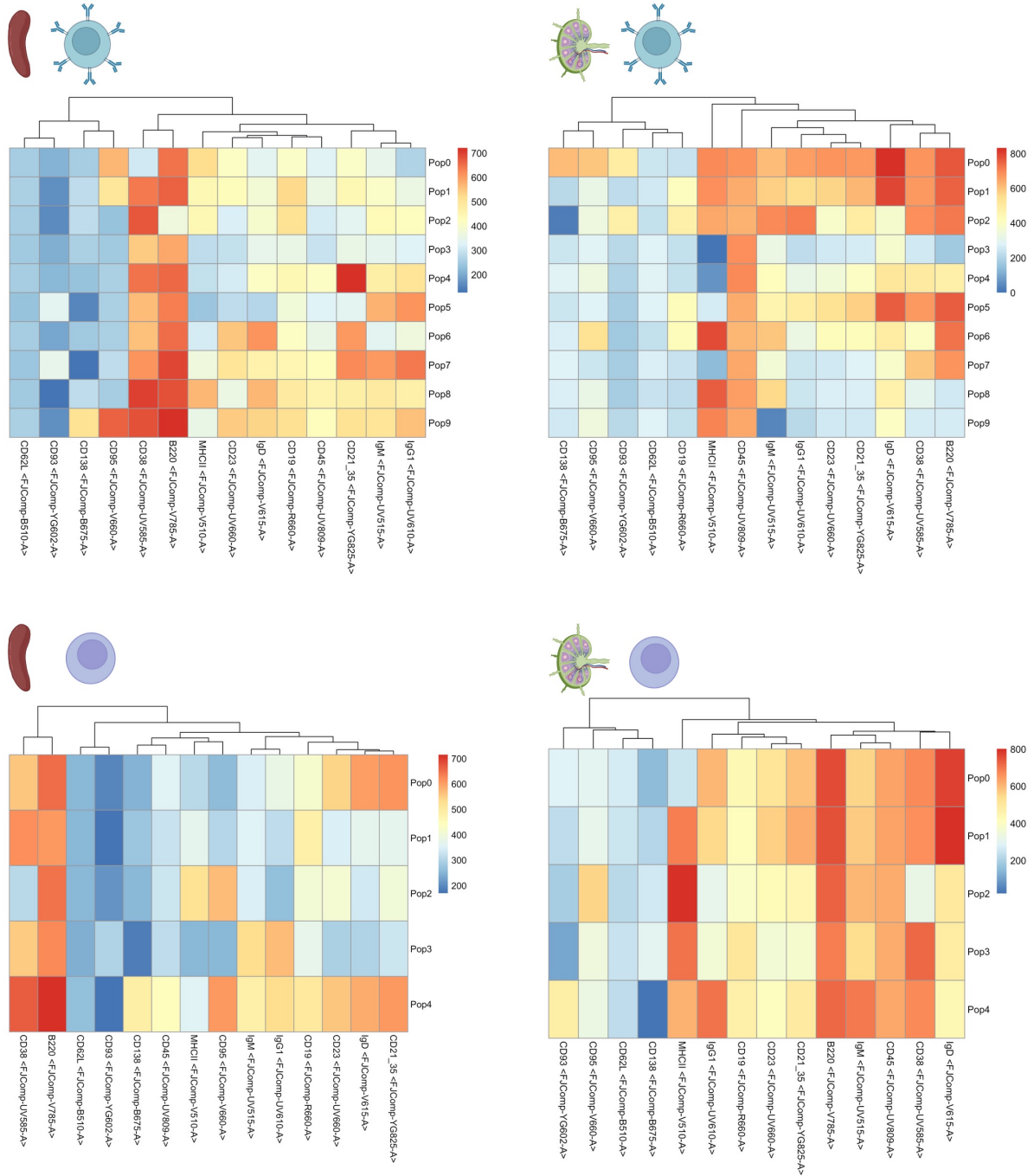


Figure 8-1: FlowSOM heat maps for tSNE cluster analysis of B and T cells shown in Figure 4-7 and Figure 4-12.

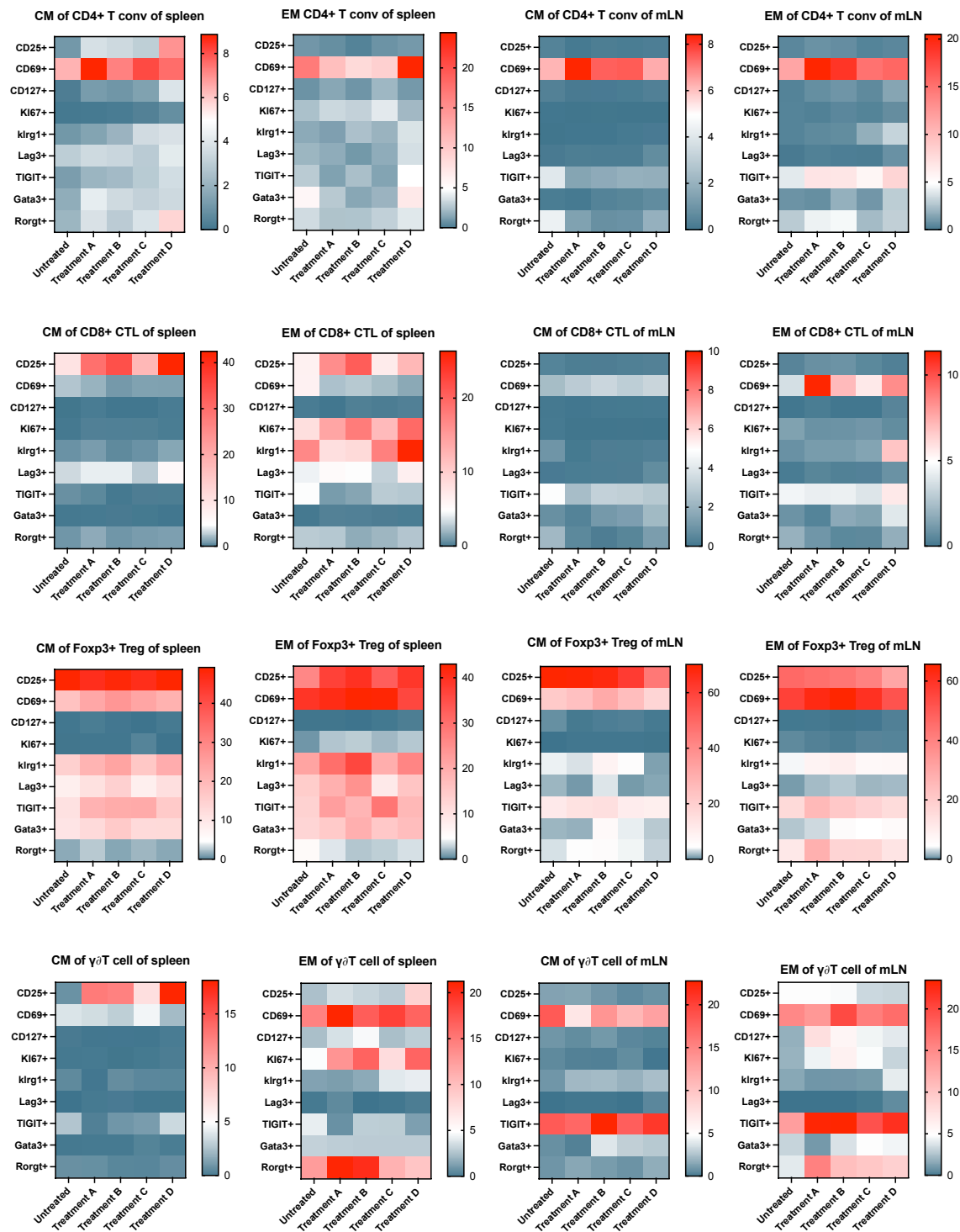


Figure 8-2: Overview of activation marker expression patterns on different T cell subsets from the spleen and mLN of untreated and vaccinated mice.

The heat map shows the percentage expression rate among each subtype. An overview was used to determine which marker should be considered for the characterization of immune responses in the different treatments.

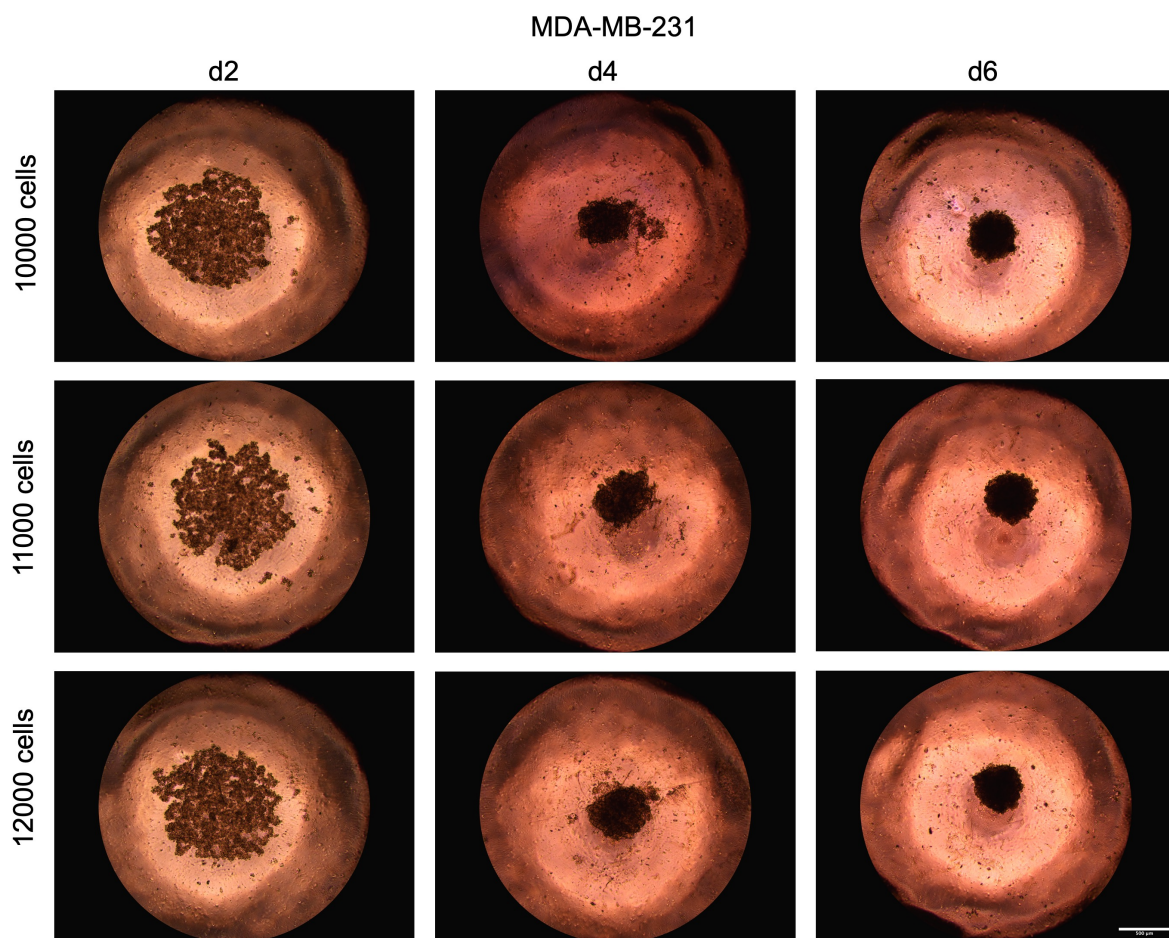


Figure 8-3: Microscopy images (Scale 16x, scale bar = 500 μ m) of spheroid formation on d2, d4, and d6 after seeding of different numbers of MDA-MD-231 cells.

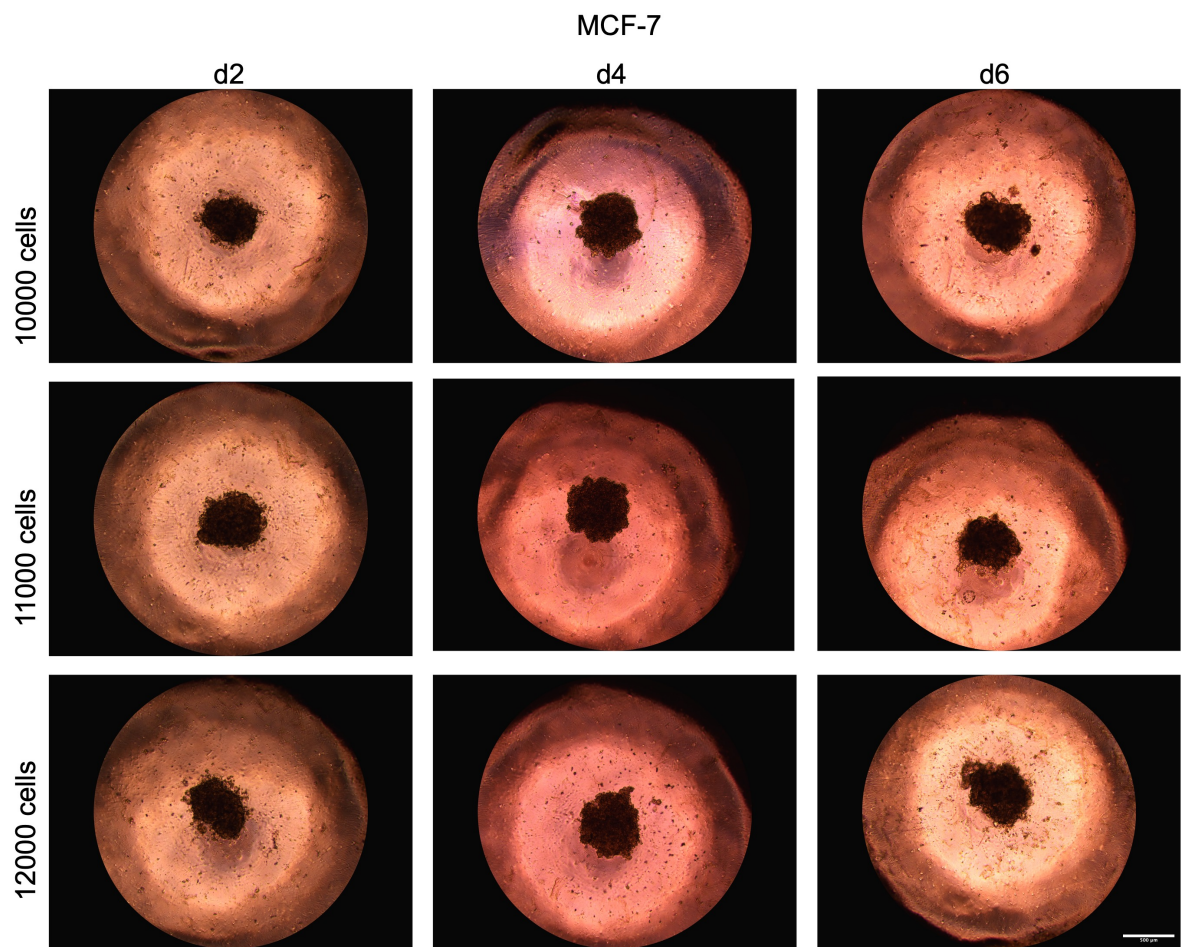


Figure 8-4: Microscopy images (Scale 16x, scale bar = 500 μ m) of spheroid formation on d2, d4, and d6 after seeding of different numbers of MCF-7 cells.

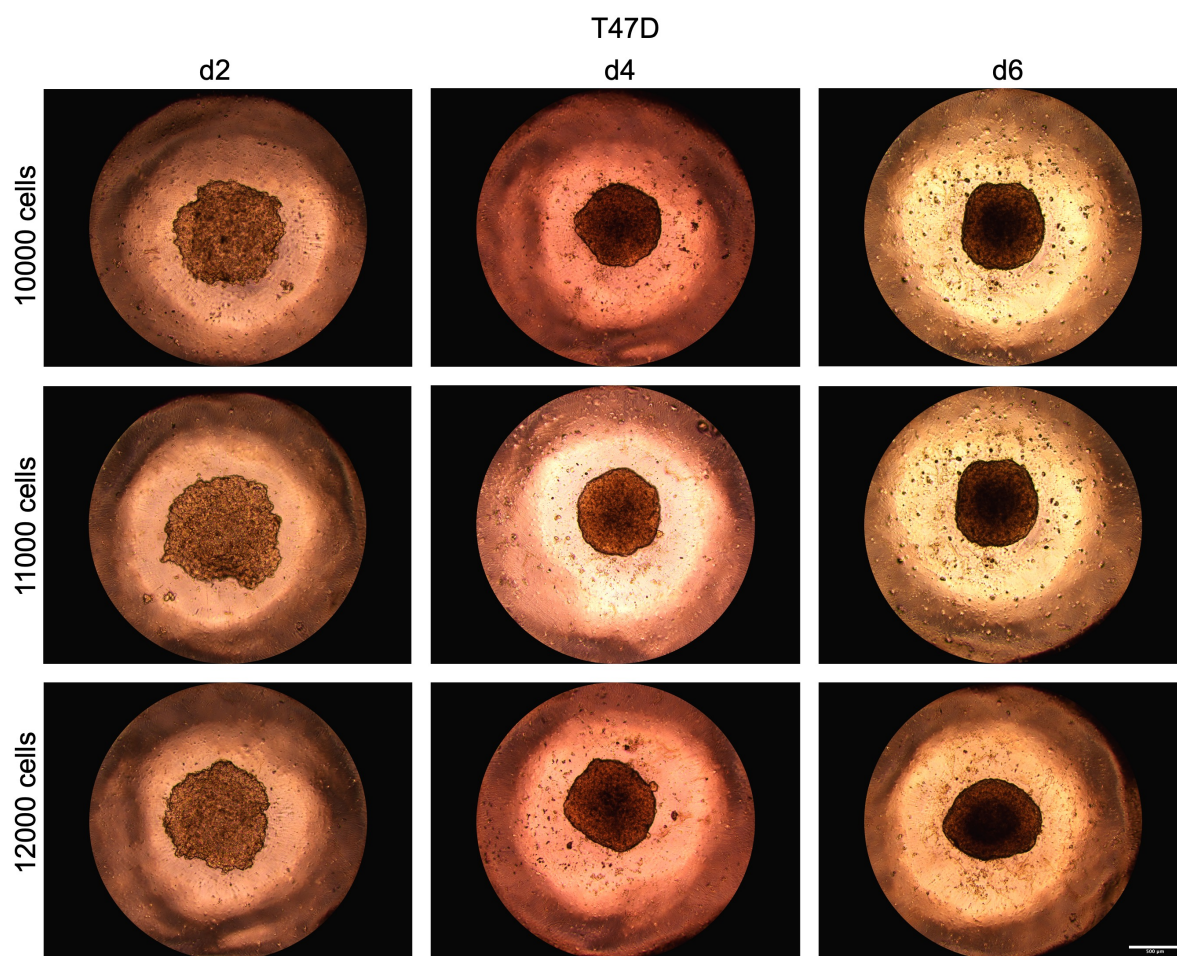


Figure 8-5: Microscopy images (Scale 16x, scale bar = 500 μ m) of spheroid formation on d2, d4, and d6 after seeding different numbers of T47D cells.

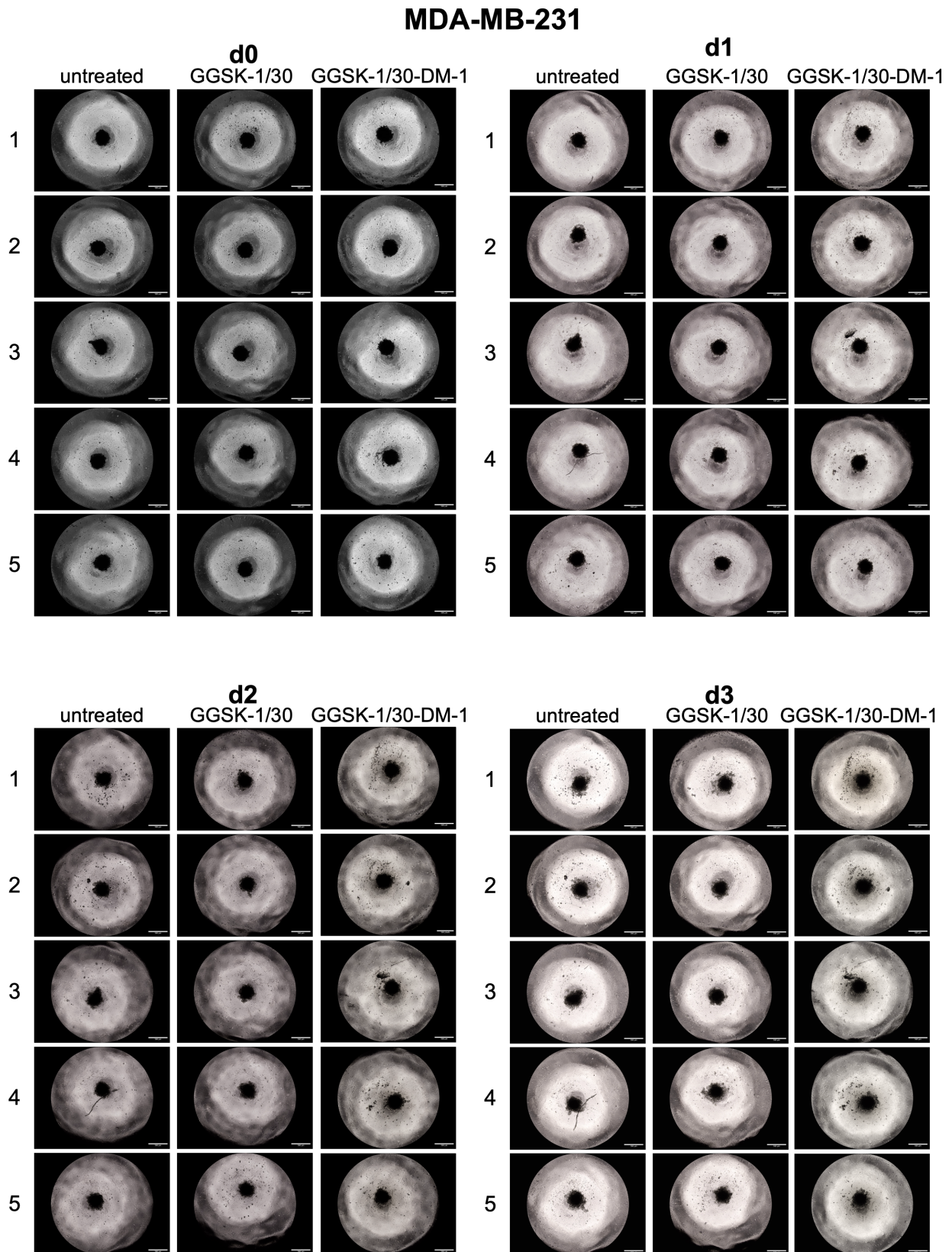


Figure 8-6:3D treatment assay of spheroids built from MDA-MB-231 human BC cells.
 Microscopic images from d0 to d3 of treatment with GGSK-1/30-DM1. Scale 16x, scale bar = 500 μ m.

MDA-MB-231

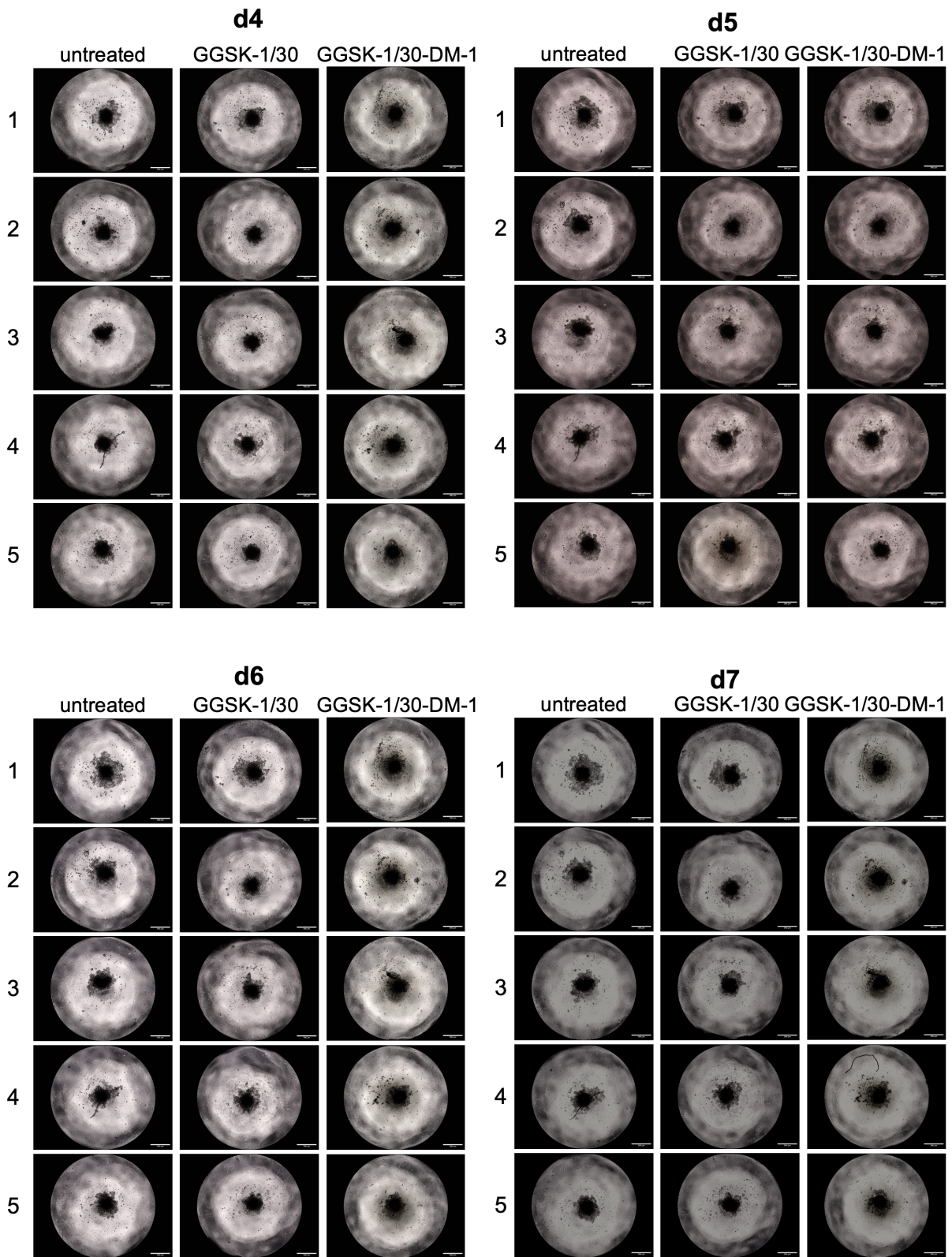


Figure 8-7:3D treatment assay of spheroids built from MDA-MB-231 human BC cells.
Microscopic images from d4 to d7 of treatment with GGSK-1/30-DM1. Scale 16x, scale bar = 500 μ m.

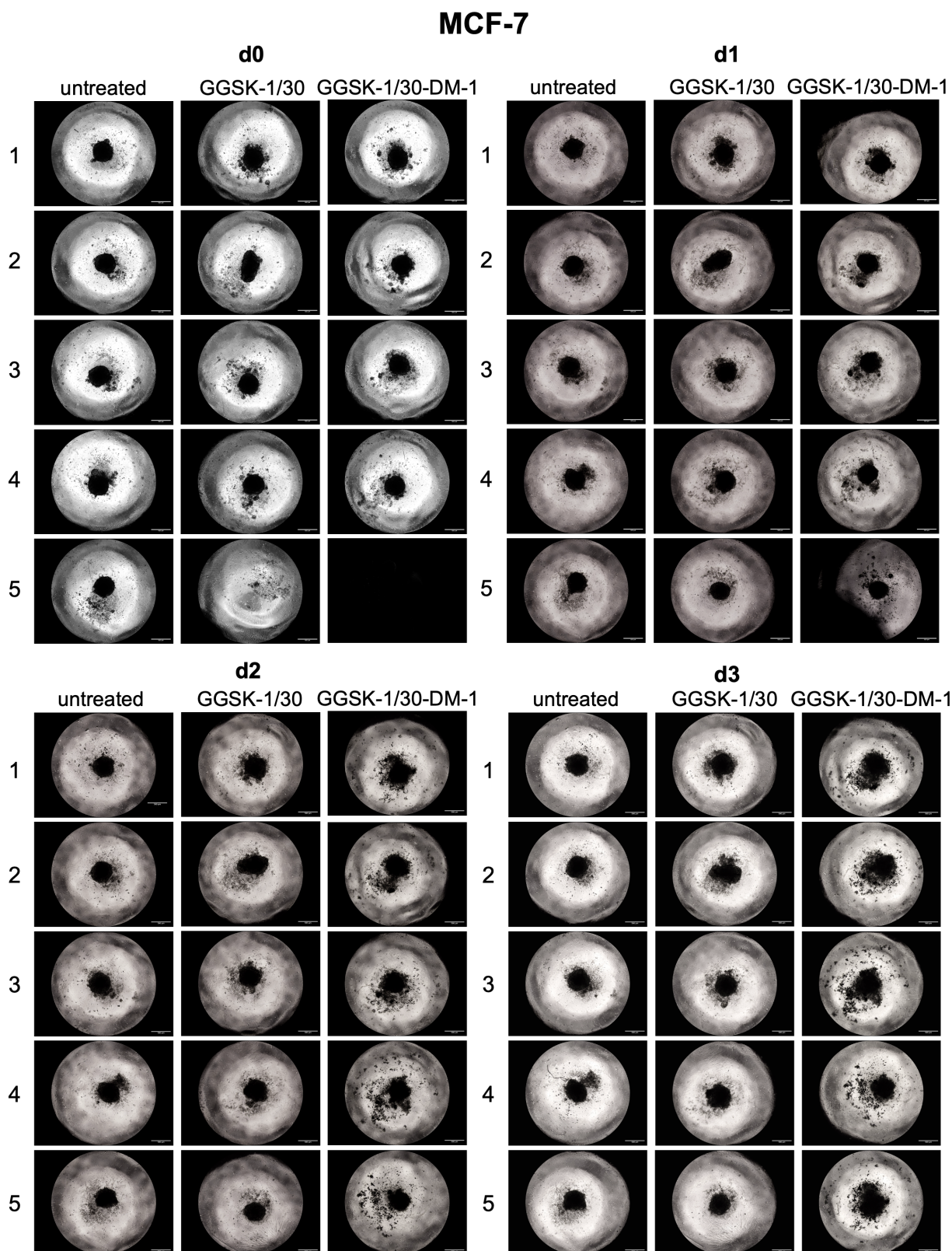


Figure 8-8:3D treatment assay of spheroids built from MCF-7 human BC cells.
 Microscopic images from d0 to d3 of treatment with GGSK-1/30-DM1. Scale 16x, scale bar = 500 μ m.

MCF-7

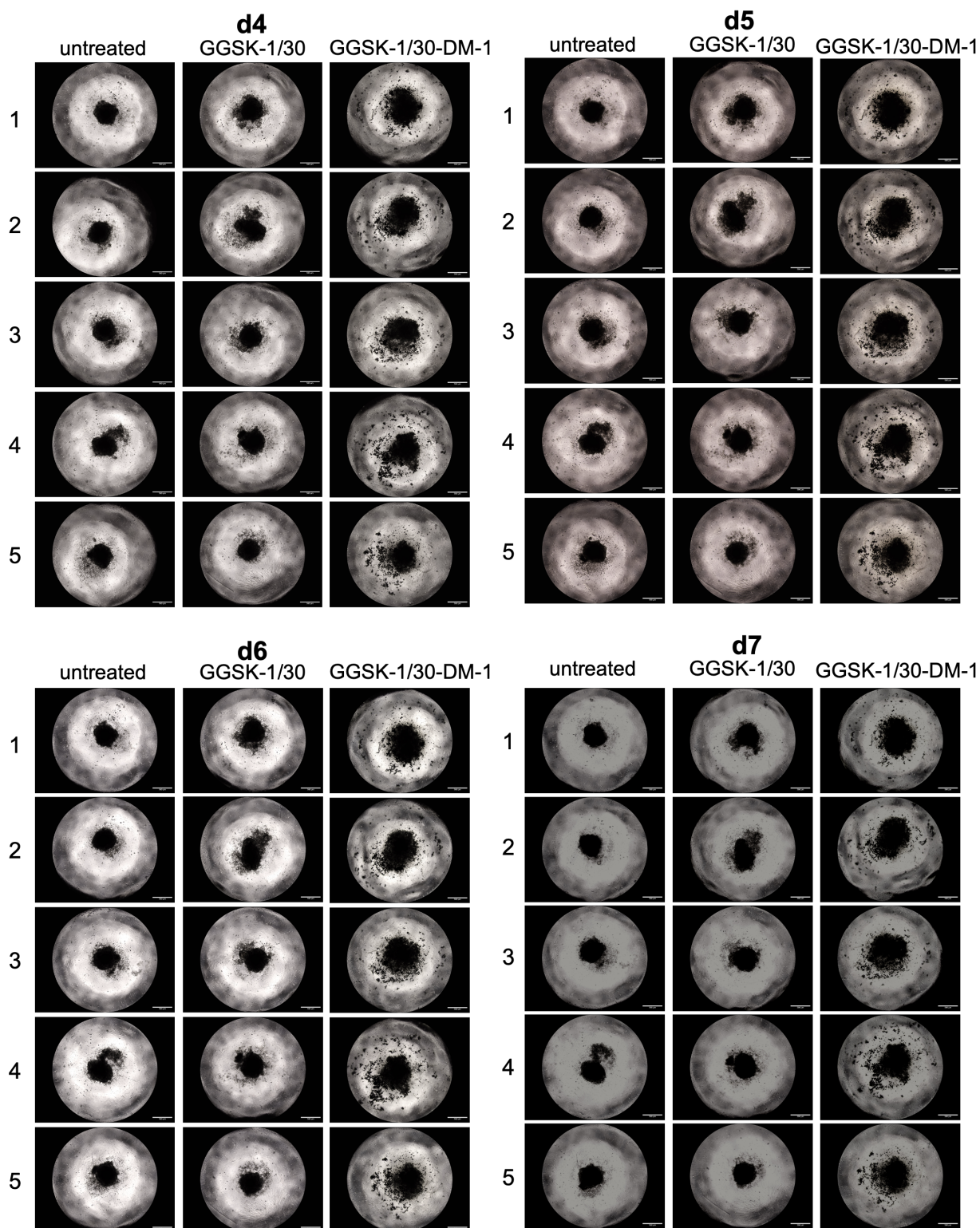


Figure 8-9:3D treatment assay of spheroids built from MCF-7 human BC cells.

Microscopic images from d4 to d7 of treatment with GGSK-1/30-DM1. Scale 16x, scale bar = 500 μ m.

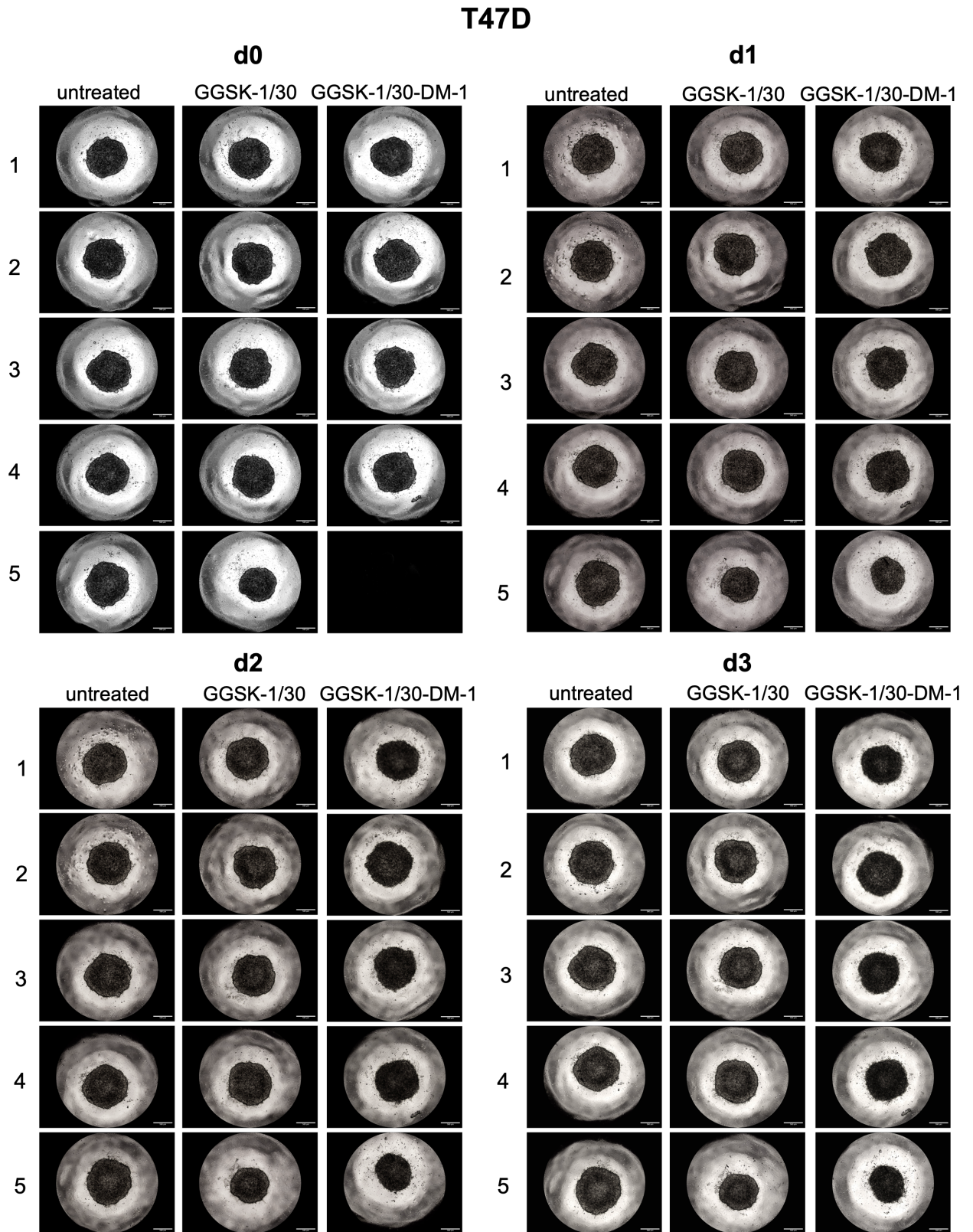


Figure 8-10:3D treatment assay of spheroids built from T47D human BC cells.
 Microscopic images from d0 to d3 of treatment with GGSK-1/30-DM1. Scale 16x, scale bar = 500 μ m.

T47D

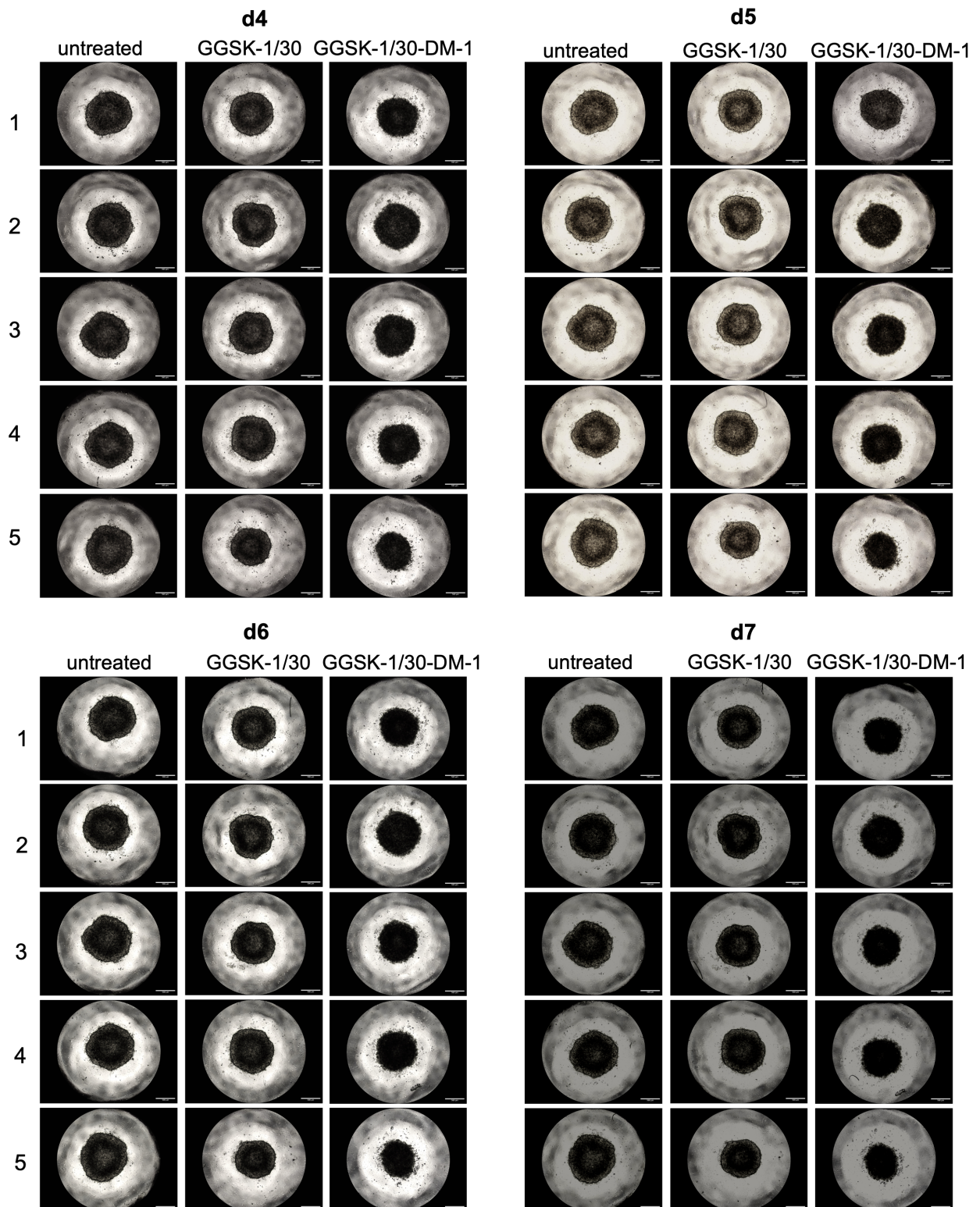


Figure 8-11:3D treatment assay of spheroids built from T47D human BC cells.

Microscopic images from d4 to d7 of treatment with GGSK-1/30-DM1. Scale 16x, scale bar = 500 μ m.

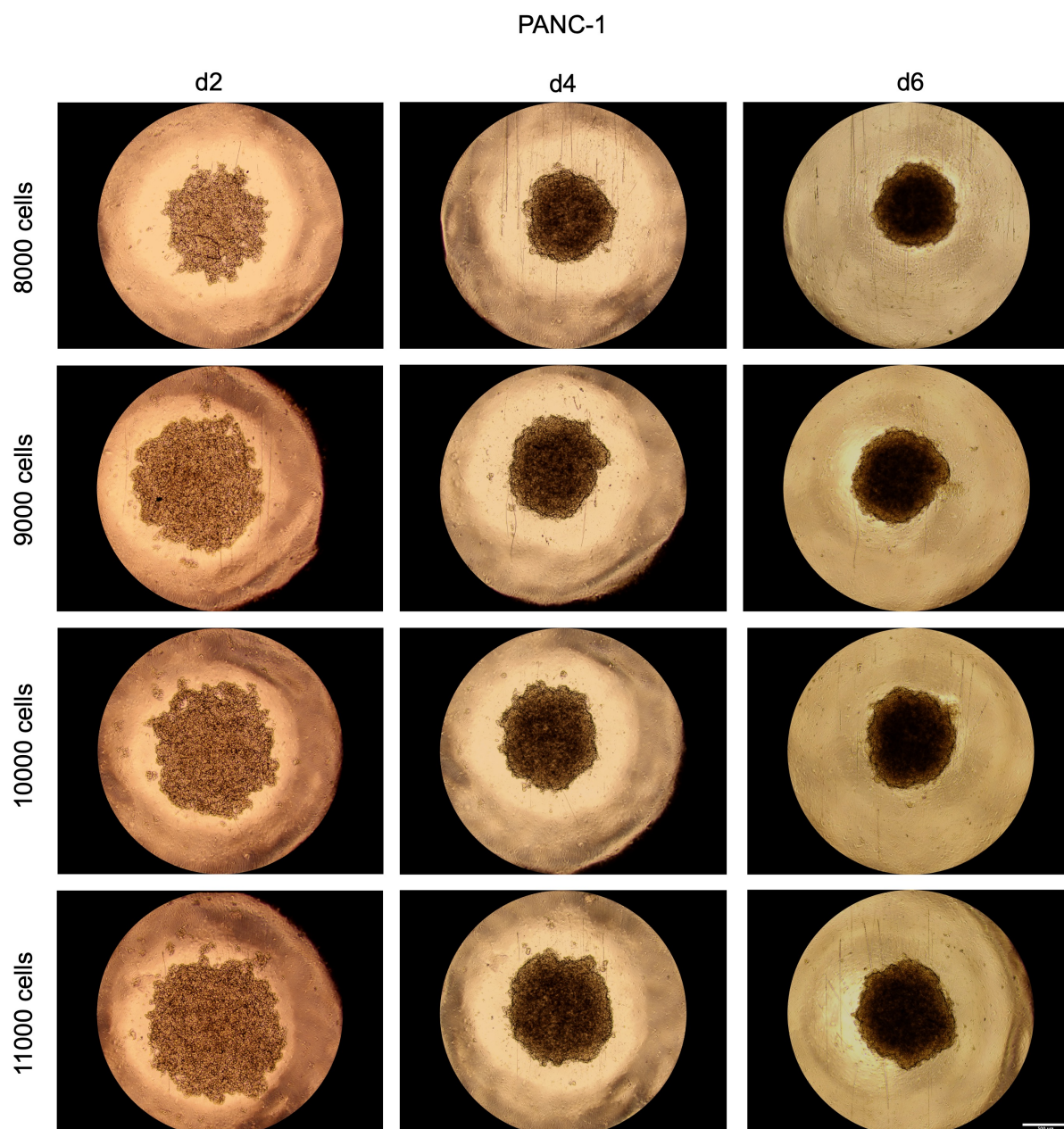


Figure 8-12: Microscopy images (Scale 16x, scale bar = 500 μ m) of spheroid formation on d2, d4, and d6 after seeding of different numbers of PANC-1 cells.

PANC-1

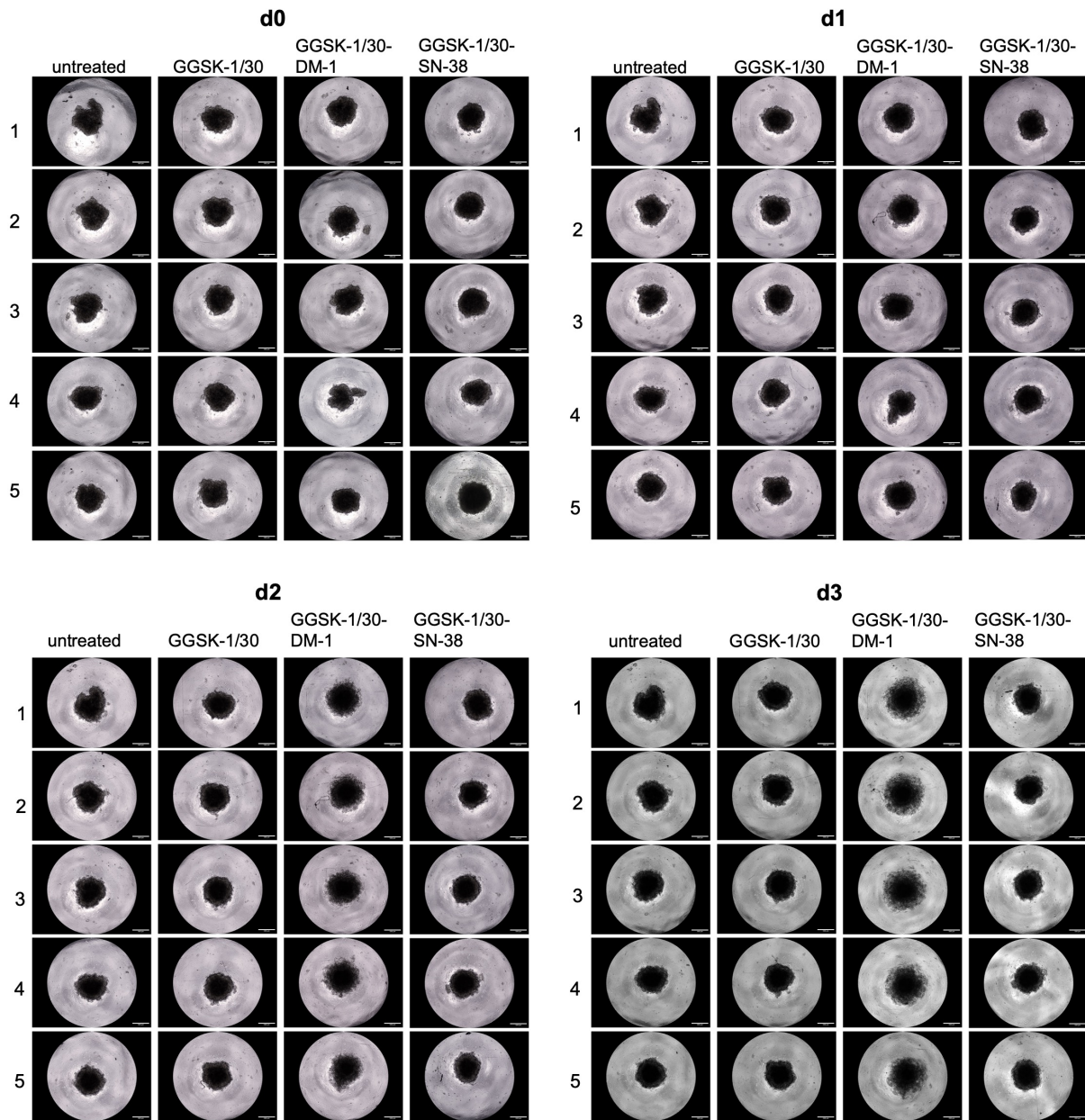


Figure 8-13:3D treatment assay of spheroids built from PANC-1 human PDAC cells.

Microscopic images from d0 to d3 of treatment with GGSK-1/30-DM1 or GGSK-1/30-SN-38. Scale 16x, scale bar = 500 μ m.

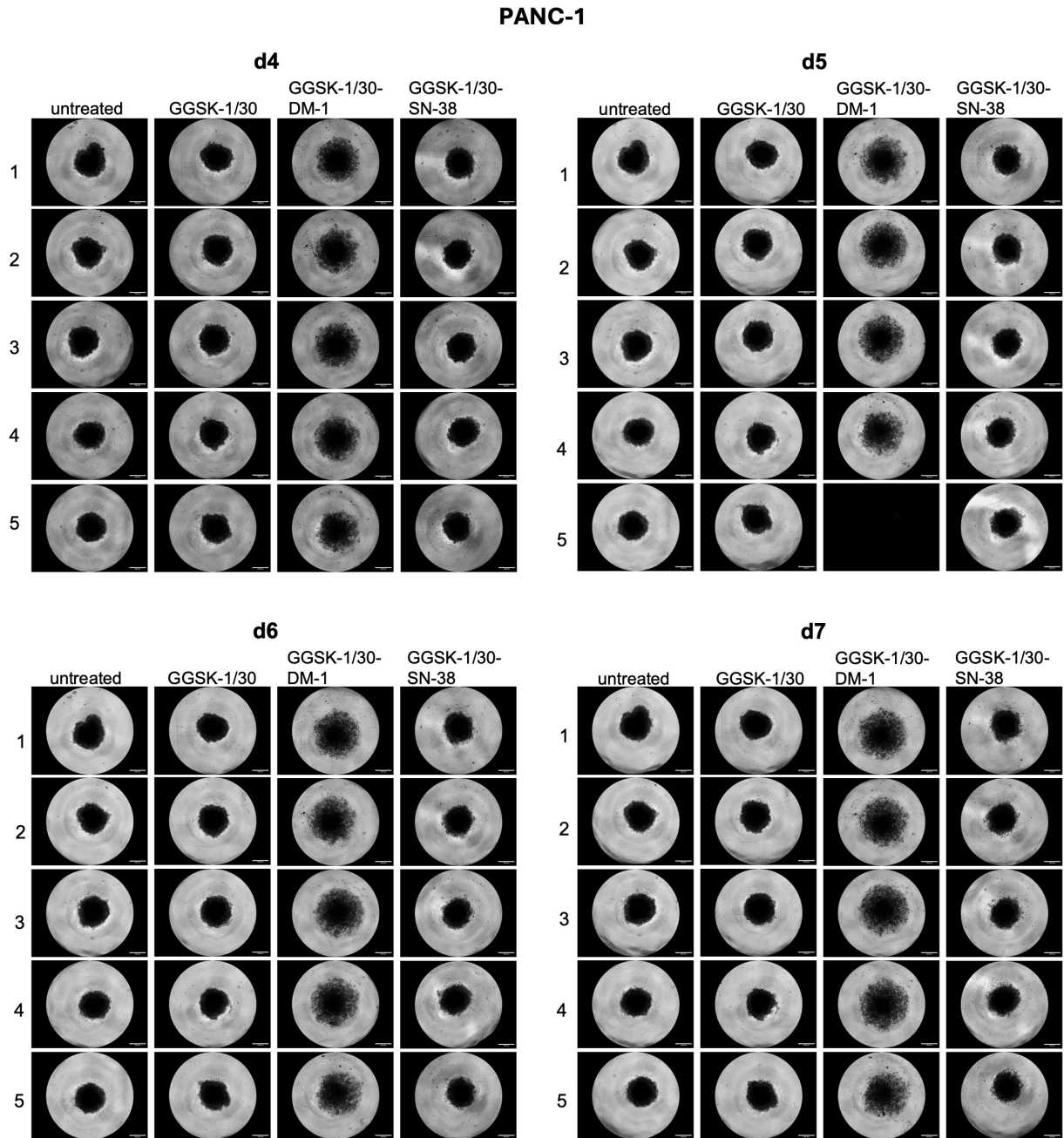


Figure 8-14:3D treatment assay of spheroids built from PANC-1 human PDAC cells.
 Microscopic images from d4 to d7 of treatment with GGSK-1/30-DM1 or GGSK-1/30-SN38. Scale 16x,
 scale bar = 500 μ m.

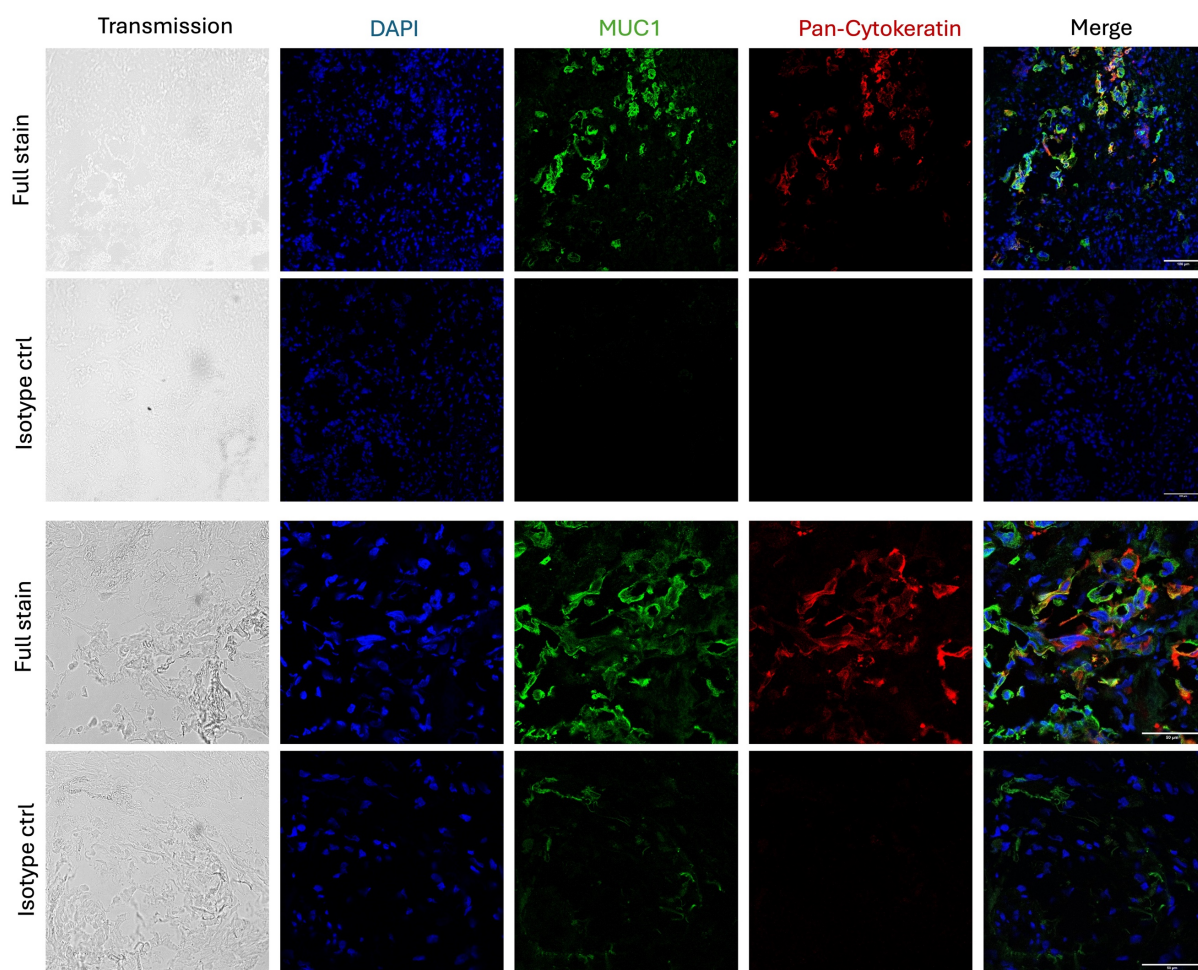


Figure 8-15 Single-channel images and isotype ctrl staining for Figure 4-34.

From left to right: transmission microscopy, fluorescence microscopy staining with DAPI (blue), GSK-1/30-AF488 for MUC1 (green), pan-cytokeratin (red), and merging of fluorescence images. The second row shows the associated isotype ctrl staining. Top: scale 20x (Scale bar = 100 μ m); bottom: scale 63x (scale bar = 50 μ m).

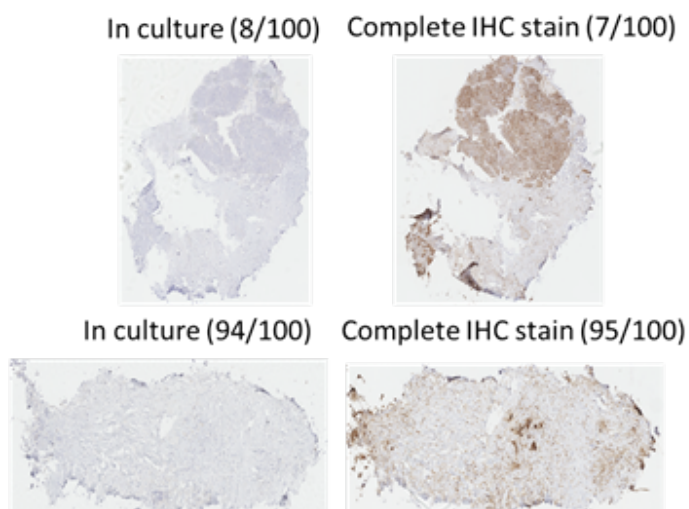


Figure 8-16: Isotype controls for IHC staining of treated PDAC samples from the tissue penetration assay.

8.2 Literature

1. Busch, W. Niederrheinische Gesellschaft für Natur- und Heilkunde in Bonn. Sitzung vom 14. März 1866. *Berliner klinische Wochenschrift* **23**, 245–246 (1866).
2. Fehleisen, F. Ueber die Züchtung der Erysipelkokken auf künstlichem Nährboden und ihre Uebertragbarkeit auf den Menschen. *Deutsche medizinische Wochenschrift* **8**, (1882).
3. Munir, M., Cheema, A. Y., Ogedegbe, O. J., Aslam, M. F. & Kim, S. William Coley: The Pioneer and the Father of Immunotherapy. *Cureus* **16**, (2024).
4. Coley, W. B. The Treatment of Inoperable Sarcoma by Bacterial Toxins (the Mixed Toxins of the Streptococcus erysipelas and the Bacillus prodigiosus). in (Surgical section, 1909).
5. Ahuja, S. & Zaheer, S. The evolution of cancer immunotherapy: a comprehensive review of its history and current perspectives. *Korean Journal of Clinical Oncology* **20**, 51–73 (2024).
6. Köhler, G. & Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495–497 (1975).
7. Köhler, G. & Milstein, C. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur J Immunol* **6**, 511–519 (1976).
8. Füreder, T. Immuntherapie in der Onkologie. *Wien Klin Wochenschr Educ* **12**, 17–33 (2017).
9. Kripp, M. Monoclonal antibodies in the fight against cancer: Around the structure, mechanism of action and therapeutic options. *CME* **10**, 63–72 (2013).
10. Schirrmacher, V. Chemotherapie und/oder Immuntherapie? **19**, 84–94 (2016).
11. Fan, T. *et al.* Therapeutic cancer vaccines: advancements, challenges and prospects. *Signal Transduct Target Ther* **8**, 1–23 (2023).
12. Sterner, R. C. & Sterner, R. M. CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer Journal* 2021 11:4 **11**, 1–11 (2021).
13. Sahin, U. *et al.* Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature* **547**, 222–226 (2017).

14. Silvestre, I. F. Transgene Expression von humanem Chondroitinsulfat-Proteoglykan 4 (CSPG4) in der murinen Melanom-Linie B16 für die Generierung eines präklinischen Maus-Tumormodells. (2021).
15. Wirth, T. C. & Kühnel, F. Neoantigen targeting - Dawn of a new era in cancer immunotherapy? *Front Immunol* **8**, 320776 (2017).
16. Nakhaie, M., Charostad, J., Kaydani, G. A. & Faghihloo, E. The role of viruses in adenocarcinoma development. (2020) doi:10.1016/j.meegid.2020.104603.
17. ELSAN. Adénocarcinome: définition, symptômes, traitements et espérance de vie. <https://www.elsan.care/fr/pathologie-et-traitement/cancers/adenocarcinome-definition-symptomes-traitements> (2024).
18. Rhiem, K. & Schmutzler, R. K. Risikofaktoren und Prävention des Mammakarzinoms. *Onkologie* **21**, 202–210 (2015).
19. Robert Koch Institute & Zentrum für Krebsregisterdaten. *Krebs - Cancer Sites*. https://www.krebsdaten.de/Krebs/EN/Content/Cancer_sites/cancer_sites_node.html (2018).
20. Orrantia-Borunda, E., Anchondo-Nuñez, P., Acuña-Aguilar, L. E., Gómez-Valles, F. O. & Ramírez-Valdespino, C. A. Subtypes of Breast Cancer. in *Breast Cancer* (ed. Mayrovitz, H.) 31–42 (Exon Publications, Brisbane, 2022). doi:10.36255/EXON-PUBLICATIONS-BREAST-CANCER-SUBTYPES.
21. Siegel, R. L., Miller, K. D. & Jemal, A. Cancer Statistics, 2020. *CA Cancer Journal* **70**, (2020).
22. Barzaman, K. *et al.* Breast cancer: Biology, biomarkers, and treatments. *Int Immunopharmacol* **84**, (2020).
23. Burstein, H. J. *et al.* Customizing local and systemic therapies for women with early breast cancer: the St. Gallen International Consensus Guidelines for treatment of early breast cancer 2021. *Annals of oncology* **32**, 1216–1235 (2021).
24. Crystal, J., Mella-Catinchi, J., Xu, K. & Weingrad, D. Current Surgical Innovations in the Treatment of Breast Cancer. in *Breast Cancer* (ed. Mayrovitz HN) 43–62 (Exon Publications, Brisbane, 2022). doi:10.36255/EXON-PUBLICATIONS-BREAST-CANCER-SURGICAL-INNOVATION.

25. Swain, S. M. *et al.* Pertuzumab, Trastuzumab, and Docetaxel in HER2-Positive Metastatic Breast Cancer. *New England Journal of Medicine* **372**, 724–734 (2015).
26. Keam, S. J. Trastuzumab Deruxtecan: First Approval. *Drugs* **80**, 501–508 (2020).
27. Martín, M. *et al.* Trastuzumab deruxtecan in breast cancer. *Crit Rev Oncol Hematol* **198**, (2024).
28. Katz, H. & Alsharedi, M. Immunotherapy in triple-negative breast cancer. *Medical oncology* **35**, (2018).
29. Flook, R. & Van Zanten, S. V. Pancreatic Cancer in Canada: Incidence and Mortality Trends from 1992 to 2005. *Can J Gastroenterol Hepatol* **23**, 546–550 (2009).
30. Vincent, A., Herman, J., Schulick, R., Hruban, R. H. & Goggins, M. Pancreatic cancer. *The Lancet* **378**, 607–620 (2011).
31. O’Kane, G. M., Ladak, F. & Gallinger, S. Avancées dans la prise en charge de l’adénocarcinome canalaire pancréatique. *Can Med Assoc J* **193**, E1362 (2021).
32. Conroy, T. *et al.* FOLFIRINOX versus Gemcitabine for Metastatic Pancreatic Cancer. *New England Journal of Medicine* **364**, 1817–1825 (2011).
33. Jiang, S. *et al.* A comprehensive review of pancreatic cancer and its therapeutic challenges. *Aging* **14**, 7635–7649 (2022).
34. American Cancer Society. *Treating Pancreatic Cancer*. <https://www.cancer.org/cancer/types/pancreatic-cancer/treating/immunotherapy.html> (2024).
35. Buchbinder, E. I. & Desai, A. CTLA-4 and PD-1 Pathways Similarities, Differences, and Implications of Their Inhibition. *Am J Clin Oncol* **39**, (2016).
36. Schizas, D. *et al.* Immunotherapy for pancreatic cancer: A 2020 update. *Cancer Treat Rev* **86**, (2020).
37. Torphy, R. J., Zhu, Y. & Schulick, R. D. Immunotherapy for pancreatic cancer: Barriers and breakthroughs. *Ann Gastroenterol Surg* **2**, 274–281 (2018).
38. Kanji, Z. S. & Gallinger, S. Diagnosis and management of pancreatic cancer. *CMAJ. Canadian Medical Association Journal* **185**, 1219–1226 (2013).

39. Fathi, M., Ghafouri-Fard, S., Abak, A. & Taheri, M. Emerging roles of miRNAs in the development of pancreatic cancer. *Biomedicine and Pharmacotherapy* **141**, (2021).
40. Kalos, M. & June, C. H. Adoptive T cell Transfer for Cancer Immunotherapy in the Era of Synthetic Biology. *Immunity* **39**, 10.1016/j.immuni.2013.07.002 (2013).
41. Striefler, J. K. *et al.* Mucin-1 Protein Is a Prognostic Marker for Pancreatic Ductal Adenocarcinoma: Results From the CONKO-001 Study. *Front Oncol* **11**, 670396 (2021).
42. Balmaña, M. *et al.* Analysis of sialyl-Lewis x on MUC5AC and MUC1 mucins in pancreatic cancer tissues. *Int J Biol Macromol* **112**, 33–45 (2018).
43. Lo, S., Pantazopoulos, P., Medarova, Z. & Moore, A. Presentation of underglycosylated mucin 1 in pancreatic adenocarcinoma (PDAC) at early stages. *Am J Cancer Res* **6**, (2016).
44. The Human Protein Atlas. MUC1. <https://www.proteinatlas.org/ENSG00000185499-MUC1>.
45. Matsui, H. *et al.* Postoperative Adjuvant Therapy for Resectable Pancreatic Cancer With Gemcitabine and Adoptive Immunotherapy. *Pancreas* **46**, 994–1002 (2017).
46. Corfield, A. P. Mucins: A biologically relevant glycan barrier in mucosal protection. *Biochim Biophys Acta Gen Subj* **1850**, 236–252 (2015).
47. Gendler, S. J. MUC1, The renaissance molecule. *J Mammary Gland Biol Neoplasia* **6**, 339–353 (2001).
48. Rakha, E. A. *et al.* Expression of mucins (MUC1, MUC2, MUC3, MUC4, MUC5AC and MUC6) and their prognostic significance in human breast cancer. *Modern Pathology* **18**, 1295–1304 (2005).
49. McGuckin, M. A., Walsh, M. D., Hohn, B. G., Ward, B. G. & Wright, R. G. Prognostic significance of muc1 epithelial mucin expression in breast cancer. *Hum Pathol* **26**, 432–439 (1995).
50. Attariya, R. Evaluation of an Armed Monoclonal Antibody against MUC1 for Immunotherapy of Breast Cancer. (2023).

51. Qing, L., Li, Q. & Dong, Z. MUC1: An emerging target in cancer treatment and diagnosis. *Bull cancer* **109**, (2022).
52. Lan, Y., Ni, W. & Tai, G. Expression of MUC1 in different tumours and its clinical significance (Review). *Mol Clin Oncol* **17**, (2022).
53. Radziejewska, I. Tumor-associated carbohydrate antigens of MUC1-Implication in cancer development. *Int J Biol Macromol* **174**, (2024).
54. Dréau, D. *et al.* Combining the specific anti-MUC1 antibody TAB004 and LIP-MSA-IL-2 limits pancreatic cancer progression in immune competent murine models of pancreatic ductal adenocarcinoma. *Front Oncol* **9**, 330 (2019).
55. Hosseinzadeh, A., Merikhian, P., Naseri, N., Eisavand, M. R. & Farahmand, L. MUC1 is a potential target to overcome trastuzumab resistance in breast cancer therapy. *Cancer Cell Int* **22**, (2022).
56. Hiraki, M. *et al.* Targeting MUC1-C suppresses BCL2A1 in triple-negative breast cancer. *Signal Transduct Target Ther* **3**, (2018).
57. Fan, T. *et al.* Therapeutic cancer vaccines: advancements, challenges and prospects. *Signal Transduction and Targeted Therapy* 2023 8:1 **8**, 1–23 (2023).
58. Budd, G. T. *et al.* Phase I trial of an alpha-lactalbumin vaccine in patients with moderate- to high-risk operable triple-negative breast cancer (TNBC). *Journal of Clinical Oncology* **40**, TPS1125–TPS1125 (2022).
59. Mittendorf, E. A., Holmes, J. P., Ponniah, S. & Peoples, G. E. The E75 HER2/neu peptide vaccine. *Cancer Immunology, Immunotherapy* **57**, 1511–1521 (2008).
60. Mittendorf, E. A. *et al.* Final report of the phase I/II clinical trial of the E75 (nelipepimut-S) vaccine with booster inoculations to prevent disease recurrence in high-risk breast cancer patients. *Annals of Oncology* **25**, 1735 (2014).
61. Tang, C. K. *et al.* Oxidized and reduced mannan mediated MUC1 DNA immunization induce effective anti-tumor responses. *Vaccine* **26**, 3827–3834 (2008).
62. Vassilaros, S. *et al.* Up to 15-year Clinical Follow-Up of a Pilot Phase III Immunotherapy Study in Stage II Breast Cancer Patients Using Oxidized Mannan–MUC1. *Immunotherapy* **5**, 1177–1182 (2013).

63. Liu, C. *et al.* Fast DNA Vaccination Strategy Elicits a Stronger Immune Response Dependent on CD8⁺CD11c⁺ Cell Accumulation. *Front Oncol* **11**, (2021).
64. Liu, L. *et al.* Combination Immunotherapy of MUC1 mRNA Nano-vaccine and CTLA-4 Blockade Effectively Inhibits Growth of Triple Negative Breast Cancer. *Molecular Therapy* **26**, 45–55 (2018).
65. Ryan, S. O., Turner, M. S., Gariépy, J. & Finn, O. J. Tumor antigen epitopes interpreted by the immune system as self or abnormal-self differentially affect cancer vaccine responses. *Cancer Res* **70**, 5788 (2010).
66. Palitzsch, B. Entwicklung synthetischer Antitumor-Vakzine nach dem Vorbild der tumorassoziierten Struktur des Mucins MUC1 und deren immunologische Evaluierung. (2014).
67. Palitzsch, B. *et al.* Antitumor Vaccines A Fully Synthetic Four-Component Antitumor Vaccine Consisting of a Mucin Glycopeptide Antigen Combined with Three Different T-Helper-Cell Epitopes. *Angewandte Chemie* **53**, (2014).
68. Cai, H. *et al.* Antibody Induction Directed against the Tumor-Associated MUC4 Glycoprotein. *ChemBioChem* **16**, 959–967 (2015).
69. Glaffig, M., Stergiou, N., Schmitt, E. & Kunz, H. Immunogenicity of a Fully Synthetic MUC1 Glycopeptide Antitumor Vaccine Enhanced by Poly(I:C) as a TLR3-Activating Adjuvant. *ChemMedChem* **12**, 722–727 (2017).
70. Stergiou, N. *et al.* The Development of Vaccines from Synthetic Tumor-Associated Mucin Glycopeptides and their Glycosylation-Dependent Immune Response. *The chemical record* **21**, (2021).
71. Stergiou, N. Verwendung von Tumor-assoziiertem Mucin 1 zur Generierung von Anti-Tumor-Vakzinen und monoklonalen Antikörper-Derivaten als Basis für Therapie und Diagnostik. (2018).
72. Kaiser, A. *et al.* A synthetic vaccine consisting of a tumor-associated sialyl-T(N)-MUC1 tandem-repeat glycopeptide and tetanus toxoid: induction of a strong and highly selective immune response. *Angew Chem Int Ed Engl* **48**, 7551–7555 (2009).

73. Stergiou, N. *et al.* Reduced Breast Tumor Growth after Immunization with a Tumor-Restricted MUC1 Glycopeptide Conjugated to Tetanus Toxoid. *Cancer Immunol Res* **7**, (2019).
74. Seladi-Schulman, J. & Slowiczek, L. Tetanus Shot Reactions: Side Effects of the Vaccine. <https://www.healthline.com/health/vaccinations/tetanus-shot-side-effects> (2023).
75. Straßburger, D. *et al.* Mannose-Decorated Multicomponent Supramolecular Polymers Trigger Effective Uptake into Antigen-Presenting Cells. *ChemBioChem* **19**, 912–916 (2018).
76. Sievers, E. L. & Senter, P. D. Antibody-drug conjugates in cancer therapy. *Annu Rev Med* **64**, 15–29 (2013).
77. Vezina, H. E., Cotreau, M., Han, T. H. & Gupta, M. Antibody–Drug Conjugates as Cancer Therapeutics: Past, Present, and Future. *The Journal of Clinical Pharmacology* **57**, S11–S25 (2017).
78. Jain, N., Smith, S. W., Ghone, S. & Tomczuk, B. Current ADC Linker Chemistry. *Pharm Res* **32**, 3526–3540 (2015).
79. Bargh, J. D., Isidro-Llobet, A., Parker, J. S. & Spring, D. R. Chem Soc Rev Cleavable linkers in antibody-drug conjugates. *Chem. Soc. Rev* **48**, 4361 (2019).
80. Fu, Z., Li, S., Han, S., Shi, C. & Zhang, Y. Antibody drug conjugate: the “biological missile” for targeted cancer therapy. *Signal Transduction and Targeted Therapy* **2022 7:1 7**, 1–25 (2022).
81. Fu, Z., Li, S., Han, S., Shi, C. & Zhang, Y. Antibody drug conjugate: the “biological missile” for targeted cancer therapy. *Signal Transduction and Targeted Therapy* **2022 7:1 7**, 1–25 (2022).
82. Verma, S. *et al.* Trastuzumab Emtansine for HER2-Positive Advanced Breast Cancer. *New England Journal of Medicine* **367**, 1783–1791 (2012).
83. Liu, X. & Gao, W. Antibody–Drug Conjugates. in *Biomedical Nanomaterials* (ed. Yuliang Zhao, Y. S.) 149–176 (John Wiley & Sons, Ltd, Weinheim, 2016). doi:10.1002/9783527694396.CH6.

84. Loganzo, F., Sung, M. & Gerber, H.-P. Mechanisms of Resistance to Antibody-Drug Conjugates. *Mol Cancer Ther* **15**, (2016).
85. Zhu, Y., Liu, K., Wang, K. & Zhu, H. Treatment-related adverse events of antibody–drug conjugates in clinical trials: A systematic review and meta-analysis. *Cancer* **129**, 283–295 (2023).
86. Taylor, R. P. & Lindorfer, M. A. Antibody-drug conjugate adverse effects can be understood and addressed based on immune complex clearance mechanisms. *Blood* **144**, 137–144 (2024).
87. Diehl, K.-H. *et al.* A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes. *J. Appl. Toxicol* **21**, 15–23 (2001).
88. Fernández, I., Arantza, P., Del Teso, N., Perez, V. & Rodriguez-Cuesta, J. Clinical Biochemistry Parameters in C57BL/6J Mice after Blood Collection from the Submandibular Vein and Retroorbital Plexus. *Journal of the American Association for Laboratory Animal Science* **49**, (2010).
89. Hawley, T. S. & Hawley, R. G. *Flow Cytometry Protocols. Methods in Molecular Biology* vol. 699 (Springer protocols, 2011).
90. Radbruch, A. *Flow Cytometry and Cell Sorting*. (Springer Berlin Heidelberg, Berlin, Heidelberg, 2000). doi:10.1007/978-3-662-04129-1.
91. Stergiou, N., Glaffig, M., Jonuleit, H., Schmitt, E. & Kunz, H. Immunization with a Synthetic Human MUC1 Glycopeptide Vaccine against Tumor-Associated MUC1 Breaks Tolerance in Human MUC1 Transgenic Mice. **12**, 14241428 (2017).
92. Straßburger, D. *et al.* Supramolecular polymerization of sulfated dendritic peptide amphiphiles into multivalent L-selectin binders. *Beilstein Journal of Organic Chemistry* **17**, 97 (2021).
93. Gabba, A. *et al.* MUC1 Glycopeptide Vaccine Modified with a GalNAc Glycocluster Targets the Macrophage Galactose C-type Lectin on Dendritic Cells to Elicit an Improved Humoral Response. *J Am Chem Soc* **145**, 13027–13037 (2023).
94. Moritz Urschbach. Entwicklung supramolekularer Multikomponenten-Copolymere zum Aufbau depotbildender Glycokonjugatvakzine. (Mainz, 2022).

95. Marius Thomas. Untersuchung der Kinetik und Dynamik der Selbstassemblierung supramolekularer Peptidamphiphile. (Mainz, 2024).
96. Pedersen, G. K., Wørzner, K., Andersen, P. & Christensen, D. Vaccine Adjuvants Differentially Affect Kinetics of Antibody and Germinal Center Responses. *Front Immunol* **11**, 579761 (2020).
97. Ishikawa, H. & Barber, G. N. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* **455**, 674–678 (2008).
98. Ishikawa, H., Ma, Z. & Barber, G. N. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* **461**, 788–792 (2009).
99. Towner, L. D. *et al.* Synthetic STING agonists elicit powerful vaccine adjuvancy providing robust central memory and anti-tumour effects. *bioRxiv* (2023) doi:10.1101/2023.01.04.522614.
100. Belkina, A. C. *et al.* Automated optimized parameters for T-distributed stochastic neighbor embedding improve visualization and analysis of large datasets. *Nat Commun* **10**, (2019).
101. Van Gassen, S. *et al.* FlowSOM: Using self-organizing maps for visualization and interpretation of cytometry data. *Cytometry Part A* **87**, 636–645 (2015).
102. Atisha-Fregoso, Y., Zou, Y. R. & Diamond, B. B Cells and Generation of Antibodies. *Dubois' Lupus Erythematosus and Related Syndromes* 101–115 (2019) doi:10.1016/B978-0-323-47927-1.00009-8.
103. Fairfax, K. A., Kallies, A., Nutt, S. L. & Tarlinton, D. M. Plasma cell development: From B-cell subsets to long-term survival niches. *Semin Immunol* **20**, 49–58 (2008).
104. Clinicaltrials.gov. *Trastuzumab and Irinotecan in Treating Patients With HER2/Neu Positive Metastatic Breast Cancer*. <https://clinicaltrials.gov/study/NCT00303992?cond=breast%20cancer&intr=Trastuzumab-sn38&sort=StudyFirstPostDate&limit=100&rank=3> (2018).
105. Puschacher, B. & Dirschlmayer, W. Langzeitüberleben nach Resektion einer Hirnmetastase bei HER2+ Mamma CA unter Trastuzumab-Emtansin (TDM 1). in *Geburtshilfe und Frauenheilkunde* vol. 78 29 (Georg Thieme Verlag KG, 2018).

106. Ballantyne, A. & Dhillon, S. Trastuzumab emtansine: First global approval. *Drugs* **73**, 755–765 (2013).
107. G. von Minckwitz, C.-S. Huang, M.S. Mano & C.E. Geyer, J. Trastuzumab Emtansine for Residual Invasive HER2-Positive Breast Cancer. *N Engl J Med* **380**, (2019).
108. Goto, K. *et al.* Trastuzumab Deruxtecan in Patients with HER2-Mutant Metastatic Non-Small-Cell Lung Cancer: Primary Results from the Randomized, Phase II DESTINY-Lung02 Trial. *Journal of Clinical Oncology* **41**, 4852–4863 (2023).
109. Lambert, J. M. & Chari, R. V. J. Ado-trastuzumab Emtansine (T-DM1): An Antibody–Drug Conjugate (ADC) for HER2-Positive Breast Cancer. *Journal of Medical Chemistry* **57**, 6949–6964 (2014).
110. Altschuler, Y. *et al.* Clathrin-mediated Endocytosis of MUC1 Is Modulated by Its Glycosylation State. *Mol Biol Cell* **11**, 819 (2000).
111. Vincerx—next-generation antibody–drug conjugates with enhanced therapeutic windows. <https://www.nature.com/articles/d43747-021-00011-3>.
112. Lerchen, H. G. *et al.* Discovery of VIP236, an $\alpha\beta3$ -Targeted Small-Molecule–Drug Conjugate with Neutrophil Elastase-Mediated Activation of 7-Ethyl Camptothecin Payload for Treatment of Solid Tumors. *Cancers (Basel)* **15**, (2023).
113. Lerchen, H.-G. *et al.* Abstract 228: KSPi-ADCs: ADCs with novel kinesin spindle protein inhibitor payloads and a tailor-made linker chemistry. *Cancer Res* **79**, 228–228 (2019).
114. Lerchen, H. G. *et al.* Antibody-Prodrug Conjugates with KSP Inhibitors and Legumain-Mediated Metabolite Formation. *Chemistry* **25**, 8208–8213 (2019).
115. Chavez, K. J., Garimella, S. V & Lipkowitz, S. Triple negative breast cancer cell lines: One tool in the search for better treatment of triple negative breast cancer. *Breast Dis* **32**, 35–48 (2010).
116. ATCC. MDA-MB-231 - HTB-26. <https://www.atcc.org/products/htb-26#product-permits>.
117. Hegde, S. M. *et al.* Interplay of nuclear receptors (ER, PR, and GR) and their steroid hormones in MCF-7 cells. *Mol Cell Biochem* **422**, 109–120 (2016).

118. Aka, J. A. & Lin, S.-X. Comparison of Functional Proteomic Analyses of Human Breast Cancer Cell Lines T47D and MCF7. *PLoS One* **7**, 31532 (2012).
119. Lee, S. Y. *et al.* Regulation of Tumor Progression by Programmed Necrosis. *Oxid Med Cell Longev* (2018) doi:10.1155/2018/3537471.
120. TCGA research network. The Cancer Genome Atlas (TCGA) database. <https://www.cancer.gov/ccg/research/genome-sequencing/tcga>.
121. Murphy, Kenneth., Weaver, Casey. & Janeway, Charles. *Janeway's Immunobiology*. (Garland Science, 2017).
122. Dingeramn, T. & Zündorf, I. Toleranzinduktion als Option auf Heilung. *Pharmazeutische Zeitung* vol. 23 (2018).
123. Li, W., Meng, J., Ma, X., Lin, J. & Lu, X. Advanced materials for the delivery of vaccines for infectious diseases. *Biosaf Health* **4**, 95–104 (2022).
124. Roth, G. A. *et al.* Injectable hydrogels for sustained codelivery of subunit vaccines enhance humoral immunity. *ACS Cent Sci* **6**, 1800–1812 (2020).
125. Schaeffeler, E., Walz, J. & Schwab, M. Personalized cancer vaccination is emerging: lessons learnt from renal cancer and challenges for broader application. *Signal Transduction and Targeted Therapy* **2025 10:1 10**, 1–3 (2025).
126. Montin, D. *et al.* Towards personalized vaccines. *Front Immunol* **15**, 1436108 (2024).
127. Westphalen, C. B. & Olive, K. P. Genetically Engineered Mouse Models of Pancreatic Cancer. *Cancer J* **18**, 502 (2012).
128. Abbas, Z. N., Al-Saffar, A. Z., Jasim, S. M. & Sulaiman, G. M. Comparative analysis between 2D and 3D colorectal cancer culture models for insights into cellular morphological and transcriptomic variations. *Scientific Reports* | **13**, 18380 (123AD).
129. Barbaro, K. *et al.* Evaluation of estrogenic and anti-estrogenic activity of endocrine disruptors using breast cancer spheroids: a comparative study of T47D and MCF7 cell lines in 2D and 3D models. *Frontiers in toxicology* **7**, (2025).

130. Wang, J. *et al.* Tumor organoid-immune co-culture models: exploring a new perspective of tumor immunity. *Cell Death Discovery* 2025 11:1 11, 1–17 (2025).
131. Perucca, A. *et al.* Micro Immune Response On-chip (MIRO) models the tumour-stroma interface for immunotherapy testing. *Nature Communications* 2025 16:1 16, 1–20 (2025).
132. Thorel, L. *et al.* Patient-derived tumor organoids: a new avenue for preclinical research and precision medicine in oncology. *Experimental & Molecular Medicine* 2024 56:7 56, 1531–1551 (2024).
133. Beck, A., Goetsch, L., Dumontet, C. & Corvaia, N. Strategies and challenges for the next generation of antibody–drug conjugates. *Nature Reviews Drug Discovery* 2017 16:5 16, 315–337 (2017).
134. He, L. & Deng, C. Recent advances in organotypic tissue slice cultures for anticancer drug development. *Int. J. Biol. Sci* 2022,.
135. Martin, S. Z. *et al.* Ex vivo tissue slice culture system to measure drug-response rates of hepatic metastatic colorectal cancer. doi:10.1186/s12885-019-6270-4.
136. Ju, Y. *et al.* Barriers and opportunities in pancreatic cancer immunotherapy. *npj Precision Oncology* 2024 8:1 8, 1–18 (2024).
137. Krishnamurti, U. & Silverman, J. F. HER2 in breast cancer: A review and update. *Adv Anat Pathol* 21, 100–107 (2014).
138. Stergiou, N. *et al.* Evaluation of a novel monoclonal antibody against tumor-associated MUC1 for diagnosis and prognosis of breast cancer. *Int J Med Sci* 16, 1188 (2019).
139. Wuensche, T. E. *et al.* Advancing 89Zr-immuno-PET in neuroscience with a bispecific anti-amyloid-beta monoclonal antibody – The choice of chelator is essential. *Theranostics* 2022, 16 (2022).

8.3 Index of figures

Figure 1-1: Frequency of cancer types in the percentage of newly diagnosed cancer cases in Germany in 2018.	3
Figure 1-2: Correlation between MUC1 expression and patient survival.....	5
Figure 1-3: Structural changes between MUC1 in healthy tissue and taMUC1 in malignant tissue.	6
Figure 1-4: Comparison of humoral immune responses after vaccinating BALB/c mice with different synthetically prepared taMUC1 sequences in IFA.	9
Figure 1-5: Preventive vaccination against taMUC1 with a synthetic glycopeptidic MUC1 sequence shows a significant reduction in tumor growth in a preclinical BC mouse model.	11
Figure 1-6: Assumed mechanism of tumor eradication under ADC treatment.	14
Figure 3-1: Vaccination scheme for C57Bl/6 and C57Bl/6-{hMUC1} mice.	25
Figure 3-2: Schematic representation of cell detection by the laser beam using forward or side scatter. ⁸⁹	27
Figure 3-3 Gating procedure for identifying Ab- or antiserum-bound cells.	29
Figure 3-4: Gating procedure for viability and apoptosis analysis via FACS.....	30
Figure 3-5: Gating procedure for analysis of T cell subsets and activation marker expression.	32
Figure 3-6: Gating procedure for analysis of B-cell subsets and activation marker expression.	34
Figure 3-7: Reaction schemes for the synthesis of the coupling of GGSK-1/30 and the three linker-toxin conjugates SMCC-DM1 (A), MC-GGFG-DX, and MC-SN38 (B).	35
Figure 3-8: Scheme of the 2D treatment assay procedure.	37
Figure 3-9: Procedure of 3D treatment assays.	39
Figure 3-10: Transformations performed on the microscopy images with Fiji Image J to measure the cross-sectional area of the spheroids.	40
Figure 4-1: Design of self-assembling supramolecular co-polymers as vaccine formulations	42
Figure 4-2: Optimization of MUC1(22)S ¹⁷ ST _N -p30 supramolecular antitumor vaccine (II). ..	43

Figure 4-3: Optimization of the MUC1(22)S ¹⁷ ST _N -p30 supramolecular antitumor vaccine (I): Testing the effect of different ratios of the hydrogel forming monomer on the humoral response.....	45
Figure 4-4: Optimization of the MUC1(22)S ¹⁷ ST _N -p30 supramolecular antitumor vaccine (III): Comparison of IFA and hydrogel formulations.	46
Figure 4-5: Comparison of humoral responses in C57Bl6 wt and hMUC1 transgenic mice after vaccination with the new hydrogel adjuvant formulation.	48
Figure 4-6: Testing different adjuvant variations within the MUC1(22)S ¹⁷ ST _N -p30 supramolecular antitumor vaccine.	49
Figure 4-7: tSNE cluster analysis of B cells from the spleen of mice after vaccination with differently adjuvated vaccine formulations as measured by FACS.	51
Figure 4-8: Figure 4-9: tSNE cluster analysis of B cells from the mLN of mice after vaccination with differently adjuvated vaccine formulations as measured by FACS.	53
Figure 4-10: Analysis of differences in B cell immunoactivation in the spleen after vaccination with adjuvant through TLR7/8 agonist and STING agonist coupled with different linkers.....	55
Figure 4-11: Analysis of differences in B cell immunoactivation in the mLN after vaccination with adjuvant through TLR7/8 agonist and STING agonist coupled with different linkers.....	56
Figure 4-12: tSNE cluster analysis of T cells from the spleen of mice after vaccination with differently adjuvated vaccine formulations measured by FACS.	59
Figure 4-13: tSNE cluster analysis of T cells from the spleens of mice after vaccination with differently adjuvated vaccine formulations as measured by FACS.	60
Figure 4-14: Expression of T cell subsets in the spleen (A) and mLN (B) after different treatments.	61
Figure 4-15: Activation state and expression of activation markers of T _H cell (A), CTLs (B), and Treg (C) subsets in spleens from untreated and vaccinated mice.	63
Figure 4-16: Activation state and expression of activation markers of T _H cells (A), CTLs (B), and Tregs (C) subsets in spleens from untreated and vaccinated mice.....	65
Figure 4-17: Tumor growth monitoring of mice implanted with humanized PyMTxhMUC1 or PyMT tumors and then treated with different GSK-1/30 based ADCs.	68
Figure 4-18: FACS analysis of MUC1 internalization upon GSK-1/30 binding.	69

Figure 4-19: Comparison of VIP331 toxin from Vincerx company and SN-38 coupled on GSK-1/30 in a 2D treatment assay of PyMT and PyMTxMUC1 cells.....	71
Figure 4-20: Histochemical screening for MUC1 expression in human BC samples collected in the pathological institute.	74
Figure 4-21: Correlation of (TA)MUC1 expression with the survival rate of metastasis-free and recurrence-free BC patients.	75
Figure 4-22: Treatment assay on different human BC cell lines in 2D culture with DM1 coupled GSK-1/30 ADC.	77
Figure 4-23: Treatment assay on different human BC cell lines in 2D culture with SN38 coupled GSK-1/30 ADC.	79
Figure 4-24: Procedure for spheroid formation, treatment, and analysis.....	81
Figure 4-25: Microscopic imaging of MDA-MB-231 human BC cell spheroids and induced size changes of spheroids during the 7 d of treatment with DM1 coupled GSK-1/30 ADC.....	82
Figure 4-26: Microscopy imaging of MCF-7 human BC cell spheroids and induced size changes of the spheroids during 7 d of treatment with DM1 coupled GSK-1/30 ADC.....	84
Figure 4-27: Microscopic imaging of T47D human BC cell spheroids and induced size changes of spheroids during 7 d of treatment with DM1 coupled GSK-1/30 ADC.....	86
Figure 4-28: Percentual occurrence of tumors with high MUC1 expression among all tumors analyzed in studies for different types of cancer data sets extrapolated from PAN Cancer Atlas TCGA database ¹²⁰	88
Figure 4-29: IHC screening for taMUC1 expression in human cancer samples collected in the pathological institute of UMC Mainz.	89
Figure 4-30: FACS screening for MUC1 expression in common commercially available human cancer cell lines from different tumor entities.	91
Figure 4-31: Analysis of treatment assay on prostate carcinoma cell line LNCAP, lung carcinoma cell line A549, and PDAC cell line PANC-1 in 2D culture with SN-38 coupled with GSK-1/30 ADC.....	93
Figure 4-32: Microscopic imaging of PANC-1 human PDAC cell spheroids and induced size changes of the spheroids during 7 d of treatment with DM1 and SN-38 coupled ADCs.	96
Figure 4-33: Establishment of PDAC tissue slice culture and Ab penetration assay testing if GSK-1/30 can reach the center of the tissue slice.....	98

Figure 4-34: Testing AF488-GGSK-1/30 for fluorescence microscopy of a PDAC sample.	100
Figure 4-35: Screening for the most efficient ADC for PDAC tissue slice culture treatment.	102
Figure 5-1: Generation of a murine BC in vivo model for MUC1 therapy testing.	105
Figure 8-1: FlowSOM heat maps for tSNE cluster analysis of B and T cells shown in Figure 4-7 and Figure 4-12.	VII
Figure 8-2: Overview of activation marker expression patterns on different T cell subsets from the spleen and mLN of untreated and vaccinated mice.....	VIII
Figure 8-3: Microscopy images (Scale 16x, scale bar = 500 μ m) of spheroid formation on d2, d4, and d6 after seeding of different numbers of MDA-MD-231 cells.	IX
Figure 8-4: Microscopy images (Scale 16x, scale bar = 500 μ m) of spheroid formation on d2, d4, and d6 after seeding of different numbers of MCF-7 cells.	X
Figure 8-5: Microscopy images (Scale 16x, scale bar = 500 μ m) of spheroid formation on d2, d4, and d6 after seeding different numbers of T47D cells.	XI
Figure 8-6:3D treatment assay of spheroids built from MDA-MB-231 human BC cells.	XII
Figure 8-7:3D treatment assay of spheroids built from MDA-MB-231 human BC cells.	XIII
Figure 8-8:3D treatment assay of spheroids built from MCF-7 human BC cells.	XIV
Figure 8-9:3D treatment assay of spheroids built from MCF-7 human BC cells.	XV
Figure 8-10:3D treatment assay of spheroids built from T47D human BC cells.	XVI
Figure 8-11:3D treatment assay of spheroids built from T47D human BC cells.	XVII
Figure 8-12: Microscopy images (Scale 16x, scale bar = 500 μ m) of spheroid formation on d2, d4, and d6 after seeding of different numbers of PANC-1 cells.	XVIII
Figure 8-13:3D treatment assay of spheroids built from PANC-1 human PDAC cells.	XIX
Figure 8-14:3D treatment assay of spheroids built from PANC-1 human PDAC cells.	XX
Figure 8-15 Single-channel images and isotype ctrl staining for Figure 4-34.	XXI
Figure 8-16: Isotype controls for IHC staining of treated PDAC samples from the tissue penetration assay.....	XXI

8.4 Index of tables

Table 3-1: Overview of all devices and materials used with their suppliers.	17
Table 3-2: Overview of all materials used with their suppliers.	18
Table 3-3: Overview of used substances and their suppliers.	18
Table 3-4: List of in-house prepared solutions and their components.	19
Table 3-5: Composition of SDS-PAGE gels.	26
Table 3-6: Abs used for taMUC1 internalization experiments and taMUC1 expression controls.	28
Table 3-7: Reagents and Abs contained in the staining mixture for cell viability and apoptosis analysis.	30
Table 3-8: Reagents and Abs contained in the staining mixture for T-cell immune-activation analysis.	31
Table 3-9 Reagents and Abs contained in the staining mixture for B-cell immune-activation analysis.	33
Table 3-10: Cell density seeded out for the 2D treatment assay.	38
Table 3-11: Cell densities seeded for the 2D treatment assay.	39

8.5 The usage of AI Tools

The following table summarizes the AI tools used in this dissertation.

Name of the AI tool	Used for	Where
DeepL translate	finding the right formulation in English /review of my translations	Over the whole text
DeepL write	refinement of English drafts	Over the whole text
Paperpal	correction of English grammar	Over the whole text
ChatGPT	shortening of drafts	Introduction, Abstract, Kurzfassung section
Perplexity AI	finding further supporting literature for statements	Over the whole text

8.6 Acknowledgements

8.7 Curriculum vitae