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Precision targeting of tumour-associated macrophages in triple negative breast cancer

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Laboratory of Precision and Nanomedicine, Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia

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My contribution to the articles referred was as following:

I publication: I performed the experiments and analysis shown in figures 2-6. I participated in writing the manuscript, answering to reviewers' questions in writing and experimentally and supplemented the manuscript based on reviewers' suggestions.

II publication: I helped with biodistribution assay experimentally and analytically (fig. 4), I induced tumours for the treatment study and performed analysis with immunofluorescence to show the differences in immune cell populations, enzymes, and proteins (fig. 5). I helped in writing the manuscript, answering reviewers' questions, and supplementing the manuscript based on feedback from reviewers.

III publication: I performed experiments and analysis shown in figures 2, 3 (except b, c), 4 and 5. Also, I performed all experiments and analysis shown in supplementary figures S3-9, S12-14, S16-21. I participated fully in writing the manuscript, answering to reviewers' questions in writing and experimentally and supplemented the manuscript based on reviewers' suggestions.

ABBREVIATIONS

ADC	antibody-drug conjugate
ADCC	antibody-dependent cellular cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
Ahx	aminohexaonic
AJCC	American Joint Committee on Cancers
ALAT	alanine aminotransferase
ANOVA	analysis of variance
BC	buffy coat
BMDM	bone marrow-derived macrophages
BRCA	breast cancer gene
BSA	bovine serum albumin
CAFs	cancer-associated fibroblasts
CDC	complement-dependent cytotoxicity
CendR	C-end rule
CPPs	cell-penetrating peptides
Crea	creatinine
CSF1	colony stimulating factor 1
CSF1R	colony stimulating factor 1 receptor
CTL	cytotoxic T lymphocyte
CTLD	C-type lectin-like domain
DC	dendritic cell
DOTA	dodecanetetraacetic acid
DOX	doxorubicin
ECIS	European Cancer Information System
EDB-FN	extra domain B of fibronectin
EGFR	epidermal growth factor receptor
EPR	enhanced permeability and retention
ER	oestrogen receptor
FAM	fluorescein
FBS	foetal bovine serum
FC	flow cytometry
FDA	Food and Drug Administration
GFP	green fluorescent protein
H&E	haematoxylin and eosin
HDAC	histone deacetylase
HER2	human epidermal growth factor 2
i.p.	intraperitoneal
i.v.	intravenous
ICIs	immune checkpoint inhibitors
IF	immunofluorescence assay
IFN-γ	interferon gamma
IHC	immunohistochemistry
IL	interleukin
iNOS	inducible nitric oxide synthase

IVIS	In Vivo Imaging System
LNP	lignin nanoparticle
LPS	lipopolysaccharide
M-CSF	macrophage colony stimulating factor
Mal	maleimide
MARCO	macrophage receptor with collagenous structure
MEK	mitogen-activated protein kinase
MEM	Minimum Essential Medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n.s.	not significant
OG	Oregon Green
PAMPs	pathogen-associated molecular patterns
PARP	poly (ADP-ribose) polymerase
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCL	polycaprolactone
PD-1	programmed cell death
PD-L1	programmed cell death ligand 1
PDC	peptide-drug conjugate
PEG	polyethylene glycol
PFA	paraformaldehyde
PGA	polyglutamic acid
PI3K	phosphoinositide 3-kinase
PR	progesterone receptor
PS	polymersome
R848	resiquimod
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
s.c.	subcutaneous
SLN	sentinel lymph node
St-PGA	star-shaped polyglutamic acid
TAM	tumour-associated macrophage
TC	tumour core
TGF-β	transforming growth factor beta
TILs	tumour infiltrating lymphocytes
TLR	toll-like receptor
TME	tumour microenvironment
TNBC	triple negative breast cancer
TNF-α	tumour necrosis factor alpha
TNM	tumour-node-metastasis
TPPs	tumour-penetrating peptides
TR	tumour rim
Tregs	regulatory T lymphocytes
VEGF	vascular endothelial growth factor
Vin	vinblastine

1. INTRODUCTION

Cancer is the second leading cause of death worldwide (Bray et al., 2021) and the risk of getting a cancer during person's lifetime is around 20% (Ferlay et al., 2021). With 2.3 million new cases in 2020, breast cancer is the most commonly diagnosed cancer (Arnold et al., 2022). In Estonia, every week 16 women are getting a breast cancer diagnosis and 5 die (European Cancer Information System (ECIS)), indicating an intense need for new therapeutics. Breast cancers can be divided into subgroups based on their receptor expression, grade, and histology. Triple negative breast cancer (TNBC) is the most aggressive one (Foulkes et al., 2010; Garrido-Castro et al., 2019), it comprises up to 20% of all breast cancer cases and as it affects mostly women under 50 who do not attend routine mammography, then during the time of diagnosis it is often already at an advanced stage (Garrido-Castro et al., 2019). As TNBC is negative for all hormone receptors, patients do not benefit from hormone therapies and right now the standard-of-care still remain chemotherapy and surgical excision, if possible (Bianchini et al., 2016; Cardoso et al., 2019; Cretella et al., 2019; Tung et al., 2021). Some TNBC patients respond well to chemotherapy, some to chemotherapy in combination with platinum-based therapies and some benefit from different inhibitors (Bianchini et al., 2016) but these patients account for just up to 30% of all TNBC patients. The treatments for the remaining 70% of TNBC patients are still limited. Immune checkpoint inhibitors (ICIs) such as Programmed Cell Death 1 (PD-1) or its ligand PD-L1 blockade have been proposed as potential treatment options for TNBC (Bianchini et al., 2022; Schmid et al., 2018) but so far, the results have been disappointing (Schmid et al., 2020).

Targeted nanotherapy has been of interest for many years (Torchilin, 2007) and it has been hypothesised that nanotechnology-based drug-delivery systems could gain momentum over traditional cancer treatments now in clinical use (Luque-Michel et al., 2017). Nanosystems could offer benefits over regular drugs as they can overcome issues such as poor bioavailability and solubility and tumour induced resistance (Khan et al., 2019).

In this thesis we studied the effect of treating TNBC through targeting tumour-associated macrophages (TAMs), found abundantly in the tumour microenvironment (TME). These TAMs are responsible for tumour initiation, progression, escape and metastasis (Ambarus et al., 2012; Lopes et al., 2014; Ma et al., 2010; Mantovani et al., 2004; Vogel et al., 2014), and therefore serve as an important target. As a targeting agent we used a peptide, mUNO, that specifically targets CD206 on TAMs (Scodeller et al., 2017). We explored different ways of tackling $CD206^+$ TAMs, including their depletion or their reprogramming towards an anti-tumoural phenotype, using different peptide-guided nanosystems here developed. Here, we mostly showed the effect that monotherapies can have on TNBC but in the future we envision combination therapies with ICIs, chemotherapy or other therapies as it has been shown that combining different therapies might be an option for this clinically difficult to manage disease (A. Lee & Djamgoz, 2018).

2. LITERATURE OVERVIEW

2.1. Overview of cancer as a disease

Cancer is the second leading cause of death worldwide, just after cardiovascular disease (Bray et al., 2021). Cancer is a complex disease which develops through multiple genetical changes, such as deletions, amplifications, inversions and point mutations which in the end lead to uncontrollable proliferation (Hanahan and Weinberg, 2000; Renan, 1993; Widschwendter and Jones, 2002). There are multiple hallmarks of cancer, such as resistance to apoptosis and growth inhibiting signals, unlimited replication, genomic instability and the avoidance of immunosurveillance just to name few (Hanahan and Weinberg, 2000, 2011). Lifestyle factors, such as smoking, consuming alcohol, diet and low physical activity can also play significant roles in tumour development (Katzke et al., 2015; Kolonel et al., 2004). Moreover, increased inflammation has been shown to be linked to tumour progression through supplying bioactive molecules, such as growth factors and angiogenesis promoters (vascular endothelial growth factor (VEGF)), to the tumour microenvironment (TME), a complex system around tumours comprising of immune cells, stroma, and an extracellular matrix. High inflammation can aid invasive tumours originating from small number of cells through supplying reactive oxygen species (ROS) (Grivennikov et al., 2010; Hanahan and Weinberg, 2011).

2.2. Breast cancer

Breast cancer is a malignant cell growth in breast tissue which can metastasise to different parts in the body, such as the lungs, bones, and brain. It is the most common type of cancer in women, affecting up to 2.3 million women worldwide in 2020 (World Cancer Research Fund International, Arnold et al., 2022). Breast cancer's death rate is one of the highest, comprising over 16% of all cancer deaths in the world (Arnold et al., 2022). In Estonia in 2020, there were 835 newly diagnosed breast cancer cases and 269 breast cancer deaths (ECIS), meaning that every week 16 people are getting breast cancer diagnosis and five people die. Depending on the age group, the 5-year survival for all breast cancers is on average 64–79%, (ECIS) but that does not account for different subtypes and the time of diagnosis.

In 1925, Greenough first described varying degrees of malignancy by their differentiation dividing breast cancers into four groups (Greenough, 1925) but nowadays the most commonly used grading system is the Nottingham Grading System where grade 1 has the highest similarity to the normal tissue and grade 3 shows very little similarity (Elston and Ellis, 1991; Rakha et al., 2010). The importance of grading breast tumours for outcome prognosis has been recognised for decades (Bloom & Richardson, 1957) showing that the higher the grade, the greater the death rate and the fewer the treatment options (Ogston et al., 2003; Rakha et al., 2010).

Tumours can also be staged using the tumour-node-metastasis (TNM) system, developed by the American Joint Committee on Cancers (AJCC) in 1959, nowadays commonly used in clinic. Stage of breast cancer is an important way of determining the cancer prognosis and treatment outcome (Cserni et al., 2018). Stage of a malignant disease is based on the tumour size (T), the involvement of lymph nodes (N) and the presence of distant metastases (M). Unlike for other cancers, breast cancer diagnosis now also includes the presence of oestrogen (ER), progesterone receptor (PR), human epidermal growth factor 2 (HER2) and the evaluation of tumour grade (Hortobagyi et al., 2017).

The mammary gland has two distinctive epithelial cell lineages, basal and luminal, which can be differentiated using immunohistochemistry (IHC) or with genetical profiling as they have distinctive biomarkers and gene expression profiles, respectively (Perou et al., 2000). The luminal A breast cancer subtype is ER or PR positive, HER2 negative, whereas the luminal B subtype is positive for HER2 and ER or PR (P. L. Nguyen et al., 2008; Onitilo et al., 2009; Sorlie et al., 2001). Luminal A is the most common one, it is less aggressive due to its lower grade and can therefore be treated with endocrine therapies whereas luminal B has higher grade (grade 3), therefore is more aggressive and needs combination of different therapies. Breast cancers positive for just HER2 usually have a higher grade (grade 3) and involve lymph nodes which means they have poorer prognosis, but they respond well to chemotherapy (Brenton et al., 2005). Basal-like breast cancers express low levels of ER but they can mostly be distinguished by the expression of genes usually related with the normal basal layer of the breast (Brenton et al., 2005; Tischkowitz et al., 2007). The last classification, triple negative breast cancer (TNBC) is negative for all three, ER, PR and HER2 and expresses a high levels of genes related to cell proliferation (Brenton et al., 2005; Dent et al., 2007; Foulkes et al., 2010; Garrido-Castro et al., 2019; Onitilo et al., 2009). TNBC is the most aggressive breast cancer subtype with highest grade and the least amount of therapeutic options (Brenton et al., 2005). Patients with luminal subtypes usually live longer before metastasising than patients with basal-like or TNBC, they also relapse more in bone, whereas besides bone, TNBC and basal-like also metastasise to the lungs and brain (Smid et al., 2008; Sørlie et al., 2003). The classification of patients into subtypes is important in determining their treatment options and possible outcomes. For example, $HER2^+$ subtypes respond better to chemotherapies and they can be treated with monoclonal antibodies against HER2, such as Herceptin® (Trastuzumab) (Onitilo et al., 2009).

2.3. TNBC – an aggressive breast cancer subtype in need of new treatment options

TNBC, first described in 2005 (Brenton et al., 2005), is an aggressive breast cancer subtype that affects mostly younger women that do not attend routine mammography (Foulkes et al., 2010; Garrido-Castro et al., 2019; Hudis and

Gianni, 2011). Since TNBC lacks the expression of ER, PR and HER2, these patients do not benefit from usual hormone therapies (L. C. Scott et al., 2019). TNBC accounts for up to 20% of all breast cancers (Garrido-Castro et al., 2019; Lehmann et al., 2011), which means that in Estonia there should have been up to 170 TNBC cases in 2020. TNBC is related to poor prognosis, the 5-year survival is under 30% for metastatic patients (Hudis and Gianni, 2011; Lehmann et al., 2011), but patients usually relapse within three years (Kassam et al., 2009; Liedtke et al., 2008) after which the survival is up to 18 months which is lower than for other breast cancer where the survival then is up to two years (Hudis and Gianni, 2011; Reddy et al., 2018; Schmid et al., 2018). Right now, the standard-of-care for TNBC is neoadjuvant and adjuvant chemotherapy and operation and/or radiation in the early stages (Cardoso et al., 2019; Tung et al., 2021) and chemotherapy in all stages (Bianchini et al., 2016; Cretella et al., 2019). All this together indicates that TNBC is an important breast cancer subtype that is in need of better treatment options.

2.4. Tumour microenvironment (TME)

The TME is a complex of cells and stroma that surround solid tumours with a role of supporting tumour initiation, progression, immunosuppression, and the formation of metastases (Anderson & Simon, 2020; Hanahan & Weinberg, 2011; M. Wang et al., 2017; Whiteside, 2008; Wu & Dai, 2017). The TME consists of immune cells (for example tumour infiltrating lymphocytes (TILs) and tumour-associated macrophages (TAMs)), stromal cells (such as cancer-associated fibroblasts (CAFs)), blood vessels, an extracellular matrix, and secreted molecules, such as growth factors, cytokines, and chemokines. These factors are needed for the crosstalk between the TME cells themselves and between the TME and cancer cells (Anderson & Simon, 2020; Bejarano et al., 2021; Mantovani et al., 2008). It has been recognised that the infiltration of TILs corresponds to a better therapy response; in opposition, more TAMs and regulatory T lymphocytes (Tregs) correspond to worse therapy outcomes and a more aggressive disease (Bejarano et al., 2021; Whiteside, 2008). Important players in the TME are the tumour endothelial cells and pericytes as they take part in angiogenesis, fuelling tumour progression even further (Hanahan & Weinberg, 2011; Pietras & Östman, 2010). Besides mature cells, TME also has precursor cells that in response to signals coming from tumour, such as VEGF and hypoxia, can differentiate into their mature form, allowing the tumour to hijack the immune system for its own benefit, thus enabling the body's own immune system to fail to recognise the cancer (Hanahan & Weinberg, 2011; Murdoch et al., 2008). The illustration of anti-tumoural and immune suppressive TME has been depicted on Fig.1.

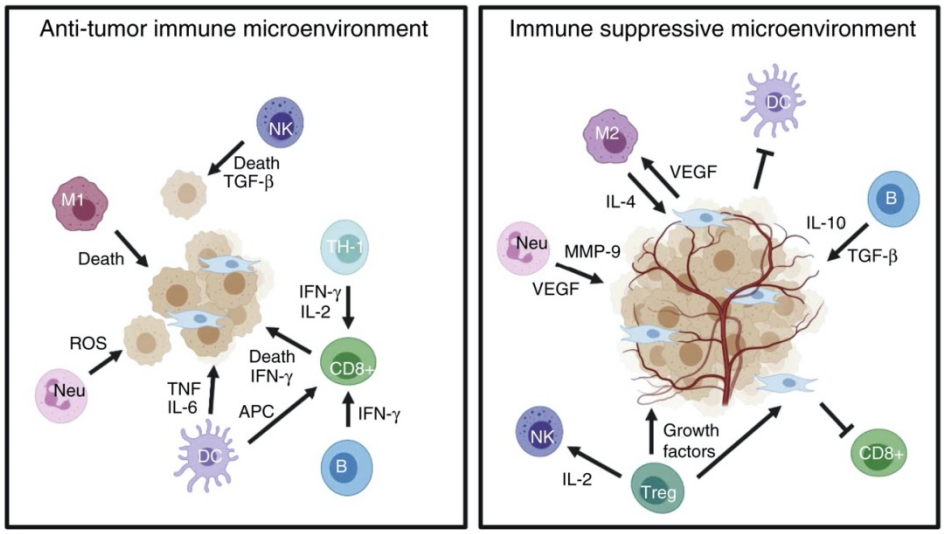


Fig. 1. Immune suppressive and anti-tumoural TME. Immune cells that promote anti-tumoural TME (left) and immune cells that promote tumourigenesis (right). © 2020 Published by Elsevier Inc (Reprinted with permission from Anderson & Simon, 2020).

2.5. Tumour-associated macrophages (TAMs)

The most abundant cell population in the TME are macrophages, more specifically TAMs (R. Hughes et al., 2015; Williams et al., 2016). Macrophages are very plastic, meaning that they can respond to signals coming from the surrounding environment thus differentiating into different phenotypes, namely inflammatory (M1) or anti-inflammatory (M2) (Edwards et al., 2006; Mantovani et al., 2003; Ohlsson et al., 2014). In non-cancer situations, the anti-inflammatory phenotype helps to resolve acute inflammation, takes part in tissue repair and in fighting with pathogens (Ambarus et al., 2012; Y. Chen et al., 2017; Edwards et al., 2006) but these same functions can be hijacked by the tumour to benefit itself. TAMs are derived from circulating monocytes or from tissue resident macrophages (Hirano et al., 2023) and depending on cytokines and chemokines present in their surroundings, can be differentiated into anti-tumoural or pro-tumoural TAMs (Ambarus et al., 2012; Edin et al., 2012; Edwards et al., 2006; Lopes et al., 2014; Mantovani et al., 2004; Stein, 1992; Vogel et al., 2014). The factors that activate M1 TAMs antagonise the activation of M2 factors and *vice versa* (Raes et al., 2002). M2 macrophages in the TME are pro-tumoural and are therefore referred to as M2 TAMs (Mantovani et al., 2002; Redente et al., 2010) whereas TAMs displaying M1 phenotype are anti-tumoural (Fig. 2) (Ma et al., 2010; Vogel et al., 2014).

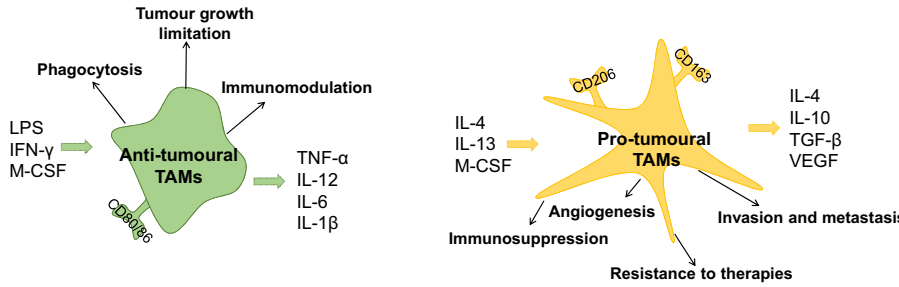


Fig. 2. Anti-tumoural TAMs (M1 phenotype) and pro-tumoural TAMs (M2 phenotype). M1 TAMs are anti-tumoural, express CD86 and CD80 on their surface and produce cytokines such as tumour necrosis factor alpha (TNF-α), IL-12, IL-6, and IL-1β. M2 TAMs are pro-tumoural, express CD163 and CD206 on their surface and produce cytokines such as interleukin 4 (IL-4), IL-10, and transforming growth factor beta (TGF-β).

Higher density of anti-tumoural TAMs correlates with a better response to therapies, and therefore a longer disease-free period and a better prognosis, hence the balance between two populations of TAMs is extremely important (Ma et al., 2010; Vogel et al., 2014). Unfortunately, in many solid tumours, including breast cancers, the balance is shifted towards pro-tumoural TAMs, which can be found in both primary and metastatic tumour sites and therefore correspond with poor prognosis and higher metastasis rate (Lopes et al., 2014; Schäfer & Werner, 2008; Vogel et al., 2014). Pro-tumoural TAMs have now become an intense area of study with promising new treatments that include their elimination or re-education (Anfray et al., 2021; Noy & Pollard, 2014; Pathria et al., 2019; Xiang et al., 2021).

2.6. Tumour homing peptides for developing targeted therapies

Tumour treatments have classically been systemic and untargeted, such as chemotherapy, which results in various side effects, such as cachexia, hair loss, fatigue, a reduction in quality of life measures and cognitive dysfunction to name few (Kayl & Meyers, 2006; Partridge et al., 2001; Tisdale, 2002). Tumours and the TME carry markers that can be quite specific to tumours which would allow the development of tumour specific drugs. Furthermore, only small percentage of administered drugs reach tumours and since tumours are heterogenous, the amount of drugs that do reach the tumours might not reach their intended place (Teesalu et al., 2009, 2013). Small cell-penetrating peptides (CPPs) have maximum of 30 amino acids and they are able to enter cells in an energy-independent way while avoiding endocytic pathways (Lundberg &

Langel, 2003). CPPs are able to target intracellular proteins and carry cargoes which means that they can offer possibly new potential for targeted therapies (Guidotti et al., 2017; Milletti, 2012). Although, CPPs are able to deliver cargo to the cells, they do not recognise a particular receptor, meaning that they need a targeting moiety, such as a peptide, to be tumour-specific (Teesalu et al., 2009). In 2009, Teesalu with colleagues described a new family of tumour binding peptides, which have a motif, R/KXXR/K (called C-end rule or CendR motif), that aids in tissue internalisation and penetration and therefore these peptides are called tumour-penetrating peptides (TPPs) (Teesalu et al., 2009). These TPPs have a vascular homing motif and a recognition site for a protease that activates and exposes the CendR motif upon the initial tumour recruitment, which provides high specificity to the tumour (Teesalu et al., 2013). The first one of these types of peptides was iRGD, developed by Sugahara, Teesalu and others (Sugahara et al., 2009). iRGD in combination with Nabpaclitaxel and Gemcitabine in metastatic pancreatic cancer has now completed a phase I clinical trial (clinical trial identifier: NCT03517176) with promising results showing that it is well tolerated by patients (Dean et al., 2022). Another TPP is LyP-1 which was discovered through phage display by Laakkonen and others (Laakkonen et al., 2002). It binds to the tumour cells and endothelial cells of tumour lymphatic vessels (Laakkonen et al., 2004).

Using peptides for developing cancer therapies has been under consideration for years (Calvo Tardón et al., 2019; Ellerby et al., 1999; Marqus et al., 2017; Reubi, 2003). As discussed in the previous section, $CD206^+$ TAMs are highly overrepresented in the TME, and they are the main players in tumour progression, immunosuppression, and metastasis. Therefore, targeting these TAMs would be an excellent option for the development of new cancer therapeutics.

2.7. mUNO – a new $CD206^+$ TAMs targeting peptide

In 2017, Scodeller and others discovered a new CD206-targeting peptide UNO and its linear version mUNO through phage display (Scodeller et al., 2017). It was shown that peritoneal cells from mice bearing orthotopic TNBC tumours overexpress CD206 compared to healthy mice (Fig. 3B) which prompted the usage of this model for intraperitoneal *in vivo* phage display. Through injecting a naïve phage library (Fig. 3A), it was discovered that sequence CSPGAKVRC (code name UNO) was highly enriched in the peritoneal cells from orthotopic TNBC mice. The peptide UNO receptor was identified through protein database search which revealed that the GSPGAK motif is present in different collagens that can bind to CD206. As this motif has high resemblance to UNO sequence, it was hypothesised that CD206 could be its receptor. CD206 is an endocytic multiligand mannose receptor which consists of three domains: a mannose binding lectin domain, a conserved fibronectin type II domain and a cysteine rich domain. It's structure was first described in 1990 (Ezekowitz, 1990; Martinez-Pomares, 2012). The hypothesis of CD206 as a receptor for UNO was

tested through fluorescent anisotropy binding studies to recombinant CD206 (Fig. 3C), discovering that the UNO peptide needs to be linear to be able to bind to the CD206 protein (Fig. 3C). Also, a minimal binding motif of the UNO peptide was identified, with the sequence CSPGAK, which was named mUNO (Fig. 3D). Through *in vivo* biodistribution assay it was shown that both fluorescein (FAM)-UNO and FAM-mUNO target $CD206^+$ TAMs in orthotopic tumour-bearing mice (Fig. 3E, F, where yellow indicates colocalisation of the peptide and CD206). Additionally, it was shown that FAM-UNO targets $CD206^+$ TAMs in glioblastoma, gastric cancer, and melanoma (Fig. 3G), therefore making it not specific to one tumour type.

The mannose receptor CD206 is an endocytic receptor expressed on macrophages. One of its functions is to recognise mannosylated ligands and pathogen-associated molecular patterns (PAMPs) on pathogens such as bacteria, virus, and fungi for their elimination by macrophages (Martinez-Pomares, 2008, 2012; Taylor et al., 2005). Many targeting agents designed to target CD206 for theranostic applications are mannose-based which makes them not specific to just $CD206^+$ TAMs as they can also target other mannose-binding receptors, such as RelB, Sirp-α, CD47 and CD209 on intestines and genital tissue (Conniot et al., 2019; Jameson et al., 2002; Jaynes et al., 2016). Asciutto and others in collaboration with Teesalu's laboratory (Asciutto et al., 2019) discovered through *in silico* analysis that the mUNO peptide binds to a previously unidentified binding site in the lectin domain of CD206 which means that it is using a different binding site than mannose-based ligands. This was confirmed with fluorescent anisotropy where the addition of mannose did not change the binding of mUNO. This finding is important as it elevates the specificity of mUNO compared to other CD206-targeting ligands which in turn would deliver the payloads specifically to the target cells sparing others.

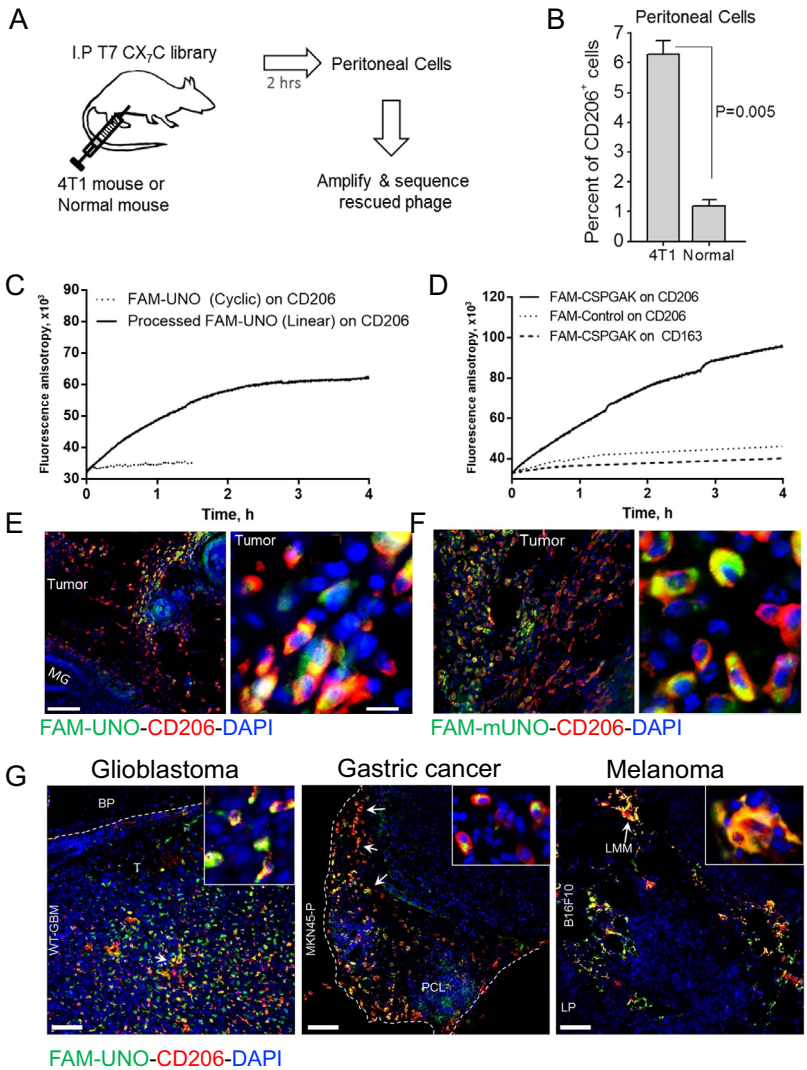


Fig. 3. The discovery and characterisation of mUNO peptide (REPRINTED from Scodeller et al., 2017). (A) Scheme displaying phage display workflow. (B) Percentage of CD206$^+$ cells in the peritoneal cavity. (C) Fluorescent anisotropy showing cyclic *vs* linear FAM-UNO binding to recombinant CD206. (D) Fluorescent anisotropy showing mUNO binding to recombinant CD206 compared to the control peptide. (E) Representative immunostained images showing FAM-UNO binding to CD206$^+$ TAMs in orthotopic TNBC. (F) Representative confocal immunofluorescence images showing systemically administered FAM-mUNO homing to CD206$^+$ TAMs in orthotopic TNBC. (G) Representative confocal immunofluorescence images showing FAM-UNO binding to CD206$^+$ TAMs in glioblastoma, gastric cancer, and melanoma. Figure kindly provided by Dr. Scodeller.

2.8. Targeted nanosystems to develop new cancer therapies

Although peptides could be used in their monomeric form as peptide-drug conjugates for therapeutic purposes (M. Cooper et al., 2021; Zhu et al., 2021), they could be also combined in a nanosystem as this would provide avidity, longer half-life, lower toxicity and increased solubility in the case that drug is hydrophobic (Greco & Vicent, 2009; Kopeček, 2013; Manandhar et al., 2021). Targeted nanocarriers to develop new cancer therapeutics have gathered a lot of attraction for many years (Torchilin, 2007) and it has been hypothesised that nanotechnology-based drugs could take over from regular drugs right now in clinical practice for treating various diseases (Luque-Michel et al., 2017). One of these synthetic nanosystems is **polymersomes (PSs)** which are small (~100 nm) polymeric vesicles that self-assemble in an aqueous solution (Discher et al., 2002; Simón-Gracia et al., 2018). They can be used as drug delivery vehicles, as they have an aqueous core and a bilayer allowing the encapsulation of both hydrophilic and hydrophobic cargoes (Meng et al., 2009; Simón-Gracia et al., 2018). **Lignin nanoparticles (LNPs)** consist of lignin which is a biopolymer that can be found in abundance in nature. Lignin has gained interest in the drug delivery field due to its nature of being easily chemically modified, its low toxicity and biocompatibility (Figueiredo et al., 2017; Siddiqui et al., 2020; Yiamsawas et al., 2017). Encapsulating cancer drugs in particles such as PSs or LNPs protects the drug from rapid degradation and lessens adverse side effects (Figueiredo et al., 2017). Another system that could be used to increase avidity and half-life is conjugating the **targeting peptide to a polymeric backbone,** such as polyglutamic acid (PGA). PGA is a water soluble, nonimmunogenic, nontoxic, biodegradable polymer that can be used in targeted cancer therapy (Richard & Margaritis, 2001; Talelli & Vicent, 2014). PGA-based polymers have a reproducible synthesis, their multivalency allows for abundant post-synthesis modifications and they accumulate in the tumour tissue through enhanced permeability and retention (EPR) effect, which makes them suitable for cancer drug development (Talelli & Vicent, 2014). Adding three polymer arms together to form a star-shaped polymer allows for control over the molecular weight of the construct (Byrne et al., 2015), allows multivalency, increases blood half-life and the cellular uptake (Duro-Castano, England, et al., 2015). Targeted nanotherapies can therefore have advantages over free drugs and they have opened a new door in cancer drug development.

2.9. TAM targeting methods for cancer therapy

As this dissertation explores TAM-targeting treatments for breast cancer therapy, different possible targeting methods available will be discussed.

TAM depletion to alleviate immunosuppression

There are multiple ongoing clinical trials that evaluate TAM depletion, many of which are in combination with immune checkpoint inhibitors (ICIs) (Pathria et al., 2019). Right now, the gold standard for depleting TAMs is to block colony stimulating factor 1 (CSF1) or its receptor CSF1R with the inhibitor PLX3397. A drawback is that normal microglia also express CSF1R, the CSF1R inhibition with PLX5622 affects M1 macrophages, and the inhibition with PLX3397 causes oedema (Bissinger et al., 2021; DeNardo et al., 2011; S. Lee et al., 2018; Mancini et al., 2019). Moreover, it has been shown that in some animal models, the CSF1R blockade actually induces tumour progression and metastases (Hollmén et al., 2016) and based on clinical data it can be said that anti-CSF1R antibodies induce only a modest effect and usually induce severe side effects that include haematologic toxicities and hepatotoxicity through targeting Kupffer cells (Papadopoulos et al., 2017; Wesolowski et al., 2019). One more promising method is to deplete TREM2-expressing TAMs with a TREM2-targeting antibody PY314 (Binnewies et al., 2021). It is right now in a phase 1a/1b clinical trial (clinical trial identifier: NCT04691375) for the treatment of advanced solid tumours, in some cases it is combined with Keytruda® (pembrolizumab, anti-programmed cell death 1(PD-1) antibody). The clinical trial is still ongoing but first results are promising showing that it is well tolerated by the patients and it can induce stable disease for weeks (Patnaik et al., 2022). Targeting CD163 on pro-tumoural TAMs with liposomes encapsulating doxorubicin (DOX, Adriamycin®) and decorated with anti-CD163 antibody has also shown promising results indicating that depleting pro-tumoural TAMs will alleviate immunosuppression and therefore promote tumour regression in combination with ICIs (Etzerodt et al., 2019). There are more depletion methods, such as targeting CCR2 and CD40, but those do not target just TAMs as these receptors are expressed by anti-tumoural TAMs and microglia (El Khoury et al., 2007; Ponomarev et al., 2006).

Re-educating pro-tumoural TAMs to become anti-tumoural TAMs

Another option is to reprogramme pro-tumoural TAMs to become anti-tumoural using compounds promoting M2→M1 switch. One of those is the compound CLEVEGEN (in collaboration with Faron Pharmaceuticals Ltd) that blocks Clever-1 on pro-tumoural TAMs (Hollmén et al., 2020; Viitala et al., 2019). It is a humanised anti-Clever-1 antibody and right now it is in phase I/II clinical trial for testing the safety, tolerability, and efficacy on metastatic or inoperable tumours (clinical trial identifier: NCT03733990). Another compound is resiquimod (R848), a low molecular weight compound, belonging to the imidazoquinoline family, showing an immunomodulatory effect both *in vitro* and *in*

vivo (Brugnolo et al., 2003; Dockrell, 2001). It is a toll-like receptor (TLR) agonist that binds to TLR7 and TLR8 (M. Lee et al., 2014; Sabado et al., 2015). R848 has been shown to promote the repolarisation of pro-tumoural TAMs to anti-tumoural TAMs (Anfray et al., 2021; Li et al., 2021; Rodell et al., 2018). Over the years there have been over 60 clinical trials using TLR agonists (H. Zhang et al., 2021) and right now there is one study using R848 for cancer treatment (clinical trial identifier: NCT04799054). Moreover, there are studies that show that pro-tumoural TAMs are positive for a macrophage receptor with collagenous structure (MARCO) (La Fleur et al., 2018; Neyen et al., 2013) and Georgoudaki et al. developed a monoclonal antibody against MARCO to show that it can re-educate pro-tumoural TAMs to become anti-tumoural ones and potentiate immunotherapy (Georgoudaki et al., 2016).

2.10. Introduction to antibody-drug conjugates, polymer-drug conjugates, peptide-drug conjugates, and monoclonal antibodies

There are various ways that drugs can be designed and targeted. One way would be a conjugation between the targeting moiety and a drug. These options include antibody-drug conjugate (ADC), polymer-drug conjugate, peptide-drug conjugate (PDC) and more.

ADCs were first developed in the second half of 20th century (Fu et al., 2022; Khongorzul et al., 2020) and have now gained a lot of attraction in the world of new cancer therapeutics. They are believed to be more potent than antibodies on their own (B. Hughes, 2010). So far there are over 10 ADCs that have been granted an approval by US Food and Drug Administration (FDA), such as Adcetris® (brentuximab vedotin) for the treatment of $CD30^+$ Hodgkins lymphoma, Kadcycla® (ado-trastuzumab emtansine) for $HER2^+$ breast cancer or Trodelvy® (sacituzumab govitecan-hziy), targeted against TROP2 for the treatment of metastatic TNBC (Beck et al., 2017; Tong et al., 2021; Zolot et al., 2013). Since ADCs are specific to certain targets, they are more selective towards cancer cells and/or cells related to tumour progression than conventional chemotherapy, therefore lessening side effects and making therapeutics more potent (Zolot et al., 2013). Although ADCs have overcome some of the issues, how to choose the appropriate target and the correct linker to avoid unspecific release of payloads, what drug to use to get correct toxicity and avoid drug resistance and how to have effective ADCs that reach the tumour are all still major concerns (Beck et al., 2017; Su et al., 2021; Zolot et al., 2013).

The first **polymer-drug conjugates** were developed back in the 1950s (Kopeček, 2013) but their use was finally confirmed in the 1970s (Ringsdorf, 1975). They consist of a drug that is covalently bound to a polymeric carrier. This enhances the solubility of the drug in aqueous solutions, increases the half-life through prolonged circulation, and reduces toxicity and immunogenicity leading to enhanced therapeutic efficacy (Canal et al., 2011; Ekladious et al.,

2019). There are multiple polymer-drug conjugates approved for the treatment of various diseases but there are still several obstacles to overcome. Immunogenicity, relying on the EPR for tumour accumulation to the tumour tissue, rapid renal clearance and potentially not improved pharmacokinetics are hampering the possibility of polymer-drug conjugates to reach the clinic (Ekladious et al., 2019). The most promising polymer-drug conjugate is Opaxio™ (paclitaxel poliglumex) that gained the FDA's approval as an orphan drug in 2012 for the treatment of glioblastoma in combination with radiotherapy (Caster et al., 2017; Talelli & Vicent, 2014; Ventola, 2017).

PDCs are constructs that have a homing peptide, a linker and a drug that can be cytotoxic, a radioligand or it can be an imaging agent (M. Cooper et al., 2021; Zhu et al., 2021). As PDCs are targeted, they allow precise drug delivery to the target cells, minimising the side effects on other cells (Zhu et al., 2021). Although ADCs have seen exponential interest as possible new drugs, PDCs are starting to show similar trend now (M. Cooper et al., 2021; Zhu et al., 2021). ^{177}Lu-dotatate (Lutathera®) is the first FDA approved PDC which is used to treat gastroenteropancreatic neuroendocrine tumours (M. Cooper et al., 2021). As a homing peptide it has somatostatin analogue Octreotide which binds to the somatostatin receptor which is overexpressed by some tumours (Hennrich & Kopka, 2019). Theratechnologies Inc. have developed two new PDCs with high potential: TH1902 that uses docetaxel and would be used to treat TNBC, and TH1904 for treating ovarian cancer, it uses DOX as a drug. TH1902 and TH1904 both target sortilin 1 which is overexpressed in many solid tumours. Designing PDCs can be hard as one needs to consider what peptide, linker and drug to use to achieve the set goals but upcoming PDCs show their promising use as pharmaceuticals, such as PDC developed by Bicycle Therapeutics currently in clinical trials (M. Cooper et al., 2021). Although there has not been too much of a success with PDCs in the clinic just yet, the case of melphalan flufenamide (Pepaxto®) being one (Olivier & Prasad, 2022), the enormous interest in them could lead to increased development of peptide-based pharmaceuticals.

Monoclonal antibodies bind to a specific epitope on the antigen they are designed against, and it can be specific antigen expressed exclusively by cancer cells or that is overexpressed in tumours. Therefore, monoclonal antibodies can induce tumour cell death through binding to growth factors, such as epidermal growth factor receptor (EGFR) or HER2, or through indirect mechanism of action, requiring complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP), such as antibodies Rituximab and Ofatumumab that bind to CD20 (Zahavi & Weiner, 2020). There are several FDA approved monoclonal antibodies used in cancer therapy, such as Atezolizumab (targets PD-1 ligand PD-L1), Cetuximab (targets EGFR), Ipilimumab (targets CTLA4) or Nivolumab (targets PD-1) (Zahavi & Weiner, 2020). Although they have shown success as cancer therapeutics for over 20 years (A. M. Scott et al., 2012), they still have drawbacks, such as adverse side effects as they modulate immune system,

resistance to them and small response rate (Melero et al., 2007; Zahavi & Weiner, 2020).

Deciding what platform to use for the development of new cancer therapeutics should take into consideration specific targeting, reduced immunogenicity and potent cytotoxic or immunomodulatory drugs.

2.11. Challenges in TNBC therapy

Therapies to treat breast cancer have gone through a massive change in the last 60 years by lowering mortality and improving the quality of life, but there are still many challenges that need to be overcome (Sledge et al., 2014). Resistance to therapies, dealing with micrometastases, enormous heterogenicity and no real actionable targets are some of those challenges (Bianchini et al., 2016; Khan et al., 2019; Sledge et al., 2014). Surprisingly, a portion of TNBC patients respond well to chemotherapies based on taxanes and anthracyclines in all treatment stages (Bianchini et al., 2016) but despite that, the 5-year survival is under 30% and metastatic TNBC leads to death in under two years (Bianchini et al., 2016; Hudis & Gianni, 2011; Schmid et al., 2018). Combining different chemotherapies has also not resulted in a significant increase in survival. Although, treating early-stage TNBC patients with platinum-based therapies in combination with taxane-anthracycline-based therapies in a neoadjuvant setting might show better results, in general platinum-based therapies have so far shown only modest effect and determining who should receive platinum therapy has not been easy, breast cancer gene (BRCA) 1 and/or 2 mutation being the only real marker (Bianchini et al., 2016). As TNBC is very heterogeneous, personalised targeted therapies might help the remaining 70% of TNBC patients who do not respond to chemotherapy. Up to 90% of those could benefit from treatments such as poly (ADP-ribose) polymerase (PARP) inhibitors, mitogen-activated protein kinase (MEK) inhibitors, histone deacetylase (HDAC) inhibitors or phosphoinositide 3-kinase (PI3K) inhibitors (Bianchini et al., 2016). The PARP inhibitor Olaparib has shown great results with minimal toxicity making it a great candidate to be explored further as a first-line monotherapy for TNBC (Bianchini et al., 2016; Cortesi et al., 2021). Immunotherapy has been proposed as an excellent cancer therapy (García-Aranda & Redondo, 2019) as modulating the immune system could play a major role in tumour progression (Bejarano et al., 2021; Whiteside, 2008). However, whether it leads to success is still under evaluation. Some treatments have been shown to turn “cold” (few TILs) immune landscapes “hot” (more TILs) leading to better prognosis (Khan et al., 2019). ICIs, such as anti-PD-1 and its ligand andi-PD-L1, have been proposed as potential treatment option for TNBC. Abraxane® (nanoformulated albumin bound Paclitaxel) in combination with Tecentriq™ (anti-PD-L1) was granted FDA approval in 2019 for the treatment of PD-L1 positive metastatic TNBC. Already in 2018, it was shown that its effect was modest (Schmid et al., 2018), and in 2021 the approval was withdrawn (Ou-Yang et al., 2022) as there was no significant difference compared to a placebo in combination with Abraxane®

(Schmid et al., 2020). Although PD-L1 based ICIs provided only a modest effect (Bianchini et al., 2022), in 2021, FDA granted the approval of Keytruda® (anti-PD-1 antibody) as a treatment for early-stage high risk TNBC in neoadjuvant and adjuvant setting (Shah et al., 2022).

2.12. Summary of the literature

TNBC, first characterised in 2005, is the most aggressive breast cancer subtype. The 5-year survival rate is under 30%, many patients relapse within three years and metastatic patients die within two years. Therefore, it is in urgent need of new treatment options. Although ICIs have given some hope for TNBC patients, chemotherapy and operations (if possible) still remain the standard-of-care. Targeted therapies with the aim of developing new cancer therapeutics have gathered a lot of attention in recent years. One of those avenues deals with targeting pro-tumoural TAMs since they are largely responsible for immunosuppression and metastasis. Targeting TAMs with the intent to eliminate them or turn them into anti-tumoural macrophages has shown to alleviate the tumour burden and lead to better treatment outcomes both in preclinical and clinical studies (as an adjuvant to existing therapies). *In vivo* peptide phage display has been used for the last 30 years to identify ligands capable of precisely homing in on a particular target or cell type of interest. Those peptidic ligands have then been used to precisely deliver a coupled therapeutic payload, including nano-formulated drugs. Nanosystems, including peptide-guided ones, through prolonged blood half-life and avidity, have shown a potentiated targeting or therapeutic efficacy over their free counterparts. In 2017, using *in vivo* phage display, Scodeller et al. developed a new peptide, mUNO, that specifically targets pro-tumoural TAMs by binding to the mannose receptor CD206 (Scodeller et al., 2017). This has opened the door to investigate how different TAM-targeted nanosystems could improve potential therapy options for TNBC. Therefore, the aim of this study was to analyse whether through targeting pro-tumoural TAMs with an mUNO-guided nanosystem, we can alleviate the tumour burden and immunosuppression for an improved therapy response in a preclinical TNBC mouse model as there have not been any breakthroughs in the disease's management since its characterisation 18 years ago.

3. AIMS OF THE STUDY

This doctoral study focused on the development and preclinical evaluation of mUNO peptide-guided therapeutic nanosystems against $CD206^+$ TAMs for TNBC, as the treatment options for TNBC are still quite limited. Preclinical validation of peptide-guided therapies is important in order to assess whether the peptide of interest is targeting the desired cells, whether the drug conjugated to it can act at its intended target site, and to test the safety and efficacy of the peptide-guided therapies. Once the safety and efficacy have been confirmed in mouse models can the therapeutic nanosystem be considered for the use in humans through clinical studies.

Therefore, the specific aims of this dissertation were:

- to evaluate mUNO peptide as a $CD206^+$ TAMs targeting peptide *in vitro* and *in vivo*,
- to evaluate different mUNO-guided nanosystems for tackling $CD206^+$ TAMs in TNBC for therapeutic effect:
  - to evaluate mUNO-guided TAM reprogramming. Using LNPs encapsulating the drug R848 with the aim of re-educating pro-tumoural TAMs to have M1-like phenotype in orthotopic TNBC,
  - to evaluate mUNO-guided TAM depletion. Using a polymer-drug conjugate where we used DOX as a drug to deplete pro-tumoural TAMs in orthotopic TNBC and experimental metastasis of TNBC.

4. MATERIALS AND METHODS

4.1. Peptides

FAM-mUNO (FAM-Ahx-CSPGAK-COOH), mUNO (sequence: CSPGAK-COOH), FAM-Control (FAM-Ahx-CAQK-NH_2) and FAM-Lyp1 (FAM-CGNKRTRGC-NH_2, disulphide cyclized) were purchased from TAG Copenhagen (Frederiksberg, Denmark), Ahx is an aminohexaonic acid linker.

4.2. Conjugates

Star-shaped polyglutamic acid (St-PGA) was kindly provided by Polypeptide Therapeutic Solutions S.L. (Valencia, Spain). St-PGA-Oregon Green (OG), St-PGA-OG-mUNO, St-PGA-DOX and St-PGA-DOX-mUNO (OximUNO) were kindly synthesised as described previously (Lepland et al., 2022) by Alessio Malfanti who was at the time part of the Maria J. Vicent's Polymer Therapeutics laboratory (CIPF, Valencia, Spain).

4.3. Polymersomes (PS)

Polymersomes (PS) were synthesised by Dr. Lorena Simón-Gracia and characterised by Valeria Sidorenko (MSc), both from Laboratory of Precision and Nanomedicine, as described previously (Lepland et al., 2020; Simón-Gracia et al., 2018). The PSs were composed of the co-polymer polyethylene glycol (PEG, ~5000 Da) – polycaprolactone (PCL, ~10 000 Da) and Maleimide (Mal)-PEG-PCL.

4.4. Cell culture

4T1 cells were purchased from ATCC (Manassas, USA) and 4T1 cells expressing green fluorescent protein (GFP) were kindly gifted by Ruoslahti's laboratory (Sanford Burnham Prebys Medical Discovery Institute, La Jolla, USA). 4T1 and 4T1-GFP cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) foetal bovine serum (FBS) and 100 IU/ml of penicillin/streptomycin in 37 °C incubator in the presence of 5% CO_2 until desired confluency was obtained. All cell lines were tested for mycoplasma with either MycoStrip™ mycoplasma detection kit or using PCR method.

4.5. Tumour models

Two TNBC tumour models were used for *in vivo* biodistribution assay: orthotopic TNBC where 1×10^6 4T1 cells were injected subcutaneously (s.c.) in the 4th mammary fat pad, and experimental metastasis of TNBC, where 5×10^5 4T1 cells were injected intravenously (i.v.) through the tail vein.

Two tumour models were used for *in vivo* treatment studies: orthotopic TNBC where 5×10^4 (Lepland et al., 2022) or 1×10^6 4T1 (Figueiredo et al., 2021) cells were injected s.c. in 4th mammary fat pad, and experimental metastasis of TNBC where 2×10^5 4T1-GFP cells were injected i.v. For all animal experiments, 8-12-week-old female Balb/c mice were used.

4.6. *In vitro* experiments

4.6.1. *In vitro* macrophages derived from human monocytes or mouse bone marrow

Cell based assays with primary monocyte derived macrophages were used to assess the binding of FAM labelled peptides to CD206 and to assess the toxicity of compounds before performing animal experiments.

Peripheral blood mononuclear cells (PBMCs) were purified from human blood buffy coat (BC) and stimulated to become M2-resembling or M1-resembling macrophages as described in our paper (Lepland et al., 2020). Briefly, purified monocytes were seeded on 24-well plate containing FBS-coated coverslips with a density of 3.5×10^5 cells per well. IL-4 and macrophage colony stimulating factor (M-CSF) were added to stimulate M2-like phenotype. M-CSF was added to produce M0 macrophages, on day 6 M-CSF, interferon gamma (IFN-γ) and lipopolysaccharide (LPS) were added to stimulate M1-like phenotype. Stimuli was replenished every other day for seven days.

Bone marrow-derived macrophages (BMDMs) were isolated from female Balb/c mouse femur and tibia bones as described previously (Figueiredo et al., 2021). Briefly, bone marrow was washed out with cold phosphate buffered saline (PBS), centrifuged, red blood cells removed with ammonium-chloride-potassium lysing buffer, suspended in M-CSF containing RPMI and plated in Petri dishes at a concentration of 3×10^5 cells/1mL. Medium with M-CSF was replenished every three days. On day 6, cells were differentiated with IL-4 to differentiate them to M2-resembling macrophages and with LPS and IFN-γ to differentiate them to M1-resembling macrophages. Cells were stimulated for 48 h for correct phenotype.

4.6.2. *In vitro* binding assays with polymersomes (PS)

FAM-mUNO-PS and FAM-PS were added to the purified and stimulated human M2-resembling and M1-resembling macrophages, incubated for 5 min after which cells were washed, fixed, immunostained for antibodies against CD206 and FAM, and imaged using Zeiss confocal microscope and 10x objective.

4.6.3. Cellular location of FAM-mUNO

Human M2-resembling macrophages were purified and stimulated as described above and 3.5×10^5 cells were seeded on FBS-coated coverslips placed in a 24-well plate. On day 7, medium was removed and substituted with fresh medium

containing 3 nM of FAM-mUNO. Cells were incubated for 20 min, washed with medium and chased for 5, 15, 30, 90 and 180 min. Then, cells were washed twice with medium, once with PBS, fixed, immunostained with antibodies against Lamp1 (lysosomes), Rab5 (early endosomes) and Rab7 (late endosomes), mounted and imaged with Zeiss confocal microscope and 63x objective, using smallest optical thickness possible (pinhole: 60).

4.6.4. *In vitro* binding assay with LNPs

7.5×10^4 BMDMs with M0 phenotype were seeded to Lab-Tek 8-chamber slides, stimulated with IL-4 for 48 h to produce M2-resembling macrophages. 200µl/mL of LNPs-P-FAM or LNPs-P-F-mUNO was added and incubated for 10, 30 and 60 min at 37 °C. Then, cells were washed, fixed, permeabilised, immunostained for antibodies against CD206 and FAM and imaged with Leica SP5 inverted confocal microscope and 63x objective.

4.6.5. Cell viability assays

For cell viability assay, 1.2×10^5 monocytes were seeded to 96-well plate, pre-coated with FBS, and stimulated to become M2-resembling or M1-resembling macrophages as described above. On day seven, cells were incubated with OximUNO, St-PGA-DOX or DOX for 15 min, concentration 33 µM in DOX. After that, cells were washed, fresh medium added and incubated at +37 °C for 48 h, after which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and incubated up to 2.5 h. Then, medium with MTT was removed, crystals dissolved in isopropanol and absorbance read at 570 nm.

4.7. Animal experiments

Animal experiments were used to assess the effectiveness of drugs, to monitor the biodistribution of peptides or to evaluate the pharmacokinetics of peptides. All animal experiments were approved by Estonian Ministry of Agriculture.

4.7.1. Plasma half-life measurements

Healthy female balb/c mice were injected either intraperitoneally (i.p.) or i.v. with FAM-mUNO or St-PGA-OG-mUNO. Ten µl of blood was collected at different timepoints:

- for i.v. administration: 0 min, 5 min, 10 min, 15 min, 30 min, 60 min, 180 min, 360 min and 1440 min,
- for i.p. administration 0 min, 5 min, 10 min, 15 min, 30 min, 60 min, 180 min, 360 min and 1440 min.

Collected blood was added to PBS-Heparin mixture to avoid clotting. For FAM fluorescence measurements, blood-heparin mixture was centrifuged, plasma collected to a 96-well plate and measured with a plate reader (FlexStation II Molecular Devices) using the SoftMax Pro v5 programme using 480 nm as the

absorption wavelength and 520 nm as the emission wavelength. N=3 for each administration method and compound.

4.7.2. *In vivo* biodistribution studies of peptides

30 nanomoles of FAM-mUNO or FAM-CAQK were administered i.p. to mice carrying orthotopic TNBC tumours or pulmonary tumours from the experimental metastasis of TNBC model. Mice were sacrificed through anaesthetic overdose and cervical dislocation 24 h after peptide circulation, organs were collected, fixed, cryoprotected, sectioned and immunostained for antibodies against CD206 and FAM. Tissues were imaged using Zeiss confocal microscope and 20x objective.

4.7.3. *In vivo* biodistribution studies of LNPs

250 μg of R848@LNP-P-F-mUNO or R848@LNP-P-FAM dispersed in 200 μl of 5.4% glucose were injected i.p., circulated for 3 h after which mice were sacrificed through anaesthetic overdose and cervical dislocation, organs were collected, fixed, cryoprotected, sectioned and immunostained for antibodies against CD206 and FAM. Tissues were imaged using Zeiss confocal microscope and 20x objective.

4.7.4. *In vivo* biodistribution studies of polymer-drug conjugates

St-PGA-OG-mUNO (0.41 mg/0.5 mL of PBS) or St-PGA-OG (0.35 mg/0.5 mL of PBS; corresponding to 15 nanomoles of OG, absorbance measured by UV-Vis) were injected i.p. to mice carrying orthotopic TNBC tumours or pulmonary tumours from the experimental metastasis of TNBC model. Conjugates were circulated for 6 h after which mice were sacrificed through anaesthetic overdose and cervical dislocation, organs collected, fixed, cryoprotected, sectioned and immunostained for antibodies against CD206, CD86, CD11c and OG. Tissues were imaged using Zeiss confocal microscope and 20x objective.

4.7.5. *In vivo* macrophage reprogramming treatment using R848@LNP-P-F-mUNO

Orthotopic TNBC tumours were induced by injecting 1×10^6 4T1 cells to 8-12-week-old female Balb/c mice, treatments were started when tumours reached ~50 mm^3. In this instance, a combination therapy with vinblastine (Vin) was used. Vin is an anticancer drug that works through targeting tubulins. This leads to the salt-like precipitate which prevents the polymerisation of the tubulin which in turn leads to the cytotoxicity and cell necrosis (Gigant et al., 2005; Vacca et al., 1999). Mice were treated every other day with Vin, Vin+R848 (TLR agonist), Vin+R848@LNP-P-F-mUNO or Vin+R848@LNP-P-FAM. Mice were treated with Vin three times and with LNPs or free R848 for seven days. The treatment was terminated on day 18 post tumour induction, tumours and

organs were collected and immunological profiles of three tumours per group were studied using flow cytometry (FC) or immunofluorescence assay (IF). To analyse the renal toxicity, one-time i.p. administration of R848@LNP-P-F-mUNO or R848@LNP-P-FAM to healthy 8-12-week-old female Balb/c mice was done. Blood creatinine (Crea) values were analysed by Tartu University Hospital United Laboratories using a Cobas 6000 IT-MW.

4.7.6. Treatment studies with OximUNO on orthotopic TNBC and experimental metastasis of TNBC

For orthotopic TNBC, 5×10^4 4T1 cells were injected s.c. to of 8-12-week-old female Balb/c mice to induce orthotopic TNBC tumours. Treatment with OximUNO, St-PGA-DOX or DOX was started when tumours reached ~25 mm3. Mice were treated every other day for a total of nine injections, 18 mg/kg of DOX as a cumulative dose. The treatment was terminated on day 28 post tumour induction, tumours and organs were collected for immune profiling with IF. Lungs were also stained with haematoxylin and eosin (H&E) for the visualisation of pulmonary metastases. Treatment was repeated to analyse the effect OximUNO has on survival. For that, mice were treated nine times as described earlier after which they were sacrificed based on the tumour volume (not above 2000 mm^3).

For experimental metastasis of TNBC, 2×10^5 4T1-GFP cells were i.v. injected to 8-12-week-old female Balb/c mice to produce pulmonary nodules. The treatment with OximUNO, St-PGA-DOX or DOX was started on day 4 post tumour induction and drugs were injected six times in total, cumulative DOX dose of 12mg/kg. On day 18 post tumour induction, all mice were sacrificed, lungs were analysed with H&E and with the visualisation of GFP to detect pulmonary nodules, and with FC and IF to understand the differences in immune cell populations and marker expression.

To analyse the renal and hepatic toxicity of OximUNO, one-time i.p. administration of OximUNO to healthy 8–12-week-old female Balb/c mice was done. Blood Crea and alanine aminotransferase (ALAT) values were analysed by Tartu University Hospital United Laboratories using a Cobas 6000 IT-MW.

4.8. Statistical analysis

Different statistical tests and programmes were used in articles presented in this dissertation:

Article I: statistical analysis was done with Origin Pro 8 and one-way analysis of variance (ANOVA). Error bars represent standard error.

Article II: statistical analysis was done with GraphPad Prism. One way ANOVA using Bomferroni's post-hoc test was used to calculate statistical significance. For tumour weights, Newman-Keuls Mul-

tiple Comparison Test was used and for tumour curves, two-way ANOVA.

Article III: statistical analysis was done with Statistica programme (release 7). One-way ANOVA and Fisher LSD test was used, except for survival where GraphPad Prism (version 9.3.1) Mantel-Cox test was used. Survival curves were done using Kaplan-Meier estimator.

5. RESULTS

5.1. Targeting CD206$^+$ TAMs found in tumour and pulmonary metastases with mUNO peptide

5.1.1. Strategies to couple mUNO to different platforms

One important aspect in using peptides as targeting moiety to develop new pharmaceuticals is their ability to be conjugated to a system without losing their activity. In this study, we tested different ways to include mUNO peptide into a nanosystem. We have previously shown that mUNO can be conjugated through its N-terminus to FAM (Asciutto et al., 2019; Scodeller et al., 2017) but we wanted to investigate it further to see if the cysteine thiol of mUNO could be used to incorporate it into a nanosystem without losing the CD206-binding activity. For that we used PSs decorated with Mal groups on the surface and used a thiol-Mal bond to couple FAM-mUNO (scheme Fig. 4A). The incubation with human M2-resembling macrophages revealed a high binding to CD206 with targeted PSs which was not seen with untargeted FAM-PS (Fig. 4B, C, G). Moreover, the binding was reduced when CD206 was blocked with an antibody (Fig. 4D, G), indicating that FAM-mUNO-PS targeting is mediated by CD206. The same blocking effect was not seen with untargeted PSs (Fig. 4E, G). The FAM-mUNO-PS signal inside the target cells seemed to be associated with CD206$^+$ structures as it was not diffusely inside the cytosol (Fig. 4F).

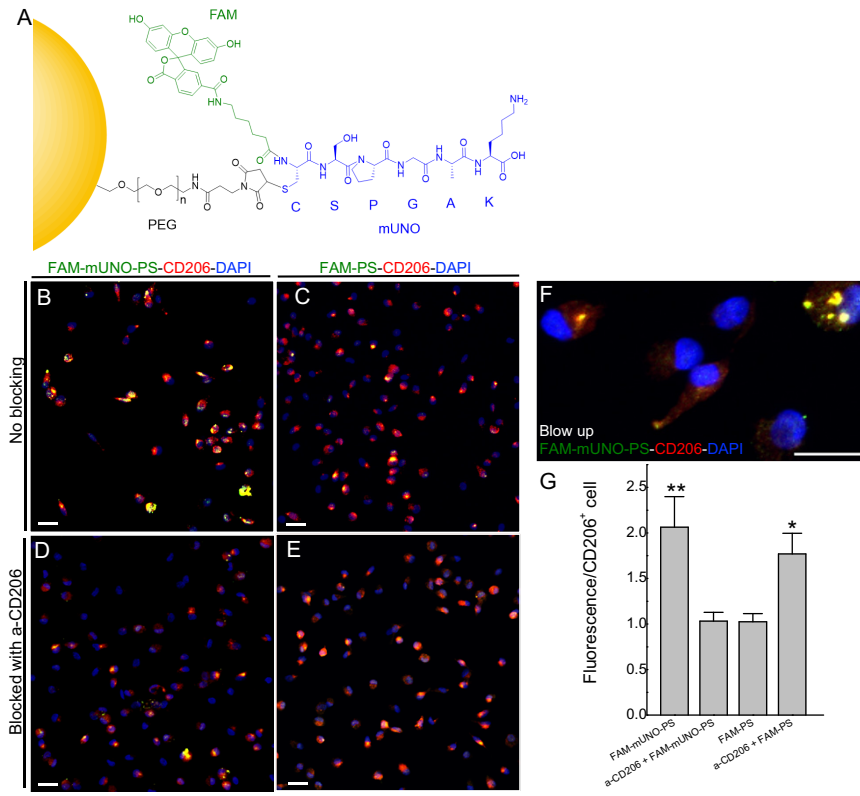


Fig. 4. mUNO coupling to the PS nanosystem. (A) Scheme showing the structure of FAM-mUNO-PS. (B-E) *In vitro* binding of (B) FAM-mUNO-PS, (C) FAM-PS on human M2-resembrling macrophages. Binding of (D) FAM-mUNO-PS was reduced when CD206 was blocked whereas increased with (E) FAM-PS. (F) Blow-up of M2-resembling macrophages showing FAM-mUNO-PS inside the cell, bound to CD206 (yellow signal). (G) Colocalisation analysis. **P ≤ 0.01, ***P ≤ 0.001, scale bars represent 20 µm.

To broaden the scope mUNO peptide could have, we used another nanoplatform, LNPs, to show that mUNO could be coupled through its cysteine without losing CD206 activity. As a collaboration project, we took LNP and functionalised them with PEG-Mal to form LNP-PEG-Mal to which we conjugated FAM or FAM-mUNO to form LNP-P-FAM or LNP-P-F-mUNO (scheme Fig. 5A). Using cultured mouse M2-resembling macrophages and fluorescent microscopy we were able to show that LNP-P-F-mUNO showed higher binding to CD206 compared to LNP-P-FAM (Fig. 5B), which was also quantified (Fig. 5C, D, E). Out of three timepoints tested, the 30 min one showed the highest CD206/LNP-P-F-mUNO colocalisation (Fig. 5C, D, E). The FC analysis revealed ~2-times higher CD206/LNP-P-F-mUNO colocalisation compared to

CD206-LNP-P-FAM (Fig. 5F) which was a clear indication that 30 min time-point would be good choice for future *in vitro* studies.

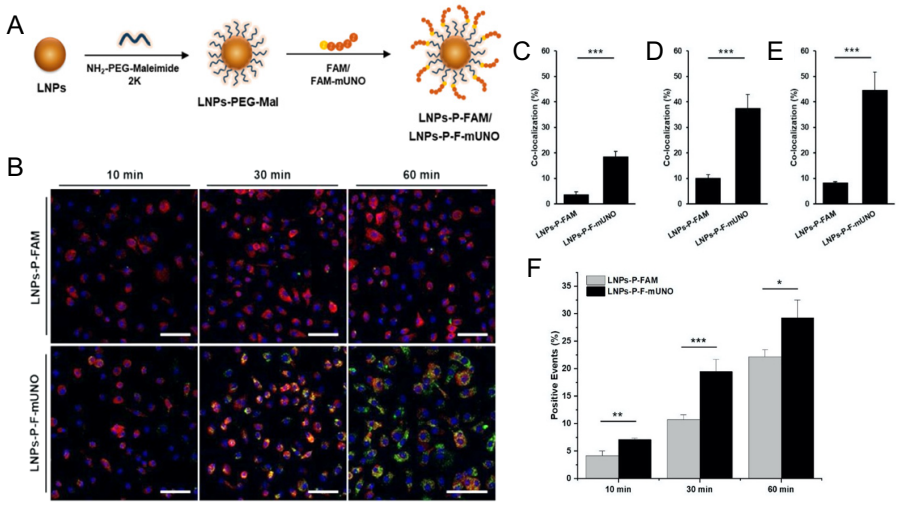


Fig. 5. LNP-P-F-mUNO binds to mouse M2-resembling macrophages with 30 min timepoint showing the highest binding. (A) Scheme of LNP-P-F-mUNO/LNP-P-FAM. (B) Binding of LNP-P-F-mUNO to mouse M2-resembling macrophages at different timepoints compared to LNP-P-FAM. Yellow signal indicates colocalisation. (C-E) The colocalisation analysis showing the FAM/CD206 colocalisation at (C) 10 min, (D) 30 min and (E) 60 min. (F) FC analysis showing LNP-P-F-mUNO/CD206 colocalisation at different timepoints compared to LNP-P-FAM. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, scale bars represent 50 µm.

5.1.2. I.p. injected FAM-mUNO has longer plasma half-life than i.v. injected FAM-mUNO

Plasma half-life is an important parameter on peptide-guided drug delivery systems. More time the compound is in the system the more chance it has to find and to bind to its receptor. Also, different administration routes can have significant effect on half-life. Therefore, we compared the i.p. and i.v. administration of FAM-mUNO as these are most common ways to administer compounds in clinic. We found that i.p. administered FAM-mUNO (Fig. 6A) has approximately four times longer half-life compared to i.v. administration (Fig. 6B). Therefore, following experiments with FAM-mUNO were performed using this administration route.

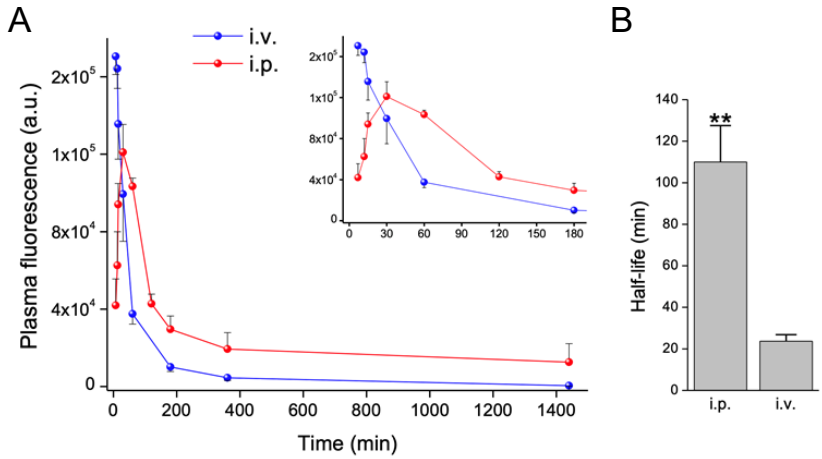


Fig. 6. FAM-mUNO shows 4x longer plasma half-life when administered i.p. (A) Plasma half-life of i.p. and i.v. administered FAM-mUNO and (B) its quantification. **P ≤ 0.01.

5.1.3. Internalised FAM-mUNO is rescued from lysosomal entrapment in CD206$^+$ macrophages *in vitro*

The development of mUNO as a precision intracellular delivery vehicle requires a detailed understanding of its subcellular fate upon cell binding and -internalisation. Endosomal and/or lysosomal entrapment could hamper peptide's use as targeting moiety. For that we performed a pulse/chase experiment with FAM-mUNO on primary human M2-resembling macrophages and found that FAM-mUNO showed a significant fraction of the peptide associated with Rab5$^+$ early endosomes (yellow signal, white arrowheads in Fig. 7A) at 5 min, which decreased as time passed. FAM-mUNO was not associated with Rab7$^+$ late endosomes (Fig. 7B) nor Lamp1$^+$ lysosomes (Fig. 7C).

These results indicate that FAM-mUNO is internalised through endosomal pathway and suggest that mUNO-targeted peptide-drug conjugate would not undergo lysosomal entrapment.

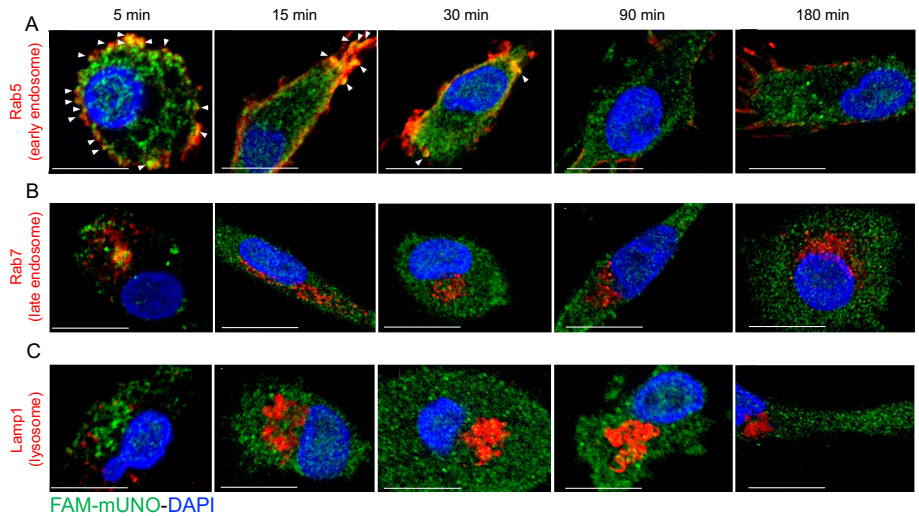


Fig. 7. FAM-mUNO escapes lysosomal entrapment in CD206$^+$ M2-resembling macrophages *in vitro*. (A) FAM-mUNO association with Rab5$^+$ early endosomes at various timepoints. (B-C) No association was observed with (B) Rab7$^+$ late endosomes or (C) Lamp1$^+$ lysosomes. Scale bars represent 10 µm.

5.1.4. FAM-mUNO homes to CD206$^+$ TAMs in early stage orthotopic TNBC and experimental metastasis of TNBC

To be able to use peptides as targeting agents for drug development, it is important to know the biodistribution of said peptide in the body. For that we used an early stage orthotopic TNBC and experimental metastasis of TNBC and i.p. administration of FAM-mUNO or FAM-Control. We chose these tumour models as they express high levels of CD206 in the tumour (Scodeller et al., 2017). With IF analysis we showed that FAM-mUNO homes to CD206$^+$ TAMs in both early stage orthotopic TNBC (Fig. 8A, E) and experimental metastasis of TNBC (Fig. 8G, K) and there was relatively low colocalisation with CD86$^+$ TAMs (M1 phenotype) (Fig. 8C, I, E, K). In contrast, For FAM-control, we observed very low colocalisation between CD206/FAM-Control (Fig. 8B, H, E, K), and the CD86$^+$ TAM targeting was similar to FAM-mUNO (Fig. 8D, J, E, K). We also looked at the i.v. availability of FAM-mUNO and were able to see that the colocalisation with CD206 is lower compared to i.p. administration (Fig. 8F), presumably because of the shorter half-life using the i.v. route. We did not observe any accumulation of FAM-mUNO in CD206$^-$ regions of lung foci (Fig. 8L) and we observed no hepatic accumulation of FAM-mUNO or FAM-Control in both models (Fig. 8M).

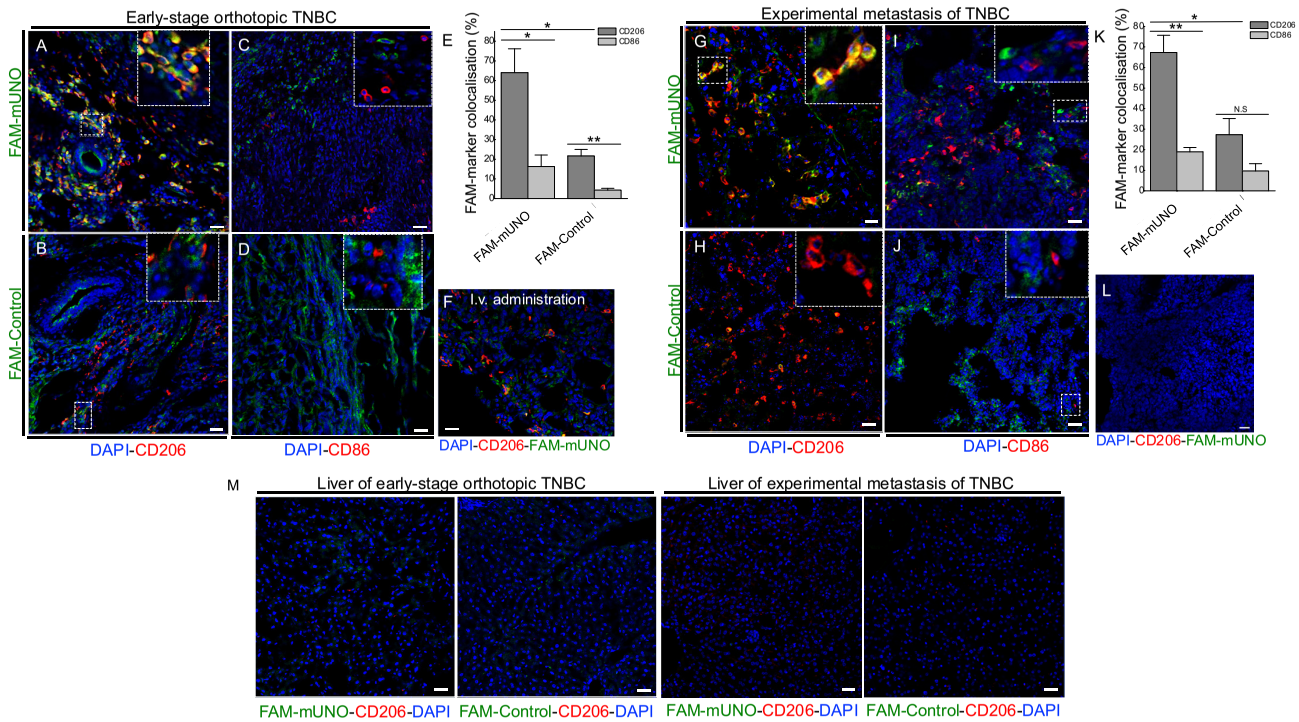


Fig. 8. FAM-mUNO shows high colocalisation with CD206+ TAMs in both early stage orthotopic TNBC and experimental metastasis of TNBC with no hepatic accumulation. (A-D) Representative immunostained images showing FAM-mUNO colocalisation with (A) CD206 and (C) CD86, and FAM-Control colocalisation with (B) CD206 and (D) CD86 in early-stage TNBC. (E) Quantification of IF signal. (F) Representative image for i.v. administration of FAM-mUNO. (G-J) Representative immunostained images showing FAM-mUNO colocalisation with (G) CD206 and (I) CD86, and FAM-Control colocalisation with (H) CD206 and (J) CD86 in lung foci of TNBC. (K) Quantification of IF signal. (L) Representative image showing no FAM-mUNO homing in $CD206^-$ areas. (M) Hepatic homing of FAM-mUNO and FAM-Control in both models. $*P \leq 0.05$, $**P \leq 0.01$, N.S. = not significant, scale bars represent 20 μm.

These results, together with the plasma pharmacokinetics, concluded that using the i.p. administration route we can see highly selective CD206 targeting with FAM-mUNO peptide sparing $CD86^+$ TAMs in both models tested. This gave us a rationale to use this administration route and these models for our future studies.

5.1.5. FAM-mUNO detects lymph node and $CD206^+$ TAMs of mice with spontaneous pulmonary metastases

We were also interested in looking at other potential application of mUNO, as an imaging peptide for lymph nodes in metastatic TNBC. Sentinel lymph node (SLN) mapping through imaging the presence of M2 macrophages in the lymph node is used in the clinic as an early predictor of relapse or metastasis (Mwagiru et al., 2022). For that we used 4T1 tumour-bearing mice and injected FAM-mUNO i.p. As a comparison, we used a well-known peptide, FAM-LyP-1 which is known to home to TAMs and to lymphatic vessels of a tumour (Kim et al., 2019; Laakkonen et al., 2002, 2004; Luo et al., 2010; Z. Wang et al., 2012; Yan et al., 2012). In ex vivo FAM fluorescence imaging, FAM-mUNO detected the tumour and SLN (Fig. 9A, D, G, H) whereas FAM-LyP-1 (Fig. 9B, E, G, H) showed similar levels to autofluorescence (Fig. 9C, F, G, H). We observed similar hepatic signal from both peptides, and it did not differ from the auto-fluorescence values (Fig. 9I). With IF analysis we were able to see that the FAM-mUNO signal was associated with $CD206^+$ TAMs in tumour (Fig. 9J) and SLN (Fig. 9L) but we did not see the same with FAM-LyP-1 (Fig. 9K, M). Importantly, we were able to show FAM-mUNO signal in spontaneous pulmonary metastases (Fig. 9N), but we did not see this at the same level with FAM-LyP-1 (Fig. 9O). Finally, the hepatic signal of each peptide was low (Fig. 9P, Q).

Although FAM is not the best imaging probe due to it high tissue absorbance and high scattering in the spectral region, this as a proof-of-concept experiment showed the possible use of mUNO as a guiding peptide to direct imaging agents to metastases better than already established TAM/SLN targeting agent LyP-1 and lays the foundation for further imaging studies using near infrared dyes such as Cy7 or Indocyanine Green instead of FAM.

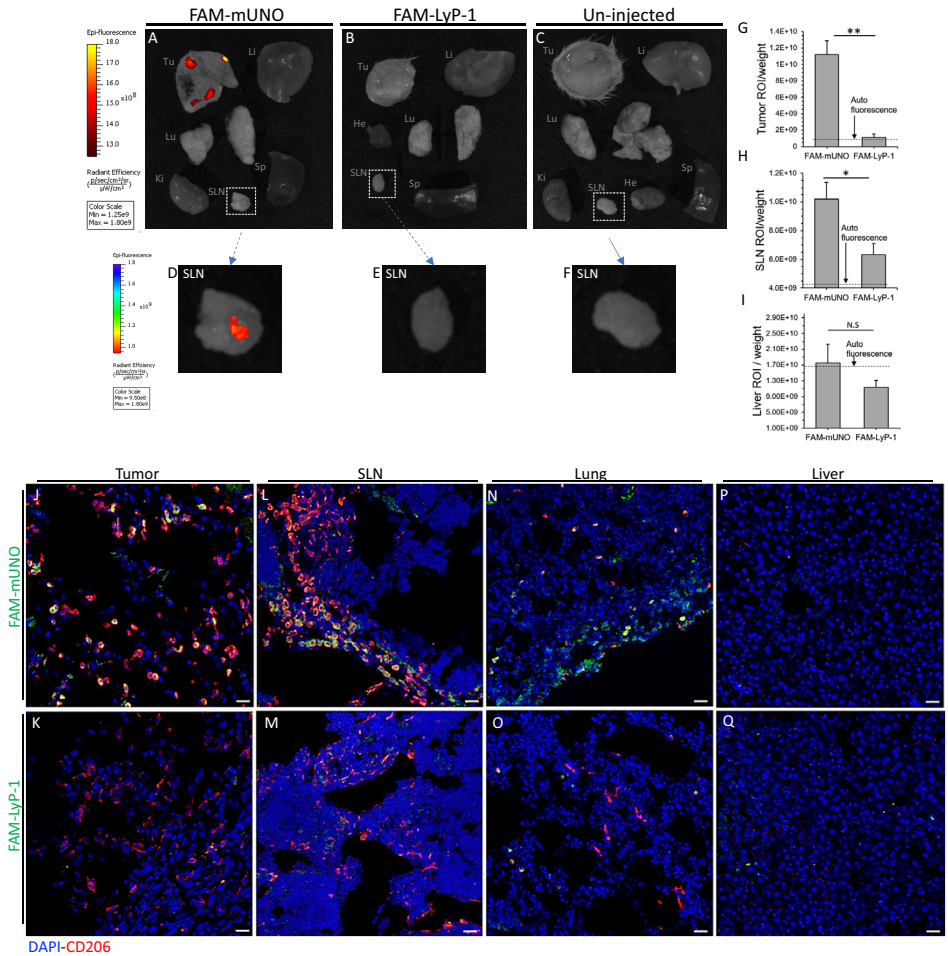


Fig. 9. FAM-mUNO detects SLN and CD206$^+$ metastatic pulmonary lesions. (A-C) Fluorescence of (A) FAM-mUNO, (B) FAM-LyP-1 and (C) noninjected control detected with In Vivo Imaging System (IVIS) Spectrum. (D-F) Close up of SLN. (G-I) Graphed FAM fluorescence normalised to organ weight. (J-Q) Representative immunostained images of (J, K) tumour, (L, M) SLN, (N, O) pulmonary lesions and (P, Q) liver. *P ≤ 0.05, **P ≤ 0.01, N.S. = not significant. Scale bars represent 20 μm.

5.2. mUNO-guided re-education of CD206$^+$ TAMs

5.2.1. R848-loaded mUNO-targeted LNPs home to CD206$^+$ TAMs in orthotopic TNBC

We were interested in tackling the high amount of immunosuppressive CD206$^+$ TAMs in different ways. One possible option would be to not eliminate these cells but to reprogramme them to become tumouricidal. Therefore, we conjugated FAM-mUNO peptide to LNPs and used R848 as a reprogramming drug: R848@LNP-P-F-mUNO where P stands for PEG-Mal and F stands for FAM. Using this nanosystem, we were able to show that i.p. administered R848@LNP-P-F-mUNO homes to CD206$^+$ TAMs in the orthotopic TNBC model (Fig. 10A, B). R848@LNP-P-F-mUNO showed similar CD206 colocalisation than untargeted R848@LNP-P-FAM (Fig. 10B, C) but when we analysed the FAM fluorescence intensity/CD206$^+$ cells, we were able to show that there is three times higher FAM/CD206$^+$ cell signal with R848@LNP-P-F-mUNO compared to R848@LNP-P-FAM (Fig. 10D). This indicates that R848@LNP-P-F-mUNO might be suitable for an *in vivo* TAM reprogramming study. Moreover, using these imaging conditions, we did not observe R848@LNP-P-F-mUNO accumulation 3 h after the administration in control organs (Fig. 10A).

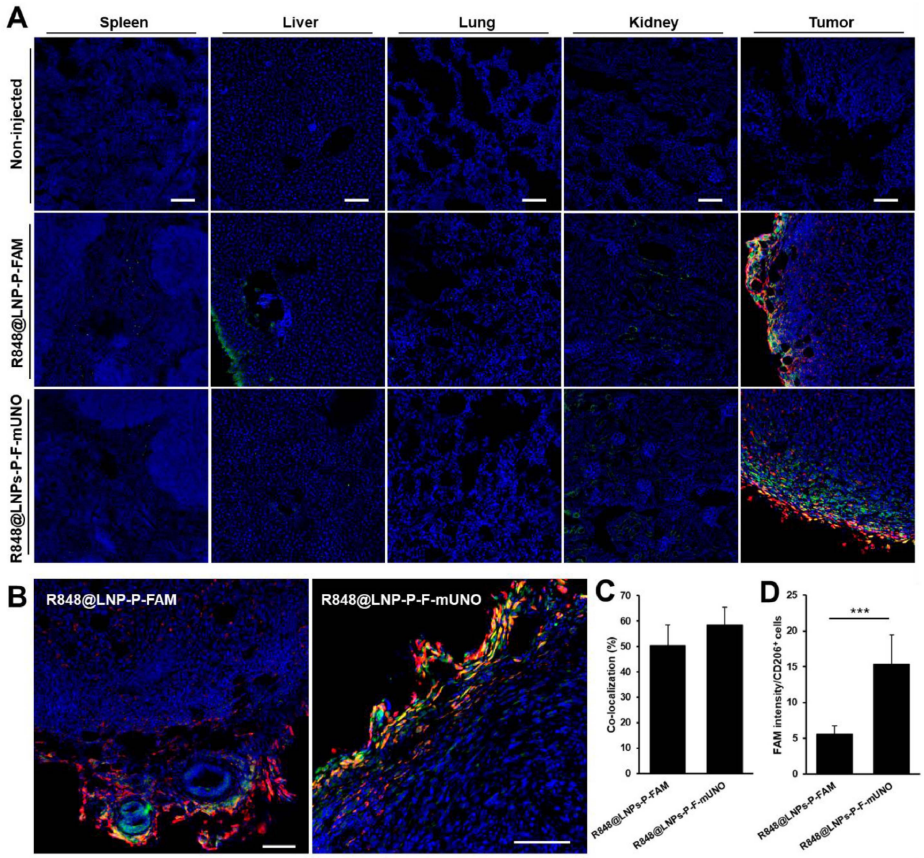


Fig. 10. R848@LNP-P-F-mUNO homes to CD206^{+} TAMs in orthotopic TNBC sparing other organs. (A) Biodistribution of i.p. administered R848@LNP-P-F-mUNO and R848@LNP-P-FAM in tumour and control organs, circulation time 3 h. (B) Close-up depicting R848@LNP-P-F-mUNO colocalisation with CD206 (yellow signal) compared to R848@LNP-P-FAM/CD206 colocalisation (yellow signal). (C) Colocalisation analysis showing similar FAM/CD206 colocalisation for both LNPs. (D) FAM signal/CD206+ cells indicating three times higher signal for R848@LNP-P-F-mUNO. ***P ≤ 0.001, scale bars represent 100 µm.

5.2.2. Combining R848@LNP-P-F-mUNO with Vin reduced tumour progression and shifted the immune landscape towards immunostimulatory

Based on *in vivo* biodistribution results, we decided to test the potential of R848@LNP-P-F-mUNO as a targeted TAM-reprogramming therapy in orthotopic TNBC model. We decided to use it in combination with an anticancer drug Vin to potentiate its effect. Treating mice bearing orthotopic TNBC tumour with Vin three times (Fig. 11A, red arrows) in combination with five injections of R848@LNP-P-F-mUNO (Fig. 11A, blue arrows) reduced tumour volume (Fig. 11B) and weight (Fig. 11C), increased M1 macrophages (Fig. 11D), $CD8^+$ cytotoxic T lymphocytes (CTLs) (Fig. 11E, I), $CD86^+$ dendritic cells (DCs) (Fig. 11F) and IFN-γ (Fig. 11J). Additionally, we also were able to reduce the level of endogenous inducible nitric oxide synthase (iNOS) (Fig. 11G) which has been associated with increased TNBC aggressiveness (Granados-Principal et al., 2015; Walsh et al., 2016). Importantly, compared to R848@LNP-P-FAM, we were able to decrease the expression of CD206 (Fig. 11H) which together with the increase in M1 macrophages indicates that we were able to reprogramme a portion of $CD206^+$ TAMs to M1 TAMs.

Altogether as a combinational therapy, R848@LNP-P-F-mUNO+Vin produced more anti-tumoural TME, indicating the use of reprogramming drugs in combination with CD206-targeting moiety as a potential treatment option for TNBC.

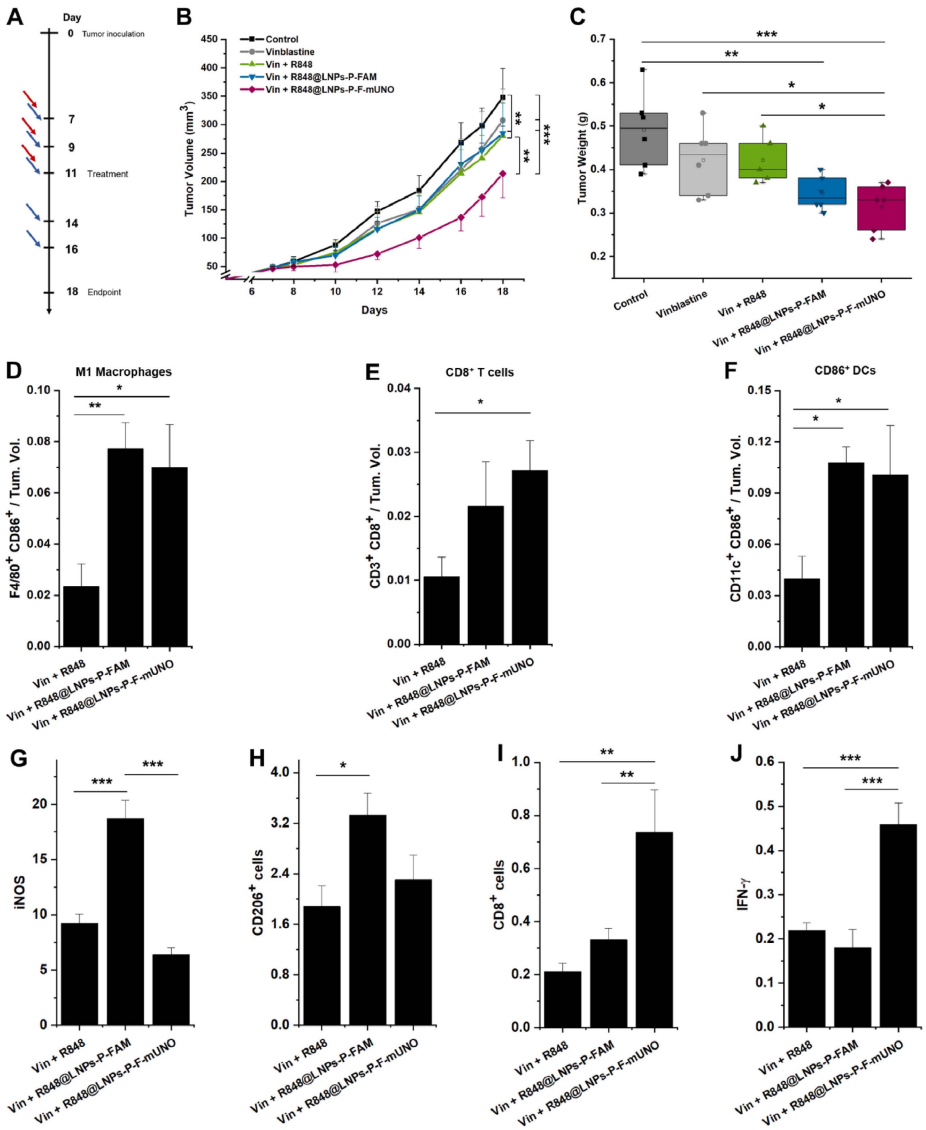


Fig. 11. Combinational therapy with Vin and R848@LNP-P-F-mUNO reduces tumour weight, increases CTLs and CD86$^+$ TAMs while decreasing CD206$^+$ TAMs. (A) Schematic representation of combination treatment. (B) Tumour volume and (C) tumour weight graphs indicating smaller volume and weight for R848@LNP-P-F-mUNO + Vin treated mice. (D-J) Analysis of immune system indicating (D) lower CD86$^+$ TAMs, (E) increased CTLs and (F) CD86$^+$ DCs, (G) lower iNOS levels, (H) decreased CD206 expression and increased (I) CD8 and (J) IFN-γ expression. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001

5.3. mUNO-guided CD206^{+} TAM depletion

5.3.1. Computational analysis of St-PGA and the structures of St-PGA-OG-mUNO and OximUNO

To continue exploring different mUNO-guided nanosystems, we next decided to explore the polymer-drug conjugate platform. To this end we used St-PGA, developed by Dr. Maria J. Vicent (CIPF, Valencia, Spain) (Docón et al., 2017), as a backbone onto which we could couple mUNO peptide and fluorescein or drug. Conjugating mUNO to a St-PGA backbone would significantly improve the targeting through the avidity effect and increased plasma half-life (Ekladious et al., 2019). Using this nanosystem allowed us to explore another biocompatible system for drug delivery. In collaboration with Dr. Asciutto (UNSAM, Buenos Aires, Argentina) and colleagues we performed computer simulations to model the structure in solution and we observed that St-PGA remains in an open structure throughout the simulation (Fig. 12A). We also saw that the addition of mUNO only slightly alters the secondary structure of PGA (Fig. 12B). The computational analysis revealed that three mUNO peptides on one PGA chain remain available to the solution, allowing rotation around 50º and 180º (Fig. 12C), which is in line with allowing mUNO peptide to remain available for receptor interaction (Asciutto et al., 2019).

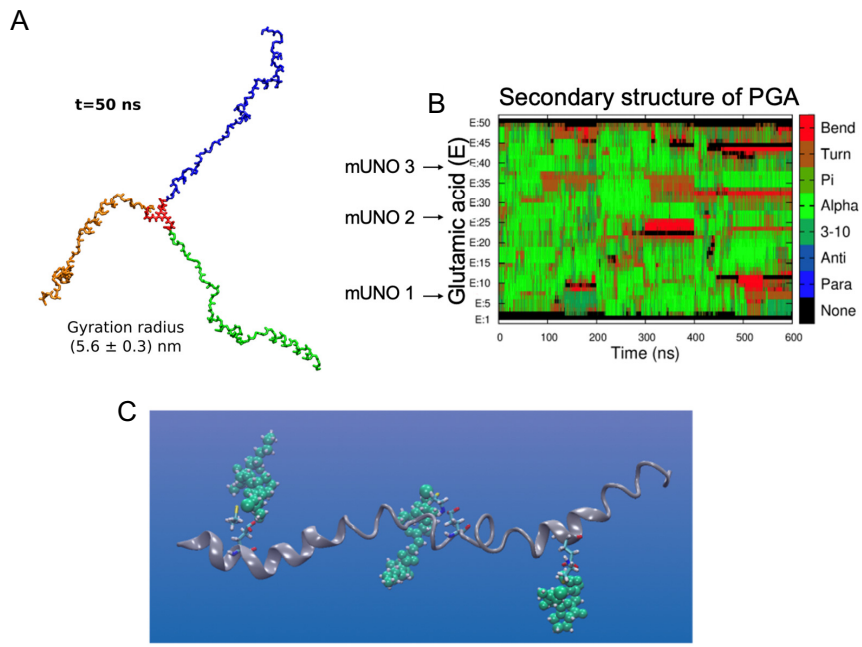
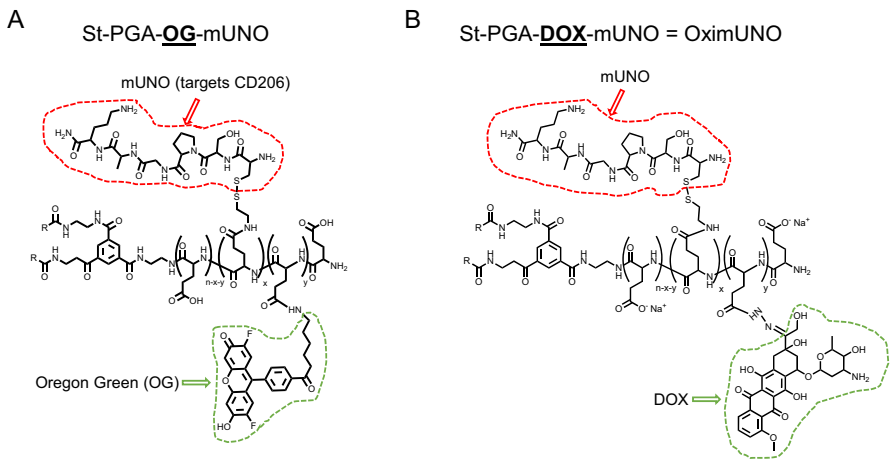


Fig. 12. Computational analysis of PGA-mUNO revealed open structure and minimal alterations to the secondary structure. (A) Representative image of open structure of St-PGA at 50ns after simulation start. (B) Addition of mUNO only minimally alters the secondary structure of PGA. (C) mUNO peptides on one PGA arm can rotate around 50º and 180º.

We then designed the conjugate St-PGA-OG-mUNO, where OG allows us to follow its fluorescence to evaluate the biodistribution *in vivo*. Schematic representation of the structure of St-PGA-OG-mUNO is shown in Scheme 1A, where mUNO was coupled through a disulphide bond (formed between the cysteine of mUNO and a pyridyldithiol linker on St-PGA) and OG was coupled using an amide bond. Next, after validating St-PGA-OG-mUNO, as we were interested in depleting $CD206^+$ TAMs, we replaced OG with the drug DOX to produce a targeted polymer-drug conjugate that we refer to as OximUNO. DOX in this case was coupled using the acid-labile hydrazone linker to allow its release from the conjugate while in endosomes. A scheme showing its structure is shown in Scheme 1B.



Scheme 1. A schematic representation of St-PGA-OG-mUNO and OximUNO. (A) St-PGA-OG-mUNO where red indicates mUNO and green OG. (B) OximUNO where red indicates mUNO and green shows DOX.

The rationale is that mUNO-guided polymer-drug conjugates could take the cytotoxic drug to $CD206^+$ TAMs found in TME and therefore, by depleting $CD206^+$ TAMs, reduce tumour size and alleviate immunosuppression.

5.3.2. St-PGA-OG-mUNO shows selective targeting of CD206$^+$ TAMs, low hepatic accumulation and better tumour penetration than a therapeutic antibody

Firstly, we were interested in the biodistribution of St-PGA-OG-mUNO inside the body to detect any possible unspecific targeting and to confirm the colocalisation with CD206 *in vivo*. Using IF and injecting St-PGA-OG-mUNO i.p. in orthotopic TNBC, we were able to show high colocalisation of St-PGA-OG-mUNO and CD206, shown in yellow, whereas untargeted St-PGA-OG showed minimum CD206 colocalisation (Fig. 13A, C) Importantly, we did not observe any targeting to CD86$^+$ M1 TAMs (Fig. 13D) and CD11c$^+$ DCs (Fig. 13F). A similar trend was observed in the experimental metastasis of TNBC where St-PGA-OG-mUNO showed significantly higher OG/CD206 colocalisation than St-PGA-OG, shown as yellow signal (Fig. 13H, J). Again, in this model, with St-PGA-OG-mUNO we did not target M1 TAMs (Fig. 13K) or DCs (Fig. 13M). The same trend was not seen with untargeted St-PGA-OG with both models. In both models, hepatic accumulation of St-PGA-OG-mUNO was low (Fig. 13O). The high tumour accumulation and low hepatic accumulation could be partially explained by the differences in the vasculature leakiness of these two tissues as shown with IgG staining (Fig. 13P). Moreover, liver has lower expression of CD206; when we used the same imaging settings for tumour and liver, tumour CD206 signal was saturated (Fig 13Q). Importantly, accumulation of St-PGA-OG-mUNO to lungs and spleen was not observed (Fig. 13R), however there was some in SLN and in the kidneys (Fig. 13R). Renal accumulation is expected as St-PGA is cleared through this route (Duro-Castano, England, et al., 2015).

We also evaluated the plasma half-life of i.p.-administered St-PGA-OG-mUNO (Fig. 13S) and found that it was two times longer compared to i.p.-administered FAM-mUNO (Fig. 6). A longer half-life is expected to translate into more effective targeting, as it gives mUNO more chance to find its receptor.

As antibody-based therapies are popular in cancer drug development, we decided to compare our St-PGA-OG-mUNO tumour homing with that of a therapeutic monoclonal antibody PD-L1. By injecting anti-PD-L1 i.v. to orthotopic TNBC bearing mice, we were able to observe that the antibody accumulated in the tumour rim (Fig. 14A, TR) but there was none detected in the tumour core (Fig. 14A, TC), even though we were able to detect the expression of PD-L1 in the tumour core (Fig. 14B, TC). This contrasts with St-PGA-OG-mUNO which showed high accumulation (Fig. 14C, TC) and CD206 colocalisation (Fig. 14D, TC) in the tumour core. That supports the implementation of our platform as an alternative to antibody-based therapies.

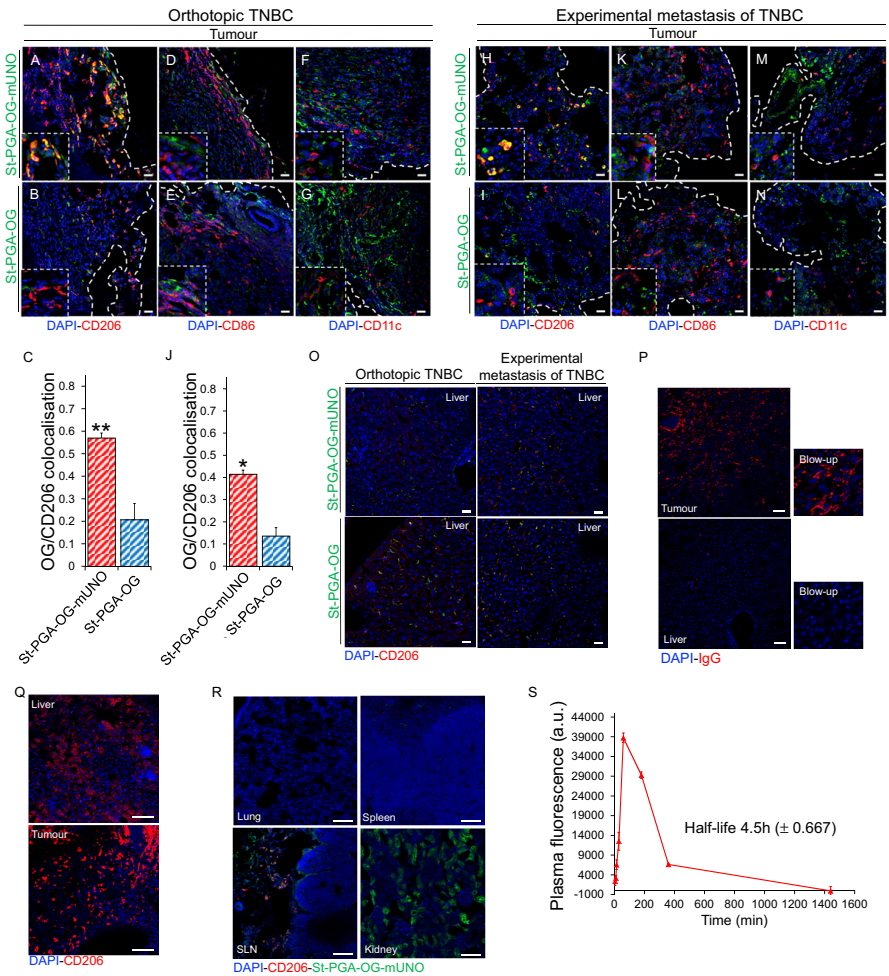


Fig. 13. St-PGA-OG-mUNO homes to CD206+ TAMS while sparing CD86$^+$ TAMs and CD11b$^+$ DCs. (A-G) Biodistribution of St-PGA-OG-mUNO/St-PGA-OG in orthotopic TNBC. (H-N) Biodistribution of St-PGA-OG-mUNO/St-PGA-OG in experimental metastasis of TNBC. (O) Hepatic accumulation of St-PGA-OG-mUNO and St-PGA-OG in both models. (P) IgG expression in tumour (top) or liver (bottom) indicating leaky vasculature. (Q) Expression of CD206 in the liver (top), using the same settings, the signal in tumour is saturated (bottom). (R) Biodistribution of St-PGA-OG-mUNO or St-PGA-OG in lung, spleen, SLN and kidneys. (S) Plasma half-life of i.p. injected St-PGA-OG-mUNO. *P ≤ 0.05, **P ≤ 0.01, scale bars represent 50 µm for A-N, R, Q, scale bars are 20 µm for O and P.

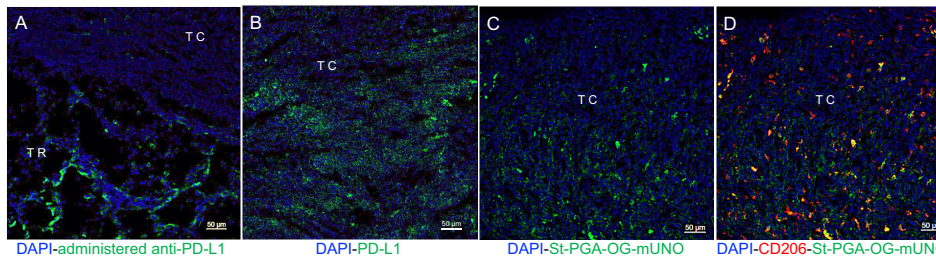


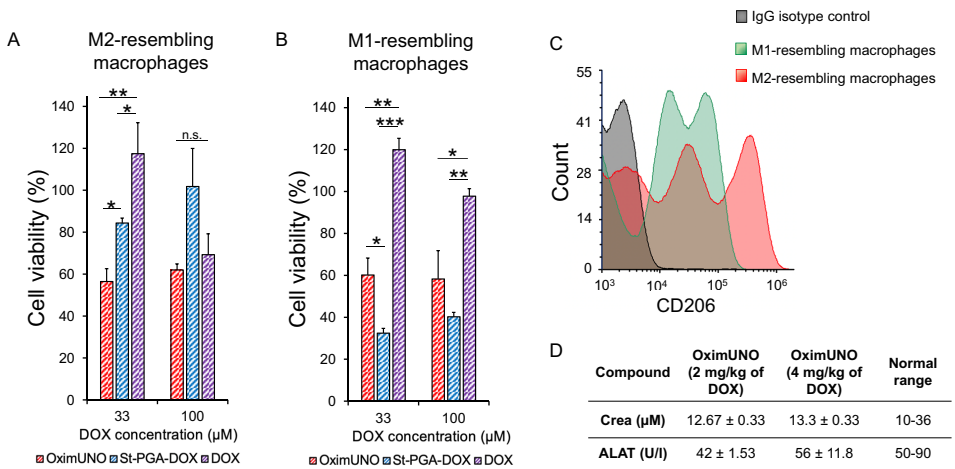
Fig. 14. St-PGA-OG-mUNO shows accumulation to the tumour core, whereas anti-PD-L1 only to the tumour rim. (A) Accumulation of anti-PD-L1 antibody to the tumour rim and tumour core. (B) Expression of PD-L1 in tumour core. (C) Accumulation of St-PGA-OG-mUNO in tumour core. (D) The expression of CD206 in the tumour core (red) and the colocalisation of OG/CD206 in the tumour core. Scale bars represent 50 µm.

As there is high colocalisation between St-PGA-OG-mUNO and CD206 in both orthotopic TNBC and experimental metastasis of TNBC and it shows accumulation in the tumour core whereas monoclonal PD-L1 antibody does not, this supports the use of polymer-drug conjugate-based therapy in the development of drugs against TNBC. Moreover, monoclonal antibodies that modulate the immune system may have drawbacks such as side effects, resistance and low response rate (Melero et al., 2007; Zahavi & Weiner, 2020).

5.3.3. OximUNO enhances the *in vitro* cytotoxicity of DOX on M2-resembling macrophages

We next evaluated the *in vitro* toxicity of OximUNO on M2- and M1-resembling macrophages derived from primary human monocytes. We were able to show that OximUNO at 33 µM concentration of DOX (similar to *in vivo* DOX concentration) showed increased toxicity to M2-resembling macrophages compared to St-PGA-DOX or DOX (cell viability 56% vs 84% or 118%, respectively) (Fig. 15A). OximUNO showed also similar level of toxicity to M1-resembling macrophages (Fig. 15B, red bars) which is expected as cytometry analysis of these cell types showed moderate expression of CD206 by M1-resembling macrophages (Fig. 15C). Since St-PGA-OG-mUNO did not target CD86+ M1 TAMs *in vivo* (Fig. 13D, K), we concluded that slight toxicity *in vitro* would not translate to significant toxicity towards CD86+ TAMs. We also evaluated the renal and hepatic toxicity with OximUNO using 2mg/kg or 4mg/kg of DOX by measuring Crea and ALAT levels in blood (Fig. 15D) and concluded that as there was no deviation from the normal range measured by The Jackson Laboratory (https://phenome.jax.org/search/details/ssmeasures?searchterm=alanine+aminotransferase+&ontavail=2) or Charles River facilities (https://www.criver.com/products-services/find-model/balbc-mouse?region=3616). Furthermore, as there was no renal toxicity, the signal in the kidneys shown in Fig. 13R

is probably related to the excretion route of St-PGA (Duro-Castano, England, et al., 2015; Duro-Castano et al., 2017).



D

Compound	OximUNO (2 mg/kg of DOX)	OximUNO (4 mg/kg of DOX)	Normal range
Crea (μM)	12.67 ± 0.33	13.3 ± 0.33	10-36
ALAT (U/l)	42 ± 1.53	56 ± 11.8	50-90

Fig. 15. OximUNO showed toxicity to M2-resembling human macrophages *in vitro*. (A) Cytotoxicity of OximUNO (red bars), St-PGA-DOX (blue bars) and DOX (purple bars) on M2-resembling human macrophages and (B) M1-resembling human macrophages at two different concentrations. (C) Expression of CD206 by M2-resembling macrophages (red), M1-resembling macrophages (green) and isotype control (black). (D) Crea and ALAT levels measured in blood serum after one time administration of OximUNO at two different concentrations. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, n.s. = not significant.

In vitro, OximUNO showed enhanced cytotoxicity compared to free DOX to M2-resembling macrophages, indicating that mUNO's conjugation to a polymer-drug nanosystem would magnify DOX's cytotoxicity and specificity to CD206+ TAMs *in vivo*.

5.3.4. OximUNO treatment on orthotopic TNBC and experimental metastasis of TNBC reduces CD206^{+} TAMs, inhibits tumour progression and attenuates immunosuppression

OximUNO suitability as a potential drug was tested in two TNBC models, orthotopic TNBC and experimental metastasis of TNBC, to evaluate its ability to suppress cancer progression as well as analyse its effect on pulmonary metastases. In orthotopic TNBC we were able to show that only the treatment with OximUNO significantly reduced the size of primary breast tumours (Fig. 16A, B) without affecting the bodyweight of these animals (Fig. 16C). We also showed that OximUNO was able to reduce pulmonary nodules in orthotopic model (Fig. 16D) with OximUNO-treated lungs having smaller metastatic area (Fig. 16E) and less pulmonary nodules (Fig. 16F), revealing its potential meta-

stasis delaying effect. OximUNO treatment did not show any significant changes in CD31 expression in tumours (Fig. 16G, H) but we did observe significantly fewer $CD31^+$ structures in this group (Fig. 16I). This might suggest that the reduction of pulmonary nodules observed with OximUNO is mediated by lower vascularisation of the primary tumour. As we are using a known cardiotoxic drug, DOX, it was important to analyse the potential cardiotoxicity of the hearts. Importantly, we did not observe any histological indication of cardiotoxicity (Fig. 16J). The IF analysis revealed that OximUNO treatment significantly reduced the expression of CD206 but the treatment with DOX upregulated CD206 expression (Fig. 16K, L) which agrees with reports showing increased number of $CD206^+$ TAMs following chemotherapy (R. Hughes et al., 2015). Importantly, only OximUNO-treated tumours showed significantly increased CD8 expression (indicating more CTLs) (Fig. 16M, N). Interestingly, St-PGA-DOX treatment increased FOXP3 expression, indicating the presence of more Tregs (Fig. 16O, P), and when we analysed the CD8/FOXP3 ratio, we were able to see that OximUNO treatment resulted in 5-fold increase compared to control groups (Fig. 16Q). This would indicate immunostimulatory shift as there are less $FOXP3^+$ Tregs and more CTLs.

Results from orthotopic TNBC treatment indicated that OximUNO might have metastasis delaying effect. To further explore this effect, we used the experimental metastasis of TNBC model where tumour cells are injected i.v. and they implant in the lungs. To monitor tumour progression, we used cells that express GFP. Analysing the whole lung fluorescence, we were able to see that OximUNO-treated mice had the lowest GFP fluorescence (Fig. 17A), indicating smaller tumour growth. This was confirmed macroscopically (Fig. 17B), with confocal microscopy (Fig. 17C) and analysing lungs histologically (Fig. 17D, E). OximUNO-treated mice showed some bodyweight loss (Fig. 17F, red line) but St-PGA-DOX and DOX-treated mice showed 19% (Fig. 17F, blue line) and 17% loss (Fig. 17F, purple line), respectively, indicating that targeting DOX to $CD206^+$ TAMs make it less toxic to the body. Using FC analysis, we observed that OximUNO treatment significantly lowered the percentage of $CD206^+$ TAMs (Fig. 17G) but did not significantly impact the percentage of M1 TAMs, CTLs or Tregs (Fig. 17H-J). As this analysis involved the whole lung, we were interested in analysing the immune cell populations locally in pulmonary nodules. For that we used IF and showed that OximUNO treatment resulted in significantly lower CD206 expression (M2 TAMs) (Fig. 17K, L), significantly increased CD8 expression (CTLs) (Fig. 17M, N) and significantly lower FOXP3 expression (Tregs) (Fig. 17O, P). This resulted in two- to three times higher CD8/FOXP3 expression ratio compared to St-PGA-DOX and DOX and seven times higher than PBS (Fig. 17Q).

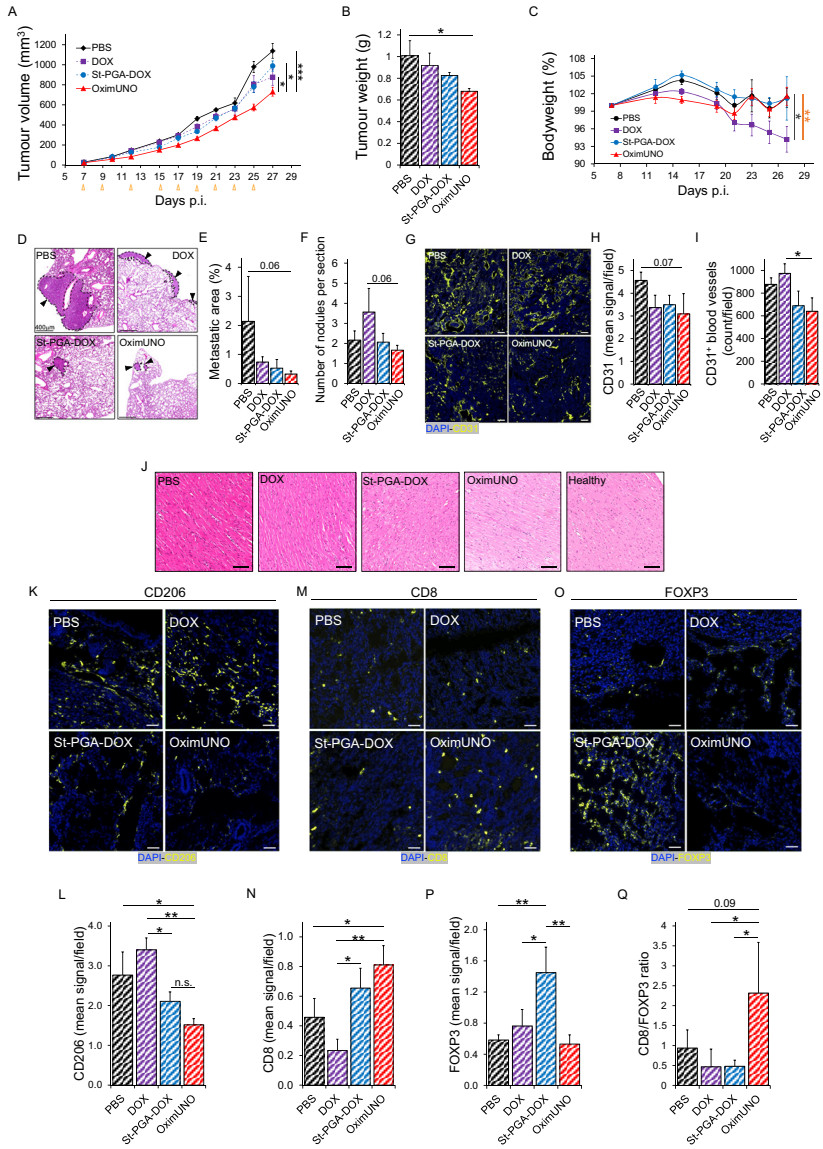


Fig. 16. Treating mice bearing orthotopic TNBC with OximUNO resulted in reduced primary tumour, reduced pulmonary metastases and alleviated immunosuppression. (A) Tumour volume measured throughout the study. (B) Tumour weights at the end of the treatment. (C) Bodyweight changes throughout the study. (D) Representative H&E images showing reduction in pulmonary metastases. (E) Quantification of metastatic lung area and (F) nodule count. (G) Representative IF images depicting $CD31^+$ blood vessels. (H) Quantification of CD31 signal and (I) the amount of $CD31^+$ blood vessels. (J) H&E of hearts after treatments. (K-O) Representative IF images for (LK CD206, (M) CD8 and (PO) FOXP3 expression. (L-P) Quantification of (L) CD206, (N) CD8 and (P) FOXP3 signal. (Q) Quantification of CD8/FOXP3 ratio. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; n.s. > 0.05 Scale bars represent 400 μm for pulmonary nodules H&E and 50 μm for immunostainings and heart H&E.

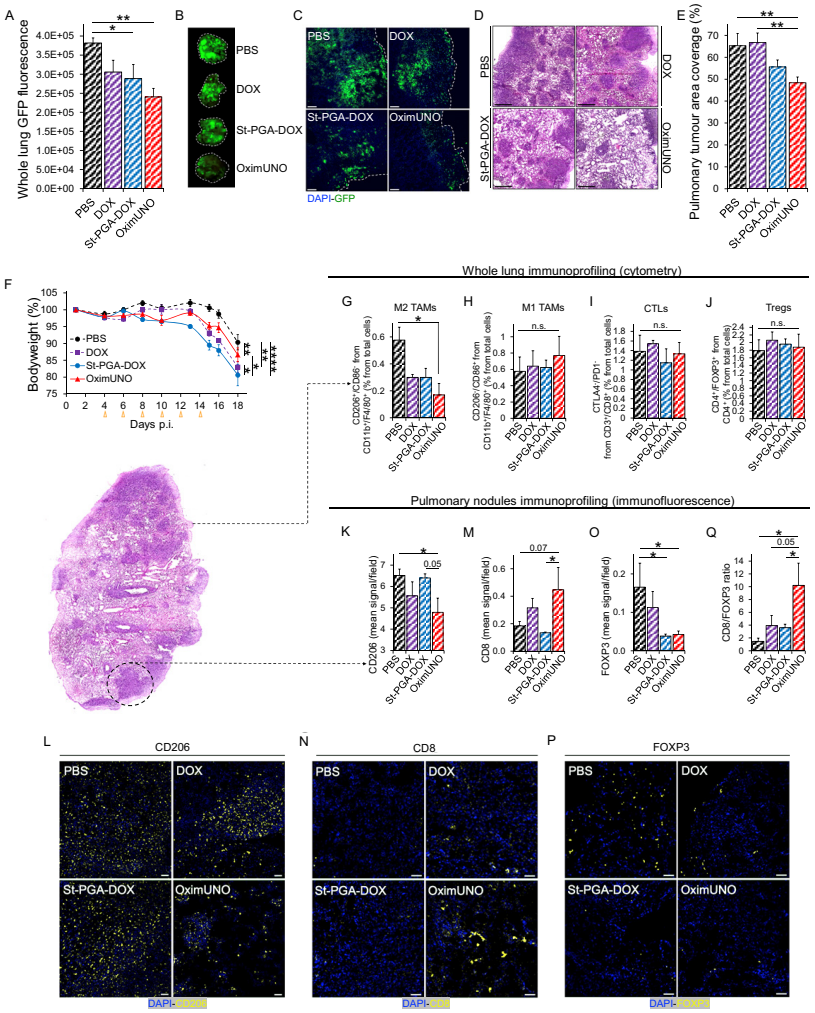


Fig. 17. OximUNO treatment in experimental metastasis of TNBC resulted in reduced pulmonary nodules and more immunogenic TME. (A) Whole lung GFP fluorescence. (B-D) Representative images of (B) macroscopic GFP, (C) GFP fluorescence measured with IF, (D) H&E showing pulmonary nodules. (E) Quantification of pulmonary nodules. (F) Measurements of bodyweights throughout study. (G-J) FC quantification of (G) M2 TAMs, (H) M1 TAMs, (I) CTLs, and (J) Tregs. (K-O) Quantification of (K) CD206, (M) CD8, (O) FOXP3 expression. (L-P) Representative immunostained images of (L) CD206, (N) CD8 and (P) FOXP3. (Q) Quantification of CD8/FOXP3 ratio. *P ≤ 0.05; **P ≤ 0.01; ****P ≤ 0.0001; n.s., > 0.05, scale bars represent 50 μm.

As OximUNO treatment in both models showed effect on primary tumour, pulmonary metastases, and immune cells, we can conclude that this peptide-guided St-PGA-based polymer-drug nanoplatform as a proof-of-concept shows promise for the development of new cancer drugs for TNBC.

6. DISCUSSION

TNBC is an aggressive breast cancer subtype with few treatment options. The 5-year survival is under 30% (Hudis & Gianni, 2011; Lehmann et al., 2011), patients relapse within three years (Kassam et al., 2009; Liedtke et al., 2008) and metastatic patients die within two years (Hudis & Gianni, 2011; Reddy et al., 2018; Schmid et al., 2018). Therefore, there is a massive need for new treatment options. As $CD206^+$ TAMs are responsible for tumour progression, metastasis, resistance to therapies and immunosuppression (Ambarus et al., 2012; Lopes et al., 2014; Ma et al., 2010; Mantovani et al., 2004; Vogel et al., 2014), tackling this population of macrophages would open new doors for TNBC treatment. So far, TAM-targeting methods include using ligands that inhibit CSF1R (inhibitors PLX3397, PLX5622), anti-CD163-coated liposomal DOX, targeting CCR2 with its inhibitor PF-04136309, inhibitor against TIE2 (rebastinib), anti-TREM2 mediated depletion (PY314), antibody blockade of Clever-1 to stimulate M2→M1 switch (CLEVEGEN) and anti-MARCO antibody that re-educates pro-tumoural TAMs to anti-tumoural TAMs. Although CSF1R blockade seemed promising at first, in clinical trials it has been shown to induce severe side effects such as haematotoxicities, hepatotoxicity and oedema (Bissinger et al., 2021; Papadopoulos et al., 2017; Wesolowski et al., 2019); it can even induce tumour progression and metastasis (Hollmén et al., 2016) and as CSF1R is expressed also by normal microglia (DeNardo et al., 2011; S. Lee et al., 2018; Mancini et al., 2019), it is not specific for just tumours. Targeting CCR2 or CD40 on TAMs is also not a good option as they are expressed by anti-tumoural TAMs and microglia (El Khoury et al., 2007; Ponomarev et al., 2006). So far, the most promising approaches appear to be targeting scavenger receptor MARCO or Clever-1 on TAMs to induce pro-tumoural TAMs to be switched to anti-tumoural TAMs (Georgoudaki et al., 2016; Hollmén et al., 2020; Viitala et al., 2019) and targeting TREM2 has shown to induce stable disease for weeks in the first clinical trial (Patnaik et al., 2022). Targeting TIE2 on pro-metastatic macrophages have also shown great promise in mouse breast cancer models (Harney et al., 2017). The overview of TAM-targeting ligands and the fact that there are several ongoing clinical trials targeting TAMs for cancer therapy and imaging or trials that analyse the changes in TAM population after treatment exhibits their great potential as target for improving cancer treatment outcomes.

Importantly, as mUNO binds to an epitope located between C-type lectin-like domain 1 (CTLD1) and CTLD2 (Asciutto et al., 2019) which is different to mannose suggesting that mUNO could have fewer off-target effects compared to mannose-based TAM-targeting methods as mannose binds also to CD209 in intestinal tissue and genital mucosa (Jameson et al., 2002). Our studies indicated that mUNO can be coupled to nanoparticles using its thiol group in the N-terminal cysteine without losing binding activity to CD206. Here we demonstrated the use of thiol-Mal bond but other bonds such as disulphide bond could be also used. How much peptide to use per PS was based on previous studies on

radiolabelled peptide-targeted PEG-PCL PSs (Simón-Gracia et al., 2018). In that study it was shown that intermediate peptide density (2.5-5 mol %) would result in optimal binding and uptake. Moreover, another study suggested that peptide density plays an important role in binding effectiveness (Wonder et al., 2018). Therefore, we decided to use peptide density between 2-3 mol % for this study. Being able to couple peptide successfully to a nanosystem can give rise to different combinations allowing more varied drug discovery. We also analysed the plasma half-life of our constructs as longer plasma half-life could result in less frequent dosing to achieve desired effect leading therefore to less exposure to the drug and lower concentrations in the bloodstream resulting in safer drug profile (Gunaydin et al., 2018; Smith et al., 2018). As in our later studies we used St-PGA-based constructs, we expected the plasma half-life to be extended compared to free mUNO peptide as it has previously been shown that larger PGA structures could have enhanced plasma half-life and higher bioavailability due to the higher hydrodynamic volume that lowers rapid renal clearance (Duro-Castano, England, et al., 2015; Duro-Castano, Movellan, et al., 2015). Increased plasma half-life is important when targeting TAMs as they are constantly replenished in the tumour TME (Cortez-Retamozo et al., 2012; Kurashige et al., 2018). In the future we would evaluate the i.v. route as it is more clinically relevant than i.p. administration.

Besides half-life, it is important to know whether constructs would be entrapped inside endosomes or lysosomes as if it happens then the cargo will also be trapped and not able to act on its intended place, hampering the peptide's use as targeting moiety. In our first study we were able to show that internalised mUNO escapes the lysosomal entrapment which is in line with literature where it has been shown that ligands binding to the CD206 lectin domain avoid lysosomal routing (Kang et al., 2005). Hence, our studies indicate that mUNO could guide a cytosolically-acting apoptotic drug, such as DOX (Kole et al., 1999) or agents that promote M2→M1 switch but their receptors are on endosomal membrane, such as TLR 7/8 agonist R848 (Lasala et al., 2003; Rodell et al., 2018), to pro-tumoural TAMs depleting or reprogramming them for enhanced therapeutic effect.

Analysing the biodistribution of a targeting peptide or construct is highly important as it would indicate if targeting was specific and if there would be any off-target effects. In all three of our studies, we showed high CD206/ peptide or CD206/nanosystem colocalisation with minimal hepatic accumulation. Some hepatic accumulation is expected as Kupffer cells express some levels of CD206 (Nielsen et al., 2020) but in contrast CD206 is highly overexpressed by pro-tumoural TAMs as seen in our studies as explored by others (Leber et al., 2019). Lower hepatic accumulation could be also explained by lower affinity of peptides compared to nanobodies for example and EPR effect observed in tumours. As mUNO targeted pulmonary lesions and primary breast tumours in TNBC model, it encourages the use of radiolabelled ligands, such as ^{177}Lu, to deplete pro-tumoural TAMs in TME. In fact, agent targeting prostate-specific membrane antigen (PSMA) coupled to ^{177}Lu conjugated to chelating agent

dodecanetetraacetic acid (DOTA) is in phase III clinical trial for castration-resistant prostate cancer (clinical trial identifier: NCT03511664) and first results have been promising (Herrmann et al., 2022). The whole organ imaging studies presented here indicate that mUNO could be used as an imaging probe to monitor metastasis when coupled to DOTA or near-infrared fluorophore such as IRDye800CW. So far, peptide-based imaging probes show translational promise (Mann et al., 2017; Scodeller & Asciutto, 2020), such as an integrin-targeted peptide or a peptide that targets extra domain B of fibronectin (EDB-FN) (Altmann et al., 2017; Han et al., 2015).

The number of polypeptide-based constructs reaching clinical trials has increased vastly in recent years. These nanocarriers have several benefits that synthetic polymer-based ones do not have, such as, lower immunogenicity, biodegradability, and no long-term accumulation (Duro-Castano et al., 2014; Melnyk et al., 2020; Moura et al., 2019). Moreover, St-PGA based nanoconjugates have met FDA criteria in terms of safety and toxicities (Duro-Castano et al., 2017). While previous studies have shown the use of St-PGA (Duro-Castano, England, et al., 2015; Duro-Castano et al., 2017) and mUNO peptide (Lepland et al., 2020), the third study presented here was the first work showing peptide-targeted St-PGA. We firstly evaluated the cytotoxicity of OximUNO *in vitro* on M2-resembling and M1-resembling human macrophages. In this study we found that OximUNO enhances the cytotoxicity of DOX on M2-resembling macrophages. Some toxicity to M1-resembling macrophages is expected as besides us, Bertani et al. have shown similar CD206 expression pattern under comparable conditions indicating that M1-resembling macrophages also express CD206 to some extent (Bertani et al., 2017). Importantly, injecting OximUNO i.p. to healthy animals did not result in acute hepatic or renal toxicity and the signal in the kidneys shown in Fig. 13R is probably related to the excretion route of St-PGA (Duro-Castano, England, et al., 2015; Duro-Castano et al., 2017).

In both treatment studies we were able to show that mUNO targeted nanosystems produced lower tumour burden and alleviated immunosuppression through increase in CTLs, DCs and decrease in $CD206^+$ TAMs and Tregs. Moreover, in the third study we saw also decrease in pulmonary metastases indicating that OximUNO would have anti-metastatic effect. These results are in line with literature where it has been shown that the presence of pro-tumoural TAMs in the TME is in correlation with poor infiltration of CTLs which results in poorer prognosis and higher possibility of relapse for breast cancer patients (Aaltomaa et al., 1992; Bianchini et al., 2016) but when TAMs were depleted, T cell migration and infiltration to the tumours were re-established (Peranzoni et al., 2018). Moreover, reducing the immunosuppressive TME can directly activate T cells and induce T cell mediated cytotoxicity (Cassetta & Pollard, 2018; Noy & Pollard, 2014). Furthermore, higher levels of IFN-γ indicates improved immunological response to the treatment (Cassetta & Pollard, 2018). Other preclinical studies assessing the effect that depleting pro-tumoural TAMs as a monotherapy could have in the 4T1 mouse model, have not shown either effect on metastases (Ramesh et al., 2020; Shan et al., 2020) or primary tumours

(Viitala et al., 2019) or they have shown to even have pro-metastatic effect as it has been shown with anti-CSF1R (Hollmén et al., 2015). Therefore, besides anti-MARCO therapy (Georgoudaki et al., 2016), OximUNO study is one of few where TAM depletion as a monotherapy affects both primary and secondary tumours in TNBC mouse model. Besides that, we show that St-PGA-mUNO is an appealing platform as a drug carrier. Possible combinations could include TLR7/8 agonists (Figueiredo et al., 2021; F. Zhang et al., 2020), ^{177}LuDOTA (Sartor et al., 2021) or photosensitisers such as verteporfin to perform photodynamic therapy (Agostinis et al., 2011; Cheah et al., 2018; V.-N. Nguyen et al., 2021). In the future we envisage the use of OximUNO in combination with chemotherapy in adjuvant or neoadjuvant setting.

To conclude, as a homing peptide iRGD has shown clinical responses, it is encouraging for other tumour homing peptides to strive higher to also reach to clinical trials and in the end to the clinic. mUNO peptide has shown great potential as one of those peptides. In the future, we envisage that combinational therapy with mUNO as a targeting moiety guiding cytotoxic or reprogramming drugs to CD206^{+} TAMs using nanoparticle platforms. Precision guided nanomedicine is a rapidly evolving field with new clinical trials every year (Z. Chen et al., 2020; Germain et al., 2020; Sunderland et al., 2017) (clinical trial identifiers NCT04718376, NCT05465590, NCT04217096, NCT04039230, NCT00609791). Future combinational studies would offer potentially more potent treatment option for TNBC than those available right now.

7. CONCLUSIONS

The data presented in this dissertation is showing the preclinical evaluation of a CD206-targeting peptide, mUNO, in TNBC with the aim of using it as a potential drug-guiding agent in a clinical setting to tackle $CD206^+$ TAMs.

7.1. Targeting pro-tumoral macrophages in early primary and metastatic breast cancer with mUNO shows its theragnostic application in TNBC

In this study we concluded that:

- the cysteine of mUNO peptide's N-terminus allows for conjugation of cargoes without altering its activity,
- mUNO peptide escapes lysosomal entrapment,
- intraperitoneal route shows superior $CD206^+$ TAMs targeting compared to intravenous route,
- mUNO precisely targets CD206+ TAMs in early-stage TNBC and those found in pulmonary metastases,
- mUNO targets SLN and CD206+ TAMs more efficiently than a validated TAM targeting peptide.

7.2. Peptide-guided resiquimod-loaded lignin nanoparticles convert tumour-associated macrophages from M2 to M1 phenotype for enhanced chemotherapy

In this collaboration study we showed that:

- mUNO can be conjugated to LNP carrying R848, without losing its targeting activity and without altering the re-educational activity of R848
- *in vivo*, R848@LNP-P-F-mUNO accumulates 3x more in $CD206^+$ TAMs compared to control untargeted R848@LNP-P-FAM,
- R848@LNP-P-F-mUNO in combination with Vin reduces tumour volume, re-educates M2-like pro-tumoural TAMs to M1-like anti-tumoural TAMs, shifts the immune landscape towards more immunostimulatory with no acute hepatic or renal toxicity.

7.3. Depletion of $CD206^+$ TAMs via a mUNO-targeted St-PGA inhibits breast cancer progression in mice

In this study we concluded that:

- mUNO can be coupled to polymeric backbone together with OG or drug to form a peptide-guided polymer-fluorophore/drug conjugate,

- St-PGA-OG-mUNO specifically targets $CD206^+$ TAMs *in vivo* in orthotopic TNBC and experimental metastasis of TNBC sparing M1 TAMs and DCs,
- Targeted polymer-drug conjugate OximUNO (St-PGA-DOX-mUNO) shows enhanced cytotoxicity to M2-resembling macrophages *in vitro* compared to free DOX,
- OximUNO reduces tumour progression with no acute hepatic or renal toxicity, decreases pulmonary metastases and shifts immune landscape towards immunostimulatory in both orthotopic TNBC and experimental metastasis of TNBC.

Based on studies shown in this thesis, it can be concluded that eliminating or re-educating pro-tumoural TAMs can alleviate immunosuppression in TNBC mouse models and therefore carve a foundation for combinational therapies with ICIs, PARP inhibitors, PI3K inhibitors and more.

8. SUMMARY IN ESTONIAN

Kasvajat toetavatele makrofaagidele suunatud täppisteraapia prekliiniline arendus, kasutades kolmiknegatiivse rinnavähi hiiremudeleid

Vähk on maailmas suuruselt teine surmapõhjus ja risk haigestuda vähki inimese elu jooksul on umbes 20%. 2020. aastal diagnoositi maailmas 2.3 miljonit uut rinnavähi juhtumit, mis teeb rinnavähist kõige sagedamini diagnoositud vähi. Eestis diagnoositakse igal nädalal keskmiselt 16 uut rinnavähi juhtu ja 5 inimest kaotavad selle vähi tõttu oma elu. Kolmiknegatiivne rinnavähk (ingl. *triple negative breast cancer*, TNBC) on kõige agressiivsem rinnavähi alatüüp, moodustades kuni 20% kõigist rinnavähi juhtudest. TNBC mõjutab peamiselt alla 50-aastaseid naisi, kes ei osale rutiinsel mammograafial ning seetõttu on tihti diagnoosimise hetkeks vähk jõudnud juba kaugele areneda. Kuna TNBC on negatiivne östrogeeni retseptori, progesterooni retseptori ja inimese epidermaalse kasvufaktor 2 (ing. *human epidermal growth factor 2,* HER2) suhtes, ei saa patsiendid kasu hormoon- ega HER2-suunatud ravist ning praegu on tavapäraseks raviks endiselt keemiaravi ja opereerimine, kui võimalik. Ligi 30% TNBC patsientidest alluvad võrdlemisi hästi keemiaravile ja ravile eri signaaliradade inhibiitoritega. Ülejäänud 70% TNBC patsientide ravi on piiratud. On pakutud, et immuunkontrollpunkti inhibiitorid, näiteks PD-1 (ingl. *programmed cell death 1*) või PD-L1 (ingl. *programmed cell death 1 ligand*) blokaad, võiksid olla potentsiaalsed uued TNBC ravivõimalused, kuid siiani on tulemused olnud pigem pettumust valmistavad. TNBC 5 aasta elulemus on alla 30%, vähk retsidiveerub kolme aasta jooksul ning pärast seda on elulemus kuni 18 kuud. Seetõttu on kolmiknegatiivne rinnavähk vähitüüp, mille puhul on suur vajadus uute ravimeetodite järele.

Käesolevas doktoritöös analüüsiti kasvajat toetavate makrofaagidele (ingl. *tumour-associated macrophages*, TAMs) suunatud kullerpeptiidi mUNO võimet suunata ravimeid sihtmärgini TNBC hiiremudelites. Töötati välja mUNO peptiidil põhinevad strateegiad kasvajat toetavate TAM-de ümberprogrammeerimiseks ja eemaldamiseks ning hinnati nende mõju primaarse TNBC arengule, metastaseerumisele ja immuunsüsteemi aktivatsioonile kasvajakoes.

8.1. Uurimistöö eesmärgid

- Hinnata mUNO peptiidi TAM-de sihtmärgistamise võimet *in vitro* ja *in vivo*.
- Hinnata mUNO-põhiste strateegiate sobivust TAM-de ümberprogrameerimiseks eesmärgiga muuta kasvaja arengut toetavad TAM-d kasvaja vastasteks TAM-deks.
- Hinnata mUNO abil suunatud peptiid-ravim konjugaatide võimet eemaldada kasvajat toetavad TAM-d kasvajate mikrokeskkonnast.

8.2. Materjal ja metoodika

Käesolevas doktoritöös analüüsiti TNBC prekliinilise ravimise võimalusi, kasutades: a) ortotoopset TNBC hiiremudelit, mis jäljendab inimese TNBC, tekitades spontaanseid kopsumetastaase: b) eksperimentaalset metastaatilist TNBC hiiremudelit, mille puhul süstiti kasvajarakke veenisiseselt eesmärgiga tekitada pulmonaarseid kasvajakoldeid. Kõik loomkatsed olid heaks kiidetud Eesti Põllumajandusministeeriumi komisjoni poolt. Töös kasutati valdavalt mUNO kullerpeptiidi, mis seondub spetsiifiliselt $CD206^+$ kasvajat toetavate TAM-dega. Lisaks kasutati võrdluseks kontrollpeptiide, millel ei ole CD206 sihtmärgistamise võimet. *In vitro* sihtmärgistamisvõime uurimiseks kasutati inimese vere *buffi coat*-st puhastatud ja stimuleeritud makrofaagide mudelit, rakkude ümberprogrammeerimise katse puhul kasutati hiire luuüdist puhastatud ja stimuleeritud makrofaagide mudelit. *In vivo* seondumisvõime uurimiseks kasutati ortotoopset või eksperimentaalset metastaatilist TNBC hiiremudelit. Tulemuste hindamiseks kasutati immunofluoresentsvärvingut, konfokaalmikroskoopiat ja läbivoolutsütomeetriat. mUNO peptiidi poolestusaja uurimiseks kasutati terveid hiiri, kellele süstiti peptiidi kas kõhuõõne- või veenisiseselt ja mõõdeti peptiidi kontsentratsiooni vereplasmas. Analüüsiti ka mUNO peptiid kasutusvõimalusi erinevatesse nanosüsteemidesse täppissuunamiseks. Selleks kasutati polümersoome, ligniini nanoosakesi (LNP) (koostöö Dr. Santose laboriga Soomes) ja polümeeril baseeruvat süsteemi (koostöö Dr. Vicenti laboriga Hispaanias). LNP-sse seoti ravim, mis võimaldab muuta kasvajat toetavad TAM-d kasvaja vastasteks. Polümeeri baasil nanosüsteemides kasutati polüglutaamhappest selgroogu, millele seoti mUNO peptiid ja kas fluorestseeruv aine või ravim. LNP ja polüglutaamhappe baasil nanosüsteemide terapeutilist toimet analüüsiti ka hiire TNBC mudelites. Kontrollkudesid ja kasvajaid analüüsiti immunofluoretsentsi, valgus- ja konfokaalmikroskoopia ning läbivoolutsütomeetria abil.

8.3. Uurimistöö peamised tulemused ja järeldused

1. mUNO peptiid seondub spetsiifiliselt $CD206^+$ TAM-ga *in vitro* ja *in vivo* ortotoopilises TNBC ja eksperimentaalses metastaatilises TNBC hiiremudelites. mUNO ei seondu kasvajavastase TAM-ga ega dendriitrakkudega, samuti näitab mUNO peptiid madalat kuhjumist maksakoes.
2. mUNO peptiidi keemiline kinnitamine nanosüsteemidesse ei vähenda peptiidi seondumisvõimet CD206 retseptorvalgu ja seda ekspresseerivate rakkudega.
3. Eksperimentaalteraapia mUNO abil suunatud terapeutiliste LNP-dega põhjustab kasvajate suuruse vähenemise, $CD206^+$ TAM-de vähenemise, tsütotoksiliste T rakkude ja kasvaja vastaste TAM-de suurenemise, mis kokku viitab leevenenud immuunsuppressioonile.
4. Eksperimentaalteraapia uudse polümeer-ravimi konjugaadiga OximUNO vähendab primaarseid TNBC kasvajaid, kopsumetastaase ja leevendab immunsuppressiooni.

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2. https://ecis.jrc.ec.europa.eu/explorer.php?$0-0$1-All$2-All$4-2$3-29$6-0,85$5-2020,2020$7-7$CEstByCountry$X0_8-3$X0_19-AE27$X0_20-No$CEstBySexByCountry$X1_8-3$X1_19-AE27$X1_-1-

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3. https://ecis.jrc.ec.europa.eu/explorer.php?$0-2$1-EE$2-All$4-2$3-29$6-0,14$5-2000,2007$7-1$CRelativeSurvivalAgeGroup$X0_14-$X0_15-RSC$CRelativeSurvivalFollow$X1_14-$X1_-1-$X1_15-RSC visited 26.01.2023

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Töökohad ja ametid

01.10.2022–...	SA Tartu Ülikooli Kliinikum, geneetika ja personaalmeditsiini kliinik, vanemspetsialist (1,00)
01.09.2020–31.12.2020	Tartu Ülikool, Meditsiiniteaduste valdkond, bio- ja siirdemeditsiini instituut, bio- ja siirdemeditsiini nooremteadur (0,50)
01.01.2017–30.08.2017	Tartu Ülikool, Loodus- ja täppisteaduste valdkond, tehnoloogiainstituut, spetsialist (1,00)
01.06.2016–31.12.2016	Tartu Ülikool, Loodus- ja täppisteaduste valdkond, tehnoloogiainstituut, spetsialist (1,00)
15.03.2016–31.05.2016	Tartu Ülikool, Loodus- ja täppisteaduste valdkond, tehnoloogiainstituut, spetsialist (0,40)
01.07.2014–31.08.2014	Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, Tartu Ülikooli Tehnoloogiainstituut, spetsialist (1,00)

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Akadeemilise väljendusoskuse õppeaine õppejõu asssistent, 2020-2022

Teaduspreemiad ja tunnustused

2020 Translatsioonilise Immunoloogia konverentsi reisigrant, Gent
2019 Parima suulise ettekande preemia Biomeditsiini instituudi teaduspäeval
2019 Liisa Kolumbuse stipendium
2019 Üliõpilaste teadustööde riiklik konkurss, tänukiri bio- ja keskkonnateaduste valdkonnas magistriastmes
2016 Üliõpilaste teadustööde riiklik konkurss, tänukiri bakalaureuse astmes

Publikatsioonid

1. **Lepland, Anni**; Malfanti, Alessio; Haljasorg, Uku; Asciutto, Eliana K.; Pickholz, Monica; Bringas, Mauro; Đorđević, Snežana; Salumäe, Liis; Peterson, Pärt; Teesalu, Tambet; Vicent, Maria J.; Scodeller, Pablo (2022). Depletion of Mannose Receptor-Positive Tumor-Associated Macrophages via a Peptide-Targeted Star-Shaped Polyglutamate Inhibits Breast Cancer Progression in Mice. Cancer Research Communications. DOI: 10.1158/2767-9764.CRC-22-0043.
2. **Lepland, Anni**; Asciutto, Eliana K.; Malfanti, Alessio; Simón-Gracia, Lorena; Sidorenko, Valeria; Vicent, Maria J.; Teesalu, Tambet; Scodeller, Pablo (2020). Targeting pro-tumoral macrophages in early primary and metastatic breast tumors with CD206-binding mUNO peptide. Molecular Pharmaceutics. DOI: 10.1021/acs.molpharmaceut.0c00226.
3. Figueiredo, Patrícia; **Lepland, Anni**; Scodeller, Pablo; Fontana, Flavia; Torrieri, Giulia; Tiboni, Mattia; Shahbazi, Mohammad-Ali; Casettari, Luca; Kostiainen, Mauri A.; Hirvonen, Jouni; Teesalu, Tambet; Santos, Hélder A. (2020). Peptide-guided resiquimod-loaded lignin nanoparticles convert tumor-associated macrophages from M2 to M1 phenotype for enhanced chemotherapy. Acta Biomaterialia. DOI: 10.1016/j.actbio.2020.09.038.
4. Asciutto, Eliana K; Kopanchuk, Sergei; **Lepland, Anni**; Simón-Gracia, Lorena; Aleman, Carlos; Teesalu, Tambet; Scodeller, Pablo (2019). Phage-Display-Derived Peptide Binds to Human CD206 and Modeling Reveals a New Binding Site on the Receptor. The Journal of Physical Chemistry B, 123 (9), 1973−1982. DOI: 10.1021/acs.jpcb.8b11876.

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