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Targeting tumour-associated macrophages in primary and metastatic breast tumours

Master's thesis

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ABSTRACT

Targeting tumour-associated macrophages in primary and metastatic breast tumours

Cancer is the second leading cause of deaths worldwide. In 2018 there were estimated to be around 17 million new cancer cases world-wide, of which breast cancer is the most common cancer among women. Breast cancers are divided into subtypes based on the markers they express on the cancer cell's surface. The most dangerous breast cancer subtype is triple negative breast cancer (TNBC), which does not express any hormonal receptors that current therapies can target. Therefore, there is no therapy option for TNBC patients. In this thesis, the evaluation of the use of a peptide (called mUNO) that targets an immune cell population with major protumoural roles, M2 tumour-associated macrophages (M2 TAMs), is shown. mUNO was validated *in vitro* using primary human M2-differentiated macrophages, and *in vivo* using two different mouse models of TNBC. It was shown that mUNO binds specifically to the M2 TAMs both *in vitro* and *in vivo*. The findings presented here may have implications for clinical management of the TNBC.

Keywords: TNBC, mUNO, M2 TAMs, targeted delivery, desmoplasia

CERES code: B200 Cytology, oncology, cancerology

Kasvajaga seotud makrofaagide märgistamine primaarsetes ja metastaatilistes rinnavähi kasvajates

Vähk on teisel kohal maailmas surmade osas. 2018. aastal arvati olevat üle 17 miljoni uue vähijuhtumi. Rinnavähk on kõige sagedasem vähiliik naiste seas. Rinnavähke jagatakse pinnamarkerite ekspressiooni alusel alamliikideks. Kolmekordselt negatiivne rinnavähk (ingl. *triple negative cancer*, TNBC) on neist kõige ohtlikum, kuna see ei ekspresseeri ühtegi hormonaalset markerit, millel põhinevad praegused ravimid. Seetõttu pole praegu ühtegi sihtmärgistatud ravi võimalust TNBC patsientidele. Käesolevas magistritöös hinnati peptiidi (mUNO), mis sihtmärgistab spetsiifiliselt immuunrakkude populatsiooni, millel on oluline kasvajat abistav roll: M2 kasvajaga seotud makrofaagid (ingl. M2 *tumour-associated macrophages*, M2 TAMs). mUNO kasutamist hinnati *in vitro*, kasutades primaarseid inimese M2 makrofaage ja *in vivo*, kasutades kahte hiire TNBC kasvajamudelit. Uurimistöös näidati, et mUNO seondub spetsiifiliselt M2 TAM-dele nii *in vitro* kui ka *in vivo*. Käesolevas töös esitatud tulemused võivad avada uusi võimalusi TNBC patsientide ravis.

Märksõnad: TNBC, mUNO, M2 TAMs, sihtmärgistatud kohaletoimetamine, desmoplaasia.

CERES kood: B200 Tsütoloogia, onkoloogia, kantseroloogia

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ABBREVIATIONS

4T1 – animal stage IV breast cancer

BSA – bovine serum albumin

CAF – cancer-associated fibroblast

cDNA – complementary DNA

CendR – C-end rule

CPP – cell-penetrating peptide

CSCLC – cancer stem cell-like cell

Cys – cysteine

DAPI – 4′,6-diamidino-2-phenylindole

DC – dendritic cells

DMSO – dimethyl sulfoxide

ECIS – European Cancer Information System

ECM – extracellular matrix

EGF – epidermal growth factor

ER – oestrogen receptor

FAM – fluorescein

FBS – foetal bovine serum

FDA – Food and Drug Administration

FGF – fibroblast growth factor

GFP – green fluorescent protein

GM-CSF – granulocyte-monocyte colony stimulating factor

H&E – haematoxylin and eosin

HA – hyaluronan

HER2 – human epidermal growth factor 2

i.p. – intraperitoneal

i.v. – intravenous

IC – immune complex

IF – immunofluorescence

IFN-γ – interferon gamma

IHC – immunohistochemistry

IL – interleukin

LPS – lipopolysaccharide

M-CSF – macrophage colony stimulating factor

MMP – matrix metalloproteases

N.S – not significant

OCT – Optimal Cutting Temperature

p.i. – post inoculation

PBMC – primary blood mononuclear cells

PBS – phosphate buffered saline

PBST – PBS containing 0.05% Tween 20

PCL – polycaprolactone

PD-1 – programmed cell death protein 1

PD-L1 – programmed cell death ligand 1

PDGF – platelet derived growth factor

PEG – polyethylene glycol

PFA – paraformaldehyde

PR – progesterone receptor

PS – polymersome

PV – perivascular

PXT – Paclitaxel

ROS – reactive oxygen species

RPMI – Roswell Park Memorial Institute

RT – room temperature

s.c. – subcutaneous

TAM – tumour-associated macrophage

TGF – transforming growth factor

TME – tumour microenvironment

TNBC – triple negative breast cancer

TNF – tumour necrosis factor

TPP – tumour penetrating peptide

T_{reg} – regulatory T-cell

VEGF – vascular epidermal growth factor

INTRODUCTION

Cancer occurs as a result of multiple genetical changes, such as deletions, genomic instability, inversions. It is a multifactorial disease, since changes in the hormonal balance, weight, lifestyle, long exposure to certain environmental factors, hereditary predisposition and age can all have an effect. One of the hallmarks of solid tumours is their cellular, structural and physiological heterogeneity, which makes them hard to treat. Tumours have developed mechanisms to avoid being recognised by the immune system, such as the suppression of immune cells that drive anticancer response, or the activation of cells signalling that the immune response needs to be terminated. In addition, the tumour microenvironment supports the tumour's growth, invasion and metastasis. The expression of different markers and receptors makes each type of cancer unique. There is a need to develop new cancer drugs that can help the patients with currently incurable cancers. One cancer type that needs more investigation is triple negative breast cancer (TNBC), which is a cancer that develops inside the mammary tissue as a result of different genetical changes. Usually, breast cancer is detected during a routine mammography. As TNBC often develops in women below the age of routine mammography screening, it can progress undetected and reach a high grade. TNBC is a deadly disease with the 5-year survival around 30% and most patients relapse within 3 years. Known monoclonal-based treatments for breast cancer target receptors which are not expressed in TNBC. If TNBC is discovered early, the therapeutic options include mastectomy, lumpectomy and/or chemotherapy. For disseminated TNBC, the patient's expected survival is 18 months or less. Therefore, there is a high need for detecting and treating TNBC in both early and late stages. The Cancer Biology group at the University of Tartu is interested in developing precision diagnostics and therapeutics for TNBC. In 2017, a peptide that specifically targets cells found in the TNBC tumour's microenvironment was described. Here, the objectives of this thesis were to develop an *in vitro* cell culture system for peptide internalisation studies, to test the peptide's homing in TNBC mouse models and to evaluate the half-life of the peptide dosed via different administration routes. In addition, the treatment with a safe and approved drug to treat hypertension was used to reduce the amount of intratumoural collagen and hyaluronan and to decompress the blood vessels. As a conclusion, this thesis gives an insight on how peptides can be used in cancer therapies and hopefully this approach can be one day applied to treat patients with TNBC.

1. LITERATURE

1.1. CANCER

Cancer is a complex disease, which requires multiple genetic changes in order to form. Most cancers share similar molecular, biochemical and cellular traits, since all mammals share a similar molecular machinery (Hanahan and Weinberg, 2000). Multiple steps are required for normal cells to become malignant (Renan, 1993), starting from genetic changes, which include deletions, amplifications, inversions, point mutations (Widschwendter and Jones, 2002), leading to uncontrollable proliferation (Renan, 1993). Malignant cells have defects in maintaining normal cell proliferation and homeostasis (Hanahan and Weinberg, 2000). The following six features have been classified as hallmarks of cancer (Hanahan and Weinberg, 2000):

- self-sufficiency in growth signals;
- resistance to growth inhibiting signals;
- resistance to apoptosis;
- unlimited replication;
- ability to induce tumour blood vessel growth (angiogenesis);
- ability of local and distant tissue invasion.

Tumours are not homogenous cell masses but are comprised of different types of cells and tumour-associated stroma. The tumour microenvironment (TME) is highly important for tumourigenesis (Hanahan and Weinberg, 2011). Since 2000, additional cancer hallmarks have been added (Hanahan and Weinberg, 2011) to explain how and why tumours develop, these are:

- genomic instability;
- proinflammatory state of both premalignant and malignant lesions;
- metabolic changes to sustain constant proliferation and progression;
- evasion of the immune system to avoid elimination.

Although some immune cells help to eliminate the tumour cells, others, mainly innate immune cells, play protumoural roles. Inflammation supports tumourigenesis by providing the bioactive molecules in the TME. Proinflammatory molecules include growth factors, angiogenesis promoters, survival factors and factors that promote invasion of tumour cells through the TME and metastasis (Hanahan and Weinberg, 2011). Inflammation can help to generate an invasive tumour from just a small group of premalignant cells (Hanahan and Weinberg, 2011) by supplying mutagenic agents such as reactive oxygen species (ROS) (Grivennikov *et al*., 2010)

and inflammatory cells in malignant lesions help to deactivate or alter checkpoint genes, genes involved in DNA repair and apoptosis (Schäfer and Werner, 2008).

1.2. BREAST CANCER

Breast cancer occurs due to the deregulation of many genes (Dvorak, 1986) and other random genetical changes (Simpson *et al.*, 2005). It is one of the most common cancers among women both in developing and developed countries, comprising over 20% of all new cancer cases each year. Breast cancer has a high death rate, representing over 14% of overall cancer deaths worldwide. However, earlier detection methods and better treatment have helped to decrease the mortality rate (Jemal *et al*., 2011).

One of the drivers of breast cancer is the mutation of the p53 tumour suppressor gene *(*Deng *et al.*, 1994). The risk of developing breast cancer will be higher if there is a family history, but these comprise only a small fraction of all breast cancer cases. Longer exposure to endogenous and exogenous steroid hormones can explain the prevalence of breast cancer among older women. Also, differences in the hormonal state can affect breast cancer frequency with a decreased cancer rate among postmenopausal women (Widschwendter and Jones, 2002).

The varying degrees of malignancy were first described by Greenough in 1925. He divided them into four classes based on the degree of its differentiation/malignancy (Greenough, 1925). Currently, the most commonly used grading system for breast cancer is the Nottingham Grading System (Elston and Ellis, 1991). The tumours in the three grades of this system show (Rakha *et al*., 2010):

- grade 1: similarity to normal tissue, low mitosis, low nuclear pleomorphism;
- grade 2: intermediate differentiation and higher mitotic count;
- grade 3: low similarity to normal tissue, high number of mitosis and high degree of nuclear pleomorphism.

In the human mammary gland, there are two types of epithelial cell lines, basal and luminal, which have different gene expression profiles and markers, and therefore can be distinguished with immunohistochemistry (IHC) (Perou *et al*., 2000). Luminal breast cancers are classified as A or B according to their gene expression patterns (Sørlie *et al.*, 2001):

- Luminal A: oestrogen receptor (ER) or progesterone receptor (PR) positive, human epidermal growth factor 2 (HER2) negative,

- Luminal B: ER+ or PR+ and HER2 + (Nguyen *et al*., 2008; Onitilo *et al.*, 2009).

The luminal subtype is the most common breast cancer subtype. Luminal type A has a lower grade and better prognosis than luminal B. Since luminal A is not so highly proliferative, it is treated with endocrine therapy alone, whereas luminal B requires the combination of chemotherapy and endocrine therapy (Brenton *et al.*, 2005).

The HER2 subtype overexpress the HER2/neu antigen and related genes but is negative toward other hormone receptors (Brenton *et al.*, 2005; Onitilo *et al.*, 2009). This subtype usually presents a higher grade (grade 3) than luminal A and also involves the lymph nodes. The HER2 subtype has a poor prognosis if all tumour cells are not eliminated, but responds well to chemotherapy (Brenton *et al.*, 2005).

The triple negative breast cancer (TNBC) subtype is negative for the expression of the ER, PR or HER2/neu antigen and highly positive for the cell proliferation related genes (Brenton *et al.*, 2005; Onitilo *et al.*, 2009). This group includes breast tumours that express low levels of the BRCA1 gene (Brenton *et al*., 2005), related to repairing DNA double stranded breaks to ensure the genome's stability (Lehmann *et al.*, 2011). TNBC is more aggressive, has a higher grade and present the worst prognosis of all breast cancers with the least therapeutic options (Brenton *et al.*, 2005).

Basal-like breast tumours do not express the PR or HER2/neu antigen, and express genes usually found in the basal layer of normal breast and may express low levels of ER (Brenton *et al*., 2005; Tischkowitz *et al*., 2007).

Compared to the basal-like or TNBC, the luminal subtype patients live longer before metastasis occur (Sørlie *et al*., 2003). Subtypes also present different relapse patterns: the luminal types relapse mainly into bone, whereas the basal-like and TNBC mainly metastasise in the lungs, and the basal type presents more brain metastasis than the luminal subtype (Smid *et al*., 2008).

The classification of breast cancers is important for choosing the right therapy. Subtypes that express hormone receptors and HER2 show a high responsiveness to chemotherapy (Onitilo *et al.*, 2009). HER2+ tumours are also treated with a monoclonal antibody against HER2 (Herceptin®), causing a downregulation of the aberrant HER2 levels present in this subtype (Baselga *et al.*, 1998).

The determination of the breast cancer's subtype in patients is performed using IHC (a quick, inexpensive and standard technique) to profile the expression of receptor antigens in tumour sections (Onitilo *et al.*, 2009) and by complementary DNA (cDNA) microarray-based expression profiling (Perou *et al*., 2000). The differentiation into subtypes is summarised in Table 1.

Table 1. Breast cancer differentiation into subtypes based on markers identified with IHC and DNA microarray. The 5 main types of breast cancer based on IHC and cDNA microarray experiments, according to the descriptions from three different reports (Brenton *et al.*, 2005; Foulkes *et al.*, 2010; Onitilo *et al.*, 2009).

Receptor / Subtype	ER	PR	HER2/neu
Luminal A	+	+	-
Luminal B	+	+	+
HER2	-	-	+
Basal-like	+/-	-	-
TNBC	-	-	-

1.3. TRIPLE NEGATIVE BREAST CANCER

TNBC was first described in 2005 (Brenton *et al.*, 2005). It is the most dangerous breast cancer type and affects up to 17% of all breast cancer patients (Foulkes *et al.*, 2010). In Estonia, according to the European Cancer Information System (ECIS) there were 801 estimated cases of breast cancer comprising 10.7% of all cancer cases in 2018. Based on statistics, among all breast cancer cases there should have been up to 136 new TNBC cases in Estonia in 2018 (https://ecis.jrc.ec.europa.eu/). Since TNBC is negative for the "usual" breast cancer receptors, the targeted therapies available for other breast cancers are ineffective (Foulkes *et al.*, 2010). TNBC is more aggressive than other breast cancer subtypes, the tumours grow larger in size and their diagnosis requires evaluation of lymph node involvement (Koscielny *et al*., 1984; Lehmann *et al.*, 2011). For metastatic patients the 5 year survival rate is less than 30% (Hudis and Gianni, 2011; Lehmann *et al.*, 2011). TNBC responds poorly to endocrine or Herceptin®

therapies and typically patients relapse within three years of diagnosis (Kassam *et al*., 2009; Liedtke *et al*., 2008). Therefore there is a need for a better understanding of the molecular basis of TNBC and the molecular markers that can be used to develop more effective and targeted treatments (Lehmann *et al.*, 2011).

TNBCs are of a high grade, rapidly growing and invasive cancers which are often missed during a routine mammography (Foulkes *et al.*, 2010). Also, they typically occur among younger women, which means again that the majority of cases will not be identified during mammography (Foulkes *et al.*, 2010; Hudis and Gianni, 2011). The probability of recurrence peaks at 3 years and then quickly drops (Dent *et al*., 2007), and after 5 years the recurrence rate is at 5% (Reddy *et al.*, 2018). This is in contrast to other breast cancer subtypes, for which the probability of recurrence and death from breast cancer remains steady throughout the whole follow-up period (Dent *et al*., 2007). However, if a patient with TNBC does relapse, then the time to death is up to 18 months, whereas it is closer to two years for other subtypes (Hudis and Gianni, 2011; Reddy *et al.*, 2018; Schmid *et al*., 2018).

One of the most promising available therapeutic approaches to TNBC management is based on immune checkpoint inhibitors, such as the programmed cell death ligand 1 (PD-L1) (Schmid *et al.*, 2018). Programmed cell death protein 1 (PD-1) is a cell surface protein expressed by different immune cells, including T-cells. Tumour cells express PD-L1, which activates PD-1 (Gordon *et al*., 2017). Interaction of PD-1 and PD-L1 triggers the activation of regulatory T-cells (T_{reg}) and the inhibition of the antitumour immune response (Sabatier *et al*., 2015). The expression of PD-L1 by the TNBC cells inhibits antitumour immunity, rendering PD-L1 an attractive therapeutic target. A recent clinical study which combined nanoformulated albumin bound Paclitaxel (PTX) (Abraxane®) and anti-PD-L1 (Tecentriq™) prolonged the progression-free survival compared to Abraxane® alone. However, the effect was modest and limited to PD-L1+ TNBC patients (Schmid *et al.*, 2018).

1.4. TUMOUR MICROENVIRONMENT (TME)

The tumour is a complex "organ" that contains, besides malignant cancer cells, other cell types as well (Hanahan and Weinberg, 2011). Cancer stem cell-like cells (CSCLCs) are a population of the tumour driving cells that proliferate unstoppably (Reya *et al*., 2001) and can transdifferentiate into other cell types required for the tumour's progression, such as tumour-derived endothelial cells (Hida *et al.*, 2013). CSCLCs are more resistant to chemotherapeutics

and hence will remain in the body even after treatments, contributing to a relapse. Since CSCLCs differ from normal stem cells, they can be specifically targeted to eliminate them (Al-Hajj *et al.*, 2003; Cho and Clarke, 2008).

Other important populations are the tumour endothelial cells and pericytes, that take part in the formation of tumour neovessels, something that is highly important for tumours to grow and progress. Pericytes are mesenchymal cells that wrap the endothelial cells and are, along with endothelial cells, responsible for the deposition of the vessels' basement membrane (Hanahan and Weinberg, 2011). Both endothelial cells and pericytes are implicated in the tumour's angiogenesis (Pietras and Östman, 2010).

An important component of the TME are the immune cells, which can have both pro- and antitumour effects (Hanahan and Weinberg, 2011). These immune cells are macrophages, B- and T-cells, mast cells and neutrophils (Mantovani *et al.*, 2008). Besides differentiated cells, the TME contains the precursor cells that are able to respond to signals from the TME, such as hypoxia and a vascular endothelial growth factor (VEGF). Myeloid cells are known to differentiate into protumoural cells in response to signals from the TME (Hanahan and Weinberg, 2011; Murdoch *et al.*, 2008). In many solid tumours, immune cells are hijacked by the tumour to become tumour promoting cells (Hanahan and Weinberg, 2011).

A large number of cells in the TME are fibroblasts, referred to as cancer-associated fibroblasts (CAFs). This population consists of two different cell types: myofibroblasts, which are normally rare in epithelial tissues, and epithelial tissue fibroblasts. Myofibroblasts are found also in a normal liver and pancreas, but they mostly appear during a chronic inflammation and during tissue repair (Hanahan and Weinberg, 2011). Recruitment of myofibroblasts into malignant lesions promotes the tumour's progression, since they promote cell proliferation, angiogenesis and metastasis (Hanahan and Weinberg, 2011; Pietras and Östman, 2010). The CAFs are responsible for the formation of desmoplastic stroma in the late stages (Hanahan and Weinberg, 2011) and directly help the tumours to progress by producing cytokines, growth factors and hormones (Pietras and Östman, 2010).

But the most abundant population of cells in the TME are the macrophages, which will be discussed in the following sections.

1.5. MACROPHAGES AND THEIR CLASSIFICATION

Macrophages are highly responsive to different signals from the environment and, therefore, their phenotype can be altered (Edwards *et al.*, 2006; Mantovani *et al*., 2003; Ohlsson *et al*., 2014). The circulating monocytes, the precursors of mature macrophages, need to enter the tissue to become macrophages (Ambarus *et al*., 2012; Ohlsson *et al*., 2014; Vogel *et al.*, 2014). Mature macrophages play a crucial role in acute and chronic inflammation (Ambarus *et al*., 2012; Chen *et al.*, 2017). They are also the host's defence against exogenous pathogens (Edwards *et al*., 2006). The different macrophage subtypes can be classified according to the markers they expose on their surfaces and by the mediators they express (Ambarus *et al.*, 2012; Edin *et al.*, 2012; Edwards *et al.*, 2006; Lopes *et al*., 2014; Ma *et al*., 2010; Mantovani *et al.*, 2004; Stein *et al.*, 1992; Vogel *et al*., 2014):

- classically activated macrophages or M1s: lipopolysaccharide (LPS), interferon gamma (IFN-γ) or granulocyte-monocyte colony stimulating factor (GM-CSF) are needed for their polarisation; they express CD80 and CD86 on their surface;
- alternatively activated macrophages or M2s: for their polarisation interleukin 4 (IL-4), IL-13, IL-10, macrophage CSF (M-CSF), immune complexes (IC) or glucocorticoids are needed; they overexpress the scavenger receptor CD163 and the mannose receptor CD206/MRC1, which allows them to recognise various mannosylated pathogens.

The production of M1 activators antagonise the production of M2 activators and *vice versa* (Raes *et al*., 2002).

M1 macrophages produce proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α), IL-12, IL-6, IL-1β, whereas M2 macrophages produce anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (TGF-β) (Chen *et al*., 2017; Lopes *et al*., 2014; Mills *et al.*, 2000; Vogel *et al.*, 2014).

1.6. TUMOUR-ASSOCIATED MACROPHAGES (TAMs) DISPLAYING M1 AND M2 PHENOTYPE

Macrophages are a major immune cell class found in primary and secondary tumours (Lewis and Pollard, 2006). Most cells in the TME are macrophages (Williams *et al.*, 2016) and of the M2 phenotype (Hughes *et al*., 2015). M2 macrophages are protumoural and are referred to as M2 tumour-associated macrophages (M2 TAMs) (Mantovani *et al.*, 2002) or by some authors simply as TAMs (Redente *et al*., 2010). In this thesis, they will be referred to as M2 TAMs. There is a second population of TAMs called M1 TAMs that comprise a smaller portion of all

macrophages in the tumours and the TME. M1 TAMs display antitumoural properties, are phagocytic and cytotoxic towards tumour cells. The higher the density of M1 TAMs, the better the prognosis, the response to therapies and the longer the disease-free period. Therefore the balance between M1 and M2 TAMs is highly important (Ma *et al*., 2010; Vogel *et al.*, 2014).

Tumours use the function of the M2 TAMs to their favour. Tumour cells, by secreting M2 polarising stimuli, shift the balance of tumour macrophages toward the M2 (Edin *et al*., 2012; Vogel *et al*., 2014). Different chemo attractants, such as CSF-1, VEGF and some chemokines (CC chemokines, CCL2-5) recruit M2 TAMs inside the tumours (Lewis and Pollard, 2006).

In solid tumours, such as breast carcinoma and lung tumours, M2 macrophages are highly overrepresented compared to M1 macrophages (Schäfer and Werner, 2008; Vogel *et al*., 2014). Importantly, the M2 TAMs are found in the primary tumours and metastatic lesions (Lewis and Pollard, 2006). Usually the high number of M2 macrophages correlates with metastasis and therefore a poor prognosis (Lopes *et al*., 2014; Vogel *et al*., 2014).

M2 TAMs have distinct roles in tumour progression (Fig. 1). In early stages they help to break down the basement membrane through the production of proteolytic enzymes [cathepsin B, matrix metalloproteases (MMPs)] and therefore allow the tumour to invade the normal tissue (Hagemann *et al*., 2004; Lewis and Pollard, 2006). They also help tumour cells to proliferate through the production of growth factors such as the fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). M2 TAMs take part in the regulation of the formation of new blood vessels, they secrete VEGF and other proangiogenic growth factors, ILs and proteolytic enzymes needed for tumour neovascularisation and tissue remodelling. Newly formed blood vessels are fragile and prone to collapse, resulting in hypoxia (Lewis and Pollard, 2006).

M2 TAMs participate in the initiation, progression and invasion of the tumours (De Palma and Lewis, 2013; Hughes *et al*., 2015). Perivascular (PV) macrophages are defined as the ones in direct contact with blood vessels (Faraco *et al*., 2017), accumulate during chemotherapies and are known to contribute to the tumour's relapse (Hughes *et al*., 2015). M2 TAMs also present reduced tumouricidal activity by avoiding nitric oxide production (Elgert *et al.*, 1998), inhibit the activity of T-cells and promote tissue repair and remodelling (Hagemann *et al.*, 2004; Lewis and Pollard, 2006).

In healthy humans, macrophages have the ability to present antigens to T-cells, kill tumour cells and produce cytokines that help to recruit other immune cells to promote an immune response. The capability of M2 TAMs to perform these tasks is significantly reduced upon exposure to the factors in the TME (Lewis and Pollard, 2006).

M2 TAMs inhibit the T-cells antitumour activity through the production of VEGF, IL-10 and TGF-β (Sica *et al*., 2006). IL-10 also blocks monocyte differentiation to dendritic cells (DC), which are the main antigen presenting cells (de Souza and Bonorino, 2009). M2 TAMs activate the production of the T_{reg}, which potently suppress the activity of T-cells and other inflammatory cells (Sica *et al*., 2006).

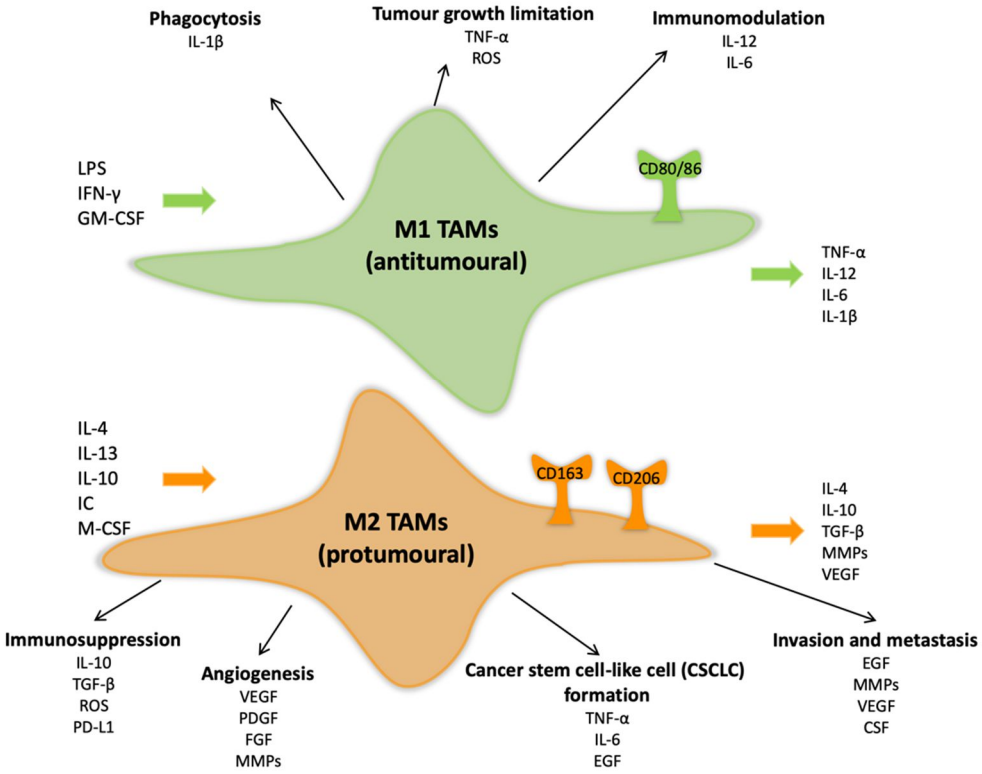


Figure 1. Differentiation and functions of M1 and M2 TAMs. Stimuli, secreted factors and roles of monocyte-derived M1 and M2 TAMs. Figure is based on (Ambarus *et al.*, 2012; Edin *et al.*, 2012; Edwards *et al.*, 2006; Lopes *et al*., 2014; Ma *et al*., 2010; Mantovani *et al.*, 2004; Stein *et al.*, 1992; Vogel *et al*., 2014).

Importantly, M2 TAMs also limit the efficacy of immunotherapies by inhibiting the migration of T-cells into the TME (Peranzoni *et al.*, 2018). Also, the more tumours secrete CSF-1, the more M2 TAMs accumulate in tumours, which in turn supports the reduced efficacy of immunotherapies even further (Gyori *et al.*, 2018; Neubert *et al*., 2018). Moreover some immunotherapies, such as the PD-1 blockade, actually increase immunosuppression through the interaction with Fc receptors on M2 TAMs leading to hyperprogression of the tumours (Lo Russo *et al*., 2019).

1.7. TUMOUR STROMA AND SOLID STRESS

Solid tumours in general, and breast tumours in particular, present an aberrant expression of stroma (Dvorak, 1986). They overexpress extracellular matrix (ECM) components like collagen, hyaluronan (HA) and fibroblasts. Stroma refers to the ECM, blood vessels, inflammatory cells and lymphatic vessels (Schäfer and Werner, 2008).

Solid stress is one of the hallmarks of the TME. It develops through two pathways (Stylianopoulos *et al.*, 2012):

- Cell migration-related pathway. During tumour growth and remodelling migrating cells extend and contract. This energy is stored inside ECM and cells. As the ECM connects all the cells in the tissue together, this force spreads.
- Pathway related to increase in cellular density. Proliferating cells represent new solid material. The TME cannot expand, due to the presence of an ECM scaffold and since it is surrounded by normal tissue. The proliferation imposes a burden on the surrounding TME, which is stored as stress through the deformation of the neighbouring structures and collapsing more delicate structures like blood vessels.

Build-up of solid stress in tumours (Stylianopoulos *et al*., 2012) contributes to hypoxia that in combination with a lower pH tumours typically have inhibits the immune cells cytotoxic properties (Goel *et al.*, 2011). The abnormal tumour vasculature contributes to poor tumour perfusion, which results in low sensitivity to therapies (Goel *et al.*, 2011; Wouters and Brown, 1997).

Hypoxia favours the tumour's progression by selecting cells that can survive under harsh conditions, by downregulating DNA repair genes and increasing genome instability through the production of ROS (Wilson and Hay, 2011). Hypoxia can also help to recruit different immune cells. For example, T_{reg} cells are recruited through the production of CC chemokines that promote tumour tolerance and angiogenesis. These T_{reg} cells diminish the T-cells response and promote angiogenesis through the production of VEGF (Facciabene *et al*., 2011).

1.8. HOMING PEPTIDES FOR CANCER THERAPY

Typically, only a small percent of injected dose of chemotherapeutic agents reaches the tumour tissue (Teesalu *et al.*, 2013). Furthermore, intratumoural distribution of nontargeted anticancer drugs is heterogeneous due to spatial differences in the tumour's structure and physiological status. Small cell-penetrating peptides (CPPs) can deliver cargo to the cells, but are not cell

type specific and hence have limited *in vivo* applications if not fused to a targeting moiety (Teesalu *et al.*, 2009). In 2009, Teesalu *et al.* described a family of peptides that contribute to tissue penetration and cell internalisation. These peptides shared the so-called C-end rule (CendR) motif: R/KXXR/K (R: arginine, K: lysine and X: any amino acid), an internalisation and tissue presentation motif that requires the exposure at the C-terminus of polypeptide chain to be active. The presence of a cryptic CendR motif defines a novel family of tumour targeting peptides, termed tumour-penetrating peptides (TPPs) (Teesalu *et al*., 2009). In addition to the internal CendR motif, the TPPs contain a vascular homing motif and a recognition site for a protease that exposes and activates CendR motif upon the initial tumour recruitment. These modules ensure the highly specific mechanism for tumour homing and penetration. The coupling of drugs to different TPPs can improve drug delivery (Teesalu *et al*., 2013). The first TPP to be identified was the prototypic CendR peptide CRGDKGPDC (termed iRGD) (Sugahara *et al.*, 2009), which is right now in phase I of clinical trials in combination with chemotherapeutic drugs to treat metastatic pancreatic cancer (clinicaltrials.gov, NCT03517176).

The use of peptides in cancer therapy against various cell types in tumours has been a hot topic for a while (Reubi, 2003). As discussed in the previous sections, M2 TAMs are highly important in the progression of tumours. During an *in vivo* phage screen on TNBC tumour macrophages, Scodeller *et al.* identified the cyclic peptide called "UNO" (sequence CSPGAKVRC) as the most highly enriched peptide. The peptide showed homing to the CD206+ M2 TAMs in a panel of solid tumour models in mice: glioblastoma, breast carcinoma, melanoma and gastric carcinoma. Also, a shorter and linear version of "UNO", "mUNO" (sequence CSPGAK) was identified and both UNO and mUNO were shown to bind to CD206 (Scodeller *et al*., 2017). CD206 is a multiligand endocytic mannose receptor composed of three domains: a mannose-binding lectin domain, a conserved fibonectin type II domain which binds collagen, and a cysteine rich domain which binds sulphated glycans (Martinez-Pomares, 2012); the structure of this protein was first described in 1990 (Ezekowitz et al., 1990). Recent computational and experimental binding studies showed that mUNO binds to a previously unidentified binding site in the lectin domain of CD206, and that it does not compete with mannose (Asciutto *et al.*, 2019), making it more specific to CD206 than mannose-based ligands reported in the literature. By coupling a model payload (fluorescein, FAM) to UNO and mUNO, it was shown that those peptides could be used for the targeted delivery of payloads to M2 TAMs (Scodeller *et al*., 2017).

Tumour homing peptides, typically identified by *in vivo* phage biopanning, are particularly well suited for guiding nanoparticle delivery. An example of a nanoparticle delivery system are polymersomes (PSs) which are ~100 nm sized polymeric vesicles formed in an aqueous solutions through self-assembly (Discher *et al*., 2002; Simón-Gracia *et al*., 2018). They are hollow nanospheres that have an aqueous core surrounded by a bilayer membrane that allow the encapsulation of both hydrophilic and hydrophobic cargo molecules, such as drugs, enzymes, or proteins (Meng *et al*., 2009). PSs can carry hydrophobic drugs, which would otherwise be insoluble, they can encapsulate and protect nuclease-sensitive cargo (Simón-Gracia *et al*., 2018) and can have a long blood half-life (Lee and Feijen, 2012).

1.9. CHALLENGES IN BREAST CANCER THERAPY

A major challenge in the clinical management of breast cancer is to normalise the stroma, since the tumour stroma compresses the blood vessels and attenuates drug delivery (Stylianopoulos *et al*., 2012). The disturbed balance between pro- and antiangiogenic signals in tumour vessels results in leaky and structurally abnormal vessels. This, in combination with the high stroma, hampers the blood flow (Goel *et al*., 2011). If blood vessels inside and around the tumour are compressed, then systemic therapeutics cannot reach all the tumour cells (Jain, 1997). TNBCs have a high stromal content (de Kruijf *et al*., 2011), which means that patients may benefit from treatments reducing the desmoplasia (Zhao *et al.*, 2019). One of those agents is Losartan Potassium, a Food and Drug Administration (FDA) approved angiotensin II inhibitor (Zhao *et al.*, 2019) used to treat hypertension (Goa and Wagstaff, 1996). In mouse models, Losartan Potassium reduced the amount of intratumoural collagen and HA, which resulted in the reduction of solid stress, in better perfusion, in improved drug penetration (Diop-Frimpong *et al.*, 2011; Zhao *et al*., 2019), and in an enhanced therapeutic effect (Chauhan *et al.*, 2013).

M2 TAMs have become an important target in multiple studies with the goal of their reprogramming or elimination (Noy and Pollard, 2014) for example by affinity targeting of endocytic markers of M2 TAMs such as CD206. Combining the M2 TAMs directed treatments with immunomodulatory antibodies or with desmoplasia-reducing strategies may lead to improved management of TNBC.

In this thesis it is shown that mUNO can be used for targeting M2 TAMs in primary and secondary TNBC tumours in mice. It is also shown that mUNO is translationally relevant as it

is able to deliver payloads to human M2-differentiated macrophages by binding to CD206. These findings may have implications for clinical management of TNBC.

2. EXPERIMENTAL PART

2.1 THE OBJECTIVES OF THE STUDY

TNBC is a breast cancer subtype lacking good therapeutic options. Right now, there is only one potential option, combining PD-L1 blockade with a chemotherapeutic drug, but it only benefits those patients who already have locally advanced or metastatic TNBC, and works best for PD-L1+ patients, which leaves a big portion of patients with TNBC that still cannot benefit from this therapy. Therefore, there is a high demand to find a therapy option for TNBC patients with the cancer at any stage. Based on those antecedents, the objectives of this study were:

- to evaluate the use of two TNBC tumour models;
- to establish an *in vitro* system for human M2-differentiated macrophages;
- to evaluate the specificity of FAM-mUNO for human M2-differentiated macrophages;
- to evaluate the *in vivo* specificity of FAM-mUNO for M2 TAMs;
- to study the half-life of FAM-mUNO administered intraperitoneally (i.p.) or intravenously (i.v.) and determine the best administration route;
- to establish a method for the desmoplasia depletion.

2.2 MATERIALS AND METHODS

The animal work, which, except for i.v. injections, was done by the author of the thesis under the protocols approved by the Estonian Ministry of Agriculture, Committee of Animal Experimentation (Project 48). The certificate for the animal work is included under the supplementary data.

2.2.1. Mediums, solutions and buffers used

The mediums and solutions used in the experiments:

- RPMI: RPMI-1640 (Roswell Park Memorial Institute) medium (Gibco by Life Technologies) supplemented with 100 IU/ml penicillin, streptomycin (Capricorn Scientific) and 10% V/V of foetal bovine serum (FBS, Capricorn Scientific)
- 0.5% Eosin solution: 0.5 g eosin Y (Sigma-Aldrich) taken to 100 ml with milli-Q water
- 4% PFA: 4 g paraformaldehyde (PFA, Carl ROTH) taken to 10 ml with 10x PBS, topped up with milli-Q water until 100 ml
- 15% sucrose solution: 15 g of sucrose (Sigma Life Science) taken to 100 ml with milli-Q water
- 30% sucrose solution: 30 g of sucrose taken to 100 ml with milli-Q water

- 2.5% avertin: 1.25 g tribromoethanol (Sigma-Aldrich) diluted firstly in 1.25 ml of 2-methyl-2-buthanol, topped up with 0.9% NaCl (Sigma-Aldrich) until 50 ml
- PBS + 0.2% Triton: 1 ml of Triton® X-100 (AppliChem) added to 500 ml of phosphate buffered saline (PBS, Lonza). Triton is an anionic detergent used for permeabilising the tissues and tumours
- PBST: 250 µl (0.05%) of Tween® 20 (Sigma-Aldrich) added to 500 ml of PBS. Tween is an anionic detergent used to avoid unspecific binding
- 5% blocking buffer: 0.5 g of bovine serum albumin (BSA, Capricorn Scientific) taken to 10 ml with PBST, containing 5% V/V goat serum (Life Technologies Thermo Fisher Scientific) and 5% V/V FBS
- 1% blocking buffer: 1% W/V BSA, 1% V/V FBS and 1% V/V goat serum in PBST

2.2.2. Cancer cell lines and cell culture

All *in vivo* experiments were performed using the animal stage IV breast cancer (4T1, ATCC) cell line, and its green fluorescent protein (GFP)-expressing derivative (a gift from Ruoslahti laboratory, SBP La Jolla, USA). Frozen cell aliquots were thawed from liquid nitrogen, centrifuged (Eppendorf Centrifuge 5810R) at 250 g for 6 min to remove the dimethyl sulfoxide (DMSO, Sigma Life Science) and cultured in 25 cm^2 flask (BD Falcon®, non-coated) in 6 ml of RPMI in +37 °C incubator in the presence of 5% CO_2. After 1-2 days when confluency was over 80%, the cells were washed twice with PBS, treated with 1-2 ml of trypsin (Gibco by Life Technologies) for 5 min in the incubator, then knocked on a hard surface to help the cells to detach further. 8-9 ml of RPMI was added to the cell suspension, the cells were pipetted up and down a few times to obtain a homogeneous suspension and transferred to a 75 cm^2 flask (Thermo Scientific, non-coated), and left to grow to 80% confluency in the incubator.

2.2.3. Mice and the tumour models.

8-12-week-old female Balb/c mice (inhouse bred) were used for all the animal experiments. Two different tumour models were used. For the orthotopic model, 10^6 4T1 cells in 50 µl of PBS were injected into 4^{th} or 5^{th} mammary fat pad subcutaneously (s.c.). For the metastatic model, $5x10^5$ 4T1 cells in 100 µl were injected i.v. into the mouse tail vein. For cell counting, 10 µl of cell suspension and 10 µl of 0.4% Trypan blue (Smart Mix) were mixed and counted with TC10TM Automated Cell Counter (BioRad). The tumour volume for the orthotopic model was measured using a digital calliper (Mitutoyo) and the volume was calculated based on the $(W^2 \times L)/2$ formula, where W equals to the tumour's width and L to the tumour's length.

2.2.4. Administration of the compounds.

All injections were performed as in the video by Machholz *et al.* (Machholz *et al*, 2012). For the i.v. injections, the mouse's tail was warmed in a warm water for few min before the injection and the needle was first bent at a 90° angle to facilitate the injection. For most of injections, 30G or 29G needles (BD Micro-Fine™ Plus) were used, i.v. injections were always performed with 30G needles.

2.2.5. Peptides used in the *in vitro* and *in vivo* experiments

Three different peptides were used of which two were control peptides:

- FAM-mUNO: 5-FAM-Ahx-CSPGAK-COOH (Mw = 1048 Da)
- FAM-Control1: 5-FAM-Ahx-CPMTDNE-COOH (Mw = 1295 Da)
- FAM-Control-2: 5-FAM-Ahx-CAQK-NH2 (Mw = 920 Da)

The Ahx stands for aminohexanoic acid and FAM denotes fluorescein. All the peptides were ordered from the TAG Copenhagen. The peptides were supplied as powder, stored at -20 °C and the peptide solutions were prepared fresh every time to avoid peptide dimerisation. The powder was equilibrated at room temperature (RT) with the cap on for 15 min, followed by incubation without the cap to allow the residual humidity to evaporate for an additional 15 min. The peptide was dissolved in fresh PBS aided by slight vortexing and covered with aluminium foil to avoid the degradation of the FAM label. The concentration of the FAM-labelled peptides was checked by measuring the height of the absorption peak for FAM (at 490 nm) using UV-Vis programme (NANODROP 2000c, Thermo Scientific).

2.2.6. PSs synthesis and functionalisation

PSs were synthesised by Lorena Simón-Gracia and characterised by Valeria Sidorenko (researcher and master's student, respectively, at the Laboratory of Cancer Biology, University of Tartu) as described previously (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-PEG-PCL. FAM-mUNO or FAM-cysteine (Cys) were coupled to the PSs by a maleimide-thiol bond using the thiol from the Cys of the peptides. Two different PSs were used and are denoted as PS-FAM-mUNO and PS-FAM. The final PSs samples had a concentration of ~10 mg of polymer/ml and for binding studies the concentration of PSs was normalised for FAM as described above.

2.2.7. Haematoxylin and eosin (H&E) staining

Tumours were induced as described above. Five days after the s.c. inoculation (orthotopic model), or 10 days after the i.v. inoculation (metastatic model), the mice were sacrificed by injecting 400 µl of 2.5% Avertin i.p. The organs were collected and fixed in a cold 4% PFA overnight at 4 °C. The following day, the tissues were washed with PBS for 1 h at RT to remove all of the PFA. For cryoprotection, the tissues were passed through sucrose (Sigma Life Science) solutions (15% and 30%) overnight at 4 °C. Cryoprotected and fixed tissues were mounted in the same block with an Optimal Cutting Temperature (OCT) compound (Leica Biosystems) and left at -20 °C overnight. Next day, 10 µm sections were cut using cryomicrotome (Leica CM1520). The tissue sections were placed in a coplin jar filled with a haematoxylin solution (Sigma Aldrich) and incubated at RT for 5 min. The sections were subsequently washed under running water until the water ran clear. Sections were then placed into another coplin jar filled with an eosin solution and incubated for 150 s and slides were washed again as above. For dehydration, the sections were incubated in a series of ethanol solutions as follows: 2x2 min in 96% ethanol, 2x2 min in 100% ethanol and finally incubated with a RotiClear® solution (Carl ROTH) 2x5 min to make the tissues clear. Sections were mounted with a mounting medium (Eukitt® quick-hardening mounting medium, Fluka Analytical), left to dry for 10-15 min at RT and sealed with nail polish. The tissues were imaged using whole slide scanner (Leica SCN400) and the pictures analysed with QuPath programme. Haematoxylin and eosin stain the cytoplasm in light pink, collagen in pink and nuclei in purple/blue.

2.2.8. GFP imaging

For the GFP imaging, the mice were injected with 4T1-GFP cells i.v. or s.c. as described above. To image the GFP in the tumours, the slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) in a PBS with final concentration of 1 µg/ml, mounted using medium (Fluoromount-G™ Electron Microscopy Sciences) and glass coverslips (Assistant Cover Glasses 40x24mm) and imaged using a Zeiss (Zeiss LSM-710), or an Olympus (Olympus FV1200MPE) confocal microscope using 20x or 10x objective, respectively.

2.2.9. *In vitro* macrophages differentiation model and binding assay

Primary blood mononuclear cells (PBMC) were purified from human donor blood obtained from Tartu University Hospital Blood Centre. The procedure is described in an Asciutto *et al*. paper along with the protocol for an *in vitro* binding assay (Asciutto *et al.*, 2019). Briefly, cells

purified from blood were sorted using CD14+ microbeads (MACSMiltenyi Biotec). 500 µl of $3.5x10^5$ cells were seeded on FBS-coated 12 mm glass cover slips (Marienfeld) placed in a 24-well plate (Thermo Scientific, non-coated). The 3 h pre-coating at +37 °C was important to obtain the optimum cell attachment. After 24 h and 72 h, 250 µl of media was replaced with a new one to favour cell maintenance. After 96 h, IL-4 was added at final concentration of 40 ng/ml (BioLegend®) and incubated for 48 h to obtain M2 phenotype. For M1 LPS (Sigma-Aldrich) and IFN-γ (BioLegend®) at a final concentration of 100 ng/ml and 20 ng/ml, respectively and incubated for 48 h. For resting macrophages phenotype (M0), no mediator was added. After differentiation, cells were divided into two groups: one blocked with human CD206 (BioLegend®) at final concentration of 10 µg/ml and the others were not. Cells were incubated with an antibody to obtain optimal blocking for 2 h. After blocking, peptides and PSs were added to the well at the final concentration of 3nM and 1 mg/ml, respectively. The cells were incubated with PSs for 5 min and with peptides for 20 min. After the peptide incubation, the cells were washed twice with RPMI and once with PBS, fixed with 4% PFA at RT for 10 min, permeabilised with PBS + 0.2% Triton and immunostained according to the immunofluorescence (IF) protocol. The antibodies used in these experiments are listed in Table 2. Cells were also counterstained with DAPI as described above. The coverslips were mounted on a glass slide (Thermo Fisher) using a mounting medium (Fluoromount-G™ Electron Microscopy Sciences), imaged with the Zeiss confocal microscope and corresponding programme and the ImageJ programme. To characterise the M0, M1 and M2 cells, they were stained for CD206 and imaged using a 20x and 63x objective. The graphs were prepared using Origin Pro 8 programme. The P values were calculated using the Origin Pro 8 and One-Way ANOVA analysis.

Table 2. Antibodies used for *in vitro* binding studies.

Marker or dye	**Primary antibody, working dilution**	**Secondary antibody, working dilution**
FAM	Anti-fluorescein/Oregon Green™ rabbit IgG fraction (Invitrogen by Thermo Fisher Scientific), 1/250	Alexa Fluor™ 647 goat anti-rabbit IgG (H+L) (Invitrogen by Thermo Fisher Scientific), 1/400
CD206	Purified anti-human CD206 (MMR) Clone:15-2 (BioLegend®), 1/200	Alexa Fluor® 546 goat anti-rat IgG (H+L) (life technologies™), 1/300

2.2.10. Peptide homing studies

The homing of FAM-mUNO and the control peptides was tested in both tumour models. For the orthotopic model, 30nmoles of peptides (30 µg for FAM-mUNO and 37 µg for FAM-Control1) dissolved in 500 µl of PBS were injected i.p. at 5 days post inoculation (p.i.) where tumour volumes were ~50 mm^3; for the metastatic model, 30nmoles of peptides dissolved in 500 µl of PBS were injected when the mice showed signs of sickness, at 10 days p.i. For both models, the peptides were circulated for 24 h, the mice were sacrificed, the organs collected, fixed, cryoprotected and sectioned as described above. 10 µm sections were cut using a cryomicrotome and slides were immunostained.

2.2.11. Immunofluorescence (IF)

The slides with tissue sections were equilibrated at RT in the dark for at least 30 min to ensure adherence to the SuperFrost™ glass (Thermo Fisher). The different primary and secondary antibodies used for the detection of markers and dye are shown in the Table 3 below. The blocking with a 5% blocking buffer was done for 1 h at RT followed by an overnight incubation with primary antibodies in a 1% blocking buffer at 4 °C. Sections were incubated with secondary antibodies in a 1% blocking buffer for 30 min at RT. Slides were counterstained with DAPI as described above. All IF steps were performed in the dark to avoid photobleaching of the FAM label. The stained sections were mounted with a mounting medium (Fluoromount-G™ Electron Microscopy Sciences), left to air-dry in the dark for 1 h at RT and sealed with nail polish. Slides were cleaned with ethanol and imaged with a Zeiss confocal microscope using 20x magnification or an Olympus confocal microscope using 10x magnification. In both cases, the images were analysed using the corresponding programme and the colocalisation analysis (Pearson's coefficient) were calculated using Fiji using at least 3 representative images from each tumour and graphs were made with Origin Pro 8.

Table 3. Primary and secondary antibodies used for the immunostaining.

Marker or dye	Primary antibody, working dilution	Secondary antibody, working dilution
FAM	Anti-fluorescein/Oregon Green™ rabbit IgG fraction (Invitrogen by Thermo Fisher Scientific), 1/100	Alexa Fluor™ 647 goat anti-rabbit IgG (H+L) (Invitrogen by Thermo Fisher Scientific), 1/200
CD31	Purified Rat anti-mouse CD31 clone MEC 13.3 (BD Biosciences), 1/100	Alexa Fluor® 546 goat anti-rat IgG (H+L) (life technologies™), 1/200
CD86	Purified anti-mouse CD86 Clone: GL-1 (BioLegend®), 1/100	Alexa Fluor® 546 goat anti-rat IgG (H+L) (life technologies™), 1/200
CD206	rat anti-mouse CD206 (Bio-Rad), 1/100	Alexa Fluor® 546 goat anti-rat IgG (H+L) (life technologies™), 1/200

2.2.12. Plasma half-life studies

The Heparin-PBS was prepared by placing 1 ml of PBS in a Heparin-coated blood collection tube (BD Vacutainer) and vortexed for ~1 min, and then added into a new blood collection tube and vortexed again for 1 min. Before the blood collection, the mouse was restrained, a ~2 mm piece of the tail was cut off to obtain the zero-point blood serum. After that 30nmoles of the FAM-mUNO was injected i.p. or i.v. Ten µl of blood was collected from warmed tail in 50 µl of PBS-Heparin. Time points used for blood sampling:

- for i.v. administration: 0 min, 6 min, 10 min, 15 min, 30 min, 1 h, 3 h, 6 h and 24 h
- for i.p. administration 0 min, 7 min, 10 min, 16 min, 30 min, 1 h, 2 h, 3 h, 6 h and 24 h

45 µl of the blood and heparin mixture was added to the 96-well plate (Thermo Scientific, non-coated) and the fluorescence of the FAM was 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. The graphs were prepared using Origin Pro 8 and fitted using a bi-exponential decay equation. The P values were calculated using Origin Pro 8 and a One-Way ANOVA analysis.

2.2.13. Desmoplasia reduction in the orthotopic tumour model

Tumours were induced as described above for the orthotopic tumour model. On day 3, 750 µl of 1.6 mg/ml of Losartan Potassium (ChemCruz) in PBS were injected i.p. The injections were repeated every day for 10 days, after which the mice were sacrificed, the organs and tumours collected, fixed, cryoprotected and sectioned as described earlier. Tissues were stained using a Movat Pentachrome Staining kit (Nordic BioSite in Life Science Research), which stains collagen in yellow to orange, hyaluronan in blue, nuclei in blue/black, elastic fibres in black to blue/black, reticular fibres in yellow, fibrin in bright red and muscle tissue in red. The staining was done according to the manufacturer's protocol (Nordic BioSite in Life Science Research) with slight modifications: elastic Stain Solution was added as drops onto the slide and kept for maximum of 7 min to not overstain the tissues; Biebrich Scarlet/Acid Fuchin Solution was added as drops onto the slide and kept for 90 s to not overstain the tissues. The slides were imaged using a Zeiss microscope (Zeiss AXIO Vert. A1) with a 20x objective.

To visualise the effect of Losartan Potassium on the tumour's blood vessels, the sections were stained with anti-CD31 using the protocol described above and imaged with a Zeiss confocal microscope.

2.3. RESULTS

2.3.1. Characterisation of tissues and tumours for overall histology and markers.

First, the characterisation of the two *in vivo* tumour models used in this thesis was performed. In the metastatic model, the H&E staining (Fig. 2B) showed the presence of tumour foci in the lungs, with the normal lung architecture destroyed (the normal structure from the lungs of healthy mice is shown in Fig. 2A). In the orthotopic model (Fig. 2C), the H&E staining showed an abundant ECM deposition in the tumours (white arrows). Both the orthotopic and metastatic models showed high amounts of M2 TAMs (CD206+) (Fig. 2D-F, in red) In the orthotopic model most of these macrophages were situated at the tumour margin, whereas in the metastatic model they were uniformly distributed throughout the entire lung tissue. The orthotopic model (Fig. 2I) showed a low amount of M1 TAMs (CD86+) compared to the metastatic model (Fig. 2H). Interestingly, the metastatic model showed a higher expression of CD86 compared to the healthy lungs (Fig. 2G). In some cases, the mice were inoculated with GFP-expressing 4T1 cells to allow for monitoring of the tumour cells. In the metastatic model, GFP+ cells formed large nodules inside the lungs (Fig. 2K). In the orthotopic model it was seen that green fluorescent cells had lower density compared to the metastatic model (Fig. 2L), suggesting that

the other cells in the tumour are immune and stromal cells. The observed GFP signal was attributed to the tumour cells as it was absent in the healthy lungs (Fig. 2J).

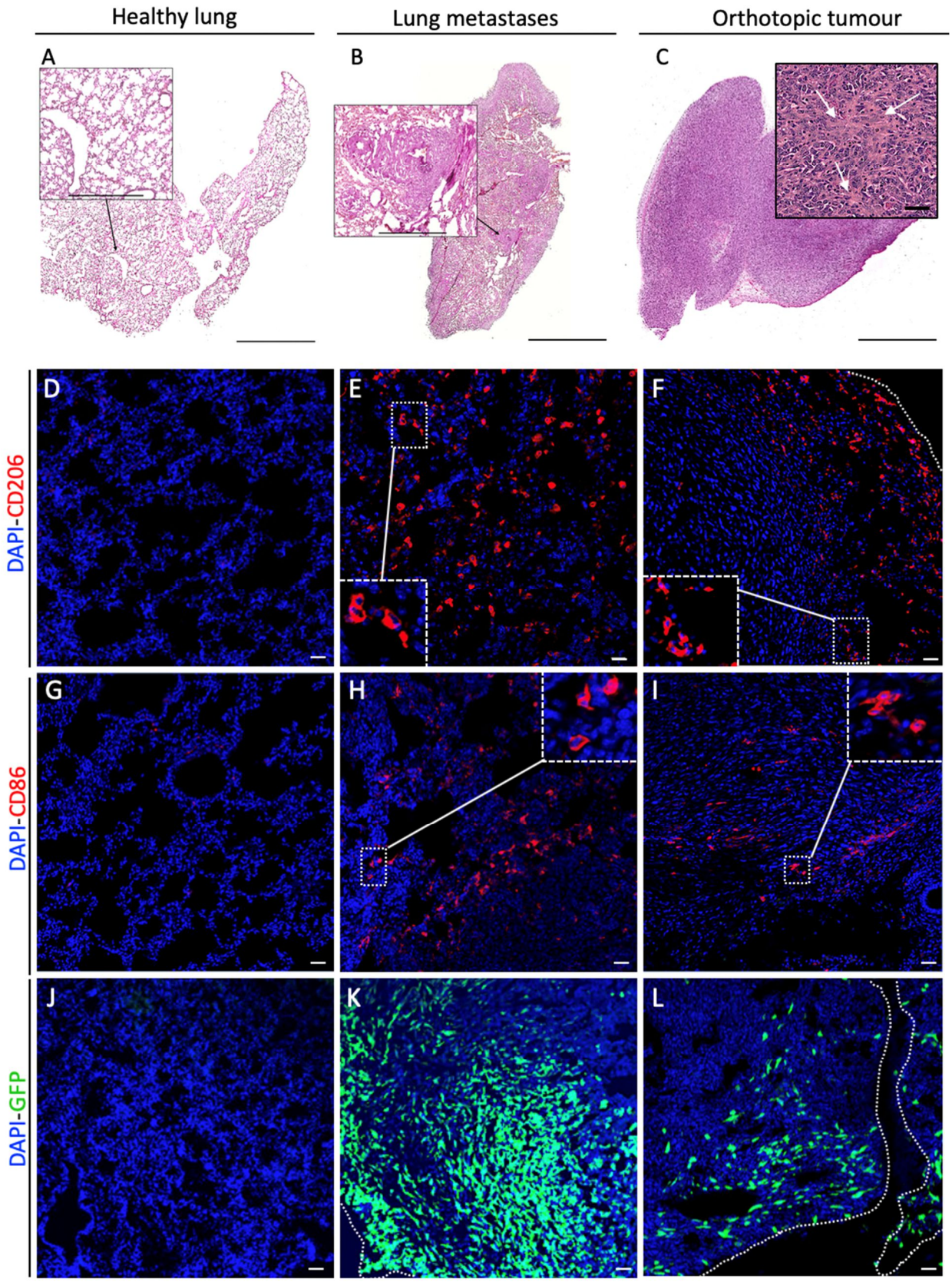


Figure 2. Characterisation of tumour models. For the orthotopic model, the mice were injected s.c. with 10^6 4T1 cells, for GFP imaging, the same amount of 4T1-GFP cells were injected s.c. and for the metastatic model

$5x10^5$ 4T1 cells were injected i.v. The mice were sacrificed either 5 days p.i. for the orthotopic model, or 10 days p.i. for the metastatic model. H&E staining of the healthy lung (A), of the the metastatic lung showing large tumour masses inside the lungs (black arrow, B) and of the orthotopic tumour showing abundant ECM (arrows, C). CD206 (D-F, red) and CD86 (G-I, red) stainings were used to characterise the M1 and M2 TAMs, and the fluorescence from the GFP channel (J-L) was used to map the tumour cells. Scale bars are 1 mm (A-C), 200 µm (insets A, B), 50 µm (inset C), 20 µm (D-L). Representative results from N=2 mice.

2.3.2. FAM-mUNO labels human M2-differentiated macrophages *in vitro.*

To evaluate the binding of FAM-mUNO to human CD206+ macrophages, the primary human macrophages were stimulated to M2 or M1 based on published procedures (Mantovani *et al.*, 2002; Martinez and Gordon, 2014). After 96 h of culture on the glass coverslips, appropriate polarising stimuli were added to the primary human macrophages to obtain M2 (IL-4), M1 (IFN-γ and LPS) phenotype (Fig. 3B, C); unstimulated resting macrophages were denoted as M0 (Fig. 3A). Quantitative immunophenotyping showed that 2% of M1 macrophages, 18% of M0 macrophages and 71% of human M2-differentiated macrophages were positive for CD206 (Fig. 3D).

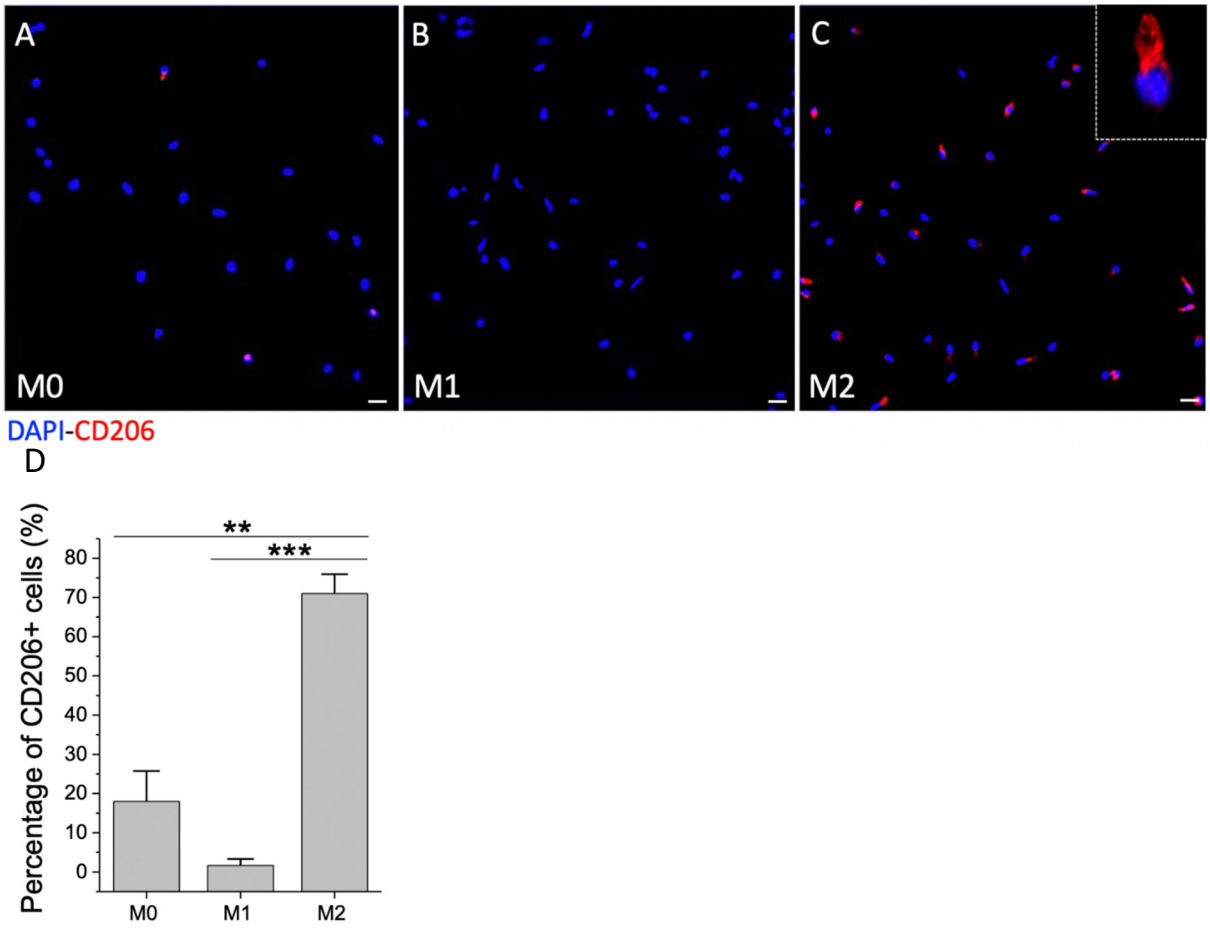


Figure 3. Characterisation of *in vitro* differentiated human macrophages. To obtain M1, M2 and M0 phenotype IL-4, LPS + IFN-γ or no stimuli at all was added to the human monocytes after 96 h of cultivation and left for 48 h. Then the cells were washed, fixed, permeabilised and stained with an antibody against CD206, and

counterstained with DAPI. Quantitative immunophenotyping showed that 2% of M1, 18% of M0 and 71% of human M2-differentiated macrophages express the CD206 (A-C). **P<0.01, ***P<0.001

After 20 min incubation the FAM-CD206 colocalisation was significantly higher for FAM-mUNO (Fig. 4A) compared with the FAM-Control1 peptide (Fig. 4B). To evaluate if the uptake of FAM-mUNO was mediated by CD206, blocking with anti-CD206 antibody was done. Blocking of CD206 with a function-blocking antibody reduced the binding of FAM-mUNO (Fig. 4C), but not of FAM-Control1 (Fig. 4D, E).

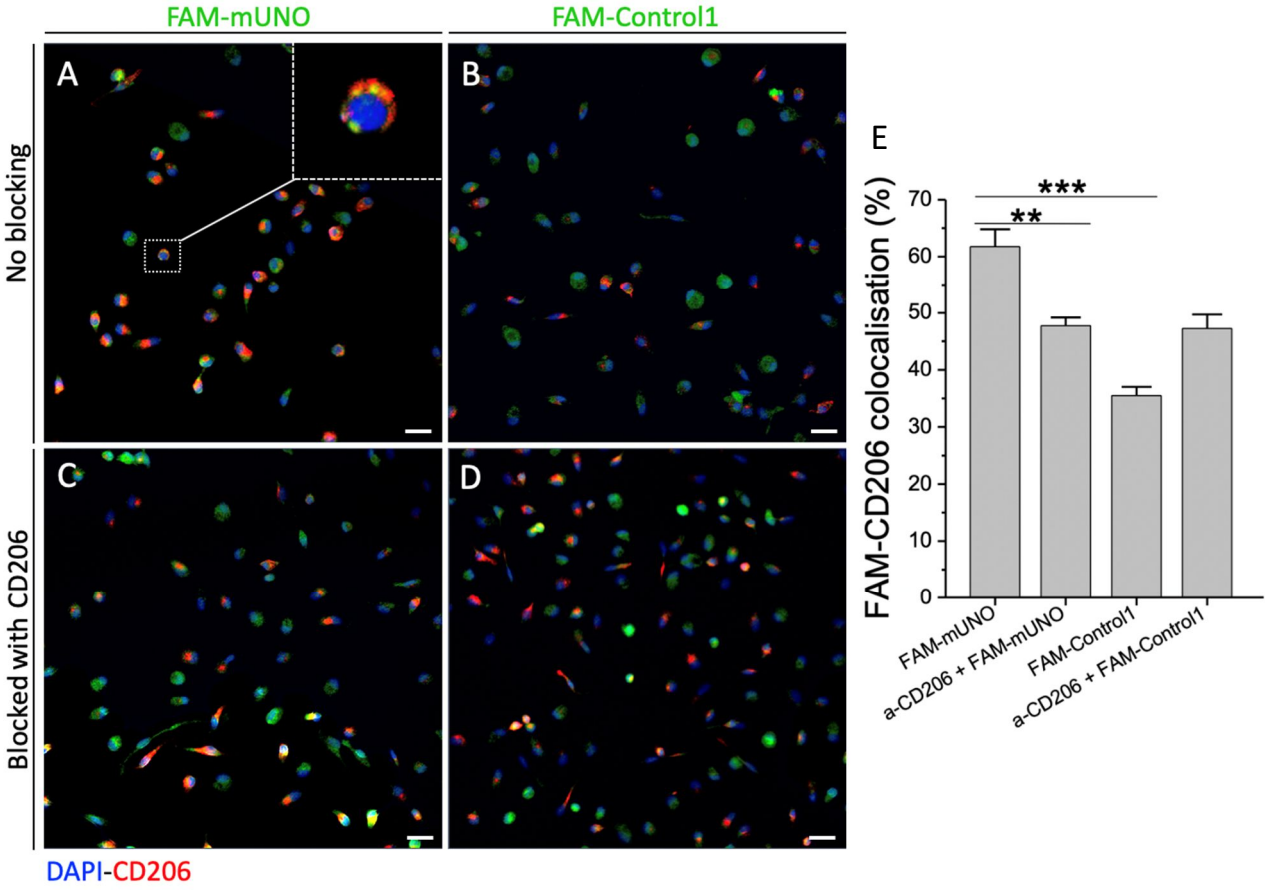


Figure 4. FAM-mUNO binding studies on human M2-differentiated macrophages. Human M2-differentiated macrophages were incubated with FAM-peptides for 20 min, the cells were washed, fixed, permeabilised, stained with antibodies against FAM and CD206, and counterstained with DAPI. FAM-mUNO binds to CD206+ cells *in vitro* (A) and the binding is reduced when blocked with anti-CD206 antibody (C). The FAM-Control1 does not specifically bind to the CD206+ cells *in vitro* (B) and the CD206 blocking had the opposite effect than with FAM-mUNO (D). The scale bars represent 20 µm. **P<0.01, ***P<0.001

To study if mUNO could be used to target nanoparticles to CD206+ macrophages, mUNO-coated PSs were used. PSs are a well-established nanoplatform in our laboratory. The PS-FAM-mUNO (Fig. 5A) showed after 5 min incubation significantly higher binding to the CD206+ cells compared to PS-FAM (Fig. 5B). Also, the blocking with anti-CD206 antibody reduced

the binding of PS-FAM-mUNO (Fig. 5C) but not of PS-FAM (Fig. 5D) to M2-differentiated macrophages, as seen from the fluorescence/cell analysis (Fig. 5E).

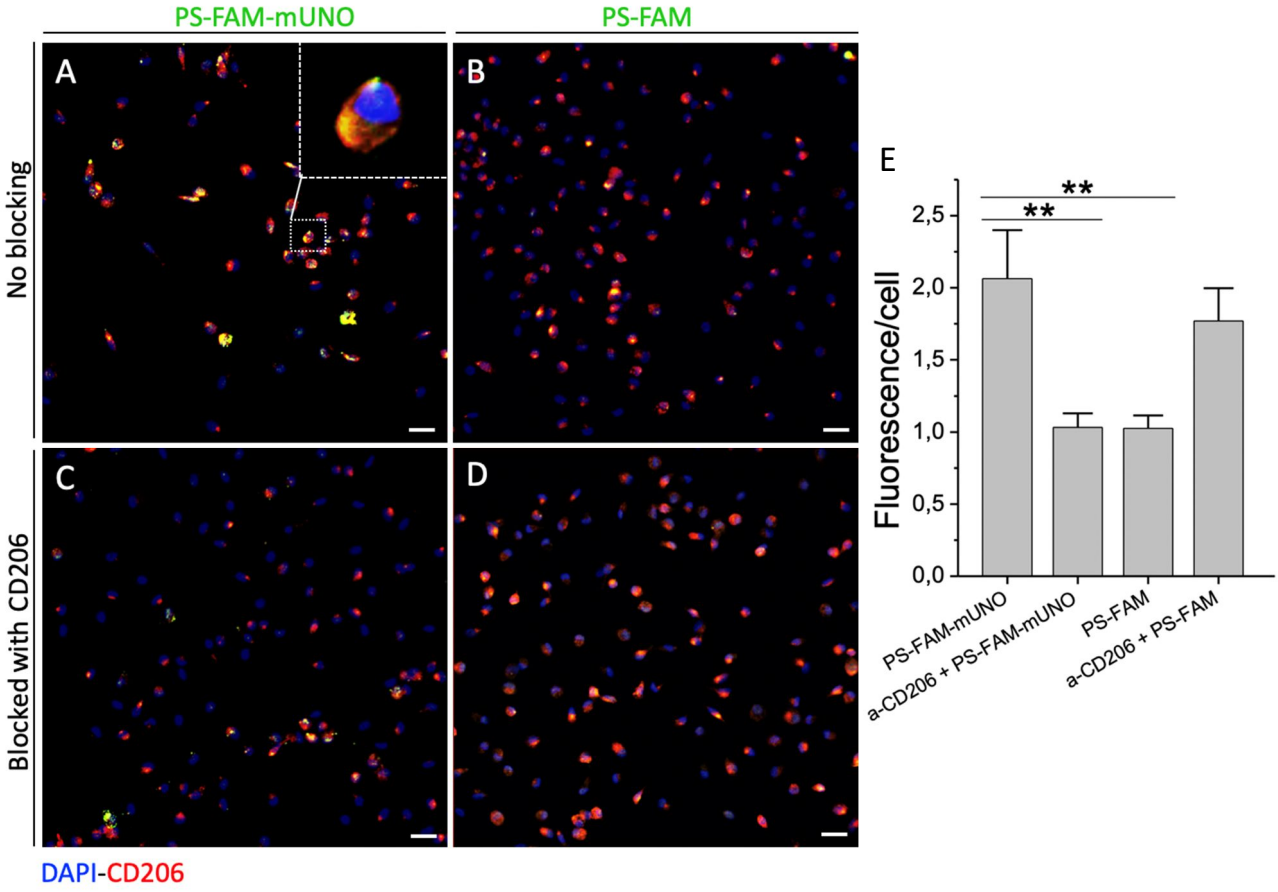


Figure 5. PS-FAM-mUNO binding studies on human M2-differentiated macrophages. Human M2-differentiated macrophages were incubated with FAM-peptide functionalised PSs for 5 min, the cells were washed, fixed, permeabilised, stained with antibodies against FAM and CD206, and counterstained with DAPI. PS-FAM-mUNO binds to CD206+ cells *in vitro* (A) and the binding is reduced when blocked with anti-CD206 antibody (C). The PS-FAM does not specifically bind to the CD206+ cells *in vitro* (B) and the CD206 blocking had the opposite effect than with PS-FAM-mUNO (D). The scale bars represent 20 μm. **P<0.01, ***P<0.001

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

Half-life of a homing peptide determines how much time it has for binding to its receptor. Also, if the peptide is coupled to therapeutic cargo, a longer half-life will translate into higher drug delivery to the tumour.

The half-life of the i.p. injected FAM-mUNO was 110 min compared to the 24 min for the i.v. route (Fig. 6B). Very different pharmacokinetics were observed for i.v. *vs* i.p. administration routes (Fig. 6A). The concentration of i.v. injected FAM-mUNO showed a biphasic exponential decay, whereas the concentration of i.p. injected FAM-mUNO displayed an initial increase, reached a maximum at 30 min, and then decayed with time. These results provided a

justification for performing the homing studies of FAM-mUNO using the i.p. administration route.

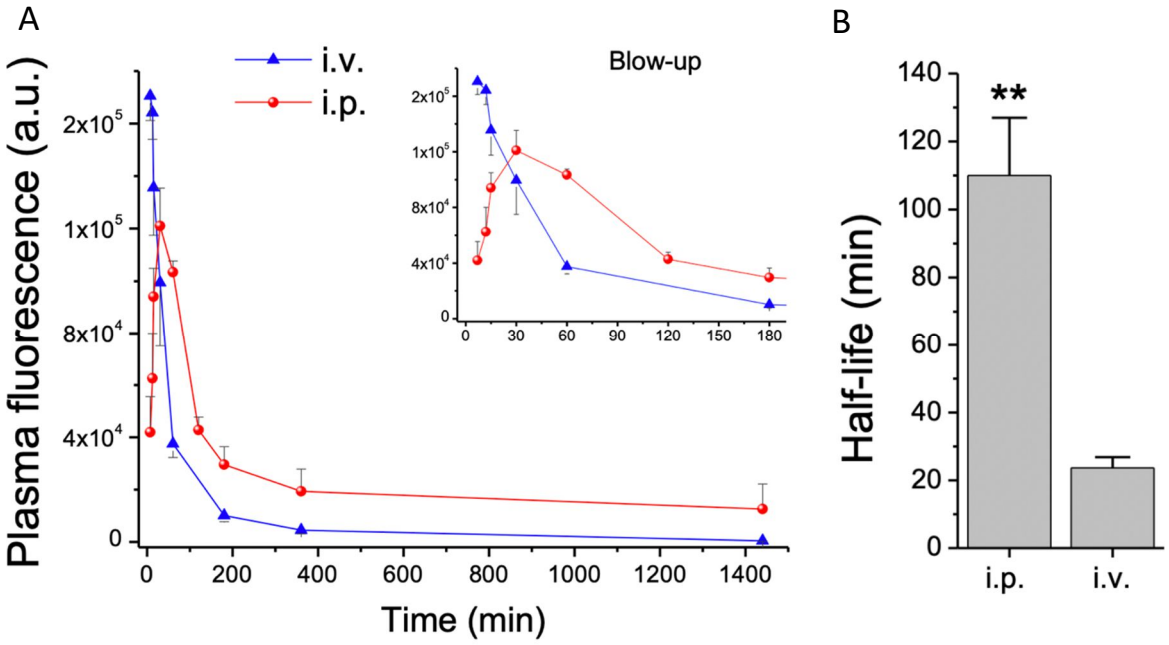


Figure 6. Plasma half-life of FAM-mUNO: i.v. *vs* i.p. administration routes. Panel A: FAM channel fluorescence of blood collected at different time points after i.v. injection (blue curve) and i.p. injection (red curve) of 30nmoles of FAM-mUNO in Balb/c mice (N=3). Panel B: half-life values obtained from the i.p. curve of panel A and from fitting i.v. data with a bi-exponential decay equation. **P<0.01

2.3.4. FAM-mUNO homes to M2 TAMs in both orthotopic and metastatic tumour models.

In the orthotopic tumour model FAM-mUNO colocalised highly with M2 TAMs (yellow signal in Fig. 7A) compared to FAM-Control1 which gave an intense signal in the tumour (Fig. 7B) but was not specific to the M2 TAMs. Colocalisation analysis showed that FAM-mUNO had 67% of FAM-CD206 colocalisation and FAM-Control1 only had 18% (Fig. 7E). The staining of tissue sections with a marker for M1 macrophages, CD86, showed no peptide uptake in this cell population (Fig 7C). The CD31 staining showed that FAM-mUNO can be used to deliver cargo inside the tumour tissue since FAM-mUNO labelled only stromal M2 TAMs (upper inset of Fig. 7F). The staining also showed that blood vessels in this tumour model are highly

compressed (lower inset of Fig. 7F). Both FAM-mUNO and FAM-Control1 did not accumulate significantly in the liver macrophages (Fig. 7G, H).

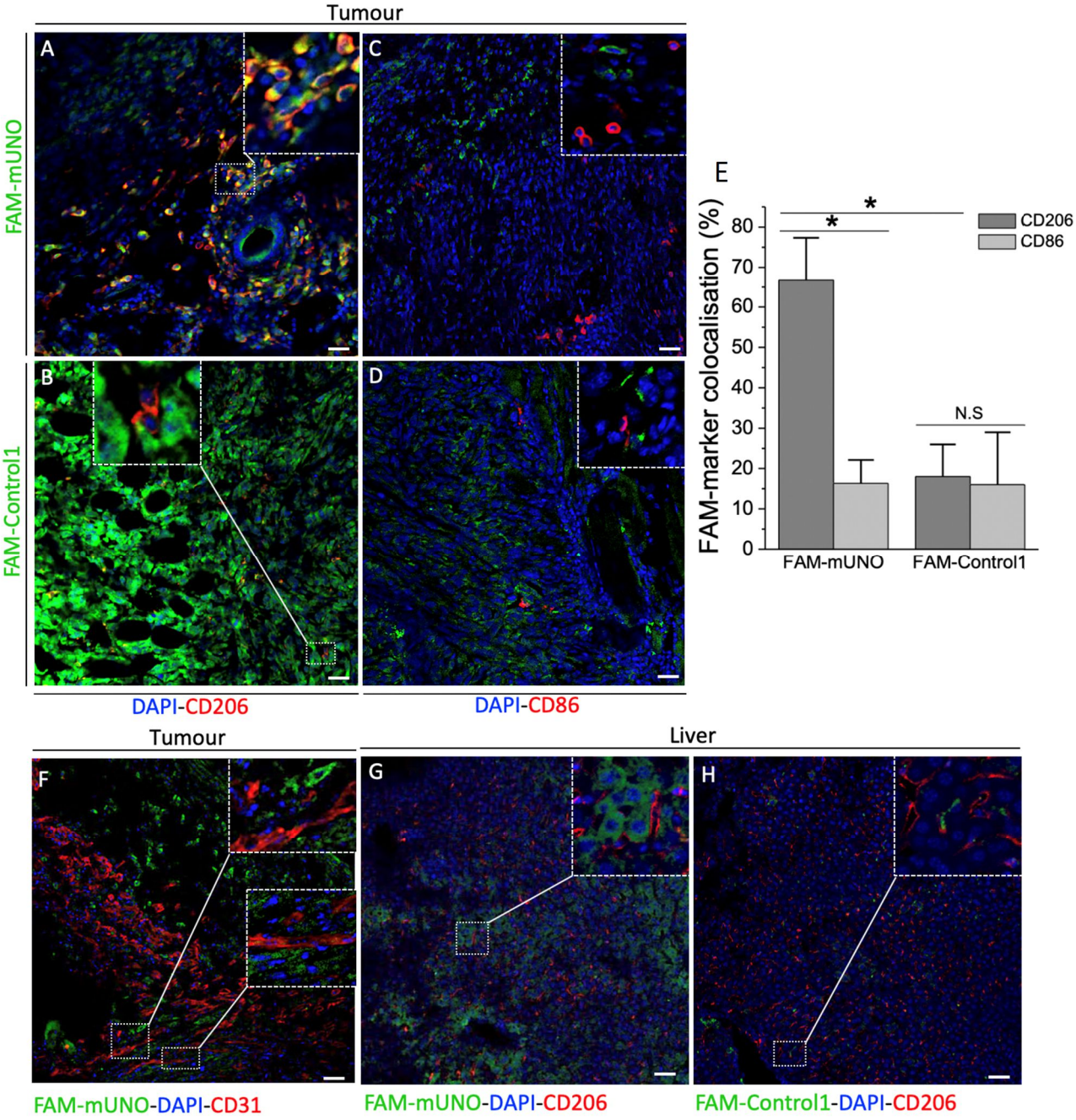


Figure 7. Targeting M2 TAMs with FAM-mUNO in the orthotopic model. 30nmoles of FAM-mUNO or FAM-Control1 peptide were injected i.p. and left to circulate for 24 h. At 24 h the mice were sacrificed, the organs collected, fixed, cryoprotected, sectioned and immunostained. FAM-mUNO colocalised with M2 TAMs on the tumour margin (A) in contrast to FAM-Control1 (B) that showed a promiscuous signal. The CD86 staining showed that FAM-mUNO did not colocalise with CD86+ cells (C). The CD31 staining showed that FAM-mUNO homed to stromal M2 TAMs (upper inset F) and that blood vessel were compressed (lower inset F). The CD206 staining of the liver sections showed that FAM-mUNO or FAM-Control1 did not colocalise with the liver resident macrophages (G, H). In the orthotopic model FAM-mUNO colocalised with M2 TAMs about 67%, compared to the FAM-Control1, where the colocalisation was only 18% (E). Scale bars represent 20 μm. *P<0.05, N.S = not significant

In the metastatic model, FAM-mUNO showed M2 TAM homing in the lungs (Fig. 8A), as opposed to FAM-Control2 (Fig. 8B), and the colocalisation analysis showed that 67% of the CD206+ cells were positive for FAM-mUNO uptake (Fig. 8E). In contrast, FAM-Control2 showed 27% overlap (Fig. 8E). CD86 staining confirmed that also in this model FAM-mUNO did not label the M1 macrophages (Fig. 8C). CD31 staining showed that metastatic lesions in the lungs were more vascularised than the orthotopic tumour tissue (Fig. 8F). It also showed that FAM-mUNO labelled stromal M2 TAMs and also PV M2 TAMs (Fig 8F inset). Again, the FAM-mUNO did not accumulate in the liver or in the Kupffer cells therein (Fig. 8G).

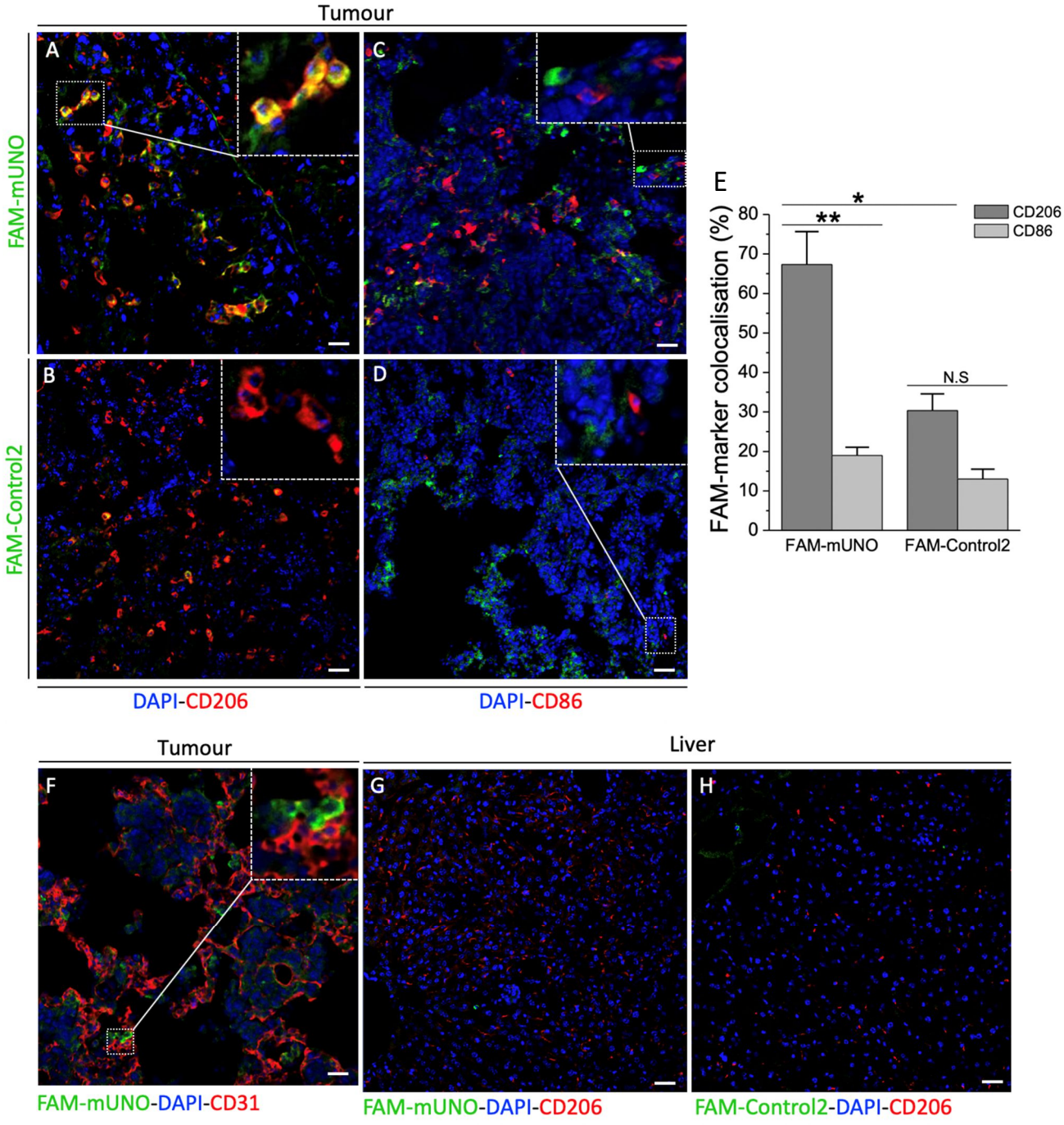
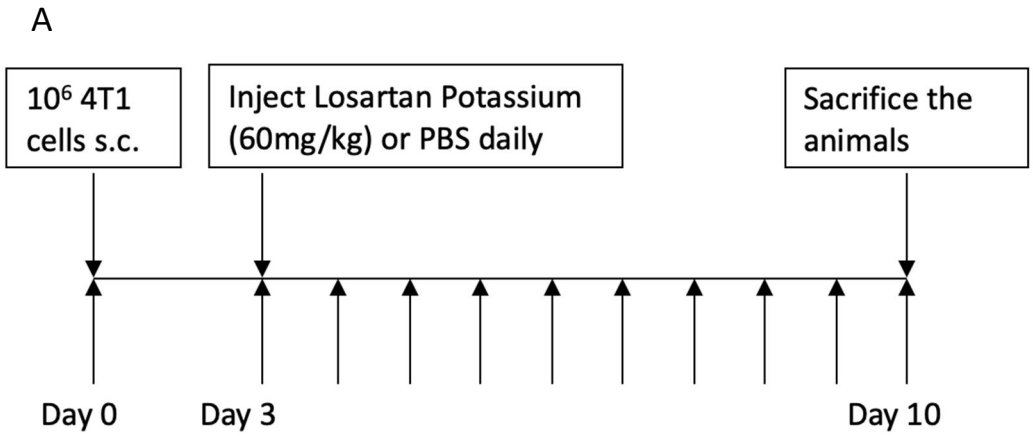


Figure 8. Targeting M2 TAMs with FAM-mUNO in the metastatic model. 30nmoles of FAM-mUNO or FAM-Control2 peptide were injected i.p., left to circulate for 24 h and then the mice were sacrificed, the organs collected,

fixed, cryoprotected, sectioned and immunostained. The FAM-mUNO, but not the FAM-Control2 (green) colocalised with CD206+ cells (red) resulting in yellow composite signal (A, B). FAM-mUNO colocalised with the M2 TAMs more (67%) than FAM-Control1 (27%) (E). CD86 staining showed that FAM-mUNO and FAM-Control2 did not label M1 macrophages (C, D). CD31 staining showed that the tumour in the metastatic model (F) is more vascularised compared to the tumour of the orthotopic model (Fig. 7F). Here, the FAM-mUNO also labelled PV cells (F inset). Neither peptide accumulated in the liver (G, H). Scale bars represent 20 μm. *P<0.05, **P<0.01, N.S = not significant

2.3.5. Treatment with Losartan Potassium reduces the desmoplasia in the orthotopic tumour model and decompresses the blood vessels.

As discussed above, abundant stroma in tumours acts as a barrier for delivery of drugs and imaging agents. The normalisation of stroma might help to relieve some of the solid stress to decompress the blood vessels and to improve drug delivery. Staining of the orthotopic tumours with a Movat Pentachrome Staining kit showed the presence of high amounts of collagen and HA (Fig. 9B). In an attempt to reduce this desmoplasia and decompress the blood vessels, a small treatment study was conducted (Fig. 9A). One group was treated with Losartan Potassium and the other group with PBS daily for 10 days. Movat Pentachrome staining showed reduced amounts of HA (in blue) and collagen (in yellow to orange) for tissues treated with Losartan Potassium (Fig. 9C), compared to the PBS group (Fig. 9B). Also, the blood vessels appeared decompressed in the Losartan Potassium-treated tumours (Fig. 9E) compared to the PBS group (Fig. 9D). It was seen that in the Losartan Potassium-treated tumours, there were more decompressed blood vessels.



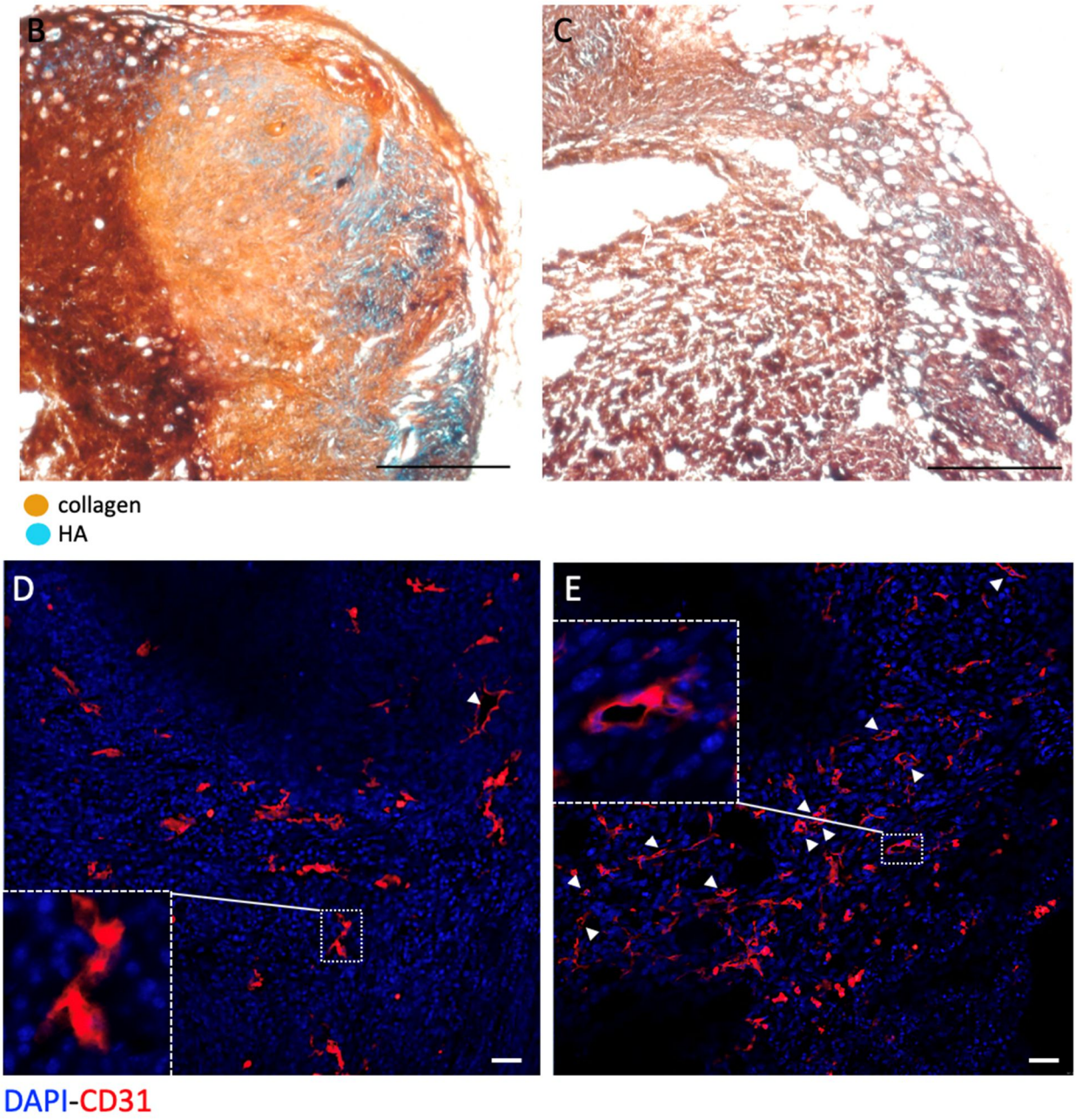


Figure 9. Collagen and HA characterisation in tumour in orthotopic model and the effects of Losartan Potassium treatment on desmoplasia and blood vessel compression. The treatment with Losartan Potassium was started on day 3 p.i., when there was already a palpable tumour. The treatment was done every day for 10 days by injecting 750 µl of Losartan Potassium (1.6mg/ml) or the same volume of PBS (A). Movat Pentachrome staining showed that the treatment with Losartan Potassium reduced the amount of intratumoural collagen and hyaluronan (C) compared to the untreated tumours (B) and decompressed the blood vessels (white arrows, E) compared to the untreated tumours (D). Scale bars for the stroma staining pictures represent 50 µm and for the CD31 staining 20 µm.

2.4. DISCUSSION

TNBC lacks the receptors targeted by available therapies and is consequently the most difficult breast cancer subtype to treat (Foulkes *et al.*, 2010). The only options for TNBC patients are mastectomy, lumpectomy and chemotherapy. TNBC contains abundant M2 TAMs: a protumoural cell population that is immunosuppressive, angiogenic, and contributing to tumour relapse and resistance to chemo- and immunotherapies (Lo Russo *et al.*, 2019; Neubert *et al.,* 2018; Peranzoni *et al*., 2018; Scodeller *et al.*, 2017). M2 TAMs have emerged as an important target for pharmacological destruction and reprogramming studies. (Noy and Pollard, 2014). However, most studies target receptors (CSF1R, CCR2, CD40) that do not precisely identify the M2 subset of macrophages and are also expressed on antitumoural M1 macrophages, microglia and/or some other cell types (El Khoury *et al.*, 2007; Lee *et al,* 2018; Mancini *et al.*, 2019; Ponomarev *et al.*, 2006). Recently, some homing peptides were reported to target M2 TAMs. However, in one study the peptide targeted a molecule not yet accepted as an M2 marker (Tang *et al.*, 2019) and the other study used a peptide with unknown receptor (Cieslewicz *et al.*, 2013; Kakoschky *et al.*, 2018).

In 2017, a peptide called UNO (cyclic CSPGAKVRC) and its linear version mUNO (CSPGAK) were described by researchers at the Laboratory of Cancer Biology at the University of Tartu (Scodeller *et al.*, 2017). UNO and mUNO specifically target CD206, a mannose receptor that identifies M2 TAMs (Lopes *et al*., 2014). In binding to CD206, mUNO does not compete with mannose and interacts with a previously unidentified site in the lectin domain (Asciutto *et al.*, 2019), rendering mUNO more specific for CD206 than mannose-based ligands that also bind to mannose-receptors other than CD206 in healthy tissues (Turner, 2003).

In this thesis, the interest was in exploring the *in vitro* and *in vivo* applications of mUNO-based targeting of TNBC and M2 macrophages.

The linear mUNO peptide used in this thesis is easier to synthesise than parental cyclic UNO, and its free thiol group allows for easy coupling to different molecular and nanoparticle cargoes. To trace mUNO, a FAM was conjugated to its N-terminus. Two different mouse models for TNBC were used: the orthotopic model, which is an aggressive and quickly progressing model, replicating the clinical TNBC situation, and the metastatic model, which enables to study the targeting of tumour metastases inside the lungs, where normal metastases would occur.

The establishment of an *in vitro* model for M2 macrophages is important, since it offers an opportunity to study the pathway of the FAM-mUNO peptide uptake in cultured cells. Here, an *in vitro* system to model human M2 macrophages was developed by polarising human blood monocytes into macrophages using appropriate stimuli. It was also decided to perform the binding studies only on the M2 macrophages, since in our hands M1 macrophages showed a very high and fast uptake irrespective of the compound. This is consistent with the reported higher phagocytic activity of M1 *vs* M2 (Gordon *et al*., 2017). Additionally, the M2-polarised samples contained a mixture of CD206+ and CD206– macrophages that reflects the situation in the tumour.

For FAM-mUNO, the highest selectivity for CD206+ macrophages was observed at 20 min, whereas for PS-FAM-mUNO it occurred at 5 min; this selectivity was lost at longer time points possibly because of the highly phagocytic nature of the macrophages together with the fact that *in vitro* the compounds are easily accessible to the macrophages. The selectivity of FAM-mUNO and PS-FAM-mUNO was mediated by CD206, as anti-CD206 antibody eliminated the difference with control peptides and control PSs. Intriguingly, when CD206 was blocked with an antibody, the binding of FAM-Control1 and PS-FAM increased. This might be again related to the phagocytic nature of the macrophages; it is possible that CD206 blocking activates another pathway for the control peptides to enter the macrophages.

The administration route can have a profound effect on systemic exposure and homing of compounds. The plasma half-life of FAM-mUNO was measured using i.p. and i.v. administration routes and it was found that both have very different pharmacokinetics. Similar i.v. *vs* i.p. pharmacokinetics profile to the ones that were observed here have been observed before (Cong *et al.*, 2016). I.p.-injected FAM-mUNO half-life was 110 min compared to the i.v. route, where the half-life was only 24 min. The half-life value obtained for i.v.-administered FAM-mUNO was in the range expected for a small peptide with a free Cys residue known to prolong the half-life through albumin binding. Without the free Cys, the half-life is expected to be even shorter (Pang *et al.*, 2014). The fact that the i.p. route showed a longer half-life justified the use of i.p. administration in the homing studies.

The homing of the peptide is highly important, since it offers an insight as to whether the peptide can be used to specifically target the cells of interest and if it allows the internalisation of its cargo. It was seen that in both *in vivo* models FAM-mUNO highly colocalised with CD206, as opposed to the control peptides, demonstrating the ability of the peptide to target M2 TAMs in the orthotopic tumours as well as in the malignant foci in the lungs. Importantly, it was found

that FAM-mUNO did not label the antitumoural M1 TAMs *in vivo*. FAM-mUNO also labelled the PV cells in the metastatic model. PV M2 TAMs contribute to tumour relapse (Hughes *et al.*, 2015) and are responsible for the local loss of vascular junctions and therefore contribute to the escape of the tumour cells and metastasis (Harney *et al.*, 2015). Therefore, even if the targeting of only a portion of PV M2 TAMs occurs, it still might play a role in the intravasation of tumour cells.

FAM-mUNO did not accumulate in the liver and did not label the resident macrophages (Kupffer cells) in the liver. This might be explained by macrophages taking up the peptide while circulating and then entering the tumour tissue. Circulating macrophages can be found in the i.p. cavity and it is possible that the peptide binds to CD206+ macrophages therein prior to tumour migration. The low accumulation of the FAM-mUNO in the liver is important to reduce the off-target effects.

These homing results presented here are novel, since previously the homing had been done with the cyclic version, FAM-UNO, and in later stages, where the tumour volumes where ~150 mm^3 (Scodeller *et al*., 2017). In contrast, experiments shown in this thesis were performed at the tumour volumes of ~50 mm^3, which is more relevant as it resembles more closely the clinical situation. The circulation time points used here are also new, since in the previous publication the peptide was left to circulate for 2 h, here it was shown that the FAM-mUNO remains inside the M2 TAMs after 24 h. This fact is important for future therapeutic applications, where compounds need to remain inside the cells for activity.

Finally, since solid tumours have abundant stroma that compresses the blood vessels and affects drug delivery, an attempt to normalise the stroma was undertaken. It was shown that after treatment with Losartan Potassium, the blood vessels decompressed, and the amount of collagen and HA was reduced. Losartan Potassium has been used by others to relieve solid stress in TNBC mouse models (Chauhan *et al.*, 2013), but in our hands the dosing regimen reported in those papers did not result in desmoplasia depletion. Therefore, the dose of Losartan Potassium was increased by 50% and the treatment was started earlier. Using this regimen, no evident toxicity or adverse effects were observed suggesting that the dose can be safely increased to obtain desmoplasia reduction in tumours.

Future steps will include the elucidation of the cellular fate of FAM-mUNO and the mechanistic uptake studies on its M2 TAM pathway. Using the mouse models shown here, it is envisioned to deliver relevant payloads conjugated to FAM-mUNO or encapsulated in PSs to reduce the

population of M2 TAMs. One payload for this objective could be Doxorubicin, which is a drug known to induce apoptosis in the macrophages (Wang *et al*., 2004). Future work will evaluate if M2 TAM-depletion strategies can potentiate existing chemo- or immunotherapies (such as Abraxane® or anti-PD-L1 antibodies). Chemotherapy alone is not effective for most TNBC patients, but the immune checkpoint inhibitors have shown promising results. TNBC cells highly express PD-L1 and therefore, this has been a main focus for the immunotherapies (Bianchini *et al.,* 2016). There are 4 ongoing phase III clinical trials on anti-PD-L1 antibodies used in combination with adjuvant or neoadjuvant chemotherapies (clinicaltrials.gov: NCT03125902, NCT03498716, NCT03197935, NCT03281954). It is hypothesised that the combination of immune checkpoint inhibitors and conventional chemotherapy will lead to increased patients' survival (Chawla *et al*., 2014). In the future, in an attempt to achieve a sustained therapeutic response, it is intended to study the preclinical efficacy of combinations of immunomodulatory and cytotoxic therapies with mUNO-based strategies for M2 depletion or reprogramming.

SUMMARY

TNBC is a breast cancer subtype lacking any good therapy options right now. If a patient has early stage TNBC, then the best option is mastectomy or lumpectomy together with chemotherapy, but if the disease has already metastasised, there is nothing that clinicians can do that can give a definite response. Therefore, it is an important medical concern that needs attention.

In this thesis the evaluation of the peptide mUNO, which binds to CD206+ M2 TAMs were done both *in vitro* and *in vivo*. The results can be summarised as follows:

- the *in vitro* system to evaluate the FAM-mUNO binding to human M2-differentiated macrophages was established
- the FAM-mUNO binds to human M2-differentiated macrophages *in vitro*
- i.p. administered FAM-mUNO has longer half-life compared to the i.v. administered FAM-mUNO
- the FAM-mUNO binds to M2 TAMs in both orthotopic and metastatic 4T1 tumour models, but does not bind to M1 TAMs
- the treatment with Losartan Potassium significantly reduced the amount of tumoural collagen and HA and decompressed blood vessels.

Based on the findings presented here it can be said that mUNO is specific to only M2 TAMs and can therefore be used in the future conjugated to cytotoxic drug(s) to eliminate M2 TAMs and hopefully potentiate chemo- and immunotherapies and prolong overall survival.

Kasvajaga seotud makrofaagide märgistamine primaarsetes ja metastaatilistes rinnavähi kasvajates

Anni Lepland

KOKKUVÕTE

Vähk on haigus, mis mõjutab igal aastal miljoneid inimesi üle maailma. Rinnavähk on vähiliik, mis ilmneb rinnakoes geneetiliste muutuste, keskkonnategurite, hormonaalsete muutuste ja/või vananemise tõttu. Kolmekordselt negatiivne rinnavähk (ingl. *triple negative breast cancer*, TNBC) on kõige ohtlikum rinnavähi alavorm. Kuna see mõjutab valdavalt nooremaid inimesi, kui teised rinnavähi liigid, ei saa seda diagnoosida rutiinse mammograafia ajal. Seetõttu on enamasti diagnoosimise hetkeks vähk jõudnud areneda juba kaugele ning tekitada kolded lümfisõlmedesse. Kuna praegusel hetkel olemasolevad ravimeetodid rinnavähi vastu on kavandatud vähemalt ühe hormonaalretseptori alusel, siis kuna TNBC ei ekspresseeri ühtegi „tavalist“ rinnavähi retseptorit, ei ole sellele ka sihtmärgistatud ravi. Kui TNBC patsient retsidiveerub, on aeg surmani vähem kui 18 kuud. Seega on olemas vajadus selle vähiliigi uurimiseks ning paremate ravivõimaluste järgi. Seetõttu olid käesoleva töö eesmärkideks uurida peptiidi, mUNO, M2 kasvajaga seotud makrofaagide (ingl. *tumour-associated macrophages*, M2 TAMs) märgistamisvõimekust *in vitro* inimese M2-differentseeritud makrofaagide ja *in vivo* kahe erineva hiiremudeli peal. Samuti uurida kõhuõõnde ja veenisiseselt süstitud mUNO poolestusaja erinevust selleks, et välja selgitada sobivaim sihtmärgistatud ravimi annustamise teekond. Viimaks, kuna kasvajatel on ümber väga palju tihket stroomat, mis kaitseb neid immuunsüsteemi ja ravimite eest, uuriti ka, et kui kasutada stroomat vähendavat ravimit, kas on võimalik seeläbi vähendada kollageeni ja hüalurooni hulka ka TNBC kasvajates.

Käesoleva uurimistöö tulemused saab kokku võtta järgmiselt:

- loodi *in vitro* süsteem uurimaks FAM-mUNO spetsiifilist märgistamisvõimet koekultuuris;
- FAM-mUNO seondub spetsiifiliselt ainult inimese M2-differentseerunud makrofaagidele *in vitro*;
- i.p. manustatud FAM-mUNO poolestusaeg on 4.5x pikem, võrreldes i.v. manustatud FAM-mUNO-ga;

- FAM-mUNO märgistab spetsiifiliselt kasvajat soodustavatele M2 TAM-e *in vivo* nii ortotoopilises kui ka metastaatilises 4T1 kasvajamudelis, kuid ei seondu kasvajavastastele M1 TAM-dele;
- töötlus Losartan-kaaliumiga vähendas kasvajate kollageeni ja hüalurooni hulka TNBC kasvajates.

Tuginedes käesolevas töös toodud infole, saab öelda, et mUNO on spetsiifiline M2 TAM-dele ning ei märgista M1 TAM-e. Tulevikus on võimalik kasutada seda koos tsütotoksiliste ravimitega kõrvaldamaks M2 TAM-id, võimendada keemia- ja immunravi ning seeläbi pikendada patsientide üleüldist elumus.

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SUPPLEMENTARY

1.1. Licence for the animal experiments.

CERTIFICATE

No 5.1-16/4760

Anni Noorem

49301266812

passed in the period of August 24th to September 3rd 2015
at the Estonian University of Life Sciences
(Register code 74001086, Kreutzwaldi 1, 51014 Tartu)
the course

Laboratory Animal Science I

code AU.441 (3 ESTS)

The purpose of the training was to educate people, in accordance with EU directive 2010/63/EU, article, 23, section 2, to perform the following tasks:
a) carrying out procedures on animals;
c) taking care of animals and
d) killing animals
and it is based on the Regulation No 22; RT I, 12.03.13;6 of Ministry of Rural Affairs

List of lecturers:
Kai Õkva, PhD, University of Eastern Finland
Külli Keerus, University of Tartu
Anne Kahru, PhD, National Institute of Chemical Physics and Biophysics
Sirje Jalakas, DVM, MSc
Mario Plaas, MSc, University of Tartu
David Arney, PhD , Estonian University of Life Sciences
Heli Säre, PhD, Estonian University of Life Sciences
Aleksandr Semjonov, Estonian University of Life Sciences
Tõnu Järveots, mag (vet-med), Estonian University of Life Sciences
Ivar Blank, Estonian University of Life Sciences
Riin Reimets, MSc, University of Tartu
Indrek Heinla, MSc,University of Tartu

Appendix: description of the course and procedures by animal species

Paavo Kaimre
Vice-Rector of Studies

Riin Kikkas
Head, Open University

Tartu, 15th of September 2015

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Appendix to the certificate No 5.1-16/4760

Anni Noorem

has passed the course of ***Laboratory Animal Science I*** in the Estonian University of Life Sciences from 24.08.2015 to 03.09.2015, AU.441 (3 EAP)

DESCRIPTION of the course:
The course consisted of following topics: current national, European and international legislation and regulatory framework concerning the breeding, usage and supply of laboratory animals; the ethical and welfare aspects of the use of animals in scientific procedures and for educational purposes, Three R's – refinement, reduction, replacement; rat and mouse biology including anatomy, physiology, reproduction, genetics and techniques of genetic modification; species-specific behaviour in animals and their environment diversification; handling and restraint methods, welfare and health monitoring of mice and rats; animal use in experimental work- dosing and sampling, recognition of signs of distress, stress and pain with a focus on mice and rats, anaesthesia and analgesia, killing of laboratory animals.

Participant has performed the following practical procedures:

Name of procedure	Mouse	Rat
Handling and fixation	Lifting up by tail Scruff method Sex determination	
Identification	With marker Ear notch (on anesthetized animal)	
Injections	Subcutaneous injections s.c. Intraperitoneal injection i.p.	
Blood sampling	Vena saphena	
	Tail vein	-
Killing of animal	Confirmation of death	
	-	Overdose of anaesthetic
	Cervical dislocation	-
Necropsy procedures	Necropsy procedures	
Cleaning work area	Cleaning work area	

Performed procedures were supervised by: Kai Õkva, Mario Plaas, Ivar Blank, Riin Reimets, Indrek Heinla

Riin Kikkas
Head, Open University

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"Targeting tumour-associated macrophages in primary and metastatic breast tumours", supervised by Pablo Scodeller (PhD), Tambet Teesalu (PhD).

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28/05/2019