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The Immunomodulation Potential of Pancreatic Cancer Exosomes

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“Que quem encontre o caminho
Para a liberdade
Tenha a fortaleza de reviver
O ponto de partida.

Tenha a fortaleza
De regressar mil vezes
Para guiar o próximo”

André Tecedeiro

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Abstract

Exosomes are extracellular vesicles produced by all cell types in the endosomal compartment and are key players in intercellular communication. Exosomes are involved in tumorigenesis, metastasis formation, angiogenesis, disease progression, drug resistance and tumor immune escape. The exosomal content contributes for the reprograming of recipient cells, namely the immune cells, which confers exosomes an immunomodulatory potential. The establishment and development of PDAC is marked by a prominent recruitment of immunosuppressive cells and the lack of cytotoxic immune cells in the tumor microenvironment. However, how PDAC exosomes modulate the immune system and how the immune system influences PDAC establishment and progression are still fields yet to explore. To address these questions, we used genetically engineered mouse models (GEMMs) of pancreatic cancer: the KPC mouse model, which develops PDAC in a spontaneous manner, recapitulating the human disease; the KPC $Rag2^{-/-}$ GEMM, which is an immunodeficient mouse model lacking B and T cells in order to study the effect of these cells in PDAC establishment and progression; and the KPC $Rag2^{-/-}IL2rg^{-/-}$ mouse models, lacking B, T and NK cells, in an attempt to understand the role of NK cells in PDAC establishment and development. Moreover, in order to study the role of PDAC exosomes in immunomodulation, we have impaired exosomes secretion in a PDAC mouse model and analysed changes in the immune landscape of the tumours. The observations collected in this thesis suggest that the immune system is not blind to PDAC, and have uncovered preliminary data that indicates that cancer exosomes are involved in the anti-tumour immune response, opening new avenues for the use of immunotherapy in PDAC patients.

Key-words: pancreatic cancer; exosomes; intercellular communication; immunomodulation

Sumário

Exossomas são vesículas extracelulares produzidas por todos os tipos celulares dentro do compartimento endossomal, desempenhando um papel importante na comunicação intercelular. Os exossomas estão envolvidos nos processos de tumorigénese, metastização, angiogénese, progressão tumoral, resistência à terapia e evasão à resposta imunológica. O conteúdo exossomal contribui para a reprogramação das células recetoras, incluindo as células do sistema imunológico, conferindo aos exossomas um potencial imunomodulatório. O desenvolvimento de adenocarcinoma ductal do pâncreas (ACDP) é caracterizado por um recrutamento proeminente de células imunossupressoras e pela fraca presença de células citotóxicas no microambiente tumoral. No entanto, a forma como exossomas derivados de ACDP modulam o sistema imunológico e como o sistema imunológico influencia o estabelecimento e progressão de ACDP são áreas a serem exploradas. Para responder a estas questões, usámos modelos animais geneticamente modificados (do inglês, genetically engineered mouse models, GEMMs) de cancro do pâncreas: o modelo KPC, que desenvolve ACDP de forma espontânea, recapitulando a doença humana; o GEMM KPC $Rag2^{-/-}$, que se trata de um modelo imunodeficiente que não possui células B nem T, de forma a estudar o efeito destas células no desenvolvimento e progressão de ACDP; e o GEMM KPC $Rag2^{-/-}$ $IL2rg^{-/-}$, que não possui células B, T nem NK, de forma a compreender o papel das células NK no desenvolvimento e progressão de ACDP. Além disso, de forma a explorar o papel dos exossomas derivados de células de ACDP no processo de imunomodulação, a secreção de exossomas foi inibida num modelo animal de ACDP e as alterações do panorama imunológico dos tumores foram analisadas. As observações coletadas ao longo desta tese sugerem que o sistema imunológico não é cego ao ACDP, e levaram à descoberta de dados preliminares que indicam que os exossomas derivados de cancro estão envolvidos na resposta anti-tumoral do sistema imunológico, abrindo novos caminhos para o uso de imunoterapia para o tratamento de pacientes com ACDP.

Palavras-Chave: cancro do pâncreas; exossomas; comunicação intercelular; imunomodulação.

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Glossary of Abbreviations and Acronyms

Ab	Antibody
ACT	Adoptive cell therapy
APC	Antigen-presenting cells
BM-MSC	Bone marrow mesenchymal stromal cells
CAF	Cancer-associated fibroblasts
CDC	Complement-dependent cytotoxicity
CML	Chronic myelogenous leukemia
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte Antigen 4
DC	Dendritic cells
DDR	DNA damage response
DGAV	Direção-Geral de Alimentação e Veterinária
Dll4	Delta-like ligand 4
DMA	Dimethyl amiloride
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
EpCAM	Epithelial cell adhesion molecules
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular vesicle
Gal-1	Galectin-1
GEMMs	Genetically engineered mouse models
GPC-1	Glypican-1
H2O2	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HEP	Humane endpoint
HSP	Heat shock protein
IFN-α	Interferon-alpha
IL-2	Interleukin-2
ILV	Intraluminal vesicles
iMVD	Intratumoral microvessel density
IPMN	Intraductal papillary mucinous neoplasm
IS	Immunological synapse
KO	Knockout
lncRNA	long noncoding RNA
mAbs	Monoclonal antibodies
MCN	Mucinous cystic neoplasm
MDR	Multi-drug resistance
MDSC	Myeloid-derived suppressor cells
MHC-I	Major histocompatibility complex class I
miRNA	microRNA

MM	Multiple myeloma
MSC	Mesenchymal stem cells
MVB	Multivesicular body
NK	Natural Killer
nSMase2	Sphingomyelinase 2
NTA	Nanoparticle Tracking Analysis
OV	Oncolytic virus
PanIN	Pancreatic intraepithelial neoplasm
PC	Pancreatic cancer
PD-1	Programmed Cell Death protein 1
PDAC	Pancreatic ductal adenocarcinoma
PD-L1	Programmed Cell Death ligand 1
Pdx1	Pancreatic-duodenal homeobox promoter
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
pre-miRNA	precursor microRNA
RBL	Red Blood cell Lysis
RFXAP	Regulatory factor X-associated protein
RISC	RNA-induced silencing complex
SNAREs	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
TAM	Tumor-associated macrophages
TCR	T cell receptor
TGFβ	Transforming growth factor β
Th1	T-helper type 1 cells
Th2	T-helper type 2 cells
TIL	Tumor infiltrating lymphocyte
TLR4	Toll-like receptor 4
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor-α
TOV	Tumor oncolytic viruses
Treg	Regulatory T cells or CD4+CD25+FOXP3+ T cells
tRNA	transfer RNA
Wnt11	Wnt family member 11

I. Introduction

I. Introduction

1. Pancreas normal anatomy and physiology

The pancreas is a regulator of glucose homeostasis. It is divided into two portions: the endocrine portion and the exocrine portion. Within the exocrine portion, which represents 80% of the tissue mass of the organ, one can find a branching network of duct and acinar cells, producing and delivering digestive proenzymes (or zymogens) into the gastrointestinal tract. The acinar cells synthesize and secrete those zymogens into the ductal lumen, as a response to the stimuli given by the stomach and the duodenum once digestion begins. Centro acinar cells can be found within the acinar units near the ducts. On the other hand, the endocrine pancreas is composed by four specialized endocrine cell types. Those endocrine cell types are organized into clusters named the Islets of Langerhans. The endocrine pancreas is a key regulator of the metabolism and of glucose homeostasis, through the secretion of hormones into the bloodstream (1), namely insulin and glucagon, which regulate the blood sugar levels. When the blood sugar levels are high, the pancreas, more specifically the islets of Langerhans, release insulin into the blood stream. Insulin stimulates sugar absorption from the bloodstream into the cells, and allows sugar storage in the liver and muscles, lowering the blood's sugar levels. However, when blood sugar drop, there is the release of glucagon into the bloodstream by the pancreas. Glucagon has the contrary effect of insulin. It stimulates the release of the sugars stored in the liver and takes part in the process of conversion of proteins in the liver into sugar (2).

2. Pancreatic Ductal adenocarcinoma (PDAC)

Pancreatic ductal adenocarcinoma (PDAC) represents the vast majority of pancreatic cancers (>85% of pancreatic cancers) and has a lethality of approximately 94% (3). The symptoms associated with PC are vague and include fatigue, indigestion and loss of appetite, abdominal and back pain, and jaundice (4). Pancreatic ductal adenocarcinoma has a poor prognosis with a 5-year survival rate of approximately 9% (5). This high mortality rate is mostly due to its late diagnosis, lack of early symptoms, early invasion of proximal vessels, early spread of metastatic disease, presence of a dense stroma, low immunogenicity, difficulties in identifying people at risk undermining effective screening (6). The majority of these tumors are unresectable at the time of diagnosis as they are already metastasized (1, 7). Only 20% of the patients diagnosed present resectable tumors

and for these patients, surgery may be a curative option, although in many cases relapse occurs within 5 years (8).

Multiple studies have established as risk factors smoking (9, 10), advanced age, type 2 diabetes mellitus, (11, 12) and chronic pancreatitis as glandular damage from bouts of acute pancreatitis can promote cell damage, such as acinar loss and extensive fibrosis (13, 14). Males were also shown to present higher risk of developing PDAC (11, 12). Obesity also appears to confer increased risk (1).

Pancreatic cancer is associated with a variety of genetic alterations as well as epigenetic factors (1, 15). It is estimated that 10% of PDAC cases are hereditary and that germline mutations may be the cause of <20% of PDAC familial cases (16). Studies show that near-ubiquitous oncogenic mutations of *KRAS* and inactivation of TP53, SMAD4 and CDKN2A tumor suppressor genes are the most prevalent mutations in PDAC (15, 17). Mutated KRAS is the driving oncogene in PDAC and is present in >90% of tumors (1), while inactivating mutations of TP53, CDKN2A and SMAD4 are present in 50-80% of PDAC tumors. KRAS mutations lead to the activation of multiple signalling pathways, having as consequence increased cell growth, proliferation, motility and survival (18, 19). Approximately 10% of PDAC tumors present mutations in other genes, such as ARID1A, MLL3 and the transforming growth factor β receptor 2 (TGFBR2) (20). Germline mutations of some DNA damage response (DDR) genes, such as BRCA1, BRCA2, PALB2, ATM, MLH1, MSH2 and MSH6, are correlated with higher probability of developing PDAC (205). Studies show that BRCA1 mutation confers increased susceptibility to PDAC, although BRCA2 mutation presents a higher associated risk (212). INK4A and BRCA2 mutations are only detected in more advanced stages of PDAC, namely in pancreatic intraepithelial neoplasm (PanIN) lesions, and are not detected in the earliest sporadic PDAC premalignant lesions (21, 22).

PDAC is characterized by the presence of an immunosuppressive microenvironment that favours immunosuppressive cells, such as tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) (23, 24), and $CD4^+CD25^+FOXP3^+$ T cells (Tregs) (25). Effector T cells, which stimulate an immune response are very scarce in PDAC. Nonetheless, effector T cells are present in low amounts in preinvasive lesions and in a subset of advanced stage tumours, although these show no signs of activation (23, 24). Immunosuppressive cells are present in the early stages of disease and persist through invasive cancer. Clark *et al.* suggested that the presence of intratumoral MDSCs

correlates with the lack of tumor-infiltrating effector T cells (24) Furthermore, *in vitro* studies showed that T-cell proliferation was suppressed by MDSCs (24). Studies performed in mouse models and observations of human tumors allowed to conclude that PDAC is associated with an inflammatory infiltrate. This can influence disease progression in contradictory manners, as the inflammatory infiltrate can help inhibit cancer growth through antigen-restricted tumoricidal immune responses, but at the same time, the inflammatory infiltrate can promote tumor progression by supressing the immune system (26). $CD8^+$ T and T-helper type 1 cells (Th1)-polarized cluster of differentiation 4 ($CD4^+$) T cells were shown to mediate antitumor responses in mouse models of PDAC. Furthermore, they also conferred an increase in survival of PDAC patients (26). On the other hand, T- helper type 2 cells (Th2)-polarized $CD4^+$ T cells were shown to be able to promote PDAC progression in murine models. Intra-tumoral $CD4^+$ Th2 infiltrates conferred a decrease in survival of PC patients (26). Moreover, $Foxp3^+$ T-regulatory cells (Tregs) were reported to facilitate tumor immune escape in this disease. Myeloid cells were shown to influence differentiation of T cells and cytotoxicity in pancreatic cancer. Tumor-infiltrating MDSCs inhibit anti-tumor responses by cytotoxic $CD8^+$ T cells, contributing to pancreatic cancer growth and metastasis (26).

2.1. PDAC: Disease Progression

Pancreatic ductal adenocarcinoma commonly arises in the head of the pancreas and it infiltrates into the surrounding tissues, namely the lymphatic organs, the peritoneal cavity, the spleen and metastasizes to the liver and lungs. PDAC is characterized by a strong desmoplastic reaction, with the presence of a dense stroma of fibroblasts and inflammatory cells (1), as well as extracellular matrix (ECM) proteins and pancreatic stellate cells (27, 28).

PC neoplasias vary from well-differentiated gland-forming carcinomas to poorly differentiated “sarcomatoid” carcinomas (15). Several studies allowed the characterization of PDAC precursor lesions: PanINs, mucinous cystic neoplasm (MCN) and intraductal papillary mucinous neoplasm (IPMN) (29, 30), with PanIN being the most common and extensively studied precursor lesion, found in approximately 30% of PDAC patients (1). MCNs are large mucin-producing epithelial cystic lesions, harbouring a distinctive ovarian-type stroma. They present a variable degree of epithelial dysplasia and

focal regions of invasion. On the other hand, IPMNs resemble PanINs at the cellular level. However, IPMNs grow into larger cystic structures (1).

PanINs are characterized by morphological alterations when compared with normal ducts and they are classified into 3 different stages depending on the dysplastic growth, which increases from stage I to III – low grade PanINs (PanIN-I and PanIN-II) and high-grade PanINs (PanIN-III) (1, 15). Grade I PanINs are characterized by a columnar, mucinous epithelium, by architectural disorganization and nuclear atypia. The architectural disorganization and nuclear atypia increase through stages II and III. The high-grade PanINs ultimately transform into PDAC tumors that invade beyond the basement membrane (1). In healthy/normal adult pancreas or in the pancreas of patients with chronic pancreatitis, it is frequent to observe low-grade lesions, being associated with a low risk of developing PDAC. On the contrary, patients with invasive PDAC present high-grade PanIN III lesions. Those high-grade lesions are almost exclusively found in those patients (31). On a macroscopic level, PDAC tumors are a solid white/yellow mass poorly demarcated. Microscopically, the surrounding normal pancreas (when existent) shows fibrosis, atrophy and dilated ducts, as the growing tumor obstructs the normal pancreatic tissue (15). Figure 1 illustrates the histological evolution of these lesions into PDAC.

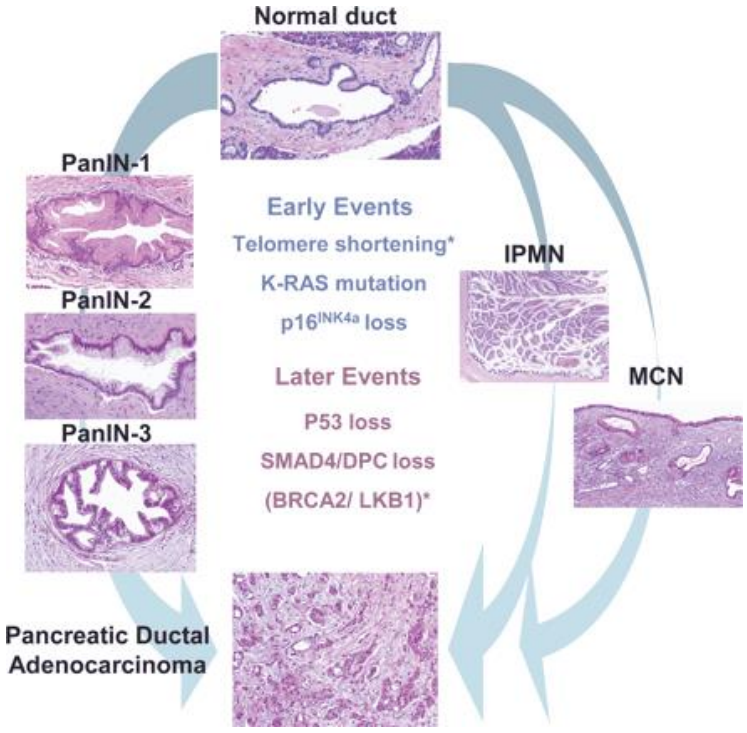


Figure 1 – PDAC precursor lesions and genetic events involved in PDAC progression. Due to the appearance of early events, as well as late events such as P53 loss, loss of SMAD4 and

mutations in BRCA2/LKB1 genes, the normal ducts begin to present signs of transformation, leading to the development of precursor lesions, such as PanIns, IPMNs and MCNs. Ultimately, these lesions develop into PDAC. Image from: *Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, Depinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. Genes Dev. 2006;20(10):1218-49.*

2.2.Treatment options for PDAC patients

The treatment approaches to PDAC are surgery, chemotherapy (6), radiation therapy (in combination with chemotherapy (7)) and targeted therapy, namely RAS effectors (MEK, PI3K components, etc.) (1). Surgery is the only curative option (7, 32), but only 20% of the patients present resectable tumors. Even so, 80% of patients that underwent surgery relapsed within 5 years (8, 33). A great part of the diagnosed patients present, at time of diagnosis, either metastatic (50%) or locally advanced cancer (30%) (7), and none of the therapeutic approaches available have shown to increase patient survival for more than a few months. For many years gemcitabine monotherapy was considered the standard of care for PDAC patients. Currently, the standard first-line treatment commonly consists of the combination therapy FOLFIRINOX or gemcitabine plus nab-paclitaxel (6). Modified FOLFIRINOX offers a disease-free survival improvement of 21.6 months in patients with resected PDAC and no metastasis, compared with gemcitabine, which conferred a disease-free survival improvement of only 12.8 months (34). Treatment of PDAC patients with the combination of gemcitabine together with nab-paclitaxel resulted in an increment of 18.8 months in the median overall survival in comparison with previous studies (35). Currently, ESMO guidelines recommend that FOLFIRINOX is used as the first option for the treatment in patients under 70 years of age. In the cases in which patients are more debilitated, the combination therapy gemcitabine-nab-paclitaxel is recommended. For more frail patients, gemcitabine alone is recommended (36).

New targeted therapeutic approaches are now being explored in an attempt to improve PDAC survival. As KRAS is mutated in almost all patients diagnosed with PDAC (~95%) (37), it has been a long-time attractive target. An ongoing phase II trial (NCT01676259) is studying the efficacy, safety, tolerability and pharmacokinetics of the combination of an siRNA drug that silences the mutant $KRAS^{G12D}$ with gemcitabine-nab-paclitaxel for the treatment of PDAC patients with locally advanced disease (6). Another approach involves the collection of the patients own effector T cells, which are then expanded *ex*

vivo and the T cells subpopulations that target tumor cells are selected for infusion into the patient (adoptive T cell transfer or ACT) (6). A recent case report in which ACT was used to target mutant $KRAS^{G12D}$ in metastatic colon cancer patients showed a substantial regression of the patient's lung metastasis (38), forecasting the revolutionary potential that this approach can have in the treatment of PDAC, as well as other tumors that harbour mutant KRAS. Furthermore, a study using iExosomes (exosomes with siRNA or shRNA targeting KRASG12D) showed very promising results as a novel therapy to target KRAS in PDAC (39). PDAC mouse models treated with iExosomes showed a reduction in tumor burden (being almost undetectable), which persisted after 200 days of treatment. iExosome treatment also suppressed PDAC progression in a highly metastatic cancer model, leading to a reduction of metastasis and an increase in survival (39). This study was the basis for a clinical trial ongoing using iExosomes – "Phase I Study of Mesenchymal Stromal Cells-Derived Exosomes With KrasG12D siRNA for Metastatic Pancreas Cancer Patients Harbouring KrasG12D Mutation" for KRAS NP_004976.2:p.G12D, metastatic pancreatic adenocarcinoma, pancreatic ductal adenocarcinoma and for Stage IV pancreatic cancer AJCC v8 conditions (40).

Because PDAC's immunosuppressive tumor microenvironment (TME) is preponderant when compared to tumor infiltration of effector immune cells (26, 41, 42), several studies have focused on targeting Tregs. Preclinical studies show that inhibition of pathways involved in Tregs homing to the TME (e.g., inhibition of CCR5, which is normally expressed on Tregs, or its ligand CCL5) (43) or Treg depletion (44) can constrain tumor progression and metastasis. Other immunotherapeutic approaches that might be useful in the fight against PDAC are checkpoint inhibitors, such as anti- Programmed Cell Death protein 1 (-PD-1), Programmed Cell Death ligand 1 (-PD-L1) and cytotoxic T-lymphocyte Antigen 4 (-CTLA-4), which aim to overcome the mechanisms explored by cancer cells to put the brakes on the immune system (6, 45). In order to stimulate an immune response against PDAC cells, different types of vaccines and other immunotherapy drugs are being studied and tested. Regarding monoclonal antibodies against CTLA4 and PD-L1, which achieved remarkable results on other solid tumors, these have not shown effective in PDAC patients (6, 26). These unfavourable results, compared to other solid cancers, may be explained by PDAC's low mutation burden, with few neoantigens, and by its immune suppressive TME (6, 26), characterized by the presence of MDSCs and Tregs, which suppress the recruitment and activation of immune

effector cells (24, 26). Recently, ongoing studies focus on the combination of these immune checkpoint therapies with cytotoxic therapy and with anti-cancer vaccines, with some preliminary promising results (6, 23). These therapeutic combinations, summarized in Figure 2 are specifically designed to enhance T cell activation, target the immunosuppressive microenvironment and to breakdown the desmoplastic barrier that characterizes PDAC (26). The use of CAR T-cells (46) and oncolytic viruses (NCT00998322) (47) alone or in combination with currently available chemotherapeutic drugs, is also under study.

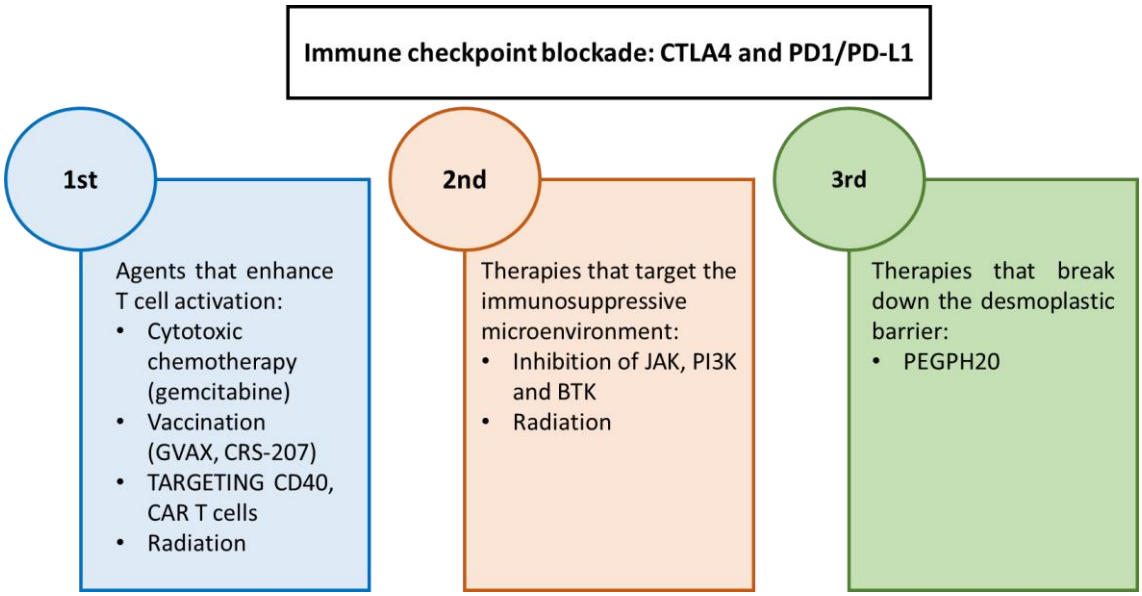


Figure 2 - Possible combinations of immune checkpoint therapies with other therapeutic approaches. Immune checkpoint therapies (CTLA4 and PD1/PD-L1) may be combined with agents that enhance T cell activation (cytotoxic chemotherapy, vaccination, CAR-T cells or with radiation), with therapies targeting the immunosuppressive microenvironment (JAK, PI3K and BTK inhibition or with radiation) and with therapies that break down the desmoplastic barrier (PEGPH20). Adapted from: *Guo S, Contratto M, Miller G, Leichman L, Wu J. Immunotherapy in pancreatic cancer: Unleash its potential through novel combinations. World J Clin Oncol. 2017;8(3):230-40.*

Despite the advances, the prognosis of PDAC patients still remains extremely poor and new therapeutic options are required. Because immunotherapy has shown the best results

in cancer care, to uncover a strategy that could potentiate the application of this therapeutic approach to PDAC would be revolutionary for these patients.

3. Cancer Immunotherapy

In the past few years, immunotherapy has become the focus of intense research, since it as demonstrated to be the most effective approach to fight cancer. Cancer immunotherapy aims to induce and amplify immune responses against cancer cells (45). This is achieved either by boosting the person's immune system or by creating, *in vitro* or *ex vivo*, immune cells that can recognize and mediate an immune response against cancer cells in a more effective way. James Patrick Allison and Tasuku Honjo won the Nobel Prize for Medicine in 2018 for his discovery of a therapeutic approach for combating cancer – the development of anti-CTLA-4 antibody – which inhibits CTLA-4, thus inhibiting the negative immune regulation (48). The success rate of cancer immunotherapy largely depends on the tumours' immunogenicity. As so, immunotherapy is combined with other treatment approaches that enhance their immunogenic potential, in order to achieve optimal results for the patients (49). There are different cancer immunotherapy treatments, including checkpoint inhibitors (45), cytokines (50), chimeric antigen receptor (CAR) T-cell therapy (51), cancer vaccines (51), oncolytic viruses (6), immunomodulators (52), and monoclonal antibodies (mAbs or MoAbs) (51), as shown in Figure 3.

Checkpoint inhibitors: Cancer immunotherapies must be able to overcome negative feedback mechanisms that regulate immune checkpoints (45), such as anti-PD-1 (expressed on T cells), -PD-L1 (commonly found expressed on cancer cells and macrophages) or -CTLA-4 (expressed on T cells) (6), which transmit inhibitory signals to effector immune cells, suppressing their activity and proliferation and arresting the immune response against tumor cells. As so, selective targeting of these rate-limiting steps could be an effective approach to fight cancer (45).

Adoptive cell therapy (ACT): Immune cells that have been collected from a patient are isolated and the tumor-specific immune cells are activated and expanded *ex vivo*, and then re-infused in the patient (51). There are different subtypes of T cells used for ACT, including tumor infiltrating lymphocytes (TILs), T cells engineered to express a cancer specific T cell receptor (TCR), and chimeric antigen receptor (CAR)-T cells (51),

which are genetically engineered T cells expressing chimeric antigen receptors that can recognize a specific antigen expressed by cancer cells (53). Dendritic cells (DCs) engineered to present specific antigens on their surface via Major histocompatibility complex (MHC) molecules are also used in ACT (51).

Cytokines: cytokines are soluble proteins, produced by some subsets of immune cells (51), that trigger differentiation, growth and inflammatory or anti-inflammatory pathways in different cell types. Consequently, cytokines are known to mediate intercellular communication. Recombinant interferon-alpha (IFN-α), that promotes the expansion of Natural Killer (NK) cells and T lymphocytes, as well as interleukin-2 (IL-2), which has a direct pro-apoptotic/anti-proliferative activity on tumor cells, was approved for the treatment of several malignancies, including cancer (50). Several cytokines impair tumor cell growth by exerting anti-proliferative or pro-apoptotic activities, as well as by stimulating the immune cells' cytotoxic activity to fight tumor cells. Cytokines are being studied to be used in combination with other immunotherapeutic agents, such as monoclonal antibodies against CTLA4 and PD-L1 and anti-CD19 CAR-T cells (50).

Monoclonal antibodies (mAbs or MoAbs): mAbs are antibodies produced in laboratory. The Fv region of the antibody recognizes the epitope region of an antigen. The antigen is present on the target cell (usually a cancer cell). The antibody's Fc region then engages the host immune system, making it easier for immune cells to recognize and kill cancer cells (51). mAbs use recombinant-DNA (r-DNA) technology: chimeric, humanized or human antibodies that have replaced mouse antibodies (51). mAbs are used in the treatment of several types of cancers, such as lymphomas, breast and colon cancer, among others. mAbs therapy success depends on three mechanisms: (1) antibody binding and inhibition/blocking of factors and receptors that activate the signal pathways used by cancer cells in division and angiogenesis, (2) the induction of antibody-dependent cellular cytotoxicity (ADCC) via binding of mAbs (formed from chimeric or full human antibody components) to specific tumor associated antigens and (3) induction of complement-dependent cytotoxicity (CDC) by complement activation (51).

Immunomodulators: Immunomodulators, also known as immunomodulatory drugs, are able to increase or decrease the response of the immune system, being designated immunostimulators and immunosuppressive, respectively (52). This section will focus only on immunostimulators. Immunomodulation is said to be selective when

the immune stimulation results in an immunoreaction against one or multiple antigens. On the other hand, immunomodulators with a non-specific activity stimulate or suppress the immune response, but the stimulated cells do not have an activity directed to a specific antigen (52). Immunomodulators act through different pathways. In case of immunostimulators (i.e.: lipopolysaccharides, Glucan, Thymosins, Levamisole, specific antibodies, IL-1, -2 and -12, IFN-γ and antigens), they are capable of activating macrophages, B, T and NK cells, proliferation of monocytes and triggering effector phase of specific immunity (52). Belimumab, vitamin D and hydroxychloroquine are immunomodulators currently available. These therapies act via numerous cellular and cytokine pathways and have shown the ability to modify aberrant immune responses (54).

Cancer vaccines: the aim of cancer vaccines is to immunize patients against tumor-associated or tumor specific antigens, thus inducing anti-tumor responses through T cells. Peptide-based vaccines consisting of immunogenic epitopes are the most used approach when it comes to cancer vaccination. Nevertheless, cancer vaccines are administrated in combination with adjuvants, such as DCs, to increase the efficacy and potency of the immune response. DCs vaccination may be used in two different modalities: transfer of antigens to DCs or production of DCs loaded *ex vivo* with specific antigens (51). DNA vaccines are also another treatment approach. In this case, the expression of tumor antigens by patients is upregulated through the administration of plasmids containing cDNAs that encode those tumor antigens, thus promoting tumor recognition and killing by T cells (51).

Oncolytic viruses: oncolytic viruses (OVs) or tumor oncolytic viruses (TOVs) are genetically engineered viruses that selectively infect and replicate in cancer cells, resulting in cancer cell death with low toxicity for the patient (6).

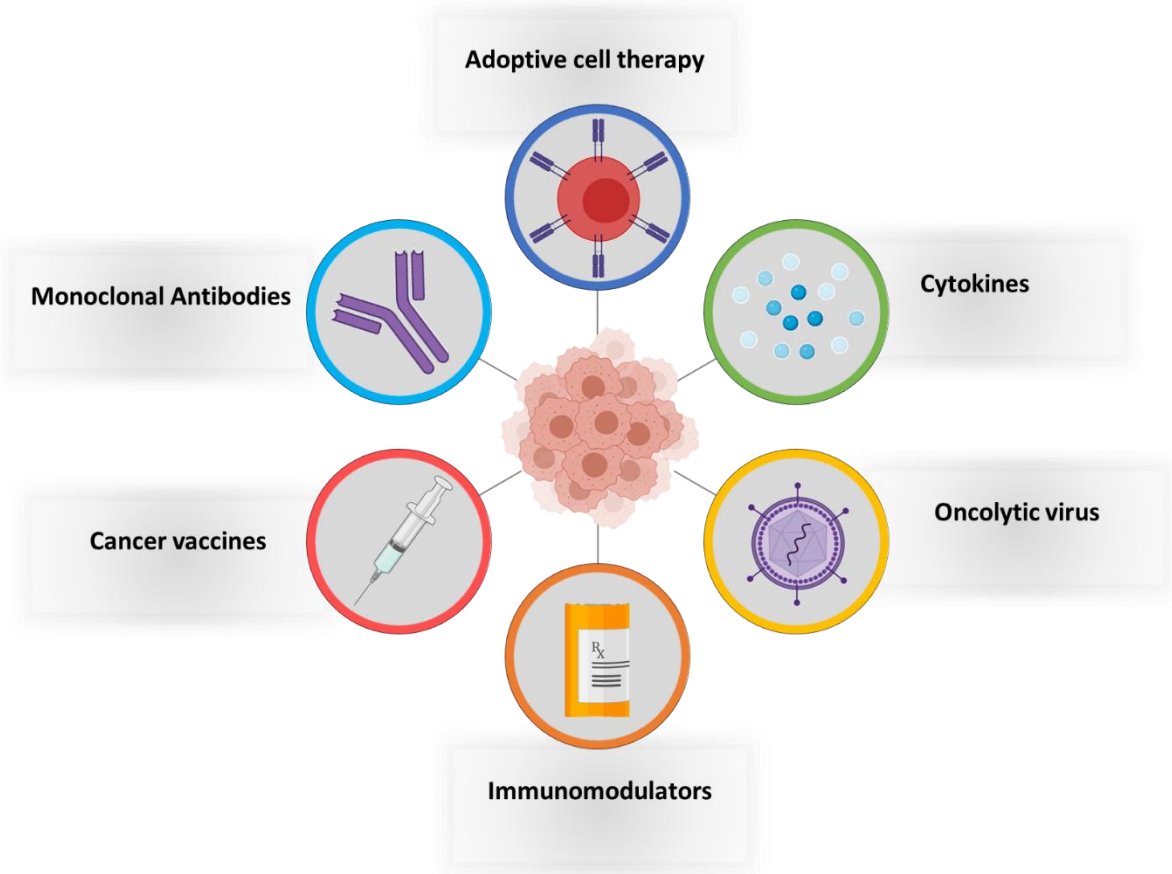


Figure 3 - Cancer immunotherapeutic strategies. Cancer immunotherapy includes: adoptive cell therapy, cytokines, oncolytic viruses, immunomodulators, cancer vaccines and monoclonal antibodies.

4. Intercellular Communication

Intercellular communication is an essential event that occurs in normal physiological conditions. In cancer, this process is used for cancer cells to communicate with neighbour cells but also at a distance, in order to adapt and survive. Extracellular vesicles are key players in intercellular communication. The term extracellular vesicles (EVs) refer to a group of heterogenous vesicles containing proteins, lipids and nucleic acids (55). EVs can have an endosomal origin (exosomes), or they can directly bud from the plasma membrane (microvesicles (56) and apoptotic bodies (57)), with a size ranging from 30 to 5000 nm (58). Findings demonstrate that exosomes in particular are important in the regulation of a wide range of cellular activities, such as the modulation of the immune system (59), in cardiovascular disease (60), infectious diseases (61), as well as in neurodegenerative diseases (62). In cancer, exosomes were implicated in disease progression, metastasis, angiogenesis, drug resistance and tumor immune escape (55, 63-65).

4.1.Exosomes

In the 1980s, the term *exosomes* was first used to describe "small vesicles of endosomal origin that are released during reticulocyte differentiation following the fusion of multivesicular bodies (MVBs) with the plasma membrane" (66). Trams *et al.* associated the term *exosome* with "the release of extracellular vesicles with 5′-nucleotidase activity from various normal and neoplastic cell lines" (67). Recent research allowed major breakthroughs in our understanding of exosomes' composition and function. Nowadays, exosomes are described as small EVs of endocytic origin, with a double lipid layer that circumscribes a small cytosol (55, 63). These EVs are characterized by their small size (from 30 to 150nm) (55, 68-70), their "cup" or "dish" shape and their content (70). During exosomes' biogenesis, several molecules are transported into the lumen of exosomes, such as DNA, mRNAs, long noncoding RNA (lncRNAs), microRNA (miRNAs) and proteins. According to the ExoCarta database, 9769 proteins, 3408 mRNAs, and 2838 miRNAs (71) have been identified in exosomes from different species (55). The exosomal content is heterogeneous and dynamic, is influenced by the cell of origin, by its pathophysiological state and even depending on the site where cellular release takes place (63). Exosomes are also characterized by their capacity to transfer these molecules to other cells, modulating the extracellular microenvironment by reprograming the recipient cells (63) (55). Exosomes can be isolated from distinct cells, either under normal

conditions, as well as stress conditions (55). Exosomes have been identified in several body fluids, such as blood (72), urine (73), saliva (74), ascites (75), semen (76), cerebrospinal fluid (77) and even breast milk (78).

4.1.1. Exosomes' biogenesis, secretion and uptake

The biogenesis of exosomes is a 4-step process: initiation, endocytosis, MVBs formation and exosomes secretion (55). This process begins with the inward invagination of the plasma membrane followed by vesicle scission (23), leading to the formation of intracellular endosomes, or early endosomes (23, 64). The inward invagination of clathrin-coated domains of the membrane of these intracellular endosomes leads to the formation of intraluminal vesicles (ILVs) (40–150 nm). This results in the maturation of early endosomes into late endosomes - also called MVBs. (23, 79). This orchestrated process of exosomes biogenesis may culminate in two distinct ways: the MVBs may fuse with lysosomes within the cell to degrade their contents or they may fuse with the plasma membrane, leading to the release of their contents into the extracellular space in the form of exosomes (63, 64), as illustrated in Figure 4.

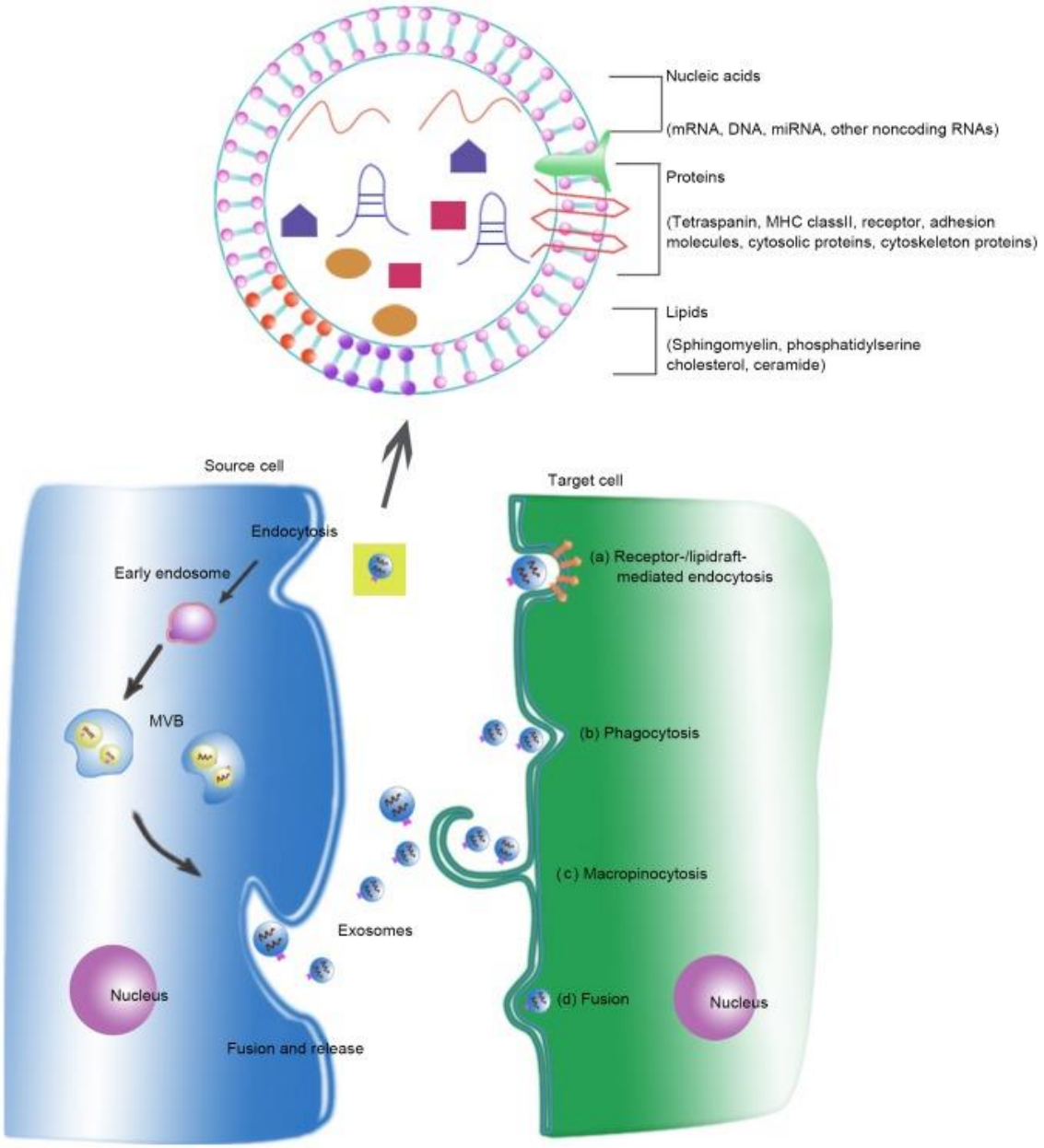


Figure 4 – Exosomes biogenesis and communication with recipient cells. Prior to invagination, the endocytic vesicles fuse with the early endosome. The early endosomes differentiate into multivesicular bodies (MVBs)/late endosomes. The multivesicular bodies fuse with the plasma membrane, releasing their content as exosomes to the extracellular space. The exosomes then enter the target cells, either by receptor-mediated endocytosis (a), phagocytosis (b), macropinocytosis (c) or by fusion (d). Taken from: *Guo W, Gao Y, Li N, Shao F, Wang C, Wang P, et al. Exosomes: New players in cancer (Review). Oncol Rep. 2017;38(2):665-75.*

An important step in exosomes' biogenesis is driven by endosomal sorting complex required for transport (ESCRT) proteins (80). ESCRT is formed by the multiprotein complexes ESCRT-0, -I, -II and -III that, together with accessory proteins (ALIX, VPS4, and VTA1). This complex participates in vesicle budding and in the MVB formation (81). Recently, the formation of MVBs was demonstrated to be controlled by the syndecan heparin sulphate proteoglycans and syntenin, which interact with ALIX, an ESCRT's accessory protein (82). The Rab GTPase family of proteins are the best described proteins involved in exosomes biogenesis (83). According to McCaffrey *et al.* and Gorvel *et al*., Rab proteins are involved in budding and mobility of endosomes, as well as in docking and fusion with the plasma membrane and consequent exosomes secretion (84, 85). Rab-4 and -5 are proteins that can be found in the early endosomes. Rab4 controls fast recycling and Rab5 is responsible for endosomal fusion (84, 85). Rab11 and Rab35 are also associated with the early endosome stage. However, Rab11 is associated with slow recycling, as illustrated in Figure 5 (65, 86). On the other hand, Rab7 is involved in the late endocytic pathway and is found in late endosomes. Rab7 is responsible for regulating the secretion of ALIX and syntenin-positive exosomes in cancer (82). Nevertheless, this protein was also shown to be involved in lysosomal degradation of MVBs (87). Recently, Ostrowski *et al.* further refined the specific roles of Rab27 isoforms in the exosomal pathway, demonstrating that Rab27a and Rab27b undertake their function in the late endosomal and secretory pathways, mediating the docking and fusion of MVBs to the plasma membrane and exosomes release (83). Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) have an important role during the release of exosomes to the extracellular space, as they help mediate the fusion of exosomes with the cell membrane (88). In Figure 5 it is demonstrated the role of Rab proteins in exosomes biogenesis.

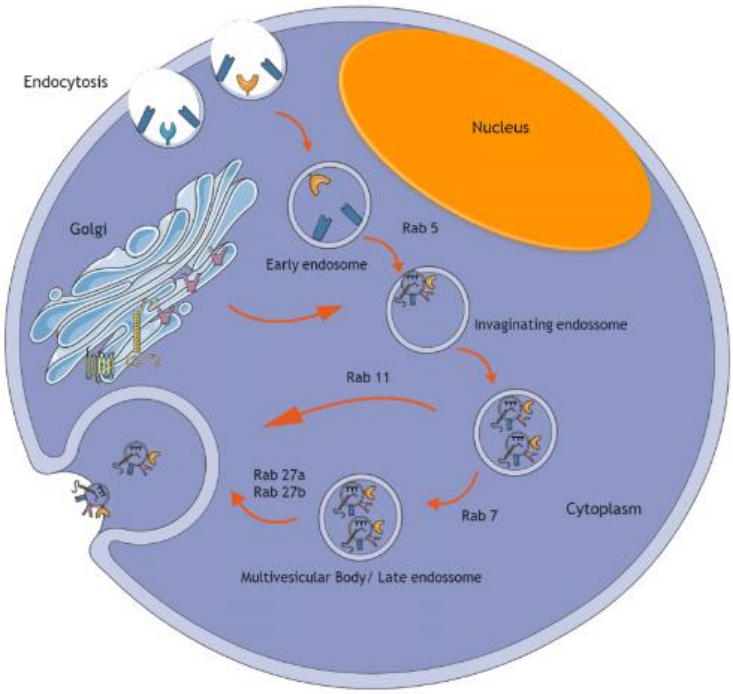


Figure 5 – Rab proteins in exosomes biogenesis. Rab5 is responsible for endosomal fusion. Rab7 is found in late endosomes and is responsible for regulating the secretion of ALIX and syntenin-positive exosomes in cancer, taking part in lysosomal degradation of MVBs. Rab11 protein is associated with the early endosomes stage and slow recycling. Rab27a and Rab27b proteins act on late endosomal and secretory pathways, mediating the docking and fusion of MVBs to the plasma membrane and exosomes release. Figure by Bastos N.

The rate of exosomes biogenesis is unknown, and it may be different according to the cell type and the cell's physiological and/or pathological status (64).

Exosomes are taken up by cells by different processes as illustrated in Figure 6: soluble signalling, endocytosis, receptor-ligand interaction or direct fusion with the surface membrane of recipient cells (55, 63, 89). Endocytosis includes a wide range of molecular pathways, being phagocytosis, macropinocytosis and clathrin-dependent or caveolae-dependent endocytosis examples of these pathways (63, 65).

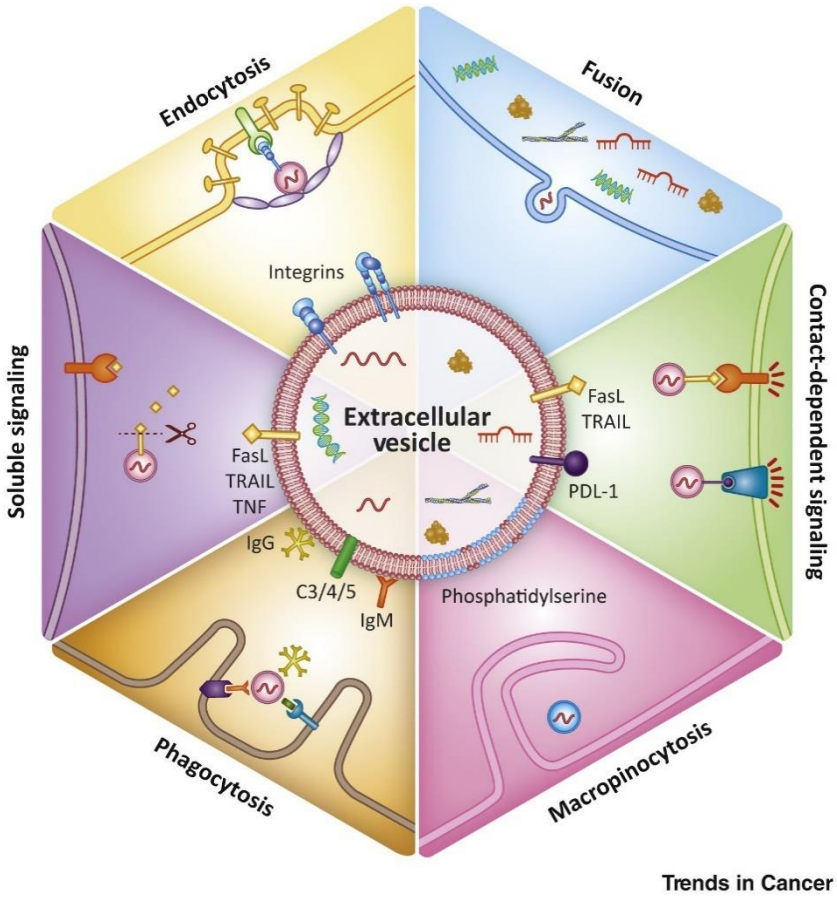


Figure 6 - Mechanisms of Extracellular Vesicle (EV) Uptake. There are different processes through which cells can do the uptake of EVs, such as endocytosis (including macropinocytosis and phagocytosis) or direct fusion of EVs with the plasma membrane of recipient cells. EVs can also transfer their information to target cells through the release of free ligands (soluble signalling) or by direct interaction with receptors present on the target cell's surface. Taken from: *Adem B, Vieira PF, Melo SA. Decoding the Biology of Exosomes in Metastasis.* ***Trends Cancer****. 2020;6(1):20-30.*

Cells that are able to uptake exosomes by phagocytosis are proven to have a greater uptake of exosomes than non-phagocytic cells (55). Regarding the uptake of exosomes by direct fusion with the plasma membrane, Parolini *et al.* demonstrated in 2009 that a portion of the secreted exosomes, under certain conditions (e.g., low pH), are capable of fusing with the membrane of the recipient cells, being internalized by them (90). However, little is known about this mechanism.

As previously mentioned, when exosomes are released from the cells, they can enter recipient cells (63), delivering their contents and modulating its activity (55). Exosomes

present a wide range of proteins from nuclear, cytosolic, endosomal and plasma origins and thus are potential mediators and regulators of key physiological processes (64). Exosomes have been reported to contain integrins, heat shock proteins (HSP70, HSP90) and tetraspanins (CD9, CD63, CD81 and CD82) (64, 91). They also contain MHC class II proteins (91), epithelial cell adhesion molecules (EpCAM) (92), proteins associated with membrane transport and fusion, such as annexins and Rab GTPases (64). Because exosomes content reflects the content of their cells of origin, they can be sources of biomarkers. Melo *et al*. presented evidence that the cell surface proteoglycan glypican-1 (GPC-1) was present on exosomes collected from the serum of patients with pancreatic cancer, being detected both early and late stages of pancreatic cancer. GPC-1 on exosomes allowed to distinguish patients with PDAC from those with non-malignant pancreas diseases, such as chronic pancreatitis (93). Exosomes also exhibit proteins on their surface which can bind to cell surface receptors of the recipient cells. This process induces intracellular signalling, directly affecting the cells' behaviour (55, 64). Nucleic acids are also enriched in exosomes, which were proven to contain DNA, mRNA, lncRNA, miRNA (55), transfer RNA (tRNA) and viral RNA (64). Kahlert *et al*., demonstrated that exosomal DNA isolated in a non-invasive way allowed the identification of driver mutations associated with PDAC, such as KRAS and TP53 mutations (94).

4.1.2. Exosomes and the tumour microenvironment (TME)

The TME comprises multiple biological components that adjoin cancer cells. Its major components are fibroblasts, endothelial cells, hematopoietic cells (both from lymphoid and myeloid origin) and the ECM, whose function is to provide physical and biochemical support (70). There is a bidirectional flow of communication between stromal cells and cancer cells. Evidence suggests that cancer cell-derived exosomes influence proximal tumour and stroma cells in the local microenvironment promoting or deaccelerating cancer progression (63).

4.1.3. Exosomes in cancer

Cancer exosomes are involved in tumorigenesis, cancer growth, angiogenesis, tumour immune escape, drug resistance, metastasis and tumour-stroma interaction (55, 63, 64, 95).

Cancer exosomes can induce transformation of non-cancer cells (55). Melo *et al.* demonstrated that breast cancer exosomes harbour precursor microRNAs (pre-miRNAs) associated with RNA-induced silencing complex (RISC)-loading proteins. This association could lead to the effective silencing of mRNAs in non-cancer epithelial cells, culminating in transformation and reprogramming of the transcriptome (96). Exosomes may also transfer oncoproteins, facilitating tumorigenesis (64). Donnarumma *et al.* demonstrated that exosomes from cancer-associated fibroblasts (CAFs) can mediate horizontal transfer of proteins and miRNAs, dictating an aggressive phenotype in breast-cancer (97). Exosomes from CAFs induce breast cancer cells' autocrine production of Wnt family member 11 (Wnt11)-associated exosomes. Those exosomes lead to the activation of signalling pathways, promoting cell motility and invasion (98). Cancer exosomes acting on the microenvironment can enhance tumor growth (55). Literature suggests that cancer cells uptake exosomes containing survinin, an anti-apoptotic protein that protects cancer cells against genotoxic stress-induced cell death (99). Corrado *et al.* showed that chronic myelogenous leukemia (CML) exosomes stimulate the production of IL-8 by the bone marrow stromal cells, thus promoting leukemia cells' growth (100). Exosomes from bone marrow mesenchymal stromal cells (BM-MSCs) from multiple myeloma (MM) patients express an increased level of molecules (oncogenic proteins, cytokines and adhesion molecules) that promote and facilitate MM cells' growth (101). In a study performed in colorectal cancer, tumor-derived exosomes enriched in mRNAs taking part in the cell-cycle promoted proliferation of endothelial cells and tumor growth (102). Zhang *et al.* provided evidence that the loss of exosomal miRNA-320a from CAFs promoted hepatocellular carcinoma (HCC) proliferation and metastasis (103). Breast cancer exosomes were shown to take part in the conversion of adipose tissue-derived mesenchymal stem cells (MSC) into myofibroblast-like phenotype via activation of the TGFβ-Smad2/3 pathway, shaping the TME to favour tumor progression (104).

Angiogenesis is the process by which new blood vessels form from pre-existing ones. This process is regulated by growth factors, signalling pathways and depends on the balance of pro and anti-angiogenic factors (105). Angiogenesis is particularly relevant in

cancer progression, because the tumour relies on new blood vessels to get oxygen, nutrients and growth factors, as well as to eliminate waste (106). In glioblastoma, it was shown that exosomes released from cancer cells grown in hypoxia stimulated angiogenesis, when compared to exosomes released from cancer cells grown in normoxic conditions (107). It has been reported that exosomes from endothelial cells might be involved in angiogenesis as they do the incorporation and transfer of Delta-like ligand 4 (Dll4; Delta 4) protein to neighbour endothelial cells (108). This results in an inhibition of Notch signalling and in an increased capillary formation both *in vitro* and *in vivo* (108). Taraboletti *et al.* suggested that endothelial-derived exosomes harbour proteins, microRNAs and mRNAs which have proangiogenic potential (109). Matrix metalloproteinases in exosomes from endothelial cells can promote endothelial cell invasion, as well as formation of new blood vessels (109). Thus, exosomes play a critical role in tumor angiogenesis, either by directly delivering angiogenic proteins into endothelial cells or by modulating the angiogenic function of endothelial cells by exosomal miRNAs.

Drug resistance has long been considered a hurdle in cancer management (63). Exosomes have an active role in the development of resistance to therapy in cancer cells and it is accomplished through a wide range of mechanisms (55), namely the transfer of multi-drug resistance (MDR)-associated proteins and miRNAs from exosomes to target cells (110, 111); for example, exosomal-miR-221/222 may be responsible for tamoxifen resistance in breast cancer, according to Wei *et al.* (111). Another mechanism by which exosomes confer drug resistance is drug efflux. Chemotherapeutic drugs and their metabolites can get enclosed and exported from the cancer cells by exosomes (112). Furthermore, exosomes may neutralize the effect of antibody drugs, inhibiting their binding to tumor cells. Lymphoma exosomes carry CD20, which binds therapeutic anti-CD20 antibodies, thus protecting target cells from antibody attack, promoting cancer survival (113). In pancreatic cancer, CAF-derived exosomes were shown to contribute to chemoresistance and, consequently, proliferation of cancer cells (114). A study conducted by Yeung *et al.* in ovarian cancer showed that CAF-derived miRNA-21 transportation in exosomes conferred paclitaxel resistance through targeting APAF1 (115).

Metastasis is partially regulated by cancer exosomes. They affect not only the cells nearby their production site, but also affect the cells from distance tissues, playing a crucial role at establishing the pre-metastatic niche and enhancing tumor cell migration

and invasion, thus promoting metastasis formation (55, 63, 64). Melanoma exosomes from melanoma cells with high metastatic potential participate in the conversion of bone marrow progenitor cells into a pre-metastatic phenotype via the MET receptor (116). Atay *et al.* showed that gastrointestinal stromal tumor cells-derived exosomes containing protein tyrosine kinase stimulate the conversion of progenitor smooth muscle cells into cells with a pre-metastatic phenotype (117). Suetsugu *et al*. present evidence on the capacity of highly metastatic breast cancer cells to transfer their own exosomes to other cancer cells as well as to normal lung tissue cells. This was observed *in vitro* and *in vivo* by fluorescent protein imaging methods (118). Exosomes derived from IL-4-activated macrophages were shown to transfer miR-223 to co-cultured breast cancer cells, increasing cell invasion (119). In PDAC, exosomes secreted by the tumor, enriched in macrophage migration inhibitory factors, recruited macrophages, establishing a pre-metastatic niche in the liver, leading to an increased hepatic metastatic burden (120).

Stroma is thought to be critical in tumor development and progression. MSCs function as precursors for tumor myofibroblasts. Zhang *et al*. also suggested that tumor-derived exosomes could induce differentiation of human MSCs to CAFs (55). Webber *et al.* suggest that prostate cancer cells could promote the differentiation of fibroblasts into myofibroblasts through exosomal TGF-β (121). Activated fibroblasts are commonly found at tumor sites, potentiating tumor progression by the secretion of chemokines, growth factors and deposition of ECM constituents (122). Furthermore, communication between the stroma and cancer cells modulates therapy response. Boelens *et al*. suggest there is an expansion of therapy-resistant tumor-initiating cells when exosomes are transferred from stromal cells to breast cancer cells, as a juxtacrine NOTCH3 pathway is constituted (123). Therefore, exosomes may mediate an interplay between stromal cells and cancer cells, thus promoting tumor progression. Figure 7 illustrates the roles of exosomes in cancer.

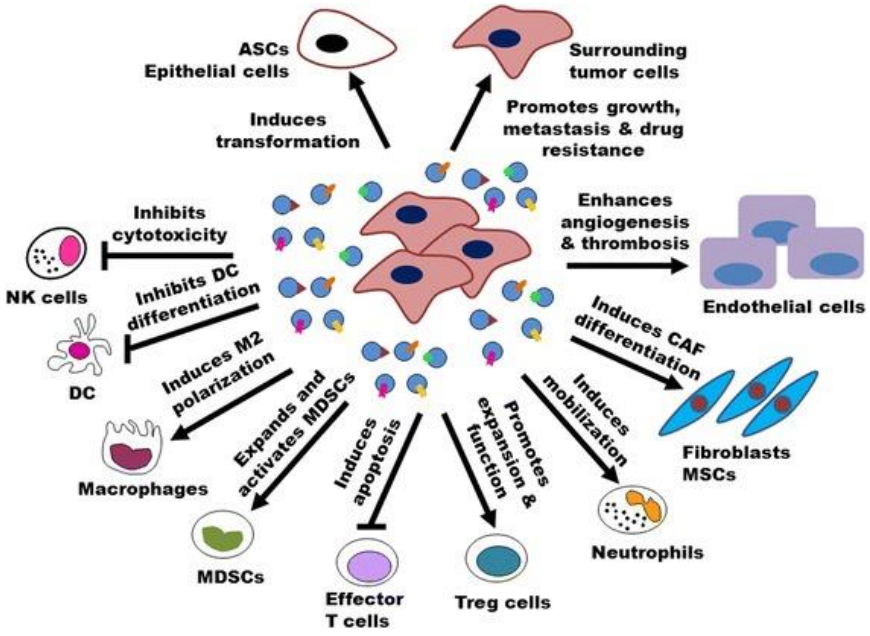


Figure 7 –Roles of exosomes in cancer. Cancer exosomes present the ability of interacting with and modulating several cell types, such as epithelial cells, ASCs, surrounding tumor cells, as well as with immune cells, namely NK cells, dendritic cells (DCs), macrophages, myeloid-derived suppressor cells (MDSCs), effector T cells, Tregs, neutrophils, fibroblasts and MSCs, and endothelial cells. These interactions potentiate multiple processes that lead to immunosuppression or to the activation of the immune system against cancer. Adapted from: *Zhang X, Yuan X, Shi H, Wu L, Qian H, Xu W. Exosomes in cancer: small particle, big player. J Hematol Oncol. 2015;8:83.*

5. Exosomes and the immune response in cancer

Cancer exosomes can interact with the majority of immune cells (70). Over the past few years, this link between exosomes and the immune system has excited the scientific community's curiosity, and many studies have revealed that exosomes modulate the tumor immune response by mediating the crosstalk between cancer cells and immune cells present in the TME (70). Evidence of this mediation via exosomes is in part provided via labelling of tumor exosomes, which allowed the observation of cancer exosomes uptake by immune cells, such as B, T and NK cells, DCs and macrophages (124-129).

To better understand the effects of the interplay between cancer and immune cells via exosomes, it is important to first understand the function of the different immune cells in cancer killing. T and B cells from the adaptive immune system are responsible for the generation of a long-term memory response (14). T-lymphocytes are classified according to their cell-surface proteins, mediating distinct effector functions. Cytotoxic T-lymphocytes (CTLs) express CD8 and activate target cell's apoptosis program, thus killing cells expressing foreign antigens. Helper T-lymphocytes express CD4 on their surface and assist in the activation of B-lymphocytes, CD8+ T-lymphocytes and macrophages, as they secrete specific cytokines, having an important role in the adaptative and innate immune responses. Tregs, a subset of CD4+ lymphocytes, inhibit autoimmune responses, protecting the body from unwanted damage caused by the immune system acting against "the self". T cells are only able to exert their immune protective function upon activation and differentiation into effector T cells, which depends on: interaction of the TCR with their specific antigen presented by MHC molecules, stimulation through co-stimulatory molecules, including cytokines, and the expression levels of inhibitory ligands, such as CTLA-4 (130-132). T cell activation occurs when the TCRs on CD4+ helper T cells and on CD8+ cytotoxic T cells binds to the antigen in the MHC-II and MHC-I complexes, respectively. Upon stimulation, $CD4^+$ Th1 cells secrete IFNγ, TNFα, TGFβ, and IL-2, which are cytokines that cooperate with cytotoxic CD8+ T cells, thus promoting cancer killing (133, 134). In contrast, Th2 cells express IL- 4, 5, 6, 10, and 13, which cause T cells' loss of cytotoxicity, while increasing the humoral immunity through the activation of B lymphocytes, paradoxically promoting humoral pro-tumorigenic responses (133, 134). On the other hand, after B cell receptors recognize an antigen, B lymphocytes become activated and undergo clonal expansion, which confers them an augmented capacity to recognize foreign antigens (135) and bind a specific antigen on the surface of cancer cells, thus exerting tumor suppressive activities by: altering or blocking the antigens function; inhibiting tumor development through the production of tumor-reactive antibodies (136); stimulating NK cells to kill cancer cells via ADCC (137, 138); and, presenting antigens to macrophages, promoting their activation (139). The activation of B lymphocytes can also induce several soluble mediators, namely diverse immunoglobulin subtypes, B-cell-derived cytokines (IL-6) and activation of complement cascades. Altogether, they trigger the recruitment of innate immune cells that are present in circulation. As so, activated B cells promote phagocytic or cytotoxic destruction of cancer cells (140). Other key players in cancer killing are NK

cells, which are able to kill tumor cells without prior sensitization, patrolling their environment using germline-encoded receptors, which integrate a variety of signals that dictate the initiation or suppression of a NK cell-mediated response (141). The molecular mechanisms that regulate NK cell cytotoxicity can be divided into three main steps: (1) target cell recognition, (2) target cell contact and immunological synapse (IS) formation, and (3) NK cell-induced target cell death. Target cell death can be achieved directly – through the release of cytotoxic granules containing granzyme and perforin by NK cells (142) – or indirectly – through NK cells ability to produce pro-inflammatory cytokines, such as IFN-γ, which amplify the immune response (143, 144). NK cells are able to distinguish 'self' from "non-self' and, then, eliminate any threat to the host (141). Although it has been known for some time that NK cells play a key role in fighting tumor development and progression, in more recent years NK cell-based immunotherapy has become a novel and promising approach to treating tumors (145). Several studies, both preclinical and clinical, have focused on NK cells and their antitumor function. As so, some of those studies report the administration of activating cytokines (IL-2 and IL-15) and their effects on cancer killing (146).

In cancer context, immunosuppressive cells play an important role in tumor progression. TAMs, MDSCs and Tregs can be widely found in the TME of most tumors (14). On the other hand, although the inflammatory process mediated by the immune system has a crucial role in protecting the body against pathogens and, consequently, disease, it can also promote carcinogenesis, being acute and chronic pancreatitis examples of inflammatory diseases that increase the risk of developing cancer, namely PDAC (14).

Some of the first studies conducted on exosomes and immunity lead to the belief that exosomes stimulated the immune system to fight cancer (64, 70). However, evidence also suggests that tumor-derived exosomes have immunosuppressive activities (55), thus presenting a dual role in mediating the crosstalk between cancer cells and immune cells (63). When exosomes were shown to carry MHC-I and MHC-II molecules (147) the idea that they could directly present antigens to cells of the immune system (alike antigen-presenting cells, APCs), such as CD4+ and CD8+ T cells (70), potentiating immune responses *in vivo* (91), was put forward. It was demonstrated that cancer exosomes can present tumor-specific antigens to DCs, which, in turn, present these antigens to CD8+ CTLs (148). Wolfers *et al.* showed that this presentation strongly induces CD8+ T cell-dependent antitumor effects on mouse tumors (149). *In vitro* and *ex vivo* studies showed

that pancreatic cancer exosomes transport Hsp70/Bag-4 that enhances the migration and the activity of NK cells against Hsp70-positive cancer cells (150, 151). Treatment with Hsp70-expressing exosomes also inhibited tumor development in mice inoculated with a mouse myeloma cells in a $CD8^+$ T cells and NK cells-dependent way (152).

Although initially reported as tumor immune response stimulators, tumor-derived exosomes have also been reported as having immunosuppressive functions (55). It is now clear that cancer exosomes have a dual role in mediating the crosstalk between cancer cells and immune cells (63, 153). Prostate cancer exosomes that carry FasL decrease T-cell proliferation in a dose-dependent manner (154). Concordantly, other EVs derived from melanoma have also been found to carry FasL, inducing Fas-dependent apoptosis of lymphocytes (155). Further evidence of the immunosuppressive function of exosomes was provided by numerous studies. Clayton *et al.* showed that tumor-derived exosomes carried TGF-β1 and altered IL-2 responsiveness, potentiating the action of Tregs in detriment of cytotoxic cells (156). Interestingly, exosomes isolated from head and neck cancer cell lines are also able to convert cytotoxic CD8+ T cells into immunosuppressive CD8+ T cells. This switch in CD8+ T cells phenotype was accompanied by the downregulation of CD27/CD28. This effects seem to be partially dependent on Galectin-1 (Gal-1) presence on exosomes (157). Another study brought to light that PDAC tumors are rich in Gal-1, which contributes for an immunosuppressive microenvironment, preventing the immune system (specially T cells) from fighting cancer cells (158). It is, thus, reasonable to postulate that PDAC cells might also use exosomes as transporters of Gal-1 to mediate these immunosuppressive functions. There have also been reports suggesting that tumor exosomes express NKG2DLs on their surface, being able to interact with NK cells via the receptor NKG2D (159, 160). Even though NKG2D is an activating receptor, its engagement by tumor exosomes did not result in NK cell activation. Instead, several reports observe a decrease in NKG2D expression on the surface of NK cells and impaired NK cells cytotoxic function after treatment with tumor exosomes (124, 159-162). These observations might be explained by the fact that upon prolonged and persistent engagement of this receptor by its ligands NKG2D was shown to be internalized and degraded in the lysosomes (163). In support of this theory, Li *et al.* (164) have shown that, even though short exposure to myeloid leukemia exosomes induced the release of perforin and the production of IFNγ, prolonged exposure caused a decrease in the expression of NKG2D as well as NKp44 on the surface of NK cells, resulting in the

impairment of NK cells cytotoxicity (164). Tumor exosomes also suppress NK cells function via the interaction of exosomal TGFβ1 with the TGFβ receptors, which triggers the TGFβ-Smad2/3 pathway (124, 165). Exosomal microRNA from pancreatic cancer (PC) cells may also inhibit the immune response. Pancreatic cancer exosomes could transfer miR-212-3p to DCs, inhibiting the regulatory factor X-associated protein (RFXAP), an important transcription factor for MHC II, consequently decreasing the expression of MHC-II. Consequently, DCs are uncapable of activating $CD4^+$ T cells, leading to an immunotolerant microenvironment in PDAC. Therefore, inhibiting the secretion of cancer exosomes and/or downregulation of miR-212-3p could lead to an activation of the immune system against cancer cells (23, 166). PDAC exosomes also express miR-203, which inhibits the expression of toll-like receptor 4 (TLR4), tumor necrosis factor-α (TNF-α) and IL-12 in DCs. Therefore, they could impair DCs ability to present antigens on their surface (167). In fact, it was demonstrated that when TLR4 is not expressed, DCs lose their ability to present tumor-specific antigens in the context of breast cancer (168). Tumor exosomes were also shown to inhibit the differentiation of myeloid precursor cells into DCs in mice via induction of IL-6 expression (169). Tumor exosomes can also promote a switch in the differentiation of myeloid cells into MDSCs, thus accelerating the process of lung metastisation in a MyD88-dependent manner (170, 171).

MDSCs and M2 macrophages are also extensively found in the PDAC microenvironment, enhancing tumor progression and lymphangiogenesis. This leads to lymphatic metastasis, and poor survival (172). A recent study showed that exosomes from pancreatic cancer cell lines caused a change in macrophages' phenotype, leading to an immunosuppressive phenotype. The macrophages' immunosuppressive phenotype was exacerbated when macrophages were treated with exosomes isolated from the ascites-derived, highly metastatic AsPC-1 pancreatic cancer cell line, promoting angiogenesis, pancreatic cancer progression and metastasis. These treated macrophages also secreted Prostaglandin E2 (PGE2), causing the inhibition of DCs' maturation and, consequently, preventing the activation of $CD8^+$ T cells (173, 174).

6. Exosomes in Immunotherapy: Targets and/or Useful Tools?

The immune system is normally activated in response to infection caused by a foreign pathogen. In cancer, a similar immune response is initiated: tumor-specific antigens, released by tumor cells, are recognized by the cells of the immune system (45, 175), which set in motion a series of steps that culminate in the killing of cancer cells. This 7-step process is termed Cancer-immunity cycle and takes place during the first phase of Cancer immunoediting (the Elimination phase, also known as the Cancer immunosurveillance phase) (45, 176). As illustrated in Figure 8, Cancer-immunity cycle involves:

1) Cancer cell antigens release

Oncogenesis leads to the creation of cancer neoantigens, which are released and captured by DCs. Simultaneously, there is release of signalling proteins, such as proinflammatory cytokines, by dying tumor cells. Tumor immunogenicity determines how well a tumor will respond to immunotherapy treatments (45).

2) Cancer antigen presentation

DCs or other APCs, such as macrophages and B-cells, present the neoantigens that were captured on MHC I or MHC II molecules to CD8+ and CD4 + T cells, respectively, by forming a contact point between the two cells, which is named immunological synapse (45).

3) Priming and activation

Priming and activation of effector T cell responses against tumor antigens are a result of the antigen presentation, together with signals generated by costimulatory molecules, such as CD28. The nature of the immune response is determined at this point. The outcome of the immune response is determined by a balance between effector T cells and Tregs (45).

4) Trafficking of T cells to tumors

CTLs stored in the lymph nodes travel to the tumor through the blood stream (45).

5) T cell infiltration into tumors

CTLs infiltrate through the vessel endothelial cells and through the tumor stroma (fibroblasts and extracellular matrix, namely) into the tumor (45).

6) Recognition of cancer cells by T cells

T cells recognize and bind to cancer cells. This process takes place through the interaction between TCRs (on the surface of T cells) and the associated antigen that is bound to MHC I or MHC II molecules (45).

7) Killing of cancer cells
8) Additional tumor-associated antigens combined with the pro-inflammatory cytokines released by T cells are released, starting the cycle again at the first step. This increases the depth of the immune response against the tumor antigens (45).

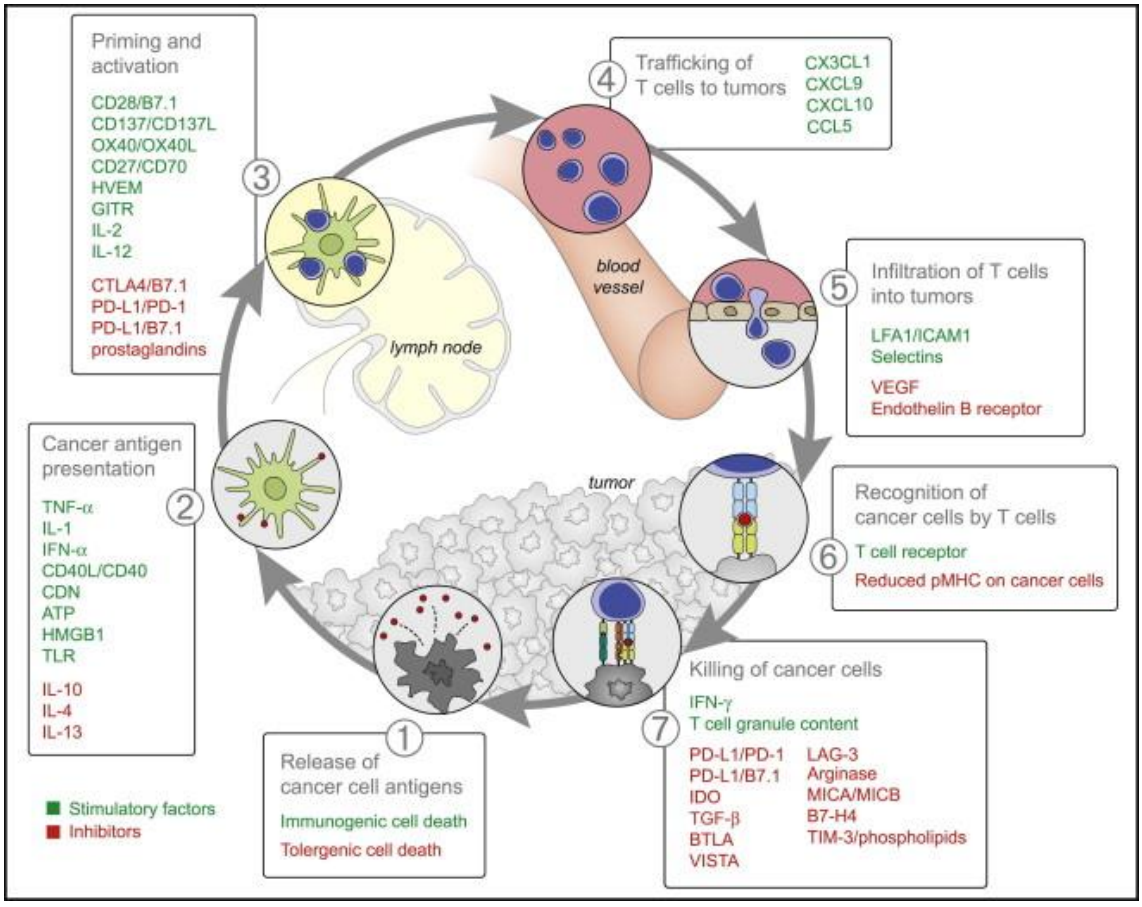


Figure 8 - The cancer-immunity cycle. The cancer immune cycle comprises: cancer cell antigens release, cancer antigen presentation, priming and activation, trafficking of T cells to tumors, T cell infiltration into tumors, recognition of cancer cells by T cells and, finally, killing of cancer cells. Taken from: *Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013;39(1):1-10.*

However, tumors are able to develop despite the surveillance by the immune system. This paradox is explained by the Cancer immunoediting theory, which is the name given to the 3-step-process that characterizes the interaction between the immune system and tumor cells, allowing tumor cells to escape the immune system and give rise to a clinically apparent tumor (175, 177, 178).

Exosomes have been explored as tools for the transport of specific factors that help in immunostimulation to fight cancer. DC exosomes contain MHC-I and -II and other costimulatory molecules and, thus, are able to promote the activation of effector T cells and NK cells, having caught the attention of the scientific community for their potential use for cancer treatment. Currently, DC exosomes are being explored as immunotherapeutic agents for the treatment of several cancer types (70, 179-181). In the context of PDAC, vaccination with DCs loaded with PDAC exosomes were combined with chemotherapeutic drugs. This combination led to a decrease in metastatic establishment and increased overall survival in mice (182). B cell exosomes were also shown to carry MHC molecules and to present antigens to cytotoxic immune cells (147). However, to my knowledge, few studies have focused their efforts on understanding the effects of B cell exosomes in cancer progression and on how these could be used in cancer treatment.

The inhibition of exosomes release could be used as a novel adjuvant therapeutic strategy for a variety of cancers (65, 183). Potential targets for the inhibition of exosomes' secretion and biogenesis are sphingomyelinase 2 (nSMase2), which mediates ceramide synthesis, and Rab proteins, which are widely involved in their biogenesis (65). Chalmin *et al.* showed that treatment of tumor bearing-mice with dimethyl amiloride (DMA), a drug that inhibits exosomes release, dampens MDSCs' suppressive effects over T cells (184). This effect was found to be a result of the inhibition of the release of Hsp72-containing exosomes, which were shown to interact with TLR2 on the surface of MDSCs and induce Stat3 phosphorylation and IL-6 secretion (184). The inhibition of MDSCs function upon treatment with DMA was also shown to potentiate the anti-tumor efficacy of cyclophosphamide (a chemotherapeutic drug with cytotoxic and immunostimulatory proprieties) (184). Cancer exosomes can also stimulate the proliferation and well as the differentiation of CD4+CD25− T cells into Tregs thereby potentiating their immune suppressive role (185, 186) (187). This data suggests that targeting exosomes biogenesis in cancer could improve the efficacy of chemotherapeutic and immunotherapeutic

strategies, particularly in PDAC, which is characterized by its extensively immunosuppressive microenvironment (23).

7. Hypothesis

Having in consideration the described roles of cancer exosomes in anti-tumour immune response, we **HYPOTHESIZE** that the immunotherapy failure in PDAC might be partially due to the fact that exosomes establish and maintain an immunosuppressive microenvironment in PDAC. Therefore, inhibition of exosomes secretion could be used as an adjuvant therapeutic strategy to potentiate the use of immunotherapy to treat PDAC patients.

To test our hypothesis, we have used genetically engineered mouse models (GEMMs) of PDAC as well as *in vitro* assays. We have started by evaluating the effects of the immune system in PDAC establishment and progression using three GEMMs: **KPC** ($Kras^{G12D/+}$; $p53^{R172H/+}$; $Pdx1^{Cre/+}$), **KPC Rag2$^{-/-}$** ($Kras^{G12D/+}$; $p53^{R172H/+}$; $Pdx1^{Cre/+}$; $Rag2^{-/-}$), **KPC Rag2$^{-/-}$IL2rg$^{-/-}$** ($Kras^{G12D/+}$; $p53^{R172H/+}$; $Pdx1^{Cre/+}$; $Rag2^{-/-}IL2rg^{-/-}$). Our initial plan intended to use the KPC-iRab27aKO ($Kras^{G12D/+}$; $p53^{R172H/+}$; $Pdx1^{Cre/+}$; $R26^{LSLFLP0ERT2/+}$; $Rab27a^{FRT/FRT}$) to inhibit cancer exosomes secretion in a conditional and inducible manner. All of these GEMM models have several alleles and thus need a very optimized and time-consuming breeding strategy to be obtained. Due to the COVID-19 pandemic the animal facility was reduced to minimal essential work, just to maintain strains. In this way, we have proceeded with our contingency plan in which we have treated the PDAC KPC mouse model with Nexinhib20, a small molecule that specifically targets Rab27a and therefore inhibits exosomes secretion.

Our **specific aims** were:

1. Determine the role of the immune response in PDAC progression;

2. Demonstrate that exosomes inhibition can open the door for the application of immunotherapy to the treatment of PDAC patients.

We **expected to demonstrate**: that the immune system plays a role in PDAC biology; and that inhibition of exosomes secretion can make PDAC tumors responsive to immunotherapy.

II. Material and Methods

II. Material and Methods

2.1.Cell culture

In this study one PDAC cell line was used: KPC cell line, which was purchased from Ximbio. KPC cell line was cultured in DMEM (Dulbecco's modified Eagle's medium) (1x) medium (Fisher Scientific, 10103542). The medium was supplemented with 10% FBS (fetal bovine serum) (Gibco®, 10500-64) and 1% penicillin-streptomycin (Gibco®, 15140122). The cells were maintained at 37 ºC in a humidified chamber with 5% CO2 and passed when they reach around 70-80% of confluency.

2.2.Exofree

The cell medium from a T180 is discarded and the T-flask is washed with 10mL of NaCl 0,9%. The solution is discarded and 5mL of trypsin-EDTA 10x (Gibco®, 15400054) are added to the T-flask. The flask is incubated at 37ºC for 2-3min (in case of working with KPC cell line). In order to neutralize the trypsin, medium should be added (at least the same volume of trypsin that was added) and the flask is washed with the medium. Then, this should be passes to a bigger flask, adding DMEM COMPLETE (Fisher Scientific, 10103542). In the next day, the medium is discarded, and the flask is washed twice with NaCl 0,9%. The NaCl 0,9% is discarded and DMEM EXOFREE (DMEM Complete medium from which exosomes were removed by ultracentrifugation) is added until making up approximately 15mL of final volume in falcons. The flask must be incubated at 37ºC for 72 hours.

2.3.Exosomes' extraction and isolation from medium

KPC cells were cultured in DMEM EXOFREE medium. After 72 hours the medium was collected and centrifuged at 2500g for 10 minutes followed by a 5 minutes centrifugation at 4000g in the centrifuge (Eppendorf, centrifuge 5810). Subsequently, the medium was filtered through a 0.2 μm filter (GE Healthcare Whatman™, 10462200) directly to an ultra-clear centrifuge tube (Beckman Coulter®, Z90408SCA). The tubes were weighted as it is necessary to calibrate them by adding the necessary volume of NaCl. The samples were then centrifuged overnight at 100000g, 4ºC using the Optima™ L-80 XP ultracentrifuge Beckman Coulter or at 24100rpm, 4ºC using the Optima™ XE 100 ultracentrifuge, Beckman Coulter®. The supernatant was then carefully discarded and the pellet was resuspended: firstly, with 300uL of NaCl 0,9% and the second time with 200uL

of NaCl 0,9%. The pellet was used to prepare samples for NTA analysis (1/100 dilution factor) with Nanoparticle Tracking Analysis (NTA) technology (NanoSight NS300 particles counter) and the remaining was kept at -20ºC for downstream analysis. These exosomes were also used to treat T cells.

2.4.Exosomes' extraction and isolation from serum

The serum samples (mice or human origin) were slowly thawed on ice. For human samples, 200uL of serum were taken to an Eppendorf and spun at 10000rpm for 2 minutes. The supernatant was recovered to a new Eppendorf and 200uL of NaCl were added. For mice samples, 50uL of serum were taken to an Eppendorf and 200uL of NaCl were added. Subsequently, the medium was filtered through a 0.2 μm filter directly to an ultra-clear centrifuge tube (Beckman Coulter®, 344061). The tubes were weighted as it is necessary to calibrate them by adding the necessary volume of NaCl. The samples were then centrifuged overnight at 100000g, 4ºC using the Optima™ L-80 XP ultracentrifuge Beckman Coulter or using the Optima™ XE 100 ultracentrifuge, Beckman Coulter. The supernatant was then carefully discarded, and the pellet was resuspended in 200uL of PBS1x (Fisher BioReagents, BP399-4) filtered. A part of the resultant exosomes sample (~10μL) was used to prepare samples for NTA analysis (1/100 dilution factor) and the remaining was stored at -20ºC for downstream analysis.

2.5.Exosome quantification by NanoSight (NTA)

Exosomes extracted from the medium of KPC cells were prepared for NTA analysis by diluting the exosomes solution obtained by ultracentrifugation in NaCl 0.9% (1/500 to 1/1000 dilution factor) and exosomes' size and concentration were measured using the NTA technology. For exosomes quantification, the samples were analysed with NTA technology (NanoSight NS300 particles counter) and three independent movies were recorded using a constant syringe pump speed of 40μL/min. The mode of exosomes size and the sample concentration provided by the NTA as particles/mL were registered. The final concentration of exosomes in our sample was calculated having into account the dilution factor used.

2.6.IHC staining

The paraffin-embedded mice organs were sectioned in 4μm slices using a microtome Microm HM335E (HEMS, i3S, Porto) and transferred to coated slides (StarFrost®,

VS1159#1FKA.0x). The slides were incubated at 37 ºC overnight. Sections were deparaffinized with xylene (VWR Bio Chemicals, 28973.363) and dehydrated in solutions of decreasing alcohol concentrations (100%, 100%, 70%). Then the slides were rinsed with running water to hydrate. Sections were subjected to antigen retrieval. To do so, the slides were placed in an antigen unmasking solution of sodium citrate buffer pH=6.0 (VECTOR Laboratories®, H-3300) at a ratio of 1:100 or of Tris-EDTA [10mM of Tris-base (Sigma-Aldrich, T6066), 1mM of EDTA (Sigma-Aldrich, Sigma Life Sciences, E5134), 0,05% of Tween20 (Sigma-Aldrich, P9416) in dH2O] with a pH=9.0 or pH=8.0 and put inside of a water vaporizer machine at approximately 99ºC for 35-40 minutes, according to the primary antibody used (see Table 3). The slides were then removed from the vaporizer machine, allowed to cool down for 20 minutes at room temperature and finally washed for 5 minutes (three times) with PBS (PBS 1X/0.1% Tween 20). The slides were then placed in a humid chamber and incubated with a solution of 3% hydrogen peroxide (H2O2) (Sigma-Aldrich, 31642) in methanol (VWR BHD Chemicals, 20903.368) for 15minutes to permeabilize the tissue and inhibit the action of endogenous peroxidases. After this step, the slides were washed twice in PBS-T as previously mentioned. Tissue sections were delimited with a hydrophobic pen (Enzo®, ADI-950-233-0001), placed in the humid chamber and incubated with Ultravision Protein-block solution (Thermofisher Scientific, TA-125-PBQ) for 30 minutes at room temperature. Sections were then incubated in an humidified chamber with the primary antibody that we wanted to test at 4ºC overnight or at room temperature for 1h (see Table 3). After primary antibody incubation, the slides were washed three times with PBS-T for 15 minutes. In the humid chamber, the secondary antibody was added to the tissue sections HRP Mouse/Rabbit (Dako, K50017). After 30 minutes incubation at room temperature, the slides were washed with PBS-T as previously described. After this step, DAB staining was performed. To do so, DAB solution (Dako, K50017) was prepared (1:50 dilution) in commercial solution available (VECTOR Laboratories). The slides were placed in the Fume Hood, once DAB is a carcinogenic, and 100uL of DAB solution were added to each slide and slides incubated for 1 minute. It is of utmost importance to discard the pipette tips and all the material that contacted with DAB solution in the red trash bin due to the biohazard associated risks. The reaction was stopped by immersing the slides in tap water and rinsing them with tap water for 5 minutes. Counterstaining was done so we can distinguish the blue stained nuclei under the microscope. Counterstaining was done in Modified Gill II Hematoxylin (Merck Millipore, 105175) for 30 seconds.

The slides were then immersed in tap water and washed with tap water between 7 to 15 minutes. The tissues were then dehydrated through 3 changes of ethanol (70%, 100%, 100%) to allow complete removal of the water, since xylene is highly hydrophobic). The slides were then incubated in xylene for 5 minutes, twice. Finally, some drops of DPX mounting medium (Sigma-Aldrich, 06522) were added in the Hotte to the slides and the glass coverslip (Normax, 5470008A or 5470003A, according to the size of the tissue) was mounted, trying to avoid bubbles between the coverslip and the slide. The slides dried in the hotte for 1-2 days and were then observed the tissue section under the microscope OLYMPUS, CX31 (i3S, Porto, Portugal). Pictures of the slides were taken in Axioskop 2 microscope (Zeiss) (IPATIMUP, Porto, Portugal).

Table 1 – Antibodies and respective conditions used for IHC staining.

Antibody (Ab)	**Dilution**	**Antigen Retrieval**	**Primary Ab incubation**	**Cells marked**	**Ab Catalog Number**
Ki67	1:500	Heat mediated - Citrate-buffer pH=6	Overnight, 4ºC	Cells in proliferation	ab15580, Abcam
Zeb-1	1:250	Heat mediated - Tris-EDTA pH=9	Overnight, 4ºC	Cells undergoing an EMT	14-9741-82, Invitrogen
CD4	1:200	Heat mediated - Tris-EDTA pH=9	Overnight, 4ºC	CD4+ T cells	ab183685, Abcam
CD31	1:50	Heat mediated - Tris-EDTA pH=9	Overnight, 4ºC	Endothelial cells	ab28364, Abcam
FoxP3	1:500	Heat mediated - Tris-EDTA pH=9	Overnight, 4ºC	Foxp3+ T cells	ab36607, Abcam

EMT, epithelial-to-mesenchymal transition; RT, Room Temperature

The quantification of cells positively marked with the antibodies in study was performed according to the following methodology:

- **Zeb-1 and Ki67** – 5 pictures are taken of the tissue in the slide after performing IHC staining, in a 400x magnification (40x objective and 10x ocular) and then positive marked cells and negative cells (unmarked cells) for the antibody are counted. Finally, the percentage of positive marked cells is calculated [(positive cells/TOTAL cells)*100].

- **CD31** – Weibner method - the tissue is scanned at low magnification (40x to 100x) and the areas of clear-cut, invasive carcinoma which have distinctly highlighted microvessels are selected. These areas are called neovascular "hotspots". One picture at 200x (20x objective and 10x ocular) is taken and all the vessels of the neovascular "hotspot" are counted. Single microvessels that can be counted were selected if they were brown-staining endothelial cells or endothelial-cell clusters, separated from adjacent microvessels, tumor cells, and other connective-tissue elements. Brown-stained microvessels or individual endothelial cells positioned on the edge of the picture at 200x were also counted as separate microvessels (188).

- **CD4 and FoxP3** – the necessary amount of pictures to have a representative depiction of the whole tissue in the slide were taken after performing IHC staining. Pictures were obtained using the 10x objective and a 10x ocular (i.e., at a 100x magnification). Then, positive marked T cells for the antibody are counted.

Table 2 - Cell quantification of stained tissues through IHC staining.

Antibody	**# of pictures taken**	**Magnification**	**Quantification method**
Zeb-1	5	40x	% positive cells (positive cells/TOTAL cells)*100
Ki67	5	40x	% positive cells (positive cells/TOTAL cells)*100

CD4	Representative of the tissue	10x	# of positive cells per field
CD31	1	200x	Weidner method (# of microvessels)
FoxP3	Representative of the tissue	10x	# of positive cells per field

2.7.H&E staining

The paraffin-embedded mice organs were sectioned in 4μm slices using a microtome Microm HM335E (HEMS, i3S, Porto) and transferred to KP frost slides (Klinipath, KP-3040). The slides were incubated at 37 ºC overnight. Sections were deparaffinized with xylene and dehydrated in solutions of decreasing alcohol concentrations (100%, 100%, 70%). Then the slides were rinsed with running water to hydrate. The sections were then stained with Modified Gill II Hematoxylin, washed in running water. The counterstain of the tissue was performed in alcoholic eosin solution (Thermo Scientific, 71204) for 1 minute, then the slides were immersed in tap water. The tissues were dehydrated through 3 changes of ethanol (70%, 100%, 100%) to allow complete removal of the water, since xylene is highly hydrophobic). The slides were then incubated in xylene for 5 minutes, twice. Finally, some drops of DPX mounting medium were added in the Hotte to the slides and the glass coverslip was mounted, trying to avoid bubbles between the coverslip and the slide. The slides dried in the hotte for 1-2 days and were then observed the tissue section under the microscope.

2.8.Isolation of T cells from spleen

Day 0 – Coating of a 6-well plate (TPP®, TPP92006) **with anti-CD3**

1. Prepare 10ug/mL of anti-CD3 (BD Pharmingen™, clone 145-2, 553057) solution in PBS 1x:
   Stock = 1mg/mL = 1ug/uL
   For 6mL of PBS 1x → 60ug = 60uL of anti-CD3

2. Add 1mL of this solution to each well of a 6-well plate and incubate O.N., at 4ºC.

Day 1 – Isolation of T cells

3. Prepare:
    - MACS buffer: 50mL PBS + 250uL FBS + 200uL EDTA 0,5M
    - FACS buffer: 49mL PBS + 1mL FBS
    - Red Blood cell Lysis (RBL): Vf= 500mL
        - 4,15g Ammonium chloride (NH4Cl) (Sigma Life Sciences, A9434)
        - 0,5g Potassium bicarbonate (KHCO3) (Sigma Life Sciences, P9541)
        - Distillate water
        - pH 7,2
        - Store 4ºC and equilibrate to RT before usage

    NOTE: After preparing these solutions, do not forget to filter them with a 0.2um filter.

Table 3 – Composition of the medium in which the T cells were kept.

FBS	20%	10mL
HEPES (VWR Life Sciences, H3375)	1%	0.5g
NaPyruvate	1%	0.5g
2-Mercaptoethanol (Sigma-Aldrich M6250)	0,01%	5uL
Pen-Strep (Gibco®, 15140-122)	1%	0.5mL
RPMI (Gibco®, 21875-034)		Up to 50mL (~39.5mL)
To 12mL of supplemented RPMI (2mL/well) add:		
Anti-CD28 (stock = 1mg/mL = 1ug/uL) (BD Pharmingen™, 553294)	8ug/mL	96uL
IL-2 (stock = 200ug/mL) (BioLegend®, 575402)		0,72uL (0,6uL to 10ml of RPMI)

Note: If it is necessary to change or add medium in the following days, do not add anti-CD28 to the medium.

The spleen is retrieved and placed on sterile PBS 1x on ice. The spleen is placed in a 70µm cell strainer and mashed with a syringe plunger. The filter is washed with FACS buffer (2% FBS in PBS) and the sample is centrifuged at 1700rpm for 5min at 4ºC. The pellet is resuspended in 5 mL of red blood cell lysis buffer (RBL buffer) and incubated for 5 min at RT. 10mL of FACS buffer are added and cells are centrifuged at 1700rpm for 5min at 4ºC. 1mL of FACS buffer is added and the sample is filtered (with the cell strainer) to a new tube. In order to calculate the quantities of Biotin-Antibody cocktail and anti-Biotin Microbeads needed in the following steps, cells are counted (1:10 in trypan-blue – 90uL trypan-blue + 10uL spleenocytes). The cells are centrifuged at 1700rpm for 5min at 4ºC.

Protocol Pan T Cell Isolation Kit II – mouse (Miltenyi Biotec, 130-095-130) (for $1x10^7$ cells, adjustments must be made according to the quantity of cells in our sample): The cell pellet is resuspended in 40uL MACS buffer + 10µL Biotin-Antibody cocktail (MACS buffer: 50mL PBS + 250uL FBS + 200uL EDTA 0,5M) and incubated in ice for 5 min. A mixture of 30uL MACS buffer + 20uL anti-Biotin Microbeads is then added to the sample and incubated on ice for 10 min. Meanwhile, the column is placed on the magnetic stand and washed with 3mL of FACS buffer in the magnetic field. A tube is then placed under the column to collect the flow-through (i.e., the T cells) and the cell suspension is added onto the column. The tube that contained the cell suspension is washed with 1 mL of FACS buffer that is also passed through the column. To assure we recover all T cells, another 2mL of FACS buffer are then added to the column. The flow-through (T cells) collected is then centrifuged at 1700rpm for 5min at 4ºC to do a pellet down. In the meanwhile, the coating solution is retrieved from the 6-well plates and the 6-well plates is washed twice with PBS (carefully so that the anti-CD3 coating does not detach from the plate surface). 100uL of the pellet is resuspended in FACS buffer and T cells are counted with trypan-blue (90uL trypan-blue + 10uL cell solution). $1x10^6$ cells are added to each well of the 6-well plate and complemented RPMI media (prepared on step 3) is added to the cells and the cells are incubated at 37ºC.

2.9.Treatment of T cells with exosomes from KPC cell line

The T cells extracted from the spleen of wild-type mice, as mentioned in section 2.10, were plated on a 6-well plate ($1x10^6$ T cells/well) and treated, on every other day, with $1x10^{11}$ exosomes isolated from the conditioned medium of KPC cell line (isolation protocol on section 2.3), as illustrated on Figure 9. The viability of the cells was accessed through FACS analysis.

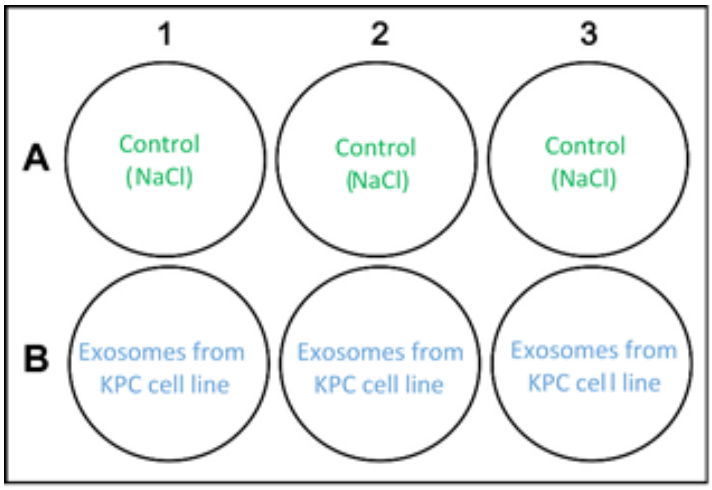


Figure 9 – Schematic representation of the well plate.

2.10. FACS staining of T cells

This protocol aims to let us understand how exosomes, added to the T cells removed from a mouse's spleen, may promote T cell differentiation. The T cells were previously plated on a 6-well plate ($1x10^6$ T cells/well) and then treated, on every other day, with $1x10^{11}$ exosomes isolated from the conditioned medium of KPC cell line. Then, the FACS staining of T cells protocol was performed.

Table 4 – Composition of Ab Mix 1 (CD4+ and CD8+ T cells).

Ab	**Dilution**	**Ab Volume**	**Channel FACS Canto**
L/D	1:1000	0,5 µL	APC

TCRβ (BD Horizon™, clone H57-597, 562839)	1:400	1,25 µL	BV421 – Pacific Blue
CD4 (eBioscience, clone GK1.5, 46-0041-82)	1:1000	0,5 µL	PerCP
CD8 (BD Biosciences, clone 53-6.7, 560776)	1:200	2,5 µL	AmCyan
CD69 (BioLegend, clone H1.2F3, 104506)	1:200	2,5 µL	FITC
TOTAL		7,2 µL Ab + 492,8 µL FACS buffer (50 µL/sample)	

Table 5 – Composition of Ab Mix 2 (CD4+ FoxP3+ Treg cells).

Ab	**Dilution**	**Ab Volume**	**Channel FACS Canto**
CD3 (Invitrogen, clone 17A2, 11-0032-82)	1/200	2,5 µL	FITC
CD4	1/1000	0,5 µL	PerCP
CD25 (Invitrogen, clone PC61.5, 25-0251-82)	1/400	1,25 µL	PE-Cy7
FoxP3* (Invitrogen, clone FJK-16s, 15-5773-82)	1/200	-	APC
TOTAL		6,7 µL Ab + 493,3 µL FACS buffer (50 µL/sample)	

*FoxP3 is incubated alone after permeabilization

Antibody monolabels: 0,5 µL of antibody + respective volume of FACS buffer (according to the dilution stipulated above)

The cells from the 6-well plate are transferred to 15mL falcons. The wells are washed with FACS buffer (1mL) and transferred to the respective 15mL falcons. 300uL are retrieved from one of the control samples to 2 wells of a 96-well plate (Alfagene, 4346907) for: one well for L/D monolabel and another one for unstained. The cells on the falcons and on the 96-well plate are centrifuged at 1700rpm for 5min at 4ºC. While the cells are centrifuging, the monolabels should be prepared (protected from the light), as well as the fixation and permeabilization solutions from the kit eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (REF: 00-5523-00):

Fixation solution –100uL Fixation/Permeabilization Concentrate diluted in 300uL Fixation/Perm Diluent (ratio 1:4).

Permeabilization buffer – 80uL Permeabilization buffer 10X diluted in 720uL dH2O (ratio 1:10).

The supernatant is removed (the pellet may not be visible). 50 µL of MIX 1 or MIX 2 are added in the falcons from control and treated T cells (up and down to mix the cells with the antibody mix should be done). 1 drop of beads should be added to 7 wells of the 96-well plate and then 50uL of monolabels should be added. 50uL of L/D monolabel are added to one of the 2 wells with T cells (prepared on step 3) and 50uL of FACS buffer to the other one.

NOTE$_1$: compensation beads for monolabels should be used in case the pellet is small. In case of a big visible pellet, cells should be used for monolabels.

NOTE$_2$: Unstained Eppendorf (control) without beads should be used in order to detect autofluorescence

NOTE$_3$: L/D without beads should be used because L/D only attaches to proteins in cells and beads do not have proteins. Use only cells for L/D monolabel.

Incubate the cells for 30min on ice protected from the light. In the meanwhile, FoxP3 solution (1:200) and the FACS tubes (blue top with filter to filter cell aggregates) should be prepared. When the incubation is finished, the cells are washed with 1 mL of FACS buffer (falcons) and centrifuged at 1700rpm for 5min at 4ºC. Approximately 200uL of FACS buffer should be added to the wells for L/D and unstained and the cells are

transferred to 2 eppendorfs and centrifuged them at 1700rpm for 5min at 4ºC. The supernatant on the cells is removed and the cells should be wash again and centrifuged. The supernatant is removed and:

- T cells incubated with MIX 1:
    - Resuspended in 400 µL of FACS buffer and added to the FACS tubes (passed through the filter on the tubes' cap). These tubes should be saved on the fridge, protected from light.
- T cells incubated with MIX 2:
    - 100uL of Fixation solution should be added and incubated for 30min on ice, protected from light.
    - 200uL of Permeabilization solution should be added and immediately centrifuged at 1700rpm for 5min at 4ºC.
    - The supernatant is discarded and 50uL of the FoxP3 solution are added.
    - The samples are incubated for 30min on ice, protected from light.
    - The samples are washed and 400uL of FACS buffer are added and this solution should be added to the FACS tubes (passed through the filter on the tubes' cap).

The samples and controls are analysed in FACS CANTO II. The number of evts/sec should be recorded. This number allows the calculation of the number of cells in the sample having into consideration that, every 1sec, 60uL of sample pass through the FACS CANTO II in the "Medium" setting and that there are 400uL of sample in total. So, if there are recorded 3000evts/sec for the sample, then the calculation of cells is:

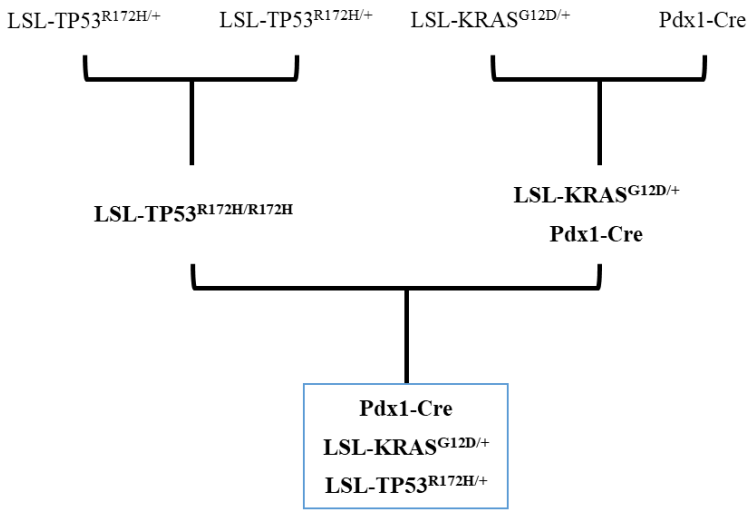
x= (3000evt/sec x 400uL)/60uL = 20000 cells

2.11. Mice related experiments and procedures

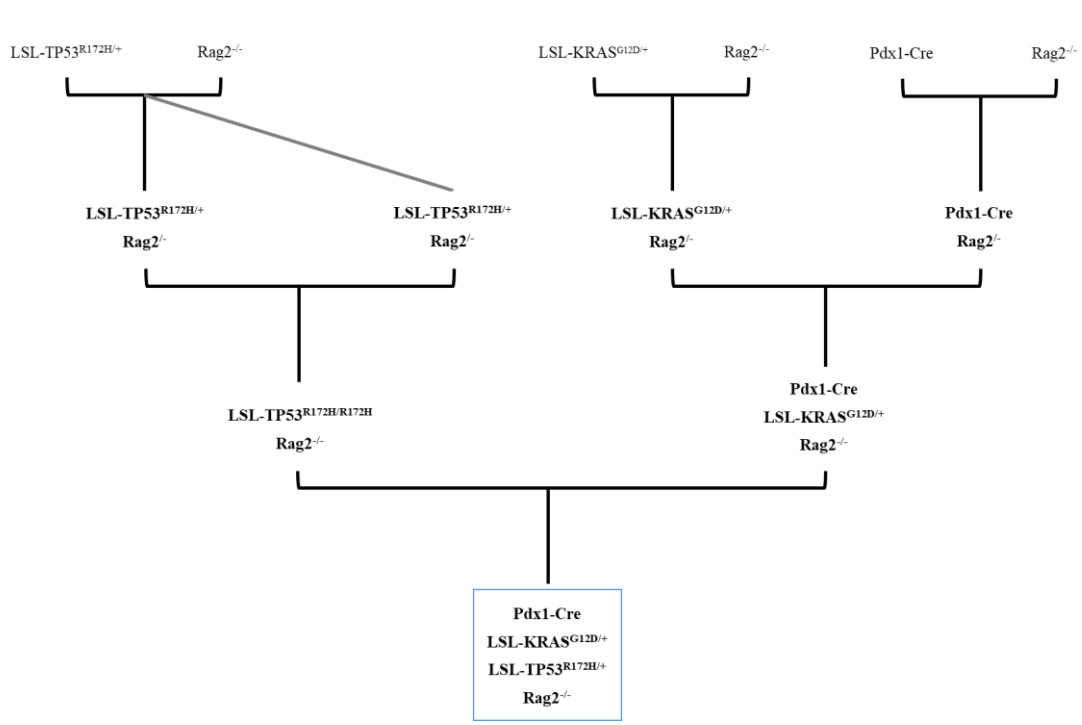
Mice are housed on an SPF animal facility, in type II and type III cages 26.5 x 20.5 x 14.5cm, approx. 545 cm2 and 42 x 26.5 x 15.5 cm, approx. 1100 cm2, respectively. The cages contained corn cob bedding, nesting material (tissue paper) and paper tunnels for environmental enrichment, increasing the animals' wellbeing. The mice were fed in the form of pellets and with water in a bottle, *ad libitum*.

i. Breeding Strategies

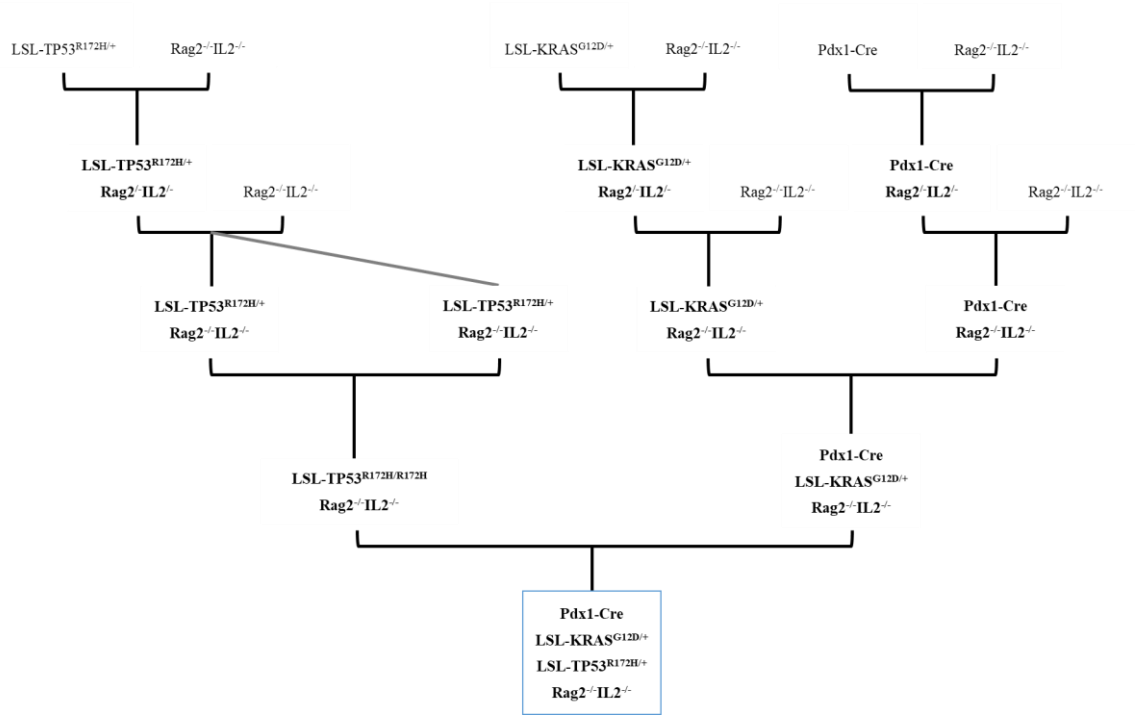
KPC mouse model (Pdx1-Cre LSL-KRAS^{G12D+}LSL-TP53$^{R172H/+}$)



KPC Rag2$^{-/-}$ mouse model (Pdx1-Cre LSL-KRAS^{G12D+}LSL-TP53$^{R172H/+}$Rag2$^{-/-}$)



KPC Rag2$^{-/-}$ Il2rg$^{-/-}$ mouse model (Pdx1-Cre LSL-KRAS^{G12D+}LSL-TP53$^{R172H/+}$Rag2$^{-/-}$ IL2rg$^{-/-}$)



ii. Mouse handling, restraint, identification

In order to begin working with the animals, it is necessary to be calm and secure, avoiding sudden movements and loud noises as they can stress the animals.

The first procedure done was mice restraining. To do so, we started by removing the cage top and the water bottle, that were placed in the bench surface, previously disinfected with alcohol 70 %. The animal was removed from the cage by grasping near the base of its tail or by removing the paper tunnel when the animal was inside of it. The animal was placed on the cage grid. It is important to remember that the animal cannot be suspended by the tail for a prolonged period, as it may stress and hurt the animal (the tail is a prolongation of the spine). By doing this, it is possible to identify the animal's sex – male, if the anogenital distance is big, and female if this distance is smaller. At this point, the animal is placed on the cage grid and is being held by its tail. Holding the animal's tail with one hand, we grasped the nape of its neck and gathered the loose skin from around the neck, using the forefinger and the thumb of the other hand, in order to restrain the

animal. The tail was placed between the ring and little finger. In this way, the animal was restrained in a painless, secure and controlled manner. We must assure that we are gathering enough skin to prevent the animal's head from turning. However, we need to make sure we are allowing the animal to breath normally (if the animal's nose is turning purple, we should free the animal, as it is asphyxiating). If the restraint was well performed, we can move on to the next procedure, using the less skilful hand to restrain the animal and the most skilful hand to execute the procedure.

The marks on the animals' ears are observed in order to identify the animal (ear notching).

iii. Anaesthesia by isoflurane inhalation

There were a few procedures that required that the animal was under general anaesthesia in order to reduce the animal's pain and distress and to guarantee the animal was completely immobilized. However, it is of upmost importance to make sure the animal is strong enough to be anesthetized before starting the procedure. As it is known, the anaesthesia has 3 major components – the so-called anaesthesia triangle - that includes analgesia, hypnosis and muscle relaxation. Concerning anaesthesia, there are 3 steps that need to be considered: induction, maintenance and recovery. To the induction phase, we placed the mouse for a few seconds in an induction chamber connected with isoflurane (IsoVet, CN5711058) at 5% and oxygen at 1L/min. The animal was removed from the chamber when loss of the postural reflexes, followed by the tail and the pedal reflex loss, were observed. After the confirmation of anaesthetic depth, the animal was transferred to a heat pad covered by a sterile waterproof pad, in order to maintain the animal's body temperature during the procedure, and the mouse's head was placed in the anaesthesia face mask. The face mask is connected to isoflurane and oxygen at 2.5% and 1L/min, respectively. In order to confirm the anaesthesia depth during the procedure, we tested the animal's reflexes by tail pinch and then pedal withdrawal pinch, which should be absent. At the end of the procedure, the animal needed to recover. As so, the isoflurane was removed (0%) and the oxygen flow was maintained. During the procedures after the anaesthesia, we always maintained the animal eyes moist (using Sicafluid).

iv. Administration of Substances

During the tutorial practice period, administration of substances by different pathways (intraperitoneal injection and subcutaneous injection) was performed.

a. Intraperitoneal Injection

This procedure was performed before surgeries, such as orthotopic injection of cancer cells in the pancreas of the animal. A combination of drugs - ketamine (Clorktam, 433382 1212) and xylazine (Baye, Rompum 2%) (100 µL/10g of a solution of 75mg/kg ketamine + 8mg/Kg xylazine) – was used, as ketamine is an NMDA (N-Methyl-D-aspartic acid) antagonist that produces dissociative anaesthesia, making it necessary to administer xylazine that has a tranquilizer effect. As we learned in the theory classes, drug combinations allow us to reduce the dose of anaesthetic administered (e.g., ketamine) and, consequently, the anaesthesia side effects. The volume of ketamine and xylazine was calculated according to the animals' weight. As so, the animals were weighted before the surgeries. To weight the animals, they should be removed from the cage as described in section i) and put in the weighting machine, inside a recipient previously disinfected with ethanol 70%.

For this procedure, we used a syringe of 1mL and a 26G needle. The administration began with the animal restraint as described in section i), slightly tilting the mice downward, in order to force the organs to move up to avoid perforating them during the procedure. To know the place in the abdominal area where we should do the injection, we need to find the point where hind paw extremity touches the mice's abdomen (right side of the abdomen). To ensure the administration is properly done, we should do 3 steps: insert the needle with the bevel up in a 45º angle, make reflux of the plunger to ensure no blood was entering (no organs were perforated) and, finally, administer the solution. After the injection, the animals were put in the cage and we evaluated the anaesthesia depth along the procedure.

During the procedures after the anaesthesia, we always maintained the animal eyes moist, using Siccafluid® 2,5 mg/g (Théa, 651516).

a1. Nexinhib20 administration

Nexinhib20 (Sigma-Aldrich, SML1919) was also administrated intraperitoneally to KPC mice, in a concentration of 20mg/kg, twice a week, until HEP. This drug impairs exosome secretion.

b. Subcutaneous Injection

Another procedure done during the tutorial practice period is subcutaneous injection of Buprenorphine (Bupaq, 60008/E), an opioid (analgesic substance) which acts at the CNS (central nervous system), allowing the reduction of pain sensation during and after the surgery. To do this administration, we grasped the mouse nape firmly, pushing the animal against the cage grid (without hurting the animal), and we administered the substance at the base of the triangle formed at the loosing skin. For this procedure, we used a syringe of 1mL and a 25G needle. To ensure the administration is properly done, we should do 3 steps: insert the needle with the bevel up, make reflux of the plunger to ensure no blood was entering (no blood vessels were perforated) and, finally, administer the solution. After the injection, the animals were put in the cage or in the heat pad, if they were already anaesthetized. The dosage of buprenorphine is 100 µL/10g of a solution at 0.08mg/Kg buprenorphine and the correct amount of substance was injected according to the drug dosage and the animal's weight. The buprenorphine was administered 30 minutes before the beginning of the procedure. Buprenorphine is administered twice a day subcutaneously, for 3 days or longer after the surgery, if mice are showing signs of discomfort.

v. Health assessment (score sheets) and monitoring of tumor volume

It is of upmost importance to monitor the animals after surgery. We monitored their health status, behaviour on the cage and tumor growth by abdominal palpation and by measuring the tumor with callipers, fulfilling a score sheet. The tumor volume was calculated using the formula ½(length x width2) and the humane endpoint (HEP) criteria was applied when the tumor volume reached a maximum of 1500mm^3. A HEP is a series of behavioural and/or pathophysiological characteristics detected in animals that indicates severe pain, distress or imminent death. HEPs should be established before the beginning of the experiment. Death is not a HEP and must be avoided at all cost.

To do the abdominal palpation, it is necessary to restrain the animal as described above and gently palp the animal's abdomen, feeling the mass at the pancreas if the tumor is developing. However, palpation should not be done until the stitches from the surgery are absorbed by the animal's organism or the staples are removed, otherwise we may open the wound. It is also important to monitor the wound to look for signs of infection (redness or pus) and to assess if the animal is not pulling the stitches. As social animals, mice should interact with cage mates. They should also be active, have a normal drinking and eating pattern, build their nests, explore, burrow, groom, have a good-looking fur, show no signs of dehydration (tested by pinching the skin over the shoulder blades – if the skin takes too long to return to the normal position, the animal is dehydrated; recessed eyes are also a sign of dehydration), have no fight wounds, etc. Those patterns should be recorded in the score sheet, which is a document that includes several parameters, which can be adapted to specific animal models and/or disease, and that allows us to score each parameter and to perform an objective animal health evaluation. Animals were also weighted. These evaluations are also important to ensure the maintenance of animal welfare.

If the animals showed signs of dehydration, a sterile saline (0.9% NaCl) solution was subcutaneously administrated with a 26G needle and a 1mL syringe as previously described, according to the animal's weight. If the animals showed signs of weakness/altered eating patterns, and were not being used in survival experiments, Anima Strath (supplement) (Bio-Strath AG, 6500280) was given orally. Some animals showed fight wounds. If not severe, we would treat the wounds and separate the animals. However, if the animals were in a critical situation, the HEP criteria would be applied.

vi. Euthanasia by cervical dislocation

An animal should be euthanized whenever we see signs that its' health and welfare are compromised or when reaching a pre-established timepoint for euthanasia (in this case, 25 weeks). When reaching the HEP, we should apply the HEP criteria and euthanize the animal. As mentioned before, the HEP established before the beginning of this tutorial included: a maximum tumor volume of 1500mm^3, along with a debilitated physiological status, severe weight loss (more than 15-20% of the initial weight), ascites, reduction of body temperature, lethargy, reduction of grooming behaviour and other abnormal physical conditions.

Once the HEP was reached, the animals were euthanized by cervical dislocation. This method of euthanasia was chosen according to future organ collection and analysis that were going to be done. There are a few steps that need to be done in order to make the euthanasia a quick, painless and effective procedure:

1. Anaesthetize the animal with isoflurane, as described above
2. Place the animal in prone position
3. Place the thumb and index finger of one hand on the neck of the animal, at the base of the skull, fixing it. With the other hand quickly pull the base of the tail. This leads to the separation of the cervical vertebrae from the skull, ceasing the animal's life
4. Confirm animal death (we confirmed it by the absence of respiratory movements and heart beat as well as feeling with our thumb and index fingers the gap in the spinal cord in the mouse's neck)

However, 1 hour before euthanising the animals, 2 solutions were administrated: FITC dextran (100uL, retro-orbital with a 300uL syringe) and Hypoxic probe (30uL/10g, intraperitoneal with a 500uL syringe; preparation: mix 50uL of NaCl + the necessary amount of hypoxic probe (60mg/Kg)). After euthanizing the animals, the organs were collected (pancreas, liver, lung, spleen, thymus, lymph nodes, brain, duodenum, kidney, ear and tail) and the animals' remains were placed in a transparent bag at -20 ºC for subsequent incineration, according to the animal facility regulation.

During the necropsy, there were taken pictures to the animal when it was closed, opened with closed peritoneum and opened with opened peritoneum in order to see the

animals' organs condition. Then, organ collection was performed. Ascites (not all animals presented ascites) collected to Eppendorf tubes and snap freeze in liquid nitrogen was done for further analysis of exosomes. Also, a piece of the tail and a piece of ear were collected to Eppendorf tubes and snap freeze in liquid nitrogen was done for genotyping purposes (in case of need to confirm the animal's genotype). The mesenteric lymph nodes, the thymus and the spleen were collected, weighted, measured with callipers and collected in formol to be included in paraffin 48h latter (HEMS, i3s, Porto). The pancreatic tumor was weighted, measured with callipers and divided into 4 portions: one portion was collected to a cassette and put in formol to be included in paraffin 48h latter (HEMS, i3s, Porto); one portion was put in paraformaldehyde 4% (PFA) (Sigma-Aldrich, 158127) to fix the cells; another portion of the tumor was divided in 2 smaller pieces, were collected into 2 Eppendorf tubes with RNA later solution: one for DNA and the other one for RNA. A portion of ear was also collected into an Eppendorf with RNA later solution (Thermo Fisher Scientific, AM7021). Finally, the last portion of the tumor was divided in 3 smaller pieces and put in 3 Eppendorf tubes: one for DNA, one for RNA and one for protein analysis and snap freeze in liquid nitrogen was done. Regarding the liver and the lungs, macro-metastasis were counted and these organs were collected and divided in 3 portions: one was collected in formol to be included in paraffin 48h latter (HEMS, i3s, Porto); one was put in PFA 4% and the other one was put in an Eppendorf and snap freeze in liquid nitrogen was done. A mix cassette was also prepared, containing the mouse's brain, one kidney and the duodenum and were put in formol to be included in paraffin 48h latter (HEMS, i3s, Porto).

Regarding tissue processing, the organs incubated in PFA 4% for 24 hours at 4ºC. on the next day, PFA 4% was discarded and the organs were washed 3 times with PBS1x for 15 minutes with agitation. The organs were incubated overnight in a solution of sucrose 30% (Sigma-Aldrich, 84097) at 4ºC and embedded in OCT (Thermo Fisher Shandon™, 6769006) on the next day and stored at -80ºC for future analysis. Finally, the organs in RNA latter were incubated at 4ºC for 24 hours and on the next day the RNA later solution was discarded, snap freeze in liquid nitrogen was done and the tubes were then stored at -80ºC.

2.12. Statistical Analysis

Statistical analysis of the results was performed using GraphPad Prism 6 Software (version 6.01). Mann-Whitney test was used to compare tumor volumes, the number of liver and lung macrometastasis, percentage of necrosis, intratumoral microvessel density between different mouse models (IHC staining for CD31), IHC staining for Ki67, Zeb1, CD4 and FoxP3, as well as the percentage of fluorescence in mice's tumors. Log-Rank (Mantel-Cox) test was used to compare survival and disease onset between different mouse models. Unpaired t test was used to access differences in T cell viability of T cells treated with cancer exosomes.

III. Results

III. Results

In order to study the impact of the immune system in PDAC progression, as well as the role of cancer exosomes in immune modulation, our lab developed a series of genetically engineered mouse models that recapitulate the histopathological characteristics of the human PDAC disease.

All procedures using mice models were approved by Direção-Geral de Alimentação e Veterinária (DGAV ref. 015225/2017-06-30), in accordance with Portuguese legislation (Decreto-lei nº 113/2013) and the i3S ethical committee. Furthermore, during my Master Thesis period I was trained and gained accredited authorization for mice handling (Functions A and D/former FELASA B accreditation, according to directive 2010/63/EU). Humane endpoints were established before the beginning of this study in order to avoid or limit pain and/or distress to the animals. Mice welfare was always assured.

1. Genetic engineered mouse models of PDAC

a. KPC mouse model

It is of great importance to study pancreatic cancer and cancer exosomes in a context that reflects the normal biological system. Therefore, the laboratory is actively generating and using the PDAC KPC (LSL-**K**ras$^{G12D/+}$; T**P**53$^{R172H/+}$; Pdx1-**C**re$^{/+}$) model that spontaneously develops PDAC, which recapitulates the clinical and histological characteristics of the human disease (189, 190). In this animal model, Cre recombinase is under the control of the mouse pancreatic-duodenal homeobox promoter (Pdx1). Kras G12D is a driver mutation gene in PDAC, as it drives neoplastic transformation (18, 191). Trp53^{R172H}, an ortholog of one of the most common TP53 mutations in human PDAC, leads to tumour progression and is one of the most prevalent mutations detected in human PDAC (189). Kras mutation can initiate PanINs, which can spontaneously progress to invasive and metastatic forms of the disease. The activation of the Kras$^{G12D/+}$ and Trp53$^{R172H/+}$ alleles are pancreas-specific, as the Cre recombinase is under the control of the Pdx promoter and are only activated at embryonic stages in the progenitor cells of the mouse pancreas in development (18, 189, 191).

The KPC mouse models present advantages over other PDAC mouse models. These animal models can live up to 52 weeks of age. Newly born KPC mice's pancreas is

histologically normal, developing precursor lesions (PanINs) only by 8 to 10 weeks of age. Most KPC mice have already developed invasive PDAC, intensely desmoplastic, at approximately 16 weeks of age, recapitulating by that time many clinical features of the human disease (ascites, bowel and biliary obstruction, jaundice and weight loss that many times leads to cachexia). Figure 10 represents the KPC disease progression. PDAC tumours can metastasize to multiple organs, including the liver, lung, peritoneum, spleen and lymph nodes. Furthermore, mice tumours express many of the immunohistochemical markers that are found in the pancreas of human PDAC patients (189), which, altogether, makes this model suitable for PDAC study.

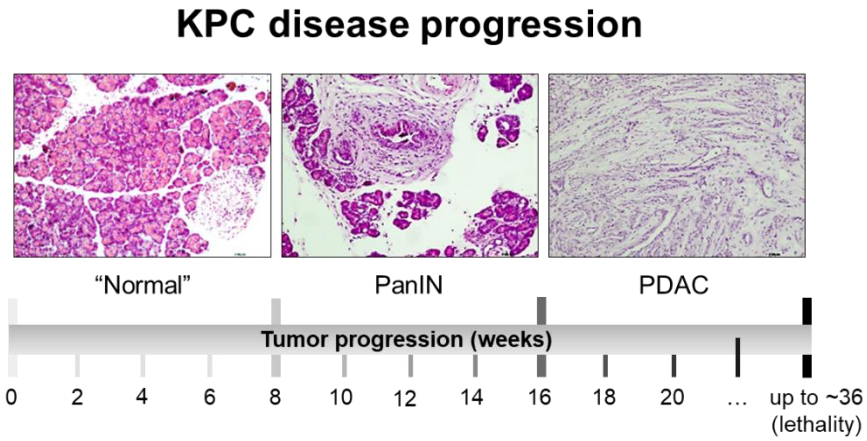


Figure 10 – KPC disease progression. Newly born KPC mice's pancreas is histologically normal. KPC mice subsequently develop precursor lesions (PanINs) by 8 to 10 weeks of age. At approximately 16 weeks of age, most KPC mice have already developed invasive PDAC, intensely desmoplastic, recapitulating by that time many clinical features of the human disease (ascites, bowel and biliary obstruction, jaundice and weight loss that many times leads to cachexia), often leading to death.

b. KPC Rag2$^{-/-}$ (first time this mouse model combination is studied in PDAC)

The KPC Rag2 KO, or KPC Rag2$^{-/-}$ (Kras$^{G12D/+}$; p53$^{R172H/+}$; Pdx1$^{Cre/+}$; Rag2$^{-/-}$), is an immunodeficient KPC mouse model that spontaneously develops PDAC. The knockout (KO) of Rag2 gene leads to a depletion of B and T lymphocytes from these animals. Rag2 gene leads to the formation of proteins called the RAG complex. The RAG complex (recombination-activating gene) has an important role at V(D)J recombination, which

allows B and T cells to acquire specific functions. The KO of Rag2 gene leads to the absence of B and T cell maturation in the thymus.

c. KPC Rag2$^{-/-}$ IL2rg$^{-/-}$ (first time this mouse model combination is studies in PDAC)

The KPC Rag2 IL2rg DKO, or KPC Rag2$^{-/-}$ IL2rg$^{-/-}$ (Kras$^{G12D/+}$; p53$^{R172H/+}$; Pdx1$^{Cre/+}$; Rag2$^{-/-}$IL2rg$^{-/-}$), spontaneously develops PDAC in an immunodeficient background without B, T and NK cells. IL2rg gene produces the common gamma chain protein, which is a component of the cytokines' receptors and immature blood-forming cells. As so, a knockout of this gene consequently leads to the lack of functional receptors for many cytokines, compromising the maturation of lymphocytes (i.e., T cells, B cells and NK cells). Together with the Rag2 KO, this mice model does not have B, T nor NK cells.

2. The Immune System is Not Blind to PDAC

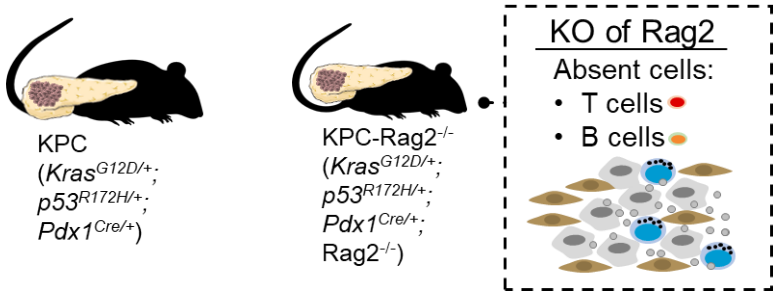
2.1. Assessment of the role of B and T cells in PDAC establishment and progression.

In order to understand how the immune system impacts disease progression (Figure 11A), we have crossed the KPC model into a Rag2$^{-/-}$ background (KPC Rag2$^{-/-}$). KPC Rag2$^{-/-}$ animals do not have T or B cells, and their controls are KPC mice. We could observe that PDAC tumours of KPC Rag2$^{-/-}$ mice grow bigger than the tumours of KPC mice during the same timeline of progression (Figures 11B and 11C). Interestingly, cancer cells in both types of tumours did not appear to have differences in proliferation, as shown by Ki67 staining (Figures 11D and 11E). This observation prompt us to study the onset of disease in the two models. We could demonstrate that KPC Rag2$^{-/-}$ mice present an earlier disease onset when compared to KPC mice (Figure 11F), explaining why at time of euthanasia immunocompromised animals present with bigger tumours, because they have been developing for longer time. This data uncovers the possibility of the role of the immune system in restraining full blown neoplastic transformation into PDAC from pre-neoplastic lesions. Once T and B cells are absent, tumours arise sooner.

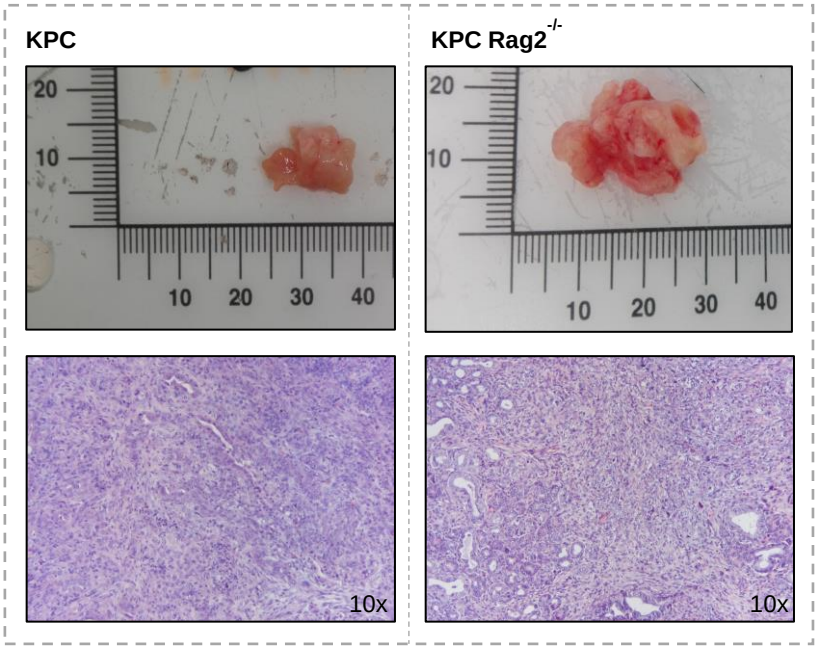
Of note, these are animal models and as such carry more heterogeneity in some of the results than when compared with groups of mice injected with cell lines. Therefore, the

data presented here needs more animals in both groups so we can confidently take conclusions.

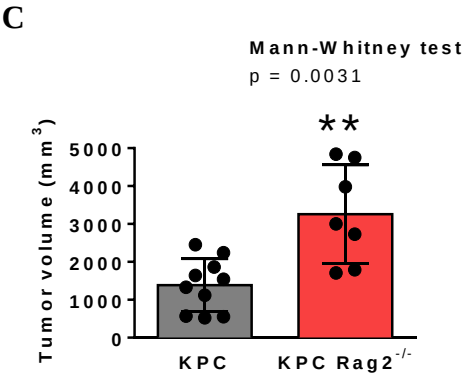
A



B



C



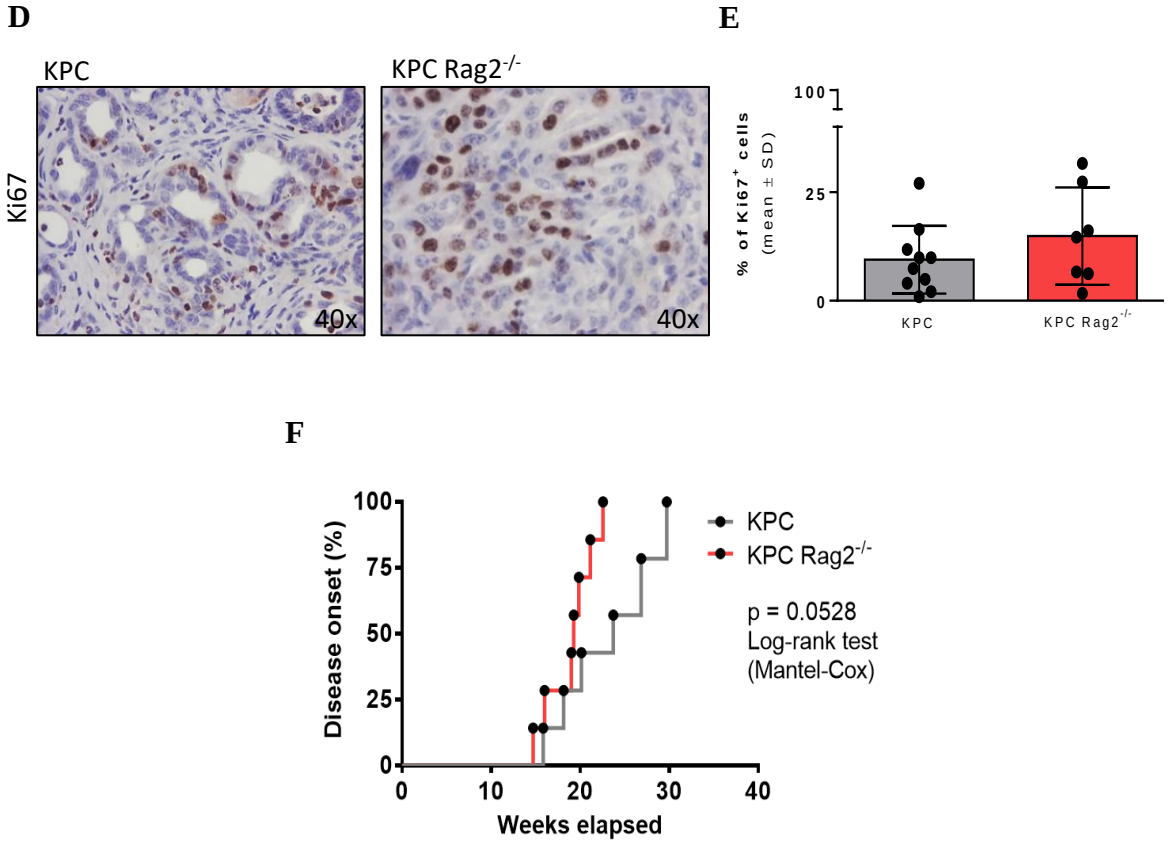
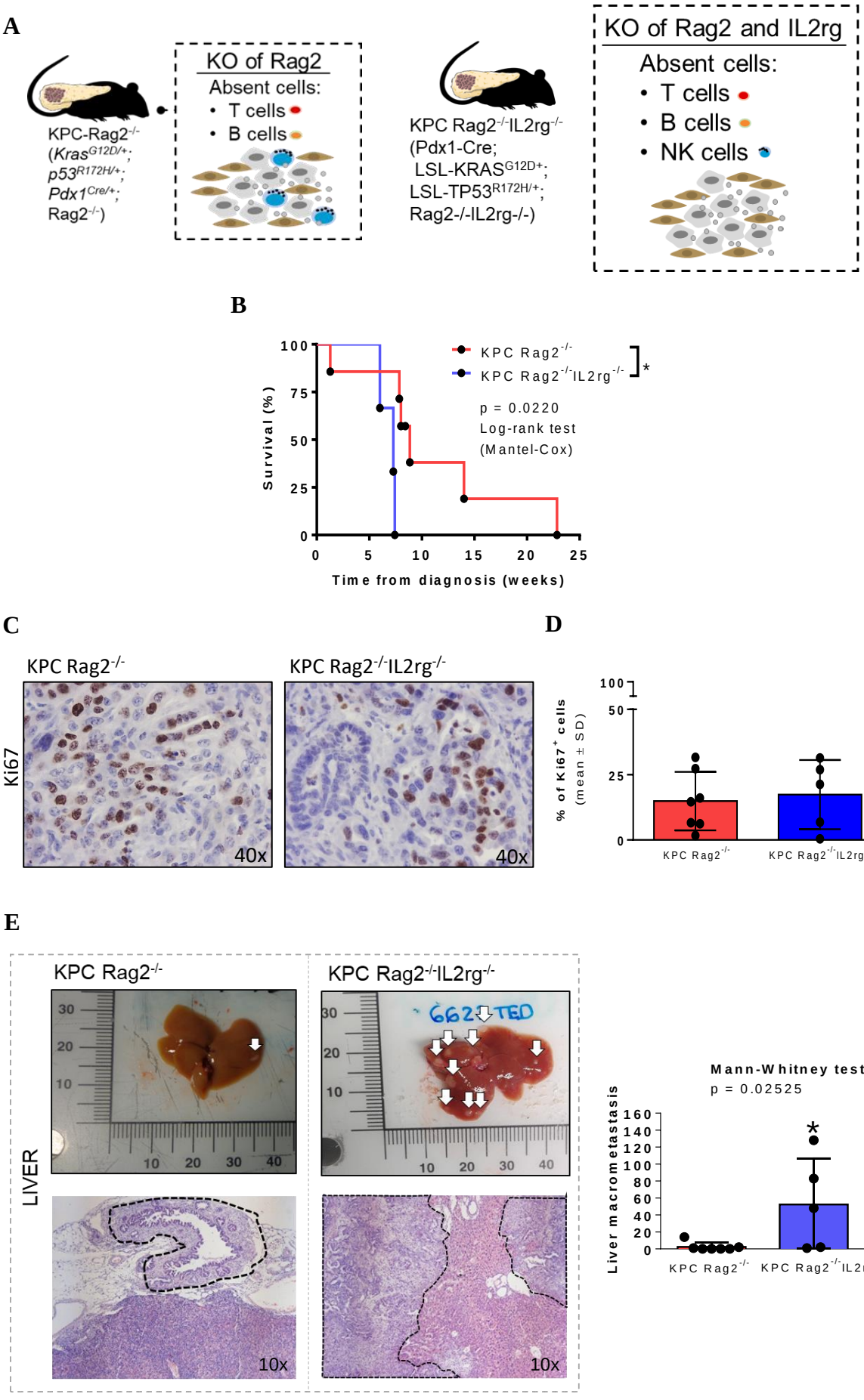


Figure 11 – Assessment of the role of B and T cells in PDAC establishment and progression. (A) Schematic representation and genotype of KPC mouse model and KPC Rag2$^{-/-}$ mouse model. (B) Representative photos of KPC and KPC Rag2$^{-/-}$ tumors and H&E stained tumors of these models. (C) Tumor volume of KPC (n=10) and KPC Rag2$^{-/-}$ (n=7) animals as measured at time of euthanasia. (D) Representative images of IHC staining for Ki67 in KPC (n=10) and KPC Rag2$^{-/-}$ (n=7) tumors, respectively. (E) Percentage of positive cells for Ki67 in KPC (n=10) and KPC Rag2$^{-/-}$ (n=7) tumors. (F) Disease onset in KPC (n=7) and KPC Rag2$^{-/-}$ (n=7) mice.

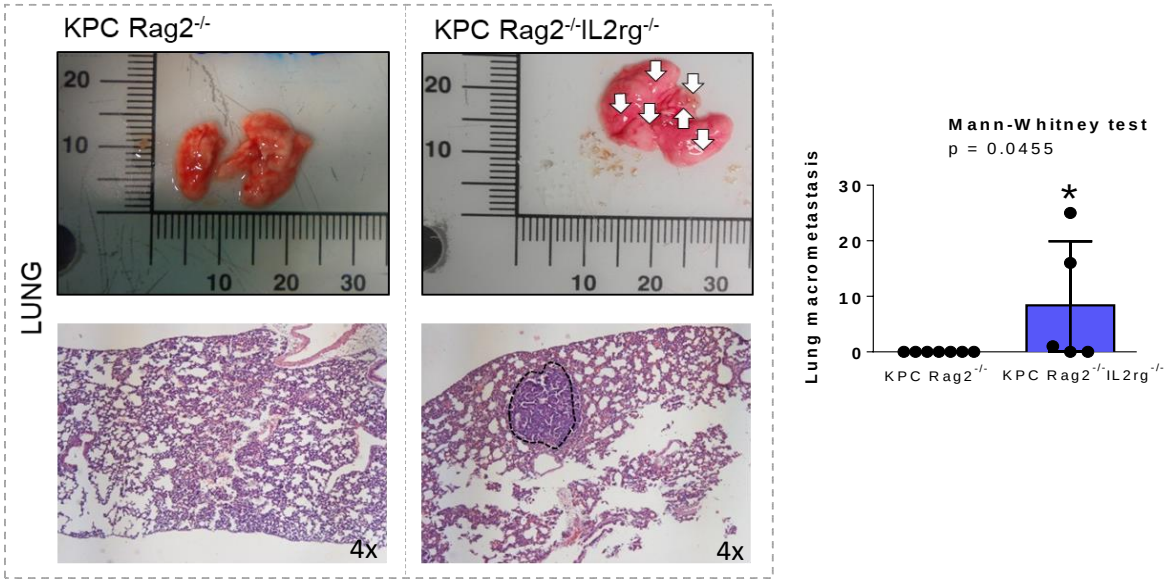
2.2. Assessment of the role of NK cells in PDAC establishment and progression.

We have used the KPC Rag2$^{-/-}$IL2rg$^{-/-}$ mouse model, and the KPC Rag2$^{-/-}$ as control, to determine if NK cells could also play a role in the anti-tumour immune response in PDAC (Figures 13A). We could demonstrate that KPC Rag2$^{-/-}$IL2rg$^{-/-}$ mice have a significantly shorter survival when compared to KPC Rag2$^{-/-}$ mice (Figure 12B). In addition, also in this case the tumours do not present distinct proliferation status as per Ki67 analysis (Figures 12C and 12D). Nonetheless, KPC Rag2$^{-/-}$IL2rg$^{-/-}$ mice develop a significantly higher number of liver and lung macrometastasis (Figures 12E and 12F, respectively) when compared to their controls (KPC Rag2$^{-/-}$), reflecting a higher disease burden in the KPC Rag2$^{-/-}$IL2rg$^{-/-}$ animals. Therefore, these results demonstrate that NK cells have an important role in PDAC metastasis.

In order to uncover the mechanism through which NK cells impair metastasis establishment in PDAC, we have looked further into the tumours in order to determine why KPC Rag2$^{-/-}$IL2rg$^{-/-}$ are more metastatic. EMT enhances cancer cells' mobility, capacity of invasion, and gives them resistance to apoptotic stimuli, being correlated with increased metastasis potential of cancer cells (192). We have started by looking at the EMT (epithelial to mesenchymal transition) marker Zeb1 (Figures 12G and 12H), but saw no significant differences between the tumours. Since we know there have been described EMT-ZEB1 independent mechanisms, we are currently looking into other markers. Furthermore, vessel density and integrity also play an important part in the metastatic capacity of tumours. Thus, we have analysed intratumoral vessel density by CD31 staining (Figures 12I). Surprisingly, KPC Rag2$^{-/-}$IL2rg$^{-/-}$ tumours present a significantly lower density of vessels (Figure 12J). This is in fact in agreement with the necrosis analysis that shows that KPC Rag2$^{-/-}$IL2rg$^{-/-}$ tumours are more necrotic (Figures 12K and 12L). This could be due to lower vessel density. We are currently looking at vessel integrity (since these animals were perfused with FITC-dextran at time of euthanasia), as well as to alternative metastatic routes present in PDAC, namely perineural invasion.

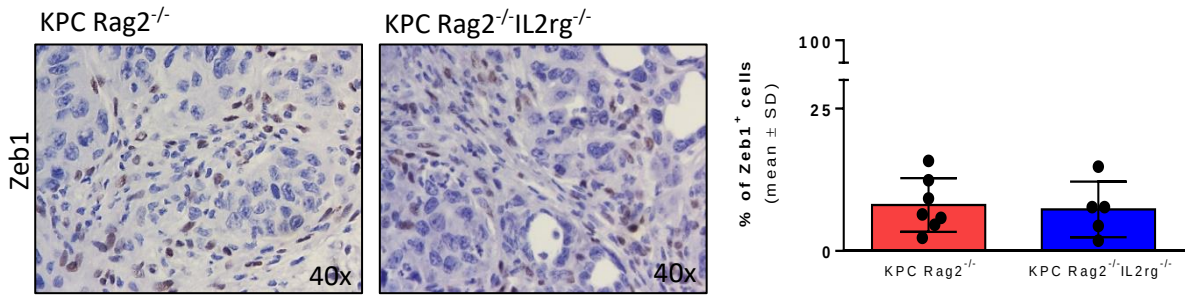

A
KPC-Rag2-/-
(KrasG12D/+;
p53R172H/+;
Pdx1Cre/+;
Rag2-/-)
KO of Rag2
Absent cells:
• T cells
• B cells
KPC Rag2-/-IL2rg-/-
(Pdx1-Cre;
LSL-KRASG12D+;
LSL-TP53R172H/+;
Rag2-/-IL2rg-/-)
KO of Rag2 and IL2rg
Absent cells:
• T cells
• B cells
• NK cells
B
KPC Rag2-/-
KPC Rag2-/-IL2rg-/-
*
p = 0.0220
Log-rank test
(Mantel-Cox)
Survival (%)
Time from diagnosis (weeks)
C
KPC Rag2-/-
KPC Rag2-/-IL2rg-/-
Ki67
40x
40x
D
% of Ki67+ cells
(mean ± SD)
KPC Rag2-/-
KPC Rag2-/-IL2rg-/-
E
KPC Rag2-/-
KPC Rag2-/-IL2rg-/-
LIVER
10x
10x
Mann-Whitney test
p = 0.02525
Liver macrometastasis
KPC Rag2-/-
KPC Rag2-/-IL2rg-/-

F


KPC Rag2-/-
KPC Rag2-/-IL2rg-/-
LUNG
20
10
10 20 30
4x
Mann-Whitney test
p = 0.0455
Lung macrometastasis
30
20
10
0
*
KPC Rag2-/-
KPC Rag2-/-IL2rg-/-

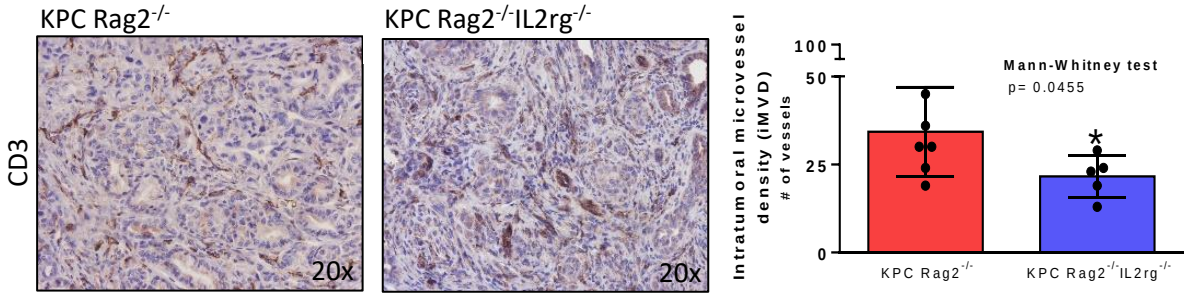
G

H


KPC Rag2-/-
KPC Rag2-/-IL2rg-/-
Zeb1
40x
40x
% of Zeb1+ cells
(mean ± SD)
100
25
0
KPC Rag2-/-
KPC Rag2-/-IL2rg-/-

I

J


KPC Rag2-/-
KPC Rag2-/-IL2rg-/-
CD3
20x
20x
Intratumoral microvessel density (iMVD)
# of vessels
100
50
25
0
Mann-Whitney test
p= 0.0455
*
KPC Rag2-/-
KPC Rag2-/-IL2rg-/-

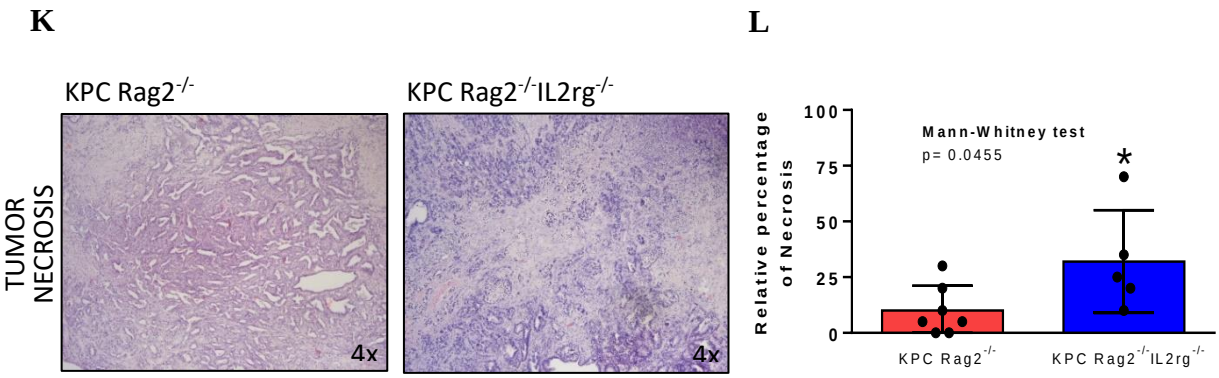


Figure 12 - Assessment of the role of NK cells in PDAC establishment and progression. (A) Schematic representation of KPC Rag2$^{-/-}$ mouse model (depleted of B and T cells) and KPC Rag2$^{-/-}$IL2rg$^{-/-}$ mouse model (depleted of B, T and Natural Killer cells). (B) Overall survival from time of diagnosis of KPC Rag2$^{-/-}$ (n=7) and KPC Rag2$^{-/-}$IL2rg$^{-/-}$ mice (n=3). (C) Representative image of IHC staining for Ki67 in KPC Rag2$^{-/-}$ (n=7) and in KPC Rag2$^{-/-}$IL2rg$^{-/-}$ (n=5) mice tumors, respectively. (D) Percentage of positive cells for Ki67 in KPC Rag2$^{-/-}$ (n=7) and in KPC Rag2$^{-/-}$ IL2rg$^{-/-}$ (n=5) mice tumors. (E) Representative image of liver metastasis, respective H&E staining and prevalence of liver metastasis observed in KPC Rag2$^{-/-}$ (n=7) and KPC Rag2$^{-/-}$IL2rg$^{-/-}$ (n=5) mice. (F) Representative image of lung metastasis, respective H&E staining and prevalence of lung metastasis observed in KPC Rag2$^{-/-}$ (n=7) and KPC Rag2$^{-/-}$IL2rg$^{-/-}$ (n=5) mice, respectively. (G) Representative image of IHC staining for Zeb1 in KPC Rag2$^{-/-}$ (n=7) and KPC Rag2$^{-/-}$IL2rg$^{-/-}$ (n=5) mice tumors, respectively. (H) Percentage of positive cells for Zeb1 in KPC Rag2$^{-/-}$ (n=7) and KPC Rag2$^{-/-}$IL2rg$^{-/-}$ (n=5) mice tumors. (I) Representative image of IHC staining for CD31 in KPC Rag2$^{-/-}$ (n=7) and KPC Rag2$^{-/-}$IL2rg$^{-/-}$ (n=5) mice tumors, respectively. (J) Intratumoral microvessel density (iMVD) in KPC Rag2$^{-/-}$ (n=7) and KPC Rag2$^{-/-}$IL2rg$^{-/-}$ (n=5) mice tumors. (K) Relative percentage of necrosis of KPC Rag2$^{-/-}$ and KPC Rag2$^{-/-}$ IL2rg$^{-/-}$ mice (histopathological score). (L) Percentage of necrosis of KPC Rag2$^{-/-}$ (n=7) and KPC Rag2$^{-/-}$ IL2rg$^{-/-}$ (n=5) mice tumors.

3. PDAC Exosomes Modulate Immune Cells

3.1. Evaluation of changes in the T cell population upon treatment with PDAC exosomes

The laboratory has data on a unique genetically engineered mouse model that spontaneously develops PDAC and secretes color-coded $CD63^{+}$ exosomes, also in a spontaneous fashion, during disease development. That work, carried out by a PhD student in the lab, clearly demonstrates *in vivo* that PDAC cells communicate with cells of the immune system by means of exosomes.

To follow up on that work, and discriminate the specific role of cancer exosomes in distinct immune cell populations, we have made *ex vivo* treatments of T cells collected from the spleen of syngeneic wild-type animals and have treated these cells with PDAC exosomes. T cells isolated from wild-type mouse's spleen were treated with exosomes from the KPC cell line, a cell line established from a tumour of a KPC mouse (Figure 13A). We have performed two biological replicates of treatments with $1x10^{11}$ exosomes/treatment, and in the first experiment we had enough cells for duplicates and in the second experiment we had enough cells for triplicates. The cells were then analysed by Flow Cytometry to evaluate T-cell subpopulations. It was possible to observe that the presence of cancer exosomes led to a significant increase in T cell viability (Figures 13B and 13C).

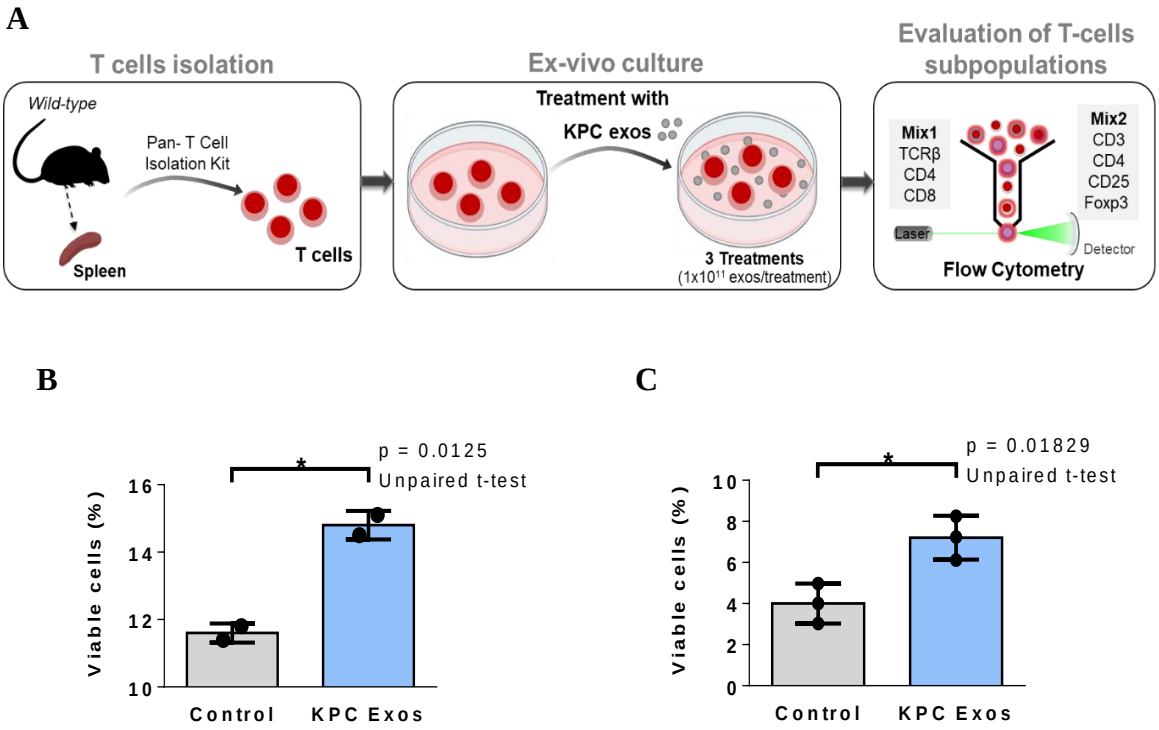
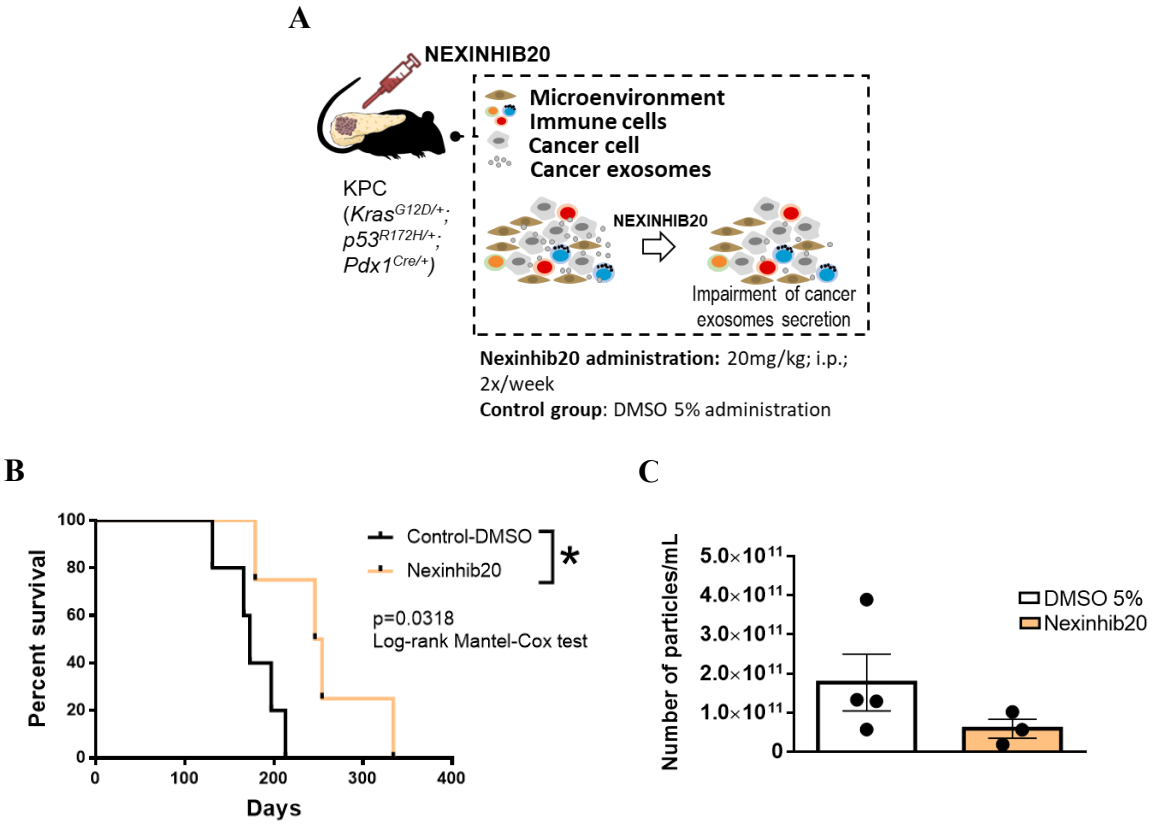


Figure 13 – Evaluation the action of PDAC exosomes on T cell population. (A) Schematic representation of murine T cells and PDAC derived exosomes isolation followed by T cells activation and exosomes treatment ex vivo. (B-D) Percentage of viable cells post-exosomes treatment at day 8 (control n=2, PDAC exosomes n=2) in (B) treatment #1 and (C) treatment #2.

4. Systemic inhibition of exosomes secretion increases survival and alters the intratumor immune landscape

With the main goal of understanding how inhibition of exosomes secretion alters survival and impacts or not the anti-tumour immune response, we have treated KPC mice with a small molecule inhibitor or Rab27a, Nexinhib20 (Figure 14A). We observed that animals treated with Nexinhib20 have a significant increase in survival when compared with the control group (vehicle – DMSO 5%; Figure 14B). The overall number of exosomes circulating in the blood of the two groups was assessed and we see a trend for a decrease in the number of exosomes in Nexinhib20 treated mice (Fig 14C). The number of mice analysed in this last experiment was smaller than the group used in the survival due to limitations of available material for analysis. We have also started to perform immune profile on these tumours, and preliminary results show that in Nexinhib20 treated animals the tumours have lower amounts of CD4+ T cells, which, according to the FoxP3 quantification, are mostly immune suppressive T cells (Figures 14D - 14G). These are still preliminary analysis, we are including more animals in the cohorts and a complete immune profile is also ongoing. Of note, these are complex models that when we achieve to have them, we still need to wait for several months to observe disease progression. To achieve these results during a specially difficult year was very significant for us.



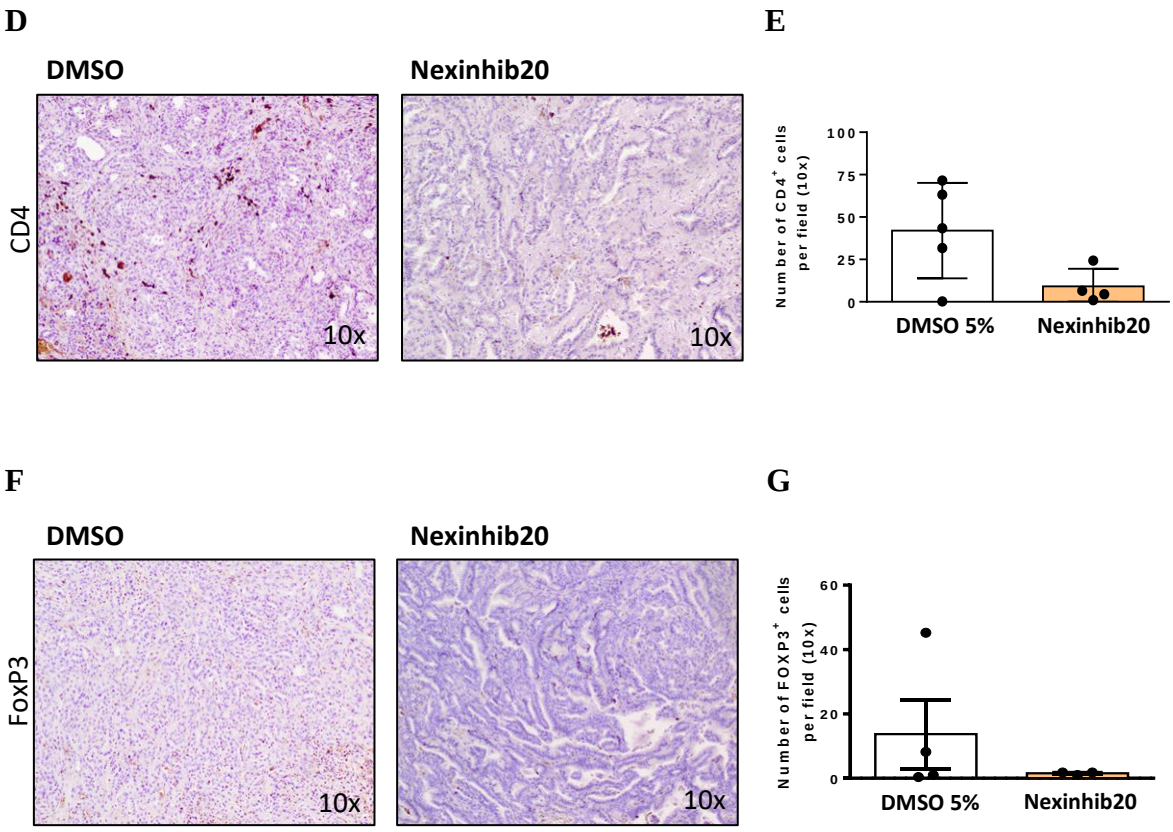


Figure 14 – Evaluation of the effects of exosomes' systemic inhibition in KPC mice survival treated with Nexinhib20 and their effects in the immune landscape. (A) Schematic representation of KPC mouse model treated with Nexinhin20. (B) Survival curve of KPC mice treated with Nexinhin20 (n=4) and with DMSO 5% (control group) (n=5). (C) Percentage of exosomes in the blood of animals treated with Nexinhib20 (n=3) and with DMSO 5% (control group) (n=4). (D) IHC staining for CD4 in KPC mice tumors treated with Nexinhib20 (n=5) and with DMSO 5% (control group) (n=4). (E) Number of positive cells for CD4 in KPC mice tumors treated with Nexinhib20 (n=5) and with DMSO 5% (control group) (n=4). (F) IHC staining for FoxP3 in KPC mice tumors treated with Nexinhib20 (n=3) and with DMSO 5% (control group) (n=4). (G) Number of positive cells for FoxP3 in KPC mice tumors treated with Nexinhib20 (n=3) and with DMSO 5% (control group) (n=4).

5. Future Perspectives

Test combination treatments of exosomes inhibition and immunotherapy (immune check point inhibitors)

KPC cancer cells were orthotopically injected into C57/BL6 wild-type animals. The aim was to follow tumour progression by ultrasound and treat groups with Nexinhib20, Vehicle (DMSO 5%), Nexinhib20 + anti-PD-L1 and anti-PD-L1, as illustrated in Figure 15. We have performed the inoculation of the cancer cells, but the experiment had to be interrupted because of the COVID-19 pandemic (animal facility required minimal services only to maintain strains).

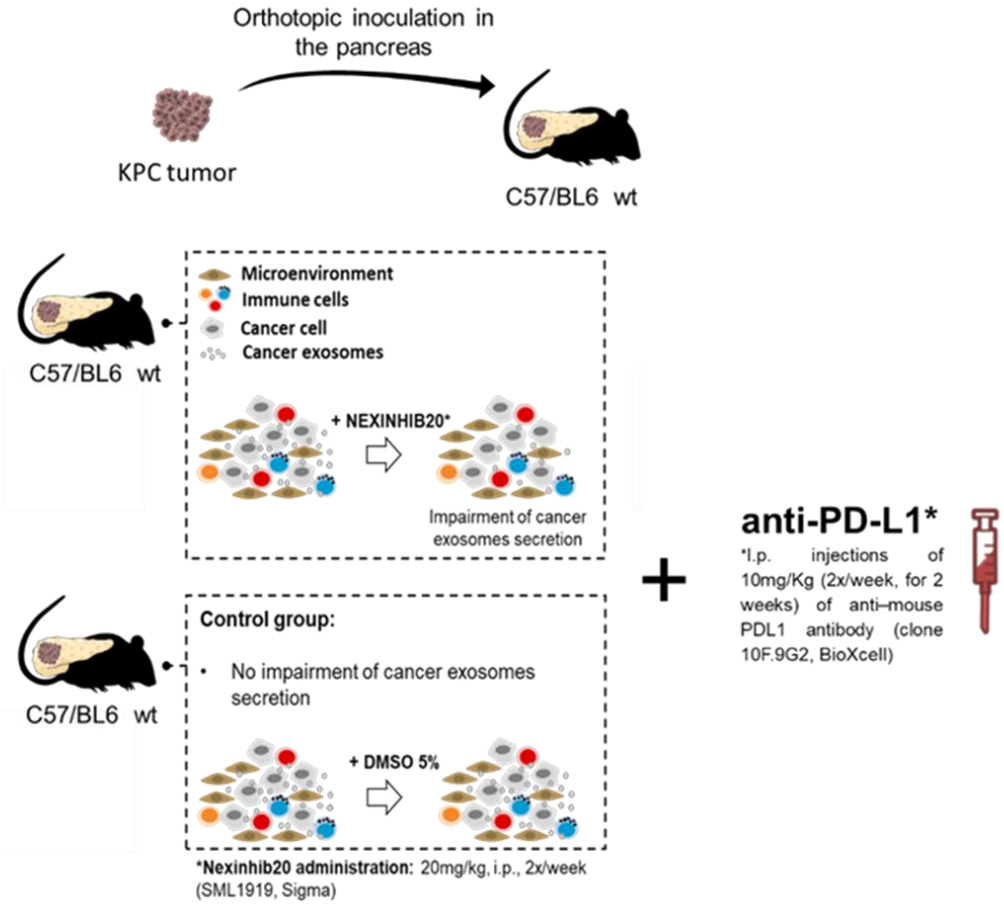


Figure 15 - Schematic representation of future administration of immunotherapy (anti-PD-L1) in animals treated with Nexinhib20, Vehicle (DMSO 5%), Nexinhib20 + anti-PD-L1 and anti-PD-L1.

IV. Discussion

IV. Discussion

Of major importance in cancer establishment and progression is the immune system, both the innate and the adaptive immune system. It is well known that the immune system is responsible for eliminating cancer cells (14). However, the role of the immune system in PDAC progression is still a field in which very little steps were given, thus this connection remains elusive. To address this question, we have delineated a series of experiments to determine if the absence of immune cells (B, T and NK cells) had an impact in PDAC progression.

We evaluated the effect of the depletion of T and B cells in PDAC establishment and progression in KPC and in KPC Rag2$^{-/-}$ mouse models, GEMMs that I performed breeding for during the course of this thesis. Compared to KPC mice, KPC Rag2$^{-/-}$ mice presented bigger tumours, most likely as a result of an earlier disease onset, which we have also demonstrated to occur upon T and B cells depletion in PDAC mice. These results suggest that T and B cells are involved in the initial phase of transformation of preneoplastic cells into malignant neoplastic cells. Since the data gathered shows that without B and T cells tumours begin to grow earlier, and grow bigger most likely because they have a longer lifespan, we can preliminarily conclude that T and B cells restrain PDAC development at the early stages of the disease. Studies show CD8+ T cells infiltrate in low-grade premalignant pancreatic lesions, but during the progression of PanINs and IPMNs, a reduction in the amount of infiltrating CD8+ T cells was observed (193). This could corroborate our findings as a parallel with the development of the disease in KPC Rag2$^{-/-}$ mice, since they do not have T cells, thus promoting the transformation of low-grade premalignant pancreatic lesions into malignant lesions. Studies also showed that in lowest grade preinvasive lesions, there is a strong infiltration of leukocytes, but the T cell infiltrate observed in PanIN lesions was mostly composed of Foxp3+ Tregs, as well as MDSCs and M2 macrophages, persisting through invasive cancer (24). Foxp3+ Tregs, MDSCs and M2 macrophages are immunosuppressive cells, thus promoting cancer progression from preneoplastic lesions to full blown PDAC. This report also showed that the amount of effector T cells infiltrating preinvasive lesions was small, being present in only a subset of advanced cancers but in an inactivated stated (24). As so, studies demonstrate that immunosuppressive cells of the host appear at the beginning of pancreatic tumorigenesis (24). Furthermore, Tregs are present in premalignant lesion of PC, and gradually increase during PC progression (from PanIN and IPMN to invasive

ductal carcinoma) (194). As T cells are important for the initiation of PDAC, and since we do the ablation of T cells, Tregs are also not present in KPC Rag2$^{-/-}$ mice. In an immunocompetent setting, if we do not have Tregs, immunosuppression through Tregs is not possible, thus the disease should progress at a slower pace. Nevertheless, in addition to Tregs, the KPC Rag2$^{-/-}$ mice do not possess effector T and B cells, and thus the effect of Tregs here is not preponderant. In fact, here we show that the complete ablation of T and B cells leads to an early onset of the disease, showing that Tregs, as well as other immunosuppressive cells, do not mediate a complete suppression of the immune system, and that effector T and B cells are important in restraining the initiation of PDAC. The role of B cells in PDAC is conflicting, as there are reports suggesting B cells have a tumour-promoting effect, whereas others suggest B cells have a tumour-protective function. In one hand, there are studies denoting the presence of B cells in the areas near the newly established neoplastic lesions, as well as in PDAC lesions (195). Compelling evidence has been provided on the role of B cells in PC initiation and development, since B cells inhibit anti-tumour immune responses (195), thus potentiating tumour development. This corroborates our results, as mice lacking B cells have an earlier disease onset and bigger tumours. On the other hand, it was shown that CD1dhiCD5+ B cells produced IL-35, which stimulates PC proliferation (195). However, our results do not go in line with these observations, as immunodeficient mice present an earlier disease onset and bigger tumours. B cells were also suggested to be able to regulate T cell immune responses, as they promote the production of effector and memory CD4^{+} T cells (196), known for combating cancer. By doing the ablation of B cells, the production of effector CD4^{+} T cells is compromised, potentiating an earlier disease onset and bigger tumour growth in immunodeficient mice.

NK cells are crucial for the immune response against cancer. In order to evaluate the effect of those immune cells in cancer establishment and progression, we used the KPC Rag2$^{-/-}$IL2rg$^{-/-}$ mouse model, which has depleted B, T and NK cells. When comparing the KPC Rag2$^{-/-}$IL2rg$^{-/-}$ with the KPC Rag2$^{-/-}$ mouse model, we could observe a significant decrease in the survival of KPC Rag2$^{-/-}$IL2rg$^{-/-}$ mice. This could be explained by the fact that these mice lack NK cells, which are known for combatting tumour cells (141), thus accelerating tumour progression and its burden, ultimately decreasing the life span to KPC Rag2$^{-/-}$IL2rg$^{-/-}$ mice. As so, we suggest that NK cells are intimately correlated with restraining the disease aggressiveness. However, when IHC staining for Ki67 (a marker

of cell proliferation (197)) was performed in these mice in order to evaluate the proliferation index of the tumour cells, no significant differences were observed. Most interestingly, it was observed that KPC $Rag2^{-/-}IL2rg^{-/-}$ mice present higher number of liver and lung macrometastasis. These results suggest that NK cells have an important role in metastasis. Our findings are corroborated by literature, which states that NK cells are crucial for metastasis control in preclinical models, in which NK cells were depleted genetically or by administration of monoclonal antibodies (198-200). In most cases, eliminating NK cells in these mice led to more aggressive tumour growth and metastasis (200). In addition, similar results were obtained in adoptive transfer models (199). NK cells were also shown to control PDAC growth (201). However, NK cells have a poor capacity of infiltrating those tumours (202, 203). Studies denote that in human tumours the NK cells that are able to infiltrate the TME present a poorly cytotoxic phenotype ($CD16^{dim}$ $CD56^{bright}$ or $CD16^{-}$ $CD56^{dim}$ NK cells), thus allowing metastasis as they are ineffective in cancer killing (204, 205). In order to uncover the mechanism through which NK cells impair metastasis establishment in PDAC, we looked further into the tumours in order to determine why KPC $Rag2^{-/-}IL2rg^{-/-}$ are more metastatic. We started by looking at EMT, as it is correlated with metastasis capacity of cancer cells, since EMT enhances cancer cells' mobility, capacity of invasion, and gives them resistance to apoptotic stimuli (192). As so, IHC staining for Zeb1 was performed in KPC $Rag2^{-/-}$ and in KPC $Rag2^{-/-}$ $IL2rg^{-/-}$ mouse models, in order to access the amount of cells undergoing epithelial to mesenchymal transition (EMT) (206). No significant results were observed. Nevertheless, even though Zeb1 is described as the most important EMT-stimulating transcription factor responsible for increasing the plasticity of PDAC cells, thus, promoting metastasis (207), it is possible that the effects observed are mediated by other EMT-associated factors such as Snail/Slug and Twist (208), which are currently being tested. Vessel density and integrity are also crucial for metastasis, as tumour cells from the primary tumour travel through the blood vessels, forming metastasis in distant organs (209). It is possible to observe a reduction in the formation of new blood vessels in the KPC $Rag2^{-/-}$ $IL2rg^{-/-}$ model, as demonstrated by the intratumoral microvessel density quantification performed using CD31 staining of tumours of these mice. This reduction of angiogenesis was previously reported in the literature as a direct effect of NK cell depletion. Studies demonstrated that NK cell depletion lead to a reduction in the formation of new blood vessels in mice (corneal model) (210). As so, the literature corroborates our results, being possible to correlate NK cell depletion with the prevalence of metastasis and, at the same

time, with the reduction of new blood vessel formation. Nonetheless, if the existing vessels are leaky because of endothelial injury or permissiveness, there will be extravasion of their content, compromising their integrity and therefore impairing metastasis (211). Consequently, it would be interesting to perform an analysis of FITC Dextran, as it will allow us to observe leakage and, luckily, shed light on why these mice present more metastasis, despite having low angiogenesis. Furthermore, the KPC $Rag2^{-/-}$ and the KPC $Rag2^{-/-}IL2rg^{-/-}$ mouse models also present different percentages of necrosis in their tumours. KPC $Rag2^{-/-}IL2rg^{-/-}$ mice present a higher necrotic percentage compared to KPC $Rag2^{-/-}$ mouse models, which goes in line with the reduced angiogenesis observed in KPC $Rag2^{-/-}IL2rg^{-/-}$ mice.

It is well known that exosomes have a significative impact in the cancer context. Preliminary data acquired in the lab demonstrated that PDAC exosomes communicate with the cells of the immune system, thus modulating the immune response. However, the effect of PDAC exosomes in the immune system is a poorly explored field. In order to evaluate the changes in the T cell population upon treatment with PDAC exosomes, T cells were treated with exosomes from a KPC cell line (pancreatic cancer cell line). It was observed an increase in T cell viability upon treatment with cancer exosomes in both experiments. As the literature suggests that exosomes modulate the immune system, having an immunosuppressive role (172, 212), it would be expected to observe a decrease in T cell viability upon treatment with cancer exosomes. However, this increase in T cell viability could mean that those are immunosuppressive T cells (Tregs), naïve T cells or even inactivated cytotoxic T cells, thus further analysis of the differentiation of those T cells treated with cancer exosomes are necessary. A study stated that Tregs-derived exosomes suppress CD8+ T cells' proliferation (213). Assuming that cancer exosomes mediate the same effects, this could indicate that the increase in viability of cells we observe is actually of the Tregs subpopulation and of inactivated cytotoxic T cells. EVs derived from pancreatic cancer cells that express SAMD4 were shown to increase Treg proliferation while decreasing the amount of CD8+ T cells (214). Studies also accessed that the interaction between exosomes altered the immune response against tumours. It is suggested that the intercellular transfer of miRNAs via Tregs-derived EVs may cause Tregs to regulate DC function, inhibiting immune reactions in the tissue (215). This could explain the increase in viability of inactivated cytotoxic T cells. In asthma, it was shown that MSC-derived exosomes promote Tregs proliferation through the upregulation of IL-

10 and TGF-β1 (216). Furthermore, even though we observe an increase in T cell viability *ex vivo*, this result does not give a clear indication regarding the effect of exosomes on T cells recruitment to the tumour site, which can only be tested *in vivo*.

As so, aiming to fill in those gaps and to understand how inhibition of exosomes secretion alters survival and if it impacts the anti-tumour immune response, we treated KPC mice with Nexinhib20, thus impairing exosomes secretion systemically in an *in vivo* model of PDAC. We could demonstrate that Nexinhib20 treated animals live significantly longer when compared to the control group. This increase in survival could be explained by the fact that exosomes have an immunomodulatory potential, altering the immune landscape (156, 161, 166), thus potentiating the establishment of an immunosuppressive microenvironment, in this way promoting PDAC progression (63). Once exosomes are inhibited, the immune system can function properly and efficiently combat PDAC (133, 134), which confers the mice a bigger survival. Evaluation of changes in CD4+ T cells and FoxP3+ cells was also performed, as exosomes have an immunomodulatory potential, altering the immune landscape (156, 161, 166). In the cohort of KPC animals treated with Nexinhib20, it was observed that treated mice (mice with impaired exosomes release) presented a slight reduction in CD4+ T cells, as well as in FoxP3+ cells, possibly indicating that those CD4+ T cells were FoxP3 cells, which have an immunosuppressive effect. This hypothesis is corroborated by literature, which shows that the T cell infiltrate observed in PanIN lesions was mostly composed of Foxp3+ Tregs (24), and that Tregs gradually increase during PC progression (from PanIN and IPMN to invasive ductal carcinoma) (194). These findings suggest that exosomes have an immunomodulatory potential, which has already been reported in the literature but never demonstrated in vivo (156, 161, 166).

Based on these results, our future perspectives include analysing vessel leakage in KPC $Rag2^{-/-}$ and in KPC $Rag2^{-/-}IL2rg^{-/-}$ mice to better understand the metastasis process in these animals. Moreover, we plan to treat C57/Bl6 wt mice inoculated with PDAC cells from KPC mice with immunotherapy (anti-PD-L1), along with Nexinhib20 administration to observe the effects of exosomes secretion impairment combined with immunotherapy in the immune response of these mice against PDAC. Tumour progression will be monitored by ultrasound 4 experimental groups will be formed: animals treated with Nexinhib20, with vehicle (DMSO 5%), with Nexinhib20 + anti-PD-L1 and with anti-PD-L1.

V. Conclusion and Future Perspectives

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The data gathered in this thesis has made an important contribution to determine that B and T cells have an important role in restraining disease progression at early pre neoplastic stages of PDAC. We have also demonstrated that NK cells are important cells combating metastasis in PDAC. These are seminal findings since PDAC tumours are thought to be cold tumours, meaning that it is believed that the anti-tumour immune response is absent in PDAC tumours. This is one of the main reasons why it is thought that immunotherapy has not worked in PDAC patients until now. We have demonstrated clearly that the immune system is not blind to PDAC and that distinct types of immune cells play central roles in restraining disease onset and progression.

On the other hand, we have also demonstrated *ex vivo* that cancer exosomes can modulate immune cells, and most importantly that inhibition of exosomes secretion in a systemic manner impairs disease progression increasing the survival of treated animals.

Together, we have demonstrated that there is a potential to test the application of immunotherapeutic strategies to PDAC in combination with inhibition of exosomes secretion.

VI. References

VI. References

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