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A study on tumor microenvironment: from metabolism to immunotherapy

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1. Introduction

Cancer is the one of the main causes of death worldwide second only to the cardiovascular diseases. Cancer is a disease that has become established in both developed and developing countries and is due to the continued growth and aging of the population but also to the increase in risk factors to which the population is subjected such as smoking, physical inactivity, economic development, and being overweight. Lung cancer appears to be the leading cause of death worldwide among men while breast cancer is the most prominent in women. Although statistics show that the incidence of cancer is much more abundant in developed rather than developing countries, the mortality rate is not much different. This disparity is due to the fact that in developed countries there is much more information about risk factors and preventive screening and treatment are possible.[1]

In the contest of tumor microenvironment, I carried out two different projects:

Project 1: Targeting of tumor cells mediated by custom antigen transfer as a novel approach in immunotherapy.

Project 2: Role of bisphosphoglycerate mutase (BPGM) in cells proliferation.

1.1. Tumor microenvironment

Tumor formation and progression is a complex mechanism influenced by genetic/epigenetic changes in the tumor cells and in the rearrangement of tumor microenvironment [2]. Cancer metabolism has been extensively studied over the past two decades and it is well accepted that oncogenic transformation led to a well characterized metabolic phenotype that influence the tumor microenvironment [3]. During cancer progression, cancer cells, the heart of TME [2], co-evolve with the surrounding environment thanks to an active crosstalk promoting tumor growth and development [4]. Apart from malignant cells, the TME contains immune cells, as well as fibroblasts, pericytes and sometimes adipocytes [5]. The tumor microenvironment (TME) play a relevant role in tumor differentiation, epigenetics, dissemination, and immune evasion. In fact, the TME is a highly heterogeneous contest consisting of different cell types that contribute to the production and release of many abundant molecules [6].

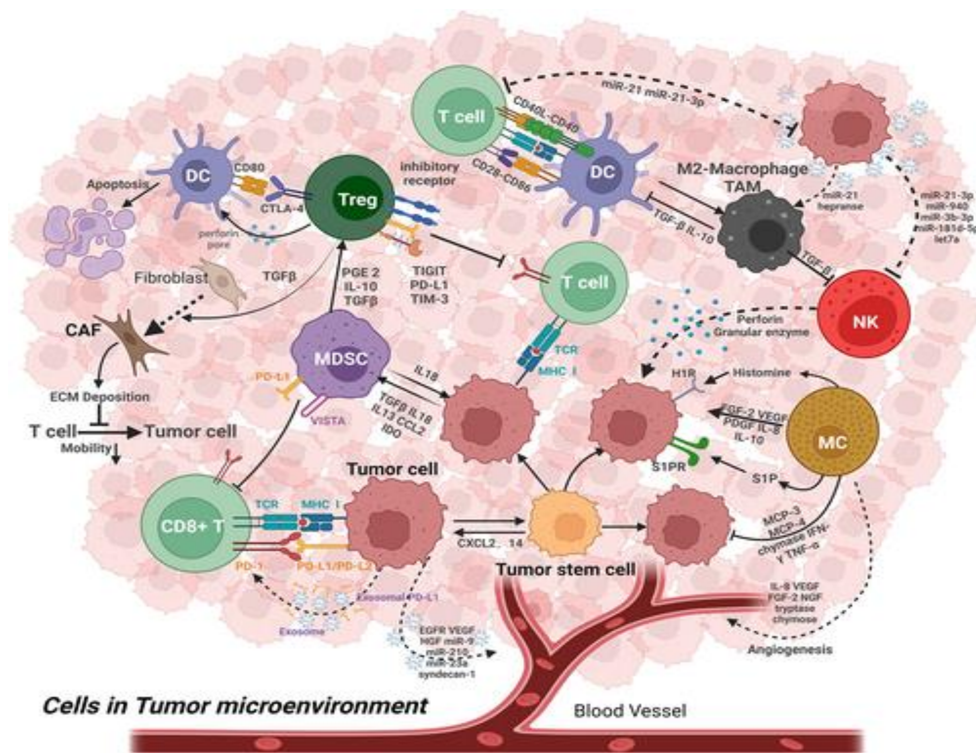

Fig 1. Structure and cellular component in tumor microenvironment [7].

It becomes clear that tumor growth is not an autonomous process but rather a complex and intricate interplay between tumor cells and their surrounding cells [8].

The acquisition and maintenance of the cancer hallmarks such as, proliferative signaling, resisting cell death, angiogenesis, invasion and metastasis, immune system evasion, depend on the contribution of TME. Given the role of TME in tumor developing, studies focused on the production of drug against the components of TME that result better targetable respect to cancer cells, characterized by drug resistance due to their highly genetic drift [9].

1.2. Tumor microenvironment component

The composition of tumor microenvironment differs in different tumor type, but it always has some constant components that comprise cellular and non-cellular component that together act as main characters in tumor onset and progression. Indeed, during early stage of tumor growth, the interaction between cancer cells and the component of TME support cancer cell survival, local invasion, and metastatic dissemination [10].

1.2.1. Immune cells

Innate immune response in TME play a pivotal role in controlling T cells fate but also sculpting the environment. Under the influence of cytokines and chemokines, immune cells modulate their function favoring tumor growth [11]. According to several studies we can assert the presence of two different population of immune cells in tumor microenvironment, tumor antagonizing and tumor promoting immune cells [12].

1.2.1.1. T cells

The presence of T cells in TME has been long studied. T cells in primary niche is correlated to a good prognosis. The presence of CD3, CD8, as well as a high CD8/FoxP3 ration, a population of CD8+ expressing FoxP3, had a positive effect on survival [13]. Actually, it is not very clear why some tumors have more infiltrating T cells than others. A study of Robert Schreiber suggested “Three Es of cancer immunoediting” explaining that the interplay between tumor and the immune system

goes through three phases characterized by immune-elimination of cancer cells, an equilibrium between cancer cells and cells of the immune system and immune escape by cancer cells [14].

CD4+ T cells may indeed exert anticancer actions by providing help to CD8+ T cells or, in some cases, by direct recognition of endogenously processed antigens presented on the surface of cancers, triggering the development of a strong immune response, or direct tumor killing [14].

1.2.1.2. B cells

TLS (tertiary limphoyd structure) are ectopic aggregates containing stromal and immune cell and also B cells. These structures originate in peripheral tissue as consequence of long-lasting exposure to inflammatory signals. TLS are areas of intense antigen presentation and where B cells can contribute to T cells activation and to the generation of effector B cells that can subsequently differentiate into antibody-releasing cells or memory B cells as well [15]. Antigen-specific interactions between T cells and B cells in the TLS and in less organized clusters of tumor-infiltrating lymphocytes seem to be crucial, and the protective role of T cells in the tumor microenvironment often depends on cooperation with B cells. In both the serum and the tumor microenvironment, antibodies are frequently found that recognize a broad array of tumor-expressed and self-antigens. If plasma cells in tumor-associated TLS produce high levels of tumor-specific antibodies in situ, these antibodies can also be detected in serum [16].

1.2.1.3. NK cells

Natural killer can be divided in CD56 bright that secrete cytokines and CD56 dim that mediate cytotoxicity. During the early stage of tumorigenesis NK are the first line of defense against tumor. Down expression of MHC-I (major histocompatibility complex class I) molecules on tumor cell surface prevent the binding of NK to killer inhibitory receptor that inhibit the initiation of killing signal [17].

Natural killer (NK) cells are belong to the innate immune system and they can be activated by interleukin (IL)-12, interferon (IFN)-α and -β, IL15, or IL-2. NK cells represent the first line of defense against infected or transformed cells. They are highly

cytotoxic, express many activating and inhibitory receptors and secrete cytokines and chemokines including tumor necrosis factor α(TNF-α), interferon γ (IFNγ), C-C motif chemokine ligand 3 (CCL3) and granulocyte–macrophage colony-stimulating factor (GM-CSF), allowing the attraction and interaction with other immune cells. The current dogma is that NK cells act early in the antitumor immune response by controlling tumor burden and stimulating adaptive T cell immune responses thereby restraining cancer cell metastasis [18].

1.2.1.4. Macrophages

Macrophages are a type of white blood cells belonging to mononuclear phagocyte immune system that play important roles in anti-infective immunity, the maintenance of tissue homeostasis, and the protection of our body thanks to their functions of engulfing and digesting foreign substances. Monocytic series in the blood are recruited in TME due to the presence of chemokines, cytokines, and other factors secreted by tumor cells, mesenchymal cells, and immune cells. The presence of local anoxia, inflammation, and high levels of lactic acid led them to become tumor-associated macrophages (TAMs). Cytokines such as CCL2, CCL11, CCL16, and CCL21 are major determinants of macrophage infiltration and angiogenesis and it has been demonstrated to function in the cancer of breast, lung, esophagus, ovary and cervix; particularly CCL2 primarily contributes to the recruitment of macrophages [19].

Monocytes derived macrophages can be polarized in two main different populations, one called classically activated macrophages (M1) and another called alternatively activated macrophages (M2), both of which can transform into each other with the changes in the internal environment [19]. M1 macrophages are characterized by the production of pro-inflammatory cytokines like TNF-α, IL-1β, IL-6 and IL-12 and for this reason are considered anti-tumor [20]. They also produce high levels of ROS and nitrogen reactive species. M2 polarized macrophages are instead considered pro-tumorigenic. M2 macrophage polarization can be induced by different stimuli: IL-4 and/or IL-13, immune complexes and toll-like receptor, IL-1 receptor ligands or IL-10 [21]. In the context of TME, immunosuppressive M2 macrophages are the main population due to the cytokines released and are considered TAM (tumor associated macrophages) in a narrow sense. Macrophages can be further classified based on the inducers molecule: IFN-γ, and LPS for M1; IL-4, IL-10, IL-13 for M2a; TLR agonists

for M2b; IL-10, TNF-α, and glucocorticoids for M2c; and TLR and adenosine A2A receptor for M2d.

1.2.1.5. Dendritic cells

Dendritic cells are considered the most efficient antigen presenting cells (APC) and they bridge the gap between innate and adaptive immune response. DCs produce inflammatory cytokines and grow factors and they can also migrate to secondary lymphoid organs to stimulate T cells. DCs can be found in two different states of immature and mature cells but the ability to trigger antigen-specific naïve T cells is a characteristic of only mature DCs [22]. They are functionally classified in different subtypes like classical DCs (cDCs), plasmacytoid DCs (pDCs), and monocyte derived inflammatory DCs (moDCs). cDC1s are able to cross present antigens thus they can present exogenous and endogenous antigens while cDC2s are able to only present exogenous antigens. In the context of TME, DCs can be divided in immunogenic and tolerogenic DCs cells. The latter are the most abundant in TME because they encounter vascular endothelial growth factor (VEGF), IL-10, transforming growth factor beta (TGF-β), prostaglandin E2 (PGE2), and other cytokines that inhibit the differentiation in immunogenic DCs favoring the other phenotype. Removing DCs from TME they regain the capability to prime T cells.

1.2.2. Stromal cells

Cancer cells recruit different type of cells from the neighbor normal tissue stroma that then start to secrete soluble factors promoting tumor growth and proliferation.

1.2.2.1. Cancer associated fibroblasts

CAFs are a sub-population of fibroblast with myofibroblast phenotype. They are normally involved in wound repair but in the context of tumor microenvironment they remain perpetually activated [23]. Cancer associated fibroblast derive from resident fibroblast that became activated in response to cytokines chemokines and other factor release within tumor microenvironment. If the activation process occurs after the initial insult, CAFs have an important impact in tumor progression due to the release of chemokines, cytokines extracellular vesicles that can induce immune evasion and drug

resistance but they can also synthesize and remodel extracellular matrix stimulating tumor invasion and metastasis due to some component of ECM such as collagen and MMP that can favor the epithelial mesenchymal transition [10].

They may also originate from bone marrow-derived mesenchymal cells or other epithelial or endothelial cells as result of epithelial-mesenchymal transition (EMT) or endothelial-mesenchymal transition (EndMT). As the physiological role of fibroblast is the structural support of connective tissues, in TME they act supporting the tumor growth as well synthesizing and releasing extracellular matrix, metalloproteinase and heparinase to remodel extracellular matrix [24].

CAF are characterized by a variety of surface marker, the most important are alpha smooth muscle actin (α-SMA), fibroblast activation protein (FAP), fibroblast specific protein 1 (FSP1), platelet-derived growth factor receptor (PDGFR)-α/β and vimentin [25].

1.2.2.2. Endothelial cells

Vascular endothelium is a thin monolayer of endothelial cells and help to orchestrate the formation of blood vessels [10]. Blood vessels in tumor microenvironment are structurally and functionally different compared to the normal vessels giving rise to hypoxia areas that can induce genetic modification in tumor cells. Angiogenesis is one of the cancer hallmarks and play a crucial role in growth and progression of tumor. Different cells in TME release factors that promote tumor growth such as fibroblast grow factor (FGF), platlets derived grow factor (PDGF), transforming grow factor alpha (TGF alfa) but VEGF (vasculare endothelial grow factor) is considered the most prototypical angiogenic factor in malignancy and a prognostic factor for poor outcome [24].

1.2.2.3. Adipocites

Adipocytes are the cells composing the adipose tissue. They are specialized cells that regulate the energy balance and store the excess energy as fat. In tumor microenvironment they secrete metabolites, enzymes, hormones, growth factors and cytokines that support tumor growth. Particularly in breast tissue the concentration of adipocytes is very high, and it is reasonable that in breast cancer, adipocytes play a pivotal role. Cancer cells release factors inducing lipid lysis, breaking down the lipid

storage and increasing the circulation of free fatty acids that can be taken up from cancer cells. Breast cancer cells use free fatty acids for energy production, cell membrane formation, lipid bioactive molecules and exosomes [10].

1.2.2.4. Stellate cells

Stellate cells are mesenchymal-derived quiescent cells located in the liver and pancreas and they have a role in the modification of ECM in tumors that affect these two organs. They can be activated with the release of pro angiogenetic factors, MMP, ECM proteins and degradation enzymes supporting tumor growth [10].

1.2.3. Non cellular component

1.2.3.1. Extracellular matrix

Extracellular matrix is a major component of tumor microenvironment, ECM constitutes up to 60% of the mass of solid tumors. It is highly dynamic and represent a physical scaffold and biochemical niche for all the different type of cells present in TME. Many cells within TME secrete components of ECM although CAF are the major source32810447. Tumor ECM is characterized by an altered balance between secretion and synthesis and altered expression of matrix remodeling enzymes making the matrix stiffer respect to the normal counterpart [26].

1.2.3.2. Extracellular vesicles

Extracellular vesicles are membrane bilayer vesicles released in TME and contribute to the cross talk between cancer cells and stromal cells. The composition of EVs reflects the make-up of origin cells. They also take part to tumor managing and drug resistance. EVs transport bioactive compounds that can affect the phenotype and features of recipient cells [27]. In TME the vesicles support tumor growth, provide nutrient and help to elude immune response.

1.3. Tumor microenvironment metabolic alteration

Metabolic alterations are considered a hallmark of cancer cells. Otto Warburg noticed that also in presence of oxygen, tumor cells increased glucose uptake with lactate formation, hypothesizing that this metabolic rearrangement was due to an impairment of the mitochondrial oxidative phosphorylation. However now it is known that aerobic glycolysis is performed also by cancer cells with functional mitochondrial machinery and is due to overexpression of glucose transporters, overexpression of key glycolytic enzymes, altered growth factor signaling, and hypoxic or normoxic hypoxia-inducible factor (HIF)-1α transcription activation [28].

1.3.1. Oncometabolites

Epigenetic dysregulation, one of the main causes of tumorigenesis, is due to the presence of oncometabolites that alter the expression of genes involved in the acquisition of malignant features. The loss-of-function mutation affecting the enzymes of TCA cycle produce an accumulation of metabolites making them act in a pro-oncogenic way.

1.3.1.1. Lactate

Lactate produced by glycolysis can directly or indirectly induce angiogenesis. In endothelial cells, lactate molecules activate nuclear factor kappa B/interleukin 8 pathway, stimulating the migration of endothelial cells and the formation of new vessels. lactate also contributes to the stabilization of HIF1a and the consequent activation of the VEGF factor leading to the activation of the NFkb pathway. Furthermore, the stabilization of HIF1a also activates the carbonic anhydrase 9 (CA9) gene which encodes the transmembrane protein carbonic anhydrases (CAIX), responsible for maintaining an acidic pH in the tumor tissue.

Some in vitro studies have also shown that lactate stimulates the pathway involving ERK1/2, increasing proliferation, migration, and angiogenesis. Furthermore, the activation of ERK and STAT1 leads to the polarization of monocytes towards a pro-angiogenic TAMs phenotype. The presence of VEGF in the tumor microenvironment, in addition to contributing to neovascularization, contributes to immune escape by

preventing the differentiation of dendritic cells and therefore antigen presentation. VEGF also increases PD-L1 expression on the surface of dendritic cells, which leads to a reduction in T-cell cytotoxicity as a result of apoptosis via interactions between PD-L1 and PD-1. It also inhibits the differentiation of progenitor cells into CD4+ and CD8+ and increases the apoptosis of NK cells due to the acidification of the microenvironment and the consequent damage to mitochondrial enzymes [29].

1.3.1.2. Glutamine

Glutamine is a donor of intermediates for the production of lipids, hexoamines, amino acids and glutathione the main scavenger of ROS in cells.

Exogenous glutamine enters cells via the SLC1A5 membrane transporter and is converted to glutamate and ammonia in the mitochondria by glutaminase 1 or 2 enzymes. Glutamate subsequently enters the TCA cycle and is ultimately converted to lactate. The stabilization of HIF causes the TCA cycle to be fueled by glutamate and not glucose. In tumor cells that use glutamine as an energy source, α-ketoglutarate derived from glutamate can be directly integrated into the TCA cycle or following the carboxylation of isocitrate. Part of the ketoglutarate can be converted into malonate which crosses the mitochondrial membrane and can be converted into lactate thanks to lactate dehydrogenase [29].

1.3.1.3. Succinate

Succinate can accumulate for various causes: the low expression of succinate dehydrogenase in tumor cells or its downregulation and inhibition by the mitochondrial chaperone TRAP1 and nuclear ARRB1 triggering a neoplastic growth. Succinate promotes tumor growth by stabilizing HIF1a and stimulate angiogenesis through ERK and STAT3 activation. Taken together these findings implicate succinate as a driver in tumor formation and progression [30].

1.3.1.4. Fumarate

Fumarate is another important metabolite produced in the TCA cycle, formed from succinate by fumarate hydratase. Dysfunctions of fumarate hydratase are closely linked to the onset of various types of tumors and the appearance of compensatory metabolisms with an increase in aerobic glycolysis and lactate production. The

accumulation of fumarate also causes an increase in ROS in tumors, with increased production of lactate by CAF despite the upregulation of antioxidant genes.

As well as the other oncometabolites, fumarate is involved in angiogenesis and immune response alteration.

Indeed, it directly stimulate angiogenesis stabilizing HIF1a and HIF2a through the inhibition of PDHs and the consequently activation of HIF-dependent genes such as VEGF and GLUT1 but also increasing the expression of VEGFA and BCL2.

Regarding the immune regulation, there are limited data about the consequence of accumulation of fumarate in tumors. Some studies demonstrated that in multiple sclerosis and psoriasis fumarate behave as inflammatory regulator downregulating T cells and B cells response. For these reasons it is possible that the same phenomenon occurs also in cancer cells [29].

Fig 2. Contribution of oncometabolites in angiogenesis. [29]

1.3.2. Metabolic enzymes

Some metabolic enzymes such as fructose-bisphosphatase 1 (FBP1), pyruvate kinase M2 (PKM2) and malate dehydrogenase 1 (MDH1) alongside their canonical function of Biochemical pathway regulation, have a non-canonical function affecting gene transcription, apoptosis and cell cycle. FBP1 act as tumor suppressor but in some cancer type is downregulated thanks to the release of the aberrant micro RNAs, miR-21, that targets FBP1. With this mechanism tumor cells have a metabolic advantage because it promotes glycolysis respect to oxidative phosphorylation.

Other enzymes are represented by PKM2 and MHD1. Pyruvate kinase catalyze the final step of glycolysis converting phosphoenolpyruvate to pyruvate. There are four isoforms, one of them PKM2, increasingly expressed in cancer cells, is involved in tumor progression. Due to its reduced enzymatic activity, the phosphorylated metabolites upstream of pyruvate in the glycolysis are addressed into different anabolic pathway to produce glycogen, triglycerides, phospholipids, nucleotides, and amino acids. Furthermore, cancer cells show a truncated TCA cycle, releasing AcetilCoA in the cytosol where it is utilized as precursor of fatty acids, cholesterol, and isoprenoids that useful for cell proliferation and growth [31]. PKM2 can also has kinase activity. For instance, during oxidative stress, PKM2, translocate into the mitochondria and it stabilize by phosphorylation the protein Bcl2, leading to anti-apoptotic signaling. PKM2 is also involved in the phosphorylation of SNARE proteins, impairing vesicular trafficking such as the release of exosomes, where the protein is present, suggesting that it can be delivered from tumor cells to the other cells in TME contributing to their reprogramming.

The other protein MHD1, in its canonical function converts malate in oxalacetate but in its nuclear form binds and stabilize in its transcriptional activity the protein p53 [32].

1.3.3. Metabolic pathway

Another feature typical of many cancers type is the aberrant activation of the (PI3K)-AKT signaling pathway and of mTORC1. The latter, a multi-subunit serine-threonine kinase, play a pivotal role in tumorigenesis; its physiological role is to control cell growth and metabolism by converting cues provided by nutrients to growth factors

signals derived from the frequently mutated (PI3K)-AKT signaling pathway and MAPK pathway. The activation of mTORC1 is due to an aberrant AKT-dependent phosphorylation of the regulator TSC2. In normal condition this protein promote GTPase activity of Rheb, that inhibit the activity of mTORC1. The phosphorylation, triggered by various growth factors, lead to the activation of both Rheb and mTORC1.

1.3.4. Non tumor cells metabolic reprogramming

Metabolic reprogramming doesn't occur only in cancer cells but rather is a process that occur also in the neighboring cells in TME. Indeed, cancer has to be considered multicellular organ depending for survival, metastasis and growth on signaling derived from the surrounding stroma and infiltrating non tumor cells.

Cancer associated fibroblasts (CAFs) are the most abundant cells in TME, representing in some cancer type up to 80% of tumor mass. The metabolic switch into a more glycolytic phenotype led to the production of high energy metabolites that fuel tumor cells. The acidification of TME and the release of ROS contribute to the metabolic reprogramming of the surrounding fibroblast and their differentiation in CAF. They release cytokines and grow factors and secrete extracellular matrix proteins. The matrix is stiffer than the normal matrix due to the release by the recruited immune cells, of extracellular matrix remodeling factor, increasing interstitial pressure, and hindering the normal activity of vascular system. Furthermore, the cytokines and grow factors impair the development of blood vessels with reduced delivery of nutrients and removal of waste products as consequence. In addition to nutrient and waste, the presence of leaky vessels limits gas exchange creating hypoxia regions. The hypoxic condition led to an increase in glycolytic activity and lactate deposition with increased environmental acidification and creating a tumor fertilized mieleu and immunosuppressive environment [33].

The alteration of immune function is also consequence of accumulation of oncometabolites but also external metabolites, such as LPA, in TME. It has been well demonstrated that the concentration of LPA is markedly higher in TME than in normal tissue. The major source of this metabolite are cancer associated adipocytes and tumor associated macrophages. LPA is involved in cell proliferation and migration, in the induction of aerobic glycolysis in the stimulation of pro-tumorigenic feature of TAM and in the impairment of t lymphocytes suppressing their immune activity [28].

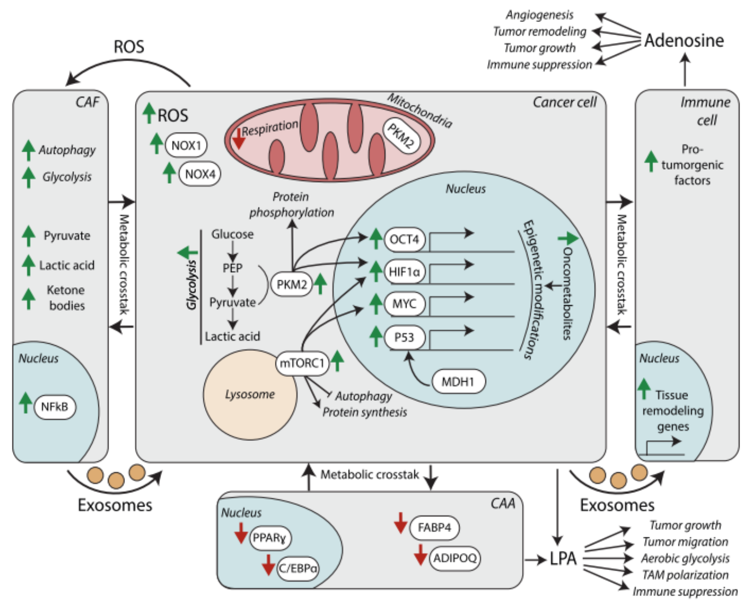

Fig 3. Schematic metabolic change in tumor microenvironment. [28]

1.4. *Tumor microenvironment crosstalk*

Intercellular communication is essential to maintain homeostasis and cellular function in multicellular organism. It can occur via autocrine and endocrine signaling, direct cell-cell contact, paracrine signaling such as release of soluble factor [chemokines and cytokines] and extracellular vesicles.

1.4.1. *Cell-cell contact*

The cell-cell contact communication is involved in different process principally embryogenesis immune responses, stem cell fate decisions and tumorigenesis. Since those process develop over the time are not easy to be traced because the available technologies are not often suitable to trace in vivo long-term experiment.

The interaction between cells and ECM occurs via receptor-ligand binding. Integrins are proteins that regulates cells adhesion migration and mechanotrasduction in a cell-ECM interaction manner and they can also trigger intracellular pathways promoting proliferation, survival, and cell growth. Ligand-bound integrin can also interact with nutrient signaling pathway such as mTOR that form two different complex, mTORC1 and mTORC2. mTORC1 controls cell growth and proliferation in response to grow factors and amino acids. mTORC2 has a role in actin organization. During nutrient starvation mTOR is retained inducing the cells to get nutrient from other sources such as autophagy [34].

1.4.2. *Long-distance cell to cell communication*

In recent decades numerous research groups have highlighted the presence of thin membranous structures interconnecting cells. These structures defined as tunneling nanotubes (TNTs), tumor microtubes (TMs), cytonemes or membrane bridges connect cells, transport cellular cargo over long distances. For those reasons, they are considered to be involved in the pathogenesis of cancer but also cancer cells resistance against the different cancer treatments [35].

1.4.3. *Cytokines, chemokines and growth factors*

The intercellular communication that occurs via cytokines and chemokines play a pivotal role in TME. Particularly, the bidirectional signaling between cancer cells and other cells mediated by cytokines, chemokines or growth factors released by CAF contribute to cancer progression and immune response evasion. CAFs are a heterogeneous cell population derived from different types of cells that become activate upon stimulation of different modulators including transforming growth factor (TGF)-β, hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), fibroblast growth factor 2 (FGF-2), stromal-derived factor-1 (SDF-1) and reactive oxygen species (ROS) [36].

Through the secretion of cytokines, chemokines and other effector molecules, CAFs can impair the antitumor activity of different types of immune cells that are overall called tumor immune microenvironment[36].

In particular, through the secretion of transforming growth factor-beta (TGF-β), interleukin-6 (IL-6), C-X-C chemokine ligand 12 (CXCL12), C–C chemokine ligand 2 (CCL2), stromal-derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF) along with indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2), CAFs can contribute to the polarization of tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), mast cells (MCs), dendritic cells (DCs) and T lymphocytes into protumorigenic cells. CAFs also contribute to the recruitment of M2-type TAMs, N2-type TANs, regulatory DCs (rDCs), regulatory T(Treg) cells and myeloid-derived suppressor cells (MDSCs) that represent immune inhibitory cells and they contribute to the restriction of cytotoxic activity and cytokine production of effector cells like natural killer cells and cytotoxic lymphocytes.

1.4.4. *Extracellular vesicles*

Extracellular vesicles, comprise exosomes, microvesicles and apoptotic bodies, are a recent discovered way of intercellular communication. EVs can act as paracrine signal or enter in blood circulation acting at a distal site as endocrine signal. All the cell of the body are able to secrete EVs that can be taken up by recipient cells remodeling their behavior. Particularly when it occurs in TME, tumor derived EVs can reprogram the neighboring cells promoting the formation of a protumorigenic niche [37].

1.5. Project 1: Targeting of tumor cells by custom defined antigen

Extracellular vesicles are secreted by all types of cells and playing fundamental roles in different physiological and pathological processes. They carry components of cytoplasm and plasmatic membrane that are selectively loaded into vesicles, reflecting the composition of the parental cells. Due to their release by all type of cells they also have been utilized as diagnostic markers and therapeutic tools in several conditions.

1.5.1. Extracellular vesicles

Extracellular vesicles are a heterogeneous family of membrane bilayer vesicles released by all cell types and can be found in body fluid like urine saliva blood but also in cell culture medium [38]. Their existence have been documented over 80 years with Pan and Jhonston as firsts researchers to describe extracellular vesicles EVs have been considered for long time a way to get out of the cells waste material, only during lasts decades it has been discovered that EVs play a role in intercellular communication exchanging proteins, RNA, DNA [39] [38]. The internalization of EVs can occur via membrane fusion or other ways such as receptor-mediated endocytosis, phagocytosis, lipid raft-dependent endocytosis and micropinocytosis [40].

The secretion of extracellular vesicles is a process conserved in the evolution and vesicles have been isolated from different body fluid including blood, urine, saliva, breast milk, amniotic fluid, ascites, cerebrospinal fluid, bile, and semen. Based on their origin and size, extracellular vesicles are classified in exosomes and microvesicles. The term exosomes was adopted in the late 1980's for small vesicles (30-100 nm) of endosomal origin released in the extracellular environment after the fusion of multivesicular bodies with the plasma membrane. The term ectosomes or microvesicles referred to 150-1000 nm released by the outward budding of plasma membrane [41].

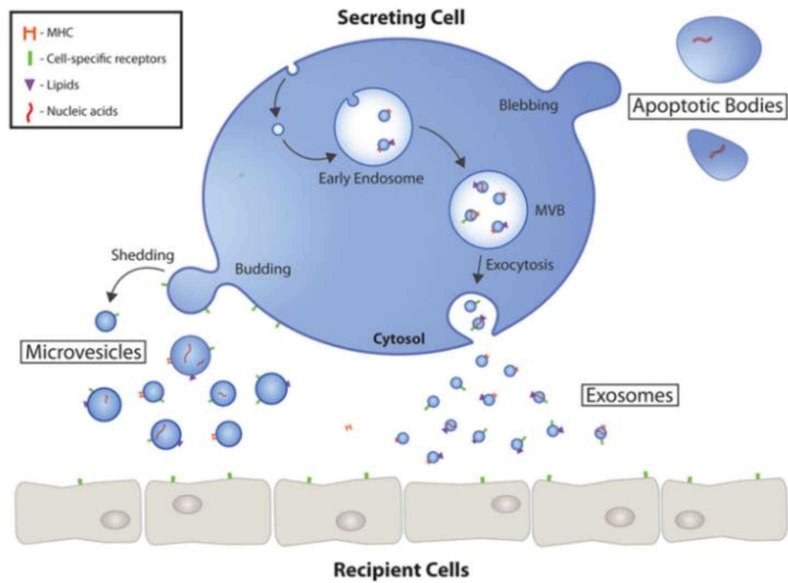

Fig 4. Schematic representation of EVs cargo, biogenesis, and secretion. [42]

1.5.1.1. Exosomes

The presence of exosomes in extracellular space is evident from early '80s but only in recent decades have been identified the role of this membrane-lipid bilayer vesicles. Initially they were considered waste material or a way to transport discard materials out of the cells, only recently have been discovered that they play a role in intercellular communication having a role in immune response, antigen presentation and signal transduction.

1.5.1.1.1. Exosomes biogenesis

Exosomes are intraluminal vesicles (ILV) of multivesicular bodies (MVB) which are 30-100 nm in diameter originated by the inward budding of MVB. Proteomic analysis of exosomes reveal that some protein arises from cells and tissue of origin and other are common to exosomes secreted by different cells. Lipids have a role in protecting exosome shape but also they take part in exosome biogenesis and regulate homeostasis in the recipient cells.

To date it is known that exosomes formation requires three steps: establishment of the endosomal system, the formation of intraluminal vesicles (ILVs) and the fusion of multivesicular bodies with plasma membrane [43].

There are two pathways for exosomes formation: ESCRT (Endosomal Sorting Complex Required for Transport) dependent pathway and ESCRT independent pathway.

According to the first pathway, ILV formation begins in EEs by inward membrane budding that leads to cargo sequestration and distribution into vesicles. The protein sorting is regulated by endosomal-sorting complex required for transport (ESCRT) machinery. ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III are four components of ESCRT machinery which ubiquitinate the substrates on the part of the inward budding endosomal membrane [44]. Experiment suggests that the membrane of EEs are enriched in PIP3 and the presence of the latter, of ubiquitinated cargos and the curved membrane typology induces the requirement of ESCRT-I and ESCRT-II driving the membrane budding and ESCRT III is necessary to complete the budding. The recruitment of ESCRT-III to the site of ESCRT-I and II occur via Alix, a protein that simultaneously binds to the TSG101 component of the ESCRT-I complex and CHMP4, a component of ESCRT-III [45]. Later, targeted ILVs can be degraded within lysosome or rescue by DUBs and after that Rab27A and Rab27B, essential mediators, lead the MVBs toward the cell periphery. Finally, SNARE complex are involved in the fusion of MVBs with plasma membrane with the release of ILVs into the extracellular space [44].

The ESCRT-independent pathway was discovered when some studies demonstrated the possibility to form exosomes even in absence of ESCRT machinery. The attention was paved on the role of lipids. It is defined that exosomes release is related to the production of ceramides promoting the budding of MVBs and can also be metabolized to produce sphingosine 1-phosphate (S1P), which binds their receptor on MVBs to enhance the production of ILVs. Another important ESCRT independent pathway for exosomes production and cargo involve tetraspanins that form tetraspanins enriched microdomains allowing the interaction with transmembrane and cytosolic signaling proteins [43].

During the formation and cargo process exosomes became enriched of several proteins fundamental during the biogenesis and other considered biomarkers such as: tetraspanins (CD9, CD63, CD81, CD82) involved in cell penetration, fusion events, and invasion; heat shock proteins HSP70 and HSP90 that are part of the stress response and are take part in antigen binding and presentation; proteins involved in exosome release (Alix and TGS101) and also proteins responsible for transport and fusion (annexin and Rab)[46].

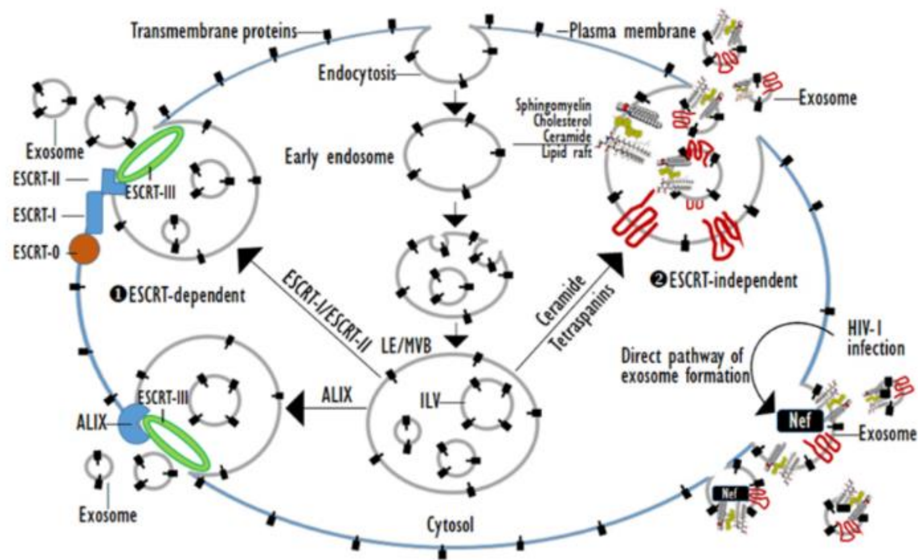

Fig 5. ESCRT-dependent or ESCRT-independent exosome biogenesis. [47]

1.5.1.2. Microvesicles

MVs, also commonly named ectosomes or microparticles, have a size comprised between 100 and 1000 μm in diameter. MVs contain information derived from parental cells from which they inherit also partial cell membrane markers. Several proteins have been proposed MVs-specific, including selectins, integrins, CD40, matrix metalloproteinase (MMP), phosphatidylserine (PS), ADP-ribosylation factor 6 (ARF6) and Rho family members [48].

1.5.1.2.1. Microvesicles biogenesis

Eukaryotic cells release heterogeneous population of membrane enclosed vesicles [49] called microvesicles that originated by outward budding of plasma membrane [50]. Microvesicular formation occur via phospholipid redistribution and cytoskeletal protein contraction. Membrane budding/vesicle formation is induced by translocation of phosphatidylserine to the outer membrane leaflet thanks to the activity of proteins called flippase and floppase, responsible of the transfer of phospholipid from one side of the membrane to the other. The budding process is completed through contraction of cytoskeletal structures by actin-myosin interactions [45].

Due to their content and their capability to transfer mRNA, proteins, miRNA, DNA they are able to modify extracellular milieu of proximal and distal recipient cells. The composition of MVs reflects the parental cells although the membrane composition remains different from that of origin cells enabling specialized functions [49]. The release of microvesicles from the plasma membrane depends on the contractile machinery present at the neck of budding vesicles. The interaction of actin and myosin with subsequent ATP-dependent contraction is the pivotal step in microvesicles release. ARF6 is the protein that activate the aforementioned contractile system, indeed, it stimulates the activation of ERK, leading to the localization of myosin heavy chain at the contractile groove and the phosphorylation of myosin light chain that regulate actin cytoskeletal rearrangement contributing to microvesicles fixation. [51]

1.5.1.3. Apoptotic bodies

Apoptotic bodies or apoptosomes are membrane encapsulated vesicles derived from the triggering of intrinsic pathways or extrinsic stimuli that are responsible of the activation of apoptotic cascade. Phagocytes are responsible of apoptotic cell removal, thanks to the expression of “eat me signals” or “find me signals”, that facilitate the engulfment of dying cells and the digestion in the phagolysosome. Apoptosis is a process that comprises different stages where chromatin and cellular content undergo to several modification with the formation of membrane-enclosed vesicles, named apoptotic bodies as final result [52].

1.5.2. Extracellular vesicles uptake

Intercellular communication is well described through cell-cell contact or soluble factor such as chemokines and cytokines metabolites. The crosstalk mediated by extracellular vesicles started to be better understood in recent decades. EVs differ in size and surface protein that make them recognizable and taken up by target cells. They are mediators of intercellular communication between cancer cells and stromal cells in the local and distant environment [53]. EVs can acting at cells surface without deliver their content or can be internalized undergoing to lysosome degradation or recycled and released in extracellular space [54].

There are several mechanism through which EVs and their cargo could be transmitted to recipient cells. EVs can fuse with the plasma membrane of recipient cells or they can be picked up with process such as phagocytosis, macropinocytosis, lipid raft-mediated endocytosis, clathrin-mediated endocytosis, and caveolin-mediated endocytosis [27].

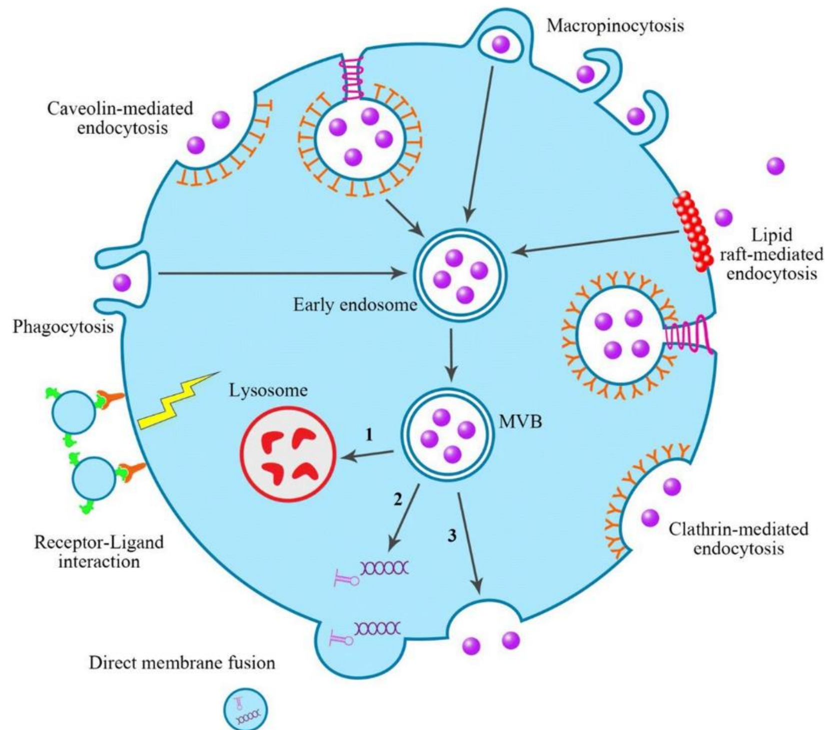

Fig 6. Extracellular vesicle uptake mechanism [55].

1.5.3. Extracellular vesicles in tumor microenvironment

1.5.3.1. Immunological control by EV

As aforementioned, metabolic alterations affecting the tumor microenvironment can affect extracellular vesicle secretion pathways leading to an increase in their production and release. The contents of the vesicles are transferred to recipient cells, and the biological effects are varied and not always easy to understand.

A surprising discovery by Raposo et al. regarded the immune role of extracellular vesicles [56]. They have been shown that EVs mimic the activity of parental cells, which, if found to be B lymphocytes, stimulate T-cell proliferation in an antigen- and MHC-restricted manner. Subsequently, numerous studies have begun to therapeutically exploit EVs as vaccines, particularly dendritic cell-derived vesicles that stimulate immune response. In parallel, the idea is to exploit tumor derived EVs directed against dendritic cells that could be a means of eliciting an immune response. Despite these promising insights, many studies have shown that tumor derived EVs contribute to an immunosuppressive environment in the contest of tumor microenvironment [57].

Immune cells, however, do not always go into death as a result of exposure to extracellular vesicles but can undergo significant functional alteration with modification of protein profiles expression adversely affecting the release of cytokines and chemokines and consequently evading immune response [58] [59].

Extracellular vesicles derived from tumor cells do not always act by direct interaction with immune cells. Sometimes naturally circulating or therapeutically administered anticancer antibodies may interact with tumor epitopes that are present on tumor-released EVs, sequestering the antibodies and impairing the antibody-dependent cytotoxicity [60][57].

1.5.3.2. EVs stimulated angiogenesis

As is well known, one of the hallmarks cancers is the ability of cancer cells to produce new vessels with which suppling oxygen demand and nutrients useful for growth. In this context, tumor-derived vesicles may contribute to angiogenesis due to their tetraspanin content. Particularly, exosomes express high levels of these proteins

compared with parental cells, and they contribute to the initiation of angiogenetic branching. Following internalization of EVs by endothelial cells, they express high levels of von Willebrand factor, VEGF and VEGF-R2 and other factors driving endothelial cell proliferation, migration, sprouting and progenitor maturation [61].

1.5.3.3. RNA delivery by EV

Extracellular vesicles contain different types of RNAs such as miRNAs and ncRNAs including small nuclear RNAs, small nucleolar RNAs (snoRNAs), rRNAs, lncRNAs, PIWI-interacting RNAs (piRNAs), transfer RNAs (tRNAs), mitochondrial RNAs, Y RNAs and vault RNAs (vtRNAs)26,27,28. miRNA precursors (pre-miRNAs). however, it is well established that the functional RNAs transported by EVs are the intact mRNAs and miRNAs. These RNAs contained in extracellular vesicles reflect the physiological or pathological state of the parental cell and play a fundamental role as biomarkers and therapeutics for numerous diseases [62].

Some studies provide evidence that EVs carry mRNAs that once entered into recipient cells are translated into proteins by changing their protein profile [63]. In addition, extracellular vesicles also carry micro RNAs (miRNAs), which can interfere with target genes in recipient cells altering the transcriptome [57].

Recently, studies proposed to exploit extracellular vesicles as a means of transporting RNA for therapeutic purposes. In fact, the EVs appear to be biocompatible therefore through an electroporation process and the siRNAs can be loaded into the EVs obtaining gene silencing in the treated cells without toxic effects [62].

1.5.3.4. Cancer EVs and metastasis

Metastatic tumors receive long-distance signals originating from primary tumors by forming a premetastatic niche in order to prepare the environment to the arrival and seeding of cancer cells. As tumor-derived vesicles circulate they may provide a medium through which long-distance signal dissemination occurs. One of the earliest evidences of the role of EVs in metastasis was the study by Hood et al. [64], who observed that the accumulation of melanoma-derived EVs at the level of sentinel lymph nodes drew tumor cells to the site by inducing changes in the matrix and subsequent establishment of the metastatic site [57].

Extracellular vesicles released by tumor cells inherit the organotropism of parental cells, disseminating through body fluids and contributing to the formation of the pre-metastatic niche.

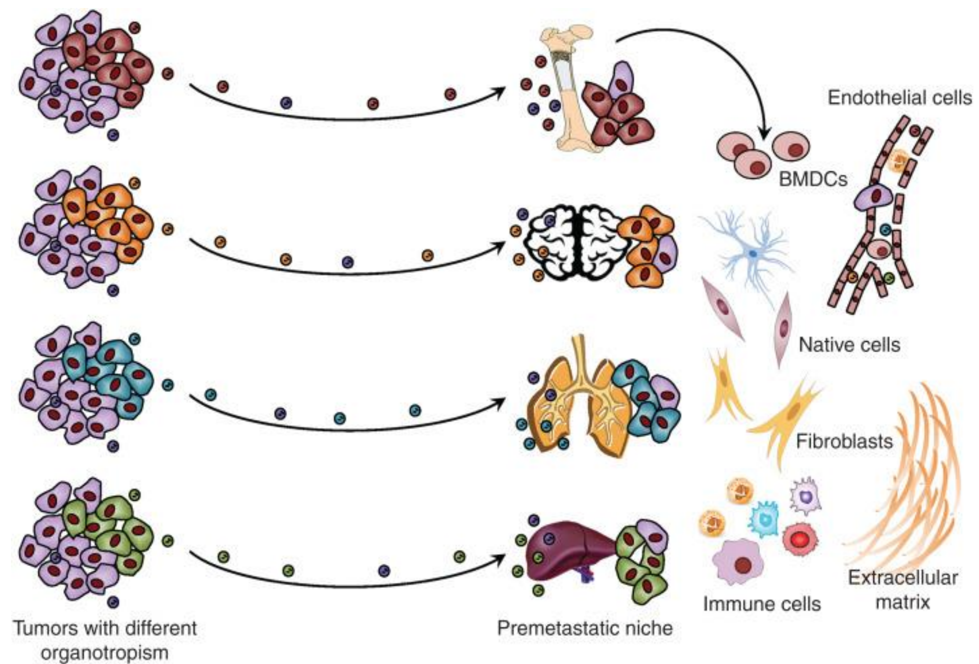

Fig 7. Effects of tumor-derived EVs on premetastatic niche formation [65].

1.5.3.5. EVs related drug resistance

The acquisition of resistance to common treatments is one of the main obstacles to the development of effective cancer therapy. In fact, cancer cells, turn out to be susceptible at first and develop tolerance through mechanisms such as alteration in drug absorption, alteration of drug metabolism, suppression of cell death, increased DNA repair. Another mechanism is vesicle-mediated sequestration of antitumor factors but also EVs-related transmission of pro-survival and anti apoptotic factors and stemness-associated genetic and protein cargo contribute to chemoresistance.

Anti-cancer drugs are designed to effectively reach the tumor site, cross the plasma membrane, and be metabolized achieving treatment success. Extracellular vesicles may provide a pathway by which tumor cells load the drug and excrete it externally through their secretion. It has been shown that some cancer cells take advantage of the

acidification of the environment to increase vesicle release resulting in increased drug excretion and treatment failure. EVs are also involved in the removal of drugs from the extracellular environment; in particular, it has been observed that many antibodies used as cancer treatments are sequestered by EVs because they express antibody-recognizing receptors on their surface, protecting cancer cells from their attack.

Not only vesicles derived from tumor cells but also vesicles derived from cells that make up the tumor microenvironment contribute to the development of chemoresistance. EVs can influence the activity of anticancer drugs as a result of their horizontal transfer of proteins and miRNAs that act at different levels in the major oncogenic cascades implicated of cell survival and apoptosis [66] [67].

1.5.4. Major histocompatibility complex I

Major histocompatibility complex, MHC-I, are molecules expressed on the surface of nucleated cells and present small peptides (8-10 aa) derived from intracellular proteins. Normally all the cells present peptides derived from their own proteins to protect themselves from the cytotoxic immune response. During an infection, the presentation of infected cells-derived peptides on the MHC-I molecules trigger a CTL immune response and the infected cells are eliminated. Given the role of MHC-I presentation pathway in the detection of viral infection, viruses developed system to impair this pathway.

1.5.4.1. Major histocompatibility complex I composition

MHC-I molecules are heterodimers of a membrane glycoprotein considered the heavy chain (45KDa) and a soluble protein the β2microglobulin (B2M) (12 KDa). The heavy chain in the extracellular region fold in three domains α1, α2, α3 and B2M represents a fourth domain. α1 and α2 domain form the groove where the small peptides dock. MHC-I molecules fold and assemble in the lumen of endoplasmic reticulum and the antigen presentation pathway involve different proteins.

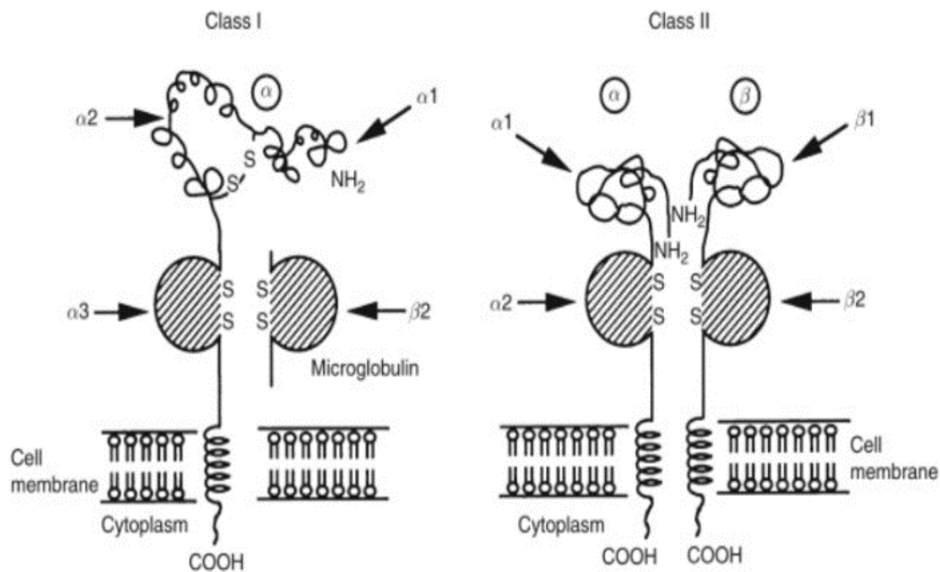

Fig 8. Structural differences between MHC-I and MHC-II. [68]

Most peptides presented by MHC-I molecules derive from defective ribosome translation product that are directly processed in the cytosol by proteasomes. Since the peptide binding occur in the ER, all the peptides have to be translocated in the lumen through the transporter associated with antigen processing (TAP).

1.5.4.2. MHC molecules polimorfism

MHC molecules are encoded by highly polymorphic genes called Human Leukocytes Antigens or HLAs. Each individual expresses different MHC-I and MHC-II molecules consequently binding many different peptides and determining the presentation of these peptides to the immune system. The heavy chain of the MHC-I molecule is encoded by three different genes called HLA-A HLA-B HLA-C while the two alpha and beta chains of MHC-II by three other pairs of genes called HLA-DR, HLA-DP, and DQ [69].

MHC polymorphism is a hallmark of these molecules. Although the extent of polymorphism varies in different vertebrate species, the total number of allelic variants is in the thousands. This diversity is sustained by evolution and selection to keep up with the evolution of pathogens [70].

1.5.5. Antigen presentation pathway

TCR of CD8+ cells can bind their ligand when they are exposed on MHC-I expressed on nucleated cells. This process is important because MHC-I can bind endogenous antigen and when cells undergo to modification such as infection or cancer transformation the antigens are expose on the surface as well, but they are recognized as foreign antigens by CD8+ and the cells are killed. Antigen presentation is a complex process in which MHC-I and B2M are synthetized in ER and are stabilized by protein such as calreticulin and tapasin. Designated intracellular proteins are targeted for degradation by ubiquitination and they are driven to proteasome to degradation into peptides. These peptides are translocated into the ER by TAP and loaded on MHC-I or after further processing by ERAP1 and ERAP2. After the binding of MHC-I and peptides the complex pass through Golgi apparatus and then to the cells surface where antigen presentation to TCR can start.

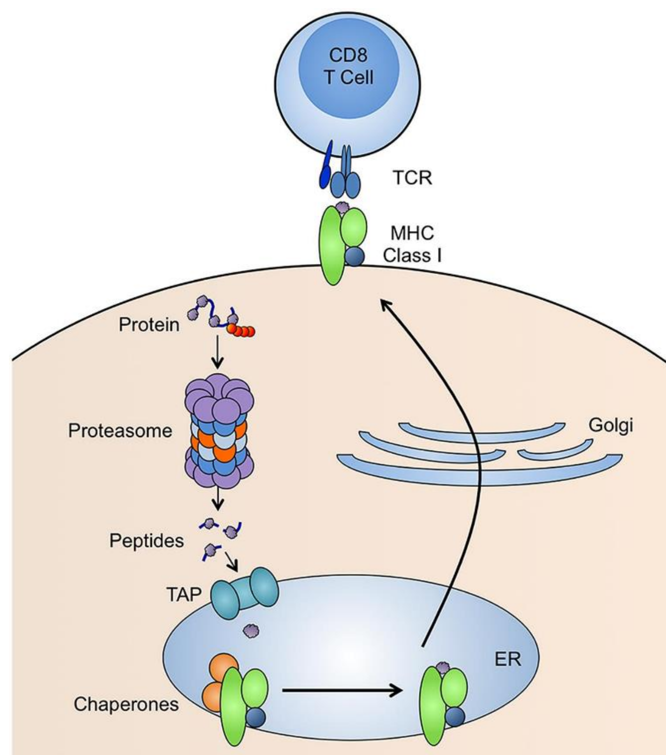

Fig 9. MHC class I antigen presentation pathway [71].

During infections, the initiation of CD8+ naive can be due to cross-presentation or direct infection of DC cells and it depends on the type and tropism of the antigen [72].

1.5.6. Major histocompatibility complex I in cancer

Tumor cells can avoid immune recognition by downregulating MHC-I surface expression, a crucial step in initiation of cytotoxic immune response. Downregulation of MHC-I has been described in 40-90% of human tumor and it is correlated with a worse prognosis. Both adult and pediatric tumor are able to reduce the expression of MHC-I on cells surface but with different ways. While adult tumors downregulate the expression of MHC-I to avoid the recognition by immune cells, pediatric tumors that arise from embryonic tissue intrinsically lack immunological features.

Downregulation of surface expression of MHC-I can avoid the recognition by CD8+ but can trigger the activity of NK cells. Indeed, MHC-I acts as inhibitory signal by binding KIRs on NK cells surface dampening their activation. Low level of MHC-I can no longer inhibit NK activation and the cells are recognized as “non self” and are killed in a mechanism known as “missing self”. Tumor cells have developed also other mechanism to avoid NK cells recognition by producing TGF β or prostaglandin that impair NK function and block their infiltration in tumor site and they also can increase MHC-I expression in some moment to avoid the recognition [73].

1.5.7. Cross presentation

In order to recognize body cells as its own, it is necessary for them to expose endogenous antigens, previously processed in the endoplasmic reticulum, on MHC-I molecules exposed on the cell surface. The antigen presentation process allows immune cells to be instructed in the recognition of self and non-self. Thus, cells infected with viruses or cancer cells will expose non-self antigens on the surface that will be the trigger for an immune response. The naïve CD8+ cells need to be activated to CTL by professional antigen presenting cells. If APCs are not directly infected it is necessary for them to acquire exogenous antigens that will be exposed in their MHC-I molecules and then presented to naive CD8+ which will be activated. This process of antigen acquisition is called cross-presentation.

Several studies demonstrated that cross presentation is a process mainly performed by DC cells exploiting two different pathway called "cytosolic" and "vacuolar" pathways. In the case of the cytosolic pathway, once the exogenous antigen is phagocytosed it is

transported to the proteasome where the generated polypeptides can be carried into the ER or back to the phagosome and loaded onto MHC-I molecules that will be targeted to the cell surface. In the vacuolar pathway, exogenous antigens are degraded in the phagosome and loaded on MHC-I molecules in the phagosome itself and addressed to the cell surface [72] [74].

1.5.8. Cross Dressing: Intercellular transfer of MHC molecules

The transfer of many molecules, including the major histocompatibility complex, costimulatory and adhesion molecules, extracellular matrix organization molecules as well as chemokines, occurs not only between APCs and T cells, but also between other cell types such as APCs–APCs, tumor cells-APCs, epithelial cells-APCs.

It was observed a horizontal transfer of complete and fully functional MHC-peptide complex from the membrane of a donor to recipient cell with a process known as cross dressing [75].

The transfer of these MHC-I and II molecule, which can be unidirectional as in the case of DCs to endothelial cells or bidirectional as in the case of DCs and T cells, is a phenomenon that occurs very rapidly, and the time in which MHC can remain stably expressed in recipients can be as long as two days. The transfer of the intact and unprocessed pMHC complex can be exploited by cross dressed cells to present antigens to T cells [75] [76].

Transfer of MHC molecules is still under debate but three main processes are the most plausible: trogocytosis, trafficking of extracellular vesicles, tunneling nanotubes.

Trogocytosis correspond to a molecular transfer of part of plasmatic membrane and require a direct cell-cell contact. It has been shown that DC cells can acquire plasma membrane from living cells. Harashyne et al. through fluorescence microscope observation showed that the membrane of a DC was pulled from another cell in coculture condition. The achievement of part of plasma membrane is a phenomenon called “nibbling” that is a receptor-mediated process. In either human or murine cell coculture, is possible to observe this transfer provided there is direct contact between cells.

Another mechanism by which cells can acquire molecules is called transsynaptic transfer and it was observed between T cells and APC. After mixing these two types

of cells it is possible to observe a clusterization of MHC-I molecule at the contact site and after a while small cluster were observed at a site distal to the contact site.

A more recent discovered mechanism of MHC-I transfer is the extracellular vesicles trafficking. It is well established that many cells secrete extracellular vesicles and that they can be internalized and processed.

What is the reason for this exchange of molecules? Why, even though professional APCs are present, we observe the transfer of costimulatory molecules and pMHC leading other cells to become antigen-presenting cells as well, risking disorder and the trigger of an uncontrolled immune response?

Transferred pMHC complexes, both I and II molecules, induce CD4+ and CD8+ activation as well as costimulatory molecules are found to be functional. Acquisition of MHC molecules, as well as chemokine receptors, may also aid trafficking of immune cells to the site of antigen presentation.

The transfer of MHC-I and II molecules can be also involved in T-cell tolerance and immune regulation. It has also been shown that MHC-II molecules transferred to T cells induce recognition id those cells by other activated T cells by inducing apoptosis and hyperresponsiveness. therefore, acquisition of MHC molecules binding self-antigen contributes to the maintenance of tolerance [76].

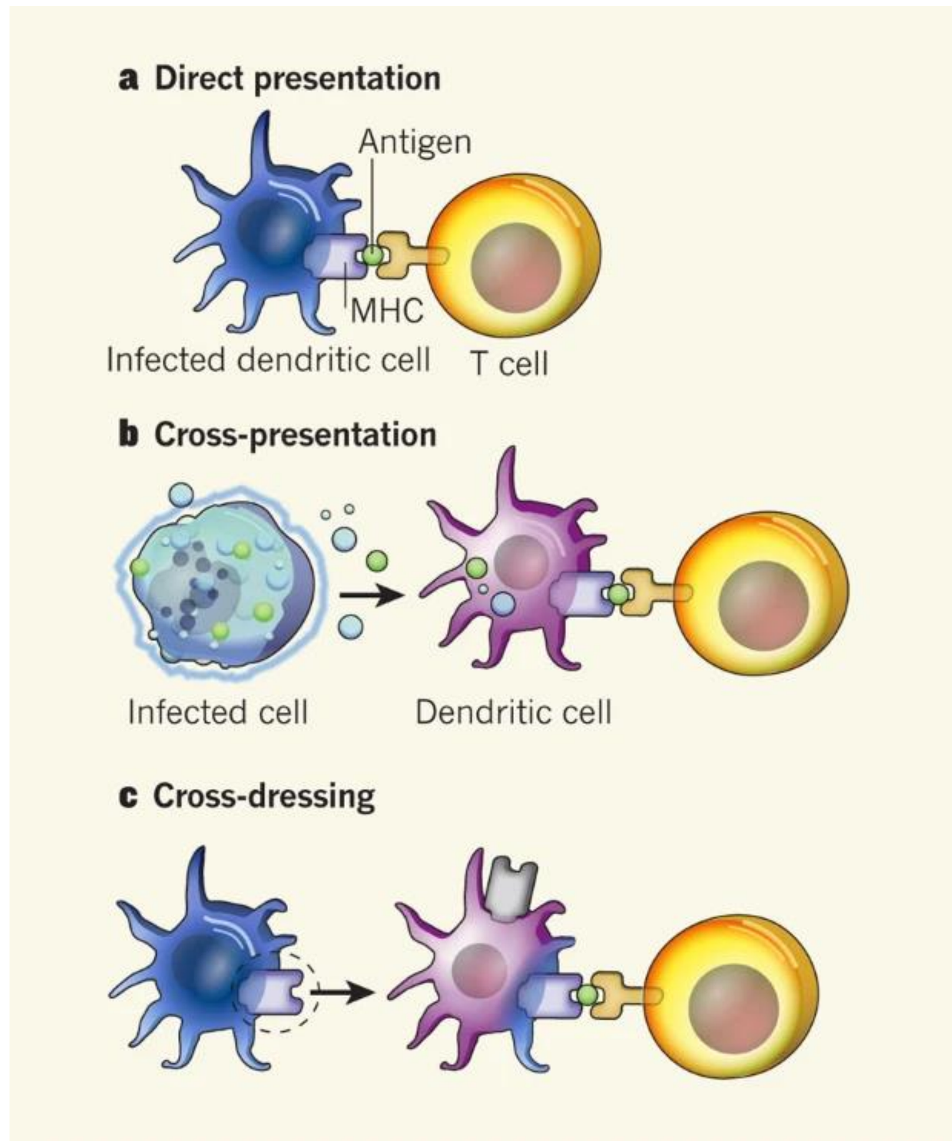

Fig 10. A) direct presentation occurs when an antigen presenting cells is infected and display a processed antigen on MHC I molecule. B) in cross presentation dendritic cells acquire antigens by infected cells with the loading on MHC I molecule. C) Cross dressing occurs when dendritic cells acquire preformed pMHC-I complex from other cells [77].

1.6. *Project 2: Role of 2,3-bisphosphoglycerate mutase (BPGM) in tumor cells proliferation*

1.6.1. *Glycolysis*

Glycolysis is a metabolic process that occurs in the cytosol of cells both under aerobic and anaerobic conditions. Starting from one molecule of glucose, two molecules of ATP are produced without the use of oxygen.

This process is characterized by 10 reactions, that can be split into 2 main phases:

- Preparatory phase: consists in the firsts 5 reaction with the consumption of 2 ATP molecules.
- Payoff phase: consist in the lasts 5 reaction with the production of 4 molecules of ATP and 2 molecules of NADH.

The yield of this metabolic pathway is 2 molecules of ATP in terms of energy production but also several other intermediates are produced, and they can participate to other biosynthetic pathway.

Particularly, the pyruvate produced by glycolysis can undergo different metabolic pathways based on the presence or absence of oxygen.

In cells that use oxidative respiration to obtain energy, the pyruvate produced by this pathway enters the TCA cycle and finally into the complexes of the respiratory chain where through the oxidative phosphorylation reaction it is oxidized to carbon dioxide and water. In this way a net or 32 molecules of ATP are produced.

In anaerobic conditions the pyruvate produced by glycolysis is converted into lactate through anaerobic respiration forming 2 molecules of ATP. In presence of little to no mitochondria such as in erythrocytes, anaerobic glycolysis is the way to obtain energy.

Taken together, glycolysis can produce large amounts of energy under oxygen presence, to contrary when oxygen is no longer available, it produces energy for cells survival.

Malignant tumor cells require big amounts of energy to supply cell division, invasion, and migration, but also to sustain excessive proliferation and the detachment from neighboring cells for metastasis. Normally to obtain large amount of energy, cells

depend on glucose oxidative phosphorylation but in the case of tumor cells they undergo to a “metabolic reprogramming”, relying on aerobic glycolysis to energy production, consuming excess glucose and forming massive quantities of lactate also in oxygenated environments [78]. This process is known as Warburg Effect or aerobic glycolysis [79].

1.6.2. Warburg effect

Otto Warburg has been a pioneer in cancer metabolism field. In 1924 he observed a particular phenomenon where tumor cells rapidly consumed glucose converting it in lactate even in presence of oxygen. But the biochemical reason and clinical significance of this choice has been under debate for long time. According to Warburg one of the possible reasons hypothesize was an impairment of oxidative phosphorylation due to mitochondria damage.

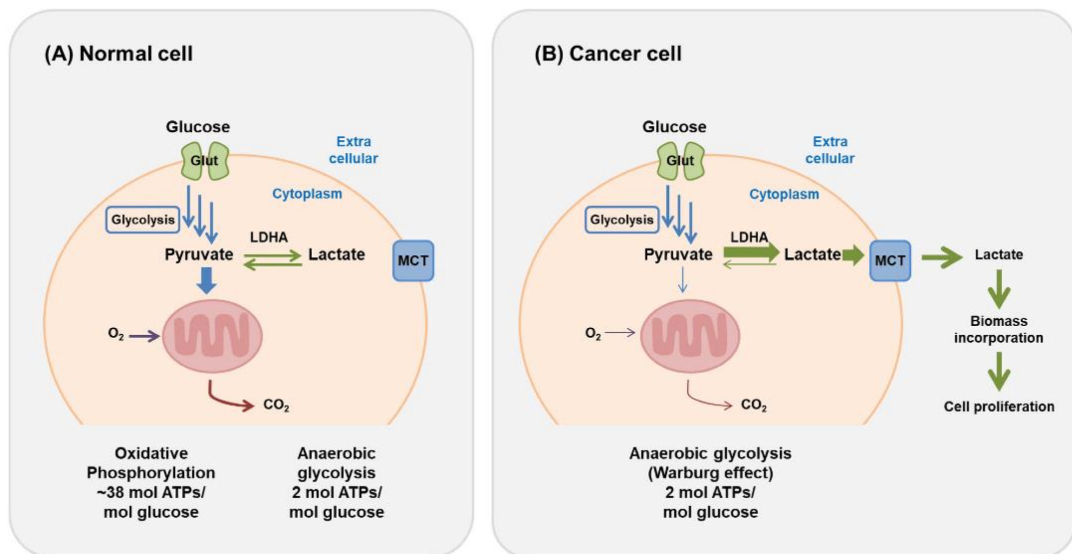

Fig 11. Differences in the glycolytic pathway between normal and cancer cells [80].

To deeply understand the biochemical nature and the clinical significance is necessary to revisit one of the hallmarks of cancer. The rapid proliferation is one of the main needs of cancer cells and to obtain energy and biomass to sustain this growth, malignant tumor cells need not only ATP but also an anabolic metabolism that provide biomass useful to grow. Glucose metabolism is considered one of the most important

aspects in cancer metabolism. The glycolysis pathway generates intermediates that provide carbon source that can enter the nucleotide, fatty acid and aminoacidic synthesis pathway [81].

Furthermore, other important hallmarks are the capability to metastasize and to avoid apoptosis. The spread of tumor cells from a primary site to a distant organ require the detachment of cells from the extracellular matrix a process that produce amounts of ROS (reactive oxygen species). The presence of ROS would let to cell death, known as anoikis, hampering metastasis. For this reason, tumor cells that display high level of ROS, have developed strong antioxidant capacity at the same time, keeping ROS level under the critical threshold. Moreover, tumor cells are also characterized by the metabolic switch avoiding mitochondrial oxidation that is the main source of ROS. Warburg effect is also involved in the branching pentose phosphate pathway that produce NADH, critical for antioxidant activity. Overall, Warburg effect promote metastasis[82].

1.6.2.1. Metabolic and oncogene reprogramming underlying Warburg effect

Metabolic reprogramming is closely related to tumor genetic. It has been understood that the altered metabolism of tumor cells is driven by activation of oncogenes and tumor suppressor. [81]

The proto-oncogene Myc, transcription factor hypoxia inducible factor 1 (HIF-1), the PI3K/Akt/mTOR pathway, and tumor suppressor p53 are considered some of the most relevant regulators.

Tumors are hypoxic due to an altered and insufficient vascular irroration. As consequence of hypoxic condition, HIF-1 and HIF-2 are stabilized leading to the transcription of genes encoding glycolytic enzymes such as hexokinase 2 (HK2), phosphofructokinase 1 (PFK1), aldolase A (ALDOA), phosphoglycerate kinase 1 (PGK1), pyruvate kinase (PK), and lactate dehydrogenase A (LDH-A) and downregulates pyruvate dehydrogenase (PDH) and glucose transporters (GLUTs) with the result of increased glucose uptake. HIF-1 also upregulate the expression of monocarboxylate transporters (MCTs), such as MCT4 for lactate transport out of cells. One more important role of HIF-1 is transcriptional activation of NADH dehydrogenase (ubiquinone) 1α subcomplex subunit 4-like 2 (NDUFA4L2), which

inhibit Complex I of the electron transport chain (ETC) downregulating OXPHOS [83].

Since not all the part of the tumor are hypoxic, there must be autonomous alteration as cause of aerobic glycolysis.

HIF-1 is stabilized not only by hypoxia but also by insulin-like growth factor, HER 2 signaling and PI3 kinase activation. In contrast it has been demonstrated that PI3K/AKT and HIF-1 are independent pathway thus indicating that AKT has HIF-1 dependent and independent effects on glycolysis. The stabilization of HIF-1 in normal oxygen conditions and hypoxic conditions contribute to aerobic glycolysis.

Oncogenes can also directly activate glycolysis. MYC oncogene is overexpressed in several tumors, and it can bind promotors of glycolytic genes (HK2, ENO1 e LDHA), glucose transporters transactivating them in normoxic conditions [84].

The transcriptional factor p53 is a tumor suppressor since it blocks the cellular cycle when a DNA damage is detected. The mutation of p53 gene, observed in numerous types of cancers, induce an increase in glycolysis rate as consequence of the loss of its function.

1.6.3. Reverse Warburg effect

The metabolic switch from higher energy providing metabolism (oxidative phosphorylation) to glycolysis observed by Warburg was a field of debate. Indeed some experiments demonstrated that there is heterogeneity within cancer cells metabolism since cancer cells can show either OXPHOS or glycolysis as energy source, indicating that the switch is not mutually exclusive [85].

In 2009 it was hypothesized the “two compartment” model, in which it was considered the strictly interplay between cancer cells and the cells present in the TME. The new theory named Reverse Warburg effect described a model in which stromal cells undergo to aerobic glycolysis to produce biomolecules that will feed cancer cells that will oxidate these molecules in the OXPHOS [85]. Of relevance, the interaction between cancer associated fibroblast and cancer cells affect growth, proliferation, metabolism and metastasis. Due to the central role of CAF in tumor progression, several studies paid attention to the metabolic switch of these cells.

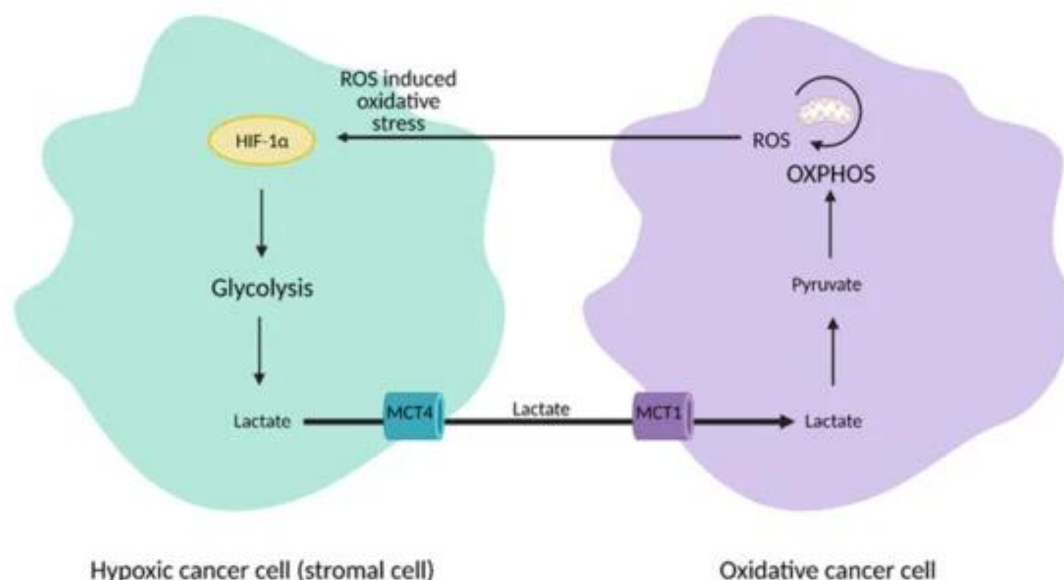

Fig 12. Reverse Warburg effect. In tumor microenvironment, substrates produced by cells can be shared with each other. Lactate produced by stromal cells can be taken up by oxidative cancer cells to fuel OXPHOS. [83]

Cells in TME are exposed to oxidative stress due to the release of H_2O_2 by cancer cells. The transformation of fibroblast into CAF is a consequence of many factors including the oxidative stress, the upregulation of HIF 1, the enhanced TGF beta signaling, the loss of CAV-; the transformation leads also a change in the metabolism. Cancer associated fibroblast, produce biomass via aerobic glycolysis, that can be used as fuel by cancer cells that will be metabolize in their mitochondria with oxidative phosphorylation.

Similar to what happen to CAF, as consequence of oxidative stress also cancer cells can give rise to hypoxic cancer cells that show aerobic glycolysis as metabolism producing lactate.

Lactate is the primary metabolites of glycolytic pathway; it is a metabolite consumed by the surrounding cancer cell to respond to energy demand. The excess of lactate accumulates in the extracellular space contributing to the acidification of TME and consequently to immune response escape.

Lactate export and uptake is possible thanks to the monocarboxylate transporter (MCTs) a family of proton-linked membrane transporter. MCT-1 is responsible of the export of lactate, and it is identified as a protein upregulated in OXPHOS cancer cells that have increase uptake of lactate while MCT4 is responsible of the uptake and is upregulated in glycolytic cells [85].

Since lactate is considered the principal fuel of cancer cells, the inhibition of the lactate shuttle has been considered as possible target of anticancer drugs. Indeed, the accumulation of lactate inside CAF or hypoxic cancer cells lead to lactic acidosis with the disruption of TME, that is beneficial for cancer therapy [86].

1.6.4. 2,3-Bisphosphoglycerate mutase

Bisphosphoglycerate mutase is a trifunctional enzyme commonly considered erythrocyte specific. It has:

- Synthase activity: catalyze the isomerization of 1,3 bisphopshoglycerate to 2,3 – bisphosphoglycerate
  1,3-DPG + 3-PGA → 2,3-DPG + 3-PGA
- Mutase activity: interconvert 2 and 3 phosphoglycerate
  3-PGA + 2,3-DPG → 2-PGA + 2,3-DPG
- Phosphatase activity: hydrolyze 2,3-bisphosphoglycerate to 3 phosphoglycerate and a phosphate.
  2,3-DPG + H20 → 3-PGA + phosphate [87]

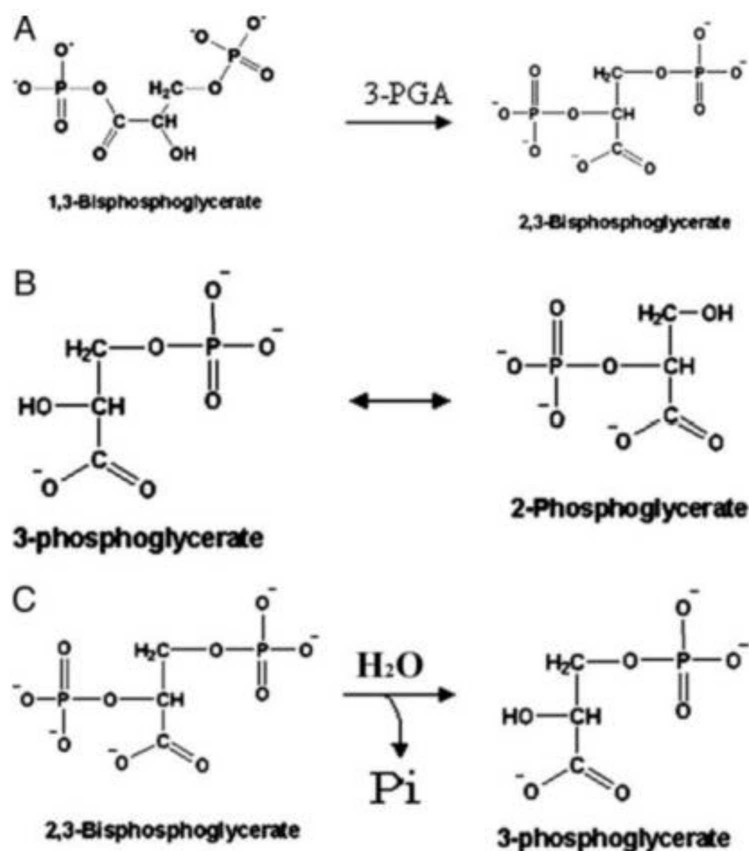

Fig 13. Bisphosphoglycerate enzyme activity [88].

The BPGM monomer contain two domains including six β strand and 10 α-helices. The α/βfold of BPGM resembles that of the dPGMs from *S. cerevisiae* and *E. coli*. The protein core consists of a six βstrand in the order βA, βB, βC, βD, and βF in parallel and strand βE in antiparallel conformations. The βsheet is flanked by six α-helices. The dimer is formed between the surface of the C strands and α3 helices of the two monomers with a non-crystallographic 2-fold symmetry similar to E. coli dPGM and S. cerevisiae dPGM. The sequence of hBPGM is 50% identical to dPGMs. The catalytic site residues Arg-10, His-11, Arg-62, Glu-89, Arg90, Arg-116, Arg-117, and His-188 are conserved in all of them. However, some residues involved in the substrate binding in dPGMs have been substituted in BPGMs [88].

Based on its enzymatic properties and amino acid sequence homology, BPGM is strictly related to the 2,3-BPG-dependent glycolytic enzyme dPGM with sequence identity of 40-50%. According to the structural comparison, the two enzyme show conserved residues in the core but they have different specificity for the substrate and catalytic activity. Indeed, BPGM has principally synthetase activity respect to dPGM with mainly mutase activity.

It has been found that the enzyme has only one active site with two different binding site one for bisphosphoglycerate and the other one for monophosphoglycerate. [88]

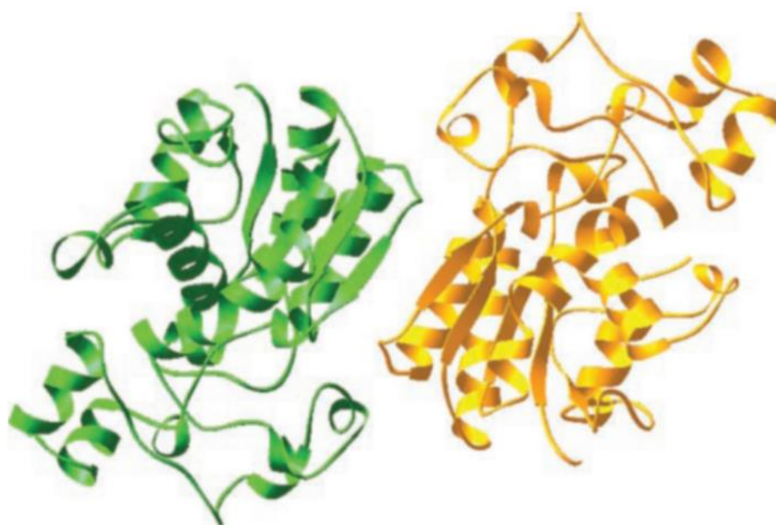

Fig 14. BPGM dimer[88].

2,3-BPG is a glycolytic intermediate firstly discovered as potent primer of PGAM1, the enzyme that convert 3-PGA to 2-PGA. During the conversion, in the catalytic pocket occur a "ping pong" mechanism by which the histidine 11 of PGAM1 is phosphorylated followed by the transfer of phosphate from H11 to C-2 of the substrate 3-PG, creating the intermediate 2,3-BPGM. The latter in turn re-phosphorylates H11of PGAM1, transferring the phosphate from C-3, creating again the active from of the enzyme and facilitating the release of the product 2-BPG. 10.1038/ncomms2759 Hence, 2,3-BPG is required for efficient glycolysis.

BPGM is the principal actor of the Rapoport-Luebering glycolytic shunt in which the intermediate 1,3 bisphosphoglycerate is converted into 2,3 bisphosphoglycerate by the BPGM mutase activity.

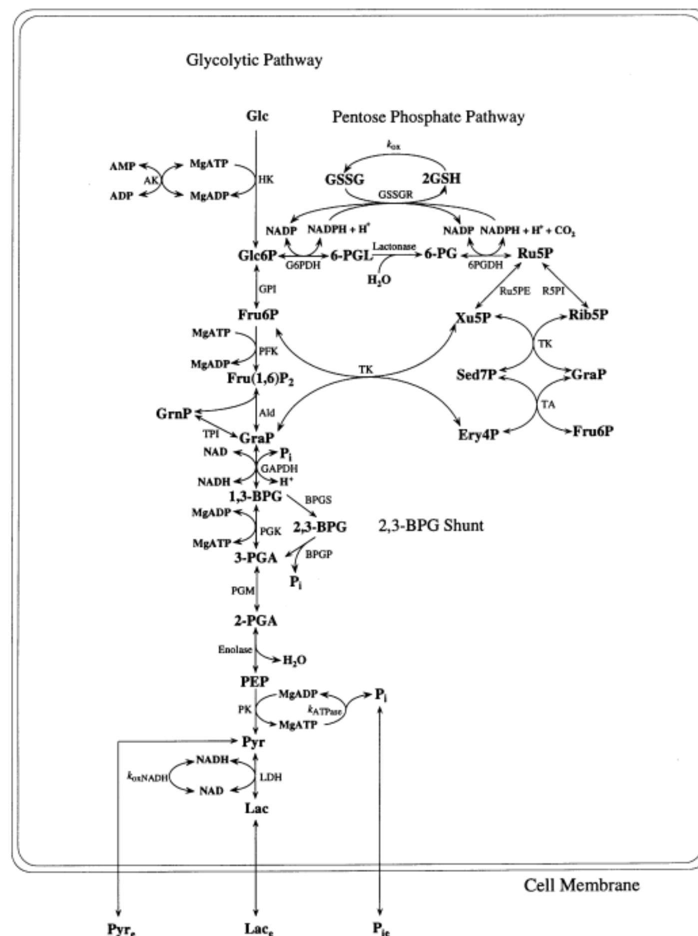

Fig 15. Reaction scheme of the metabolic pathway: Rapoport-Luebering shunt [89].

In red blood cells, 2,3-BPG has another important activity since it represents the main allosteric effector of hemoglobin, and it is regulated by the enzyme BPGM. Indeed,

2,3-BPG show high affinity for deoxyhemoglobin and lowers oxygen affinity of hemoglobin facilitating the release of oxygen from hemoglobin in the surrounding tissue. Furthermore, the regulation of 2,3 BPG is also an adaptation to oxygen deprivation for example in hypoxic condition like altitude or anemia it is possible to observe increased level of 2,3-BPG in RBC facilizing the release of oxygen in tissue when pO2 is low [90].

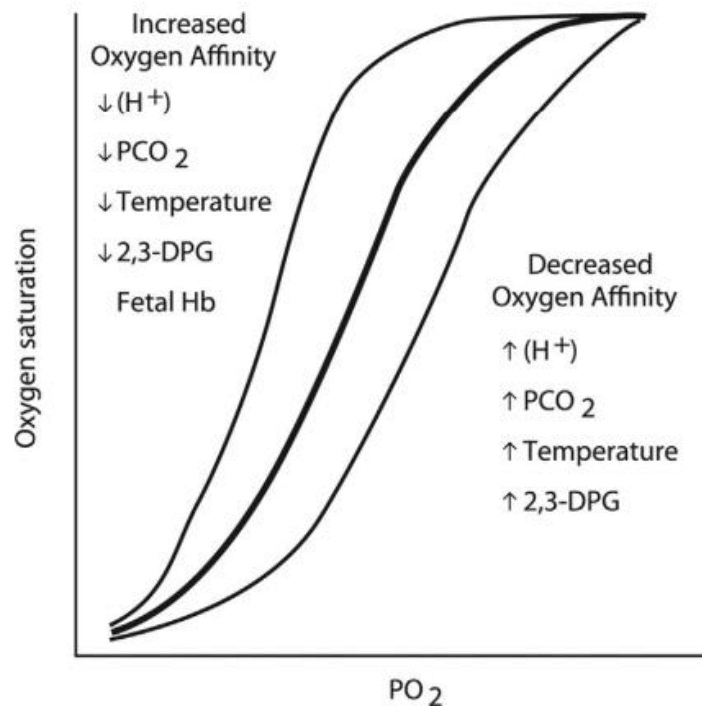

Fig 16. Oxygen dissociation curve [91].

Despite the well-defined role of BPGM in erythrocytes, less is known about its role in maintaining PGAM1 activity and glycolytic flux in all the other cells.

As aforementioned, glycolysis is a metabolic pathway used by the cells to anaerobic ATP generation and to produce biomass to support cells growth. This pathway is regulated at different points for example, one important intermediate is the 3-PG that is at a branching point since it can either go to serine biosynthesis or continue into glycolysis. The majority of 3-PG goes to lower glycolysis through PGAM1that is primed by phosphorylation on His11 thanks to the donation of phosphate group by 2,3 BPG. Very little is known about how BPGM-mediated 2,3BPG production contribute

to the activation of PGAM1. According to the study conducted in 2017 by Oslund et. al, the knockout of BPGM in HEK 293T cell line, the amount of 2,3 BPG was dramatically depleted with loss of PGAM1 phosphorylation.

This study also shows that PGAM1 can also be phosphorylated by 1,3 bisphosphoglycerate, used as backup for 2,3 BPG. The efficiency of 2,3 BPG production with this way is much lower respect to the BPGM-dependent production and this reflect the loss of most cellular 2,3 BPG after BPGM knock out, however, it is sufficient to maintain the glycolytic flux.

3-PG accumulation after BPGM deletion led to serine de novo synthesis that not only function in protein synthesis but also provide one carbon units to sustain nucleotide synthesis. Hence, BPGM results indispensable for 3-PG and serine homeostasis while it has a minor role in maintaining glycolytic flux [92].

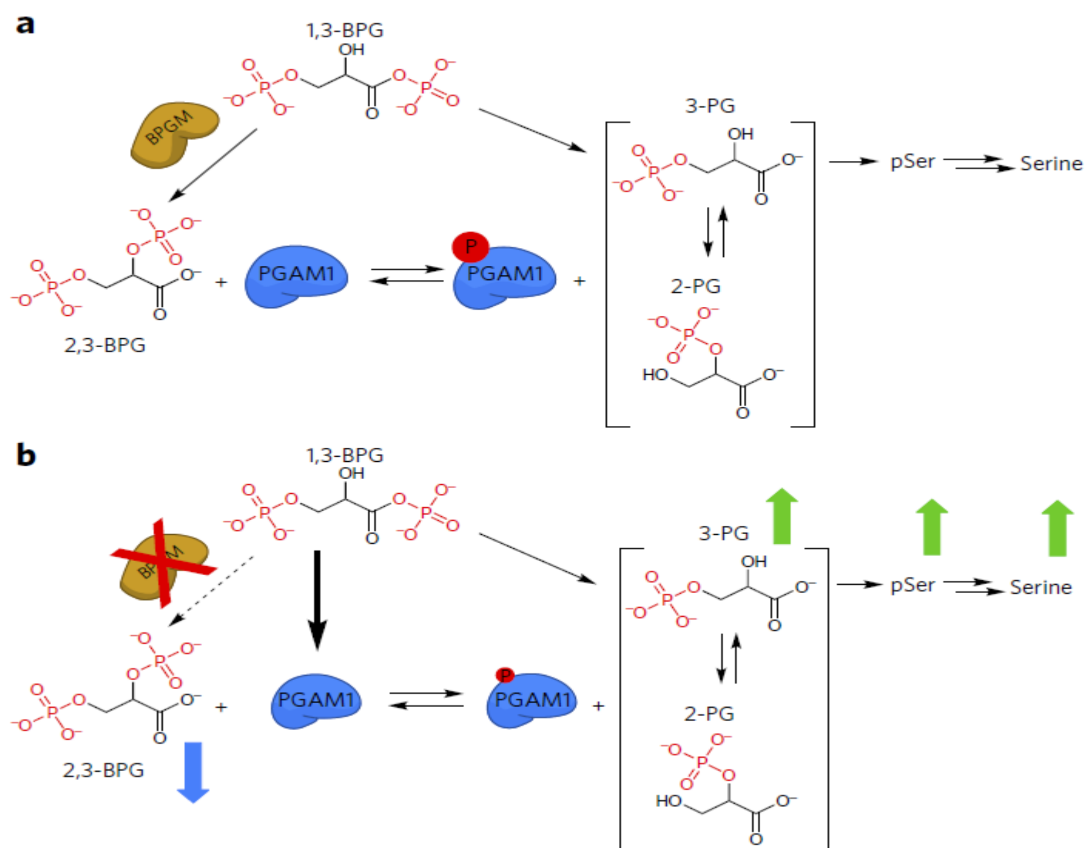

Fig 17. Effect of BPGM depletion on glycolysis. A) BPGM converts 1,3 BPG into 2,3 BPG for PGAM1 phosphorylation. B) 2,3 BPG level and PGAM1 phosphorylation

drop down after BPGM depletion with increased 3-PG, pSer and serine metabolite level.[92]

2. *Aim of the projects*

Studies conducted in recent decades have highlighted that focusing efforts only on tumor cells has not led to any conclusive results. This is due to the fact that when we talk about cancer, we must take into account that tumors are a heterogeneous collection of cells, infiltrating and resident host cells, continuously exchanging soluble factors and vesicles. In early stage of cancer development, a dynamic and bidirectional relationship is established between cancer cells and the surrounding cells that ensure tumor growth, proliferation, survival, local invasion and metastasis.

The aim of the project has been the study of the role of tumor microenvironment in tumor progression under two different point of view.

The first aspect that we took into account was the intercellular communication mediated by extracellular vesicles in tumor microenvironment and their capability to transfer proteins. Studies demonstrated that EVs can be secrete by almost all the cells of our body and they are involved in proteins, nucleic acids, lipids, RNAs transfer. The exchange can occur between APC cells, between tumor cells and also between tumor and APC cells. This aspect is very important because it suggest that EVs can transfer immunological molecules playing a pivotal role in immune response. Tumor cells elude the immune response in several ways, one of which is the downregulation of surface MHC-I molecule avoiding the trigger of the cytotoxic immune response. The goal of this part of the project was to exploit the physiological trafficking of MVs to deliver a custom defined antigen loaded onto MHC-I molecules from antigen presenting cells to tumor cells to trigger a CD8+ immune response.

The second aspect that we analyzed was the study of the role of the enzyme Bisphosphoglycerate mutase (BPGM) in the crosstalk between cells of the tumor microenvironment (TME) to highlight the way in which the activity of cells present in the tumor microenvironment influences the activity and metabolism of tumor cells and vice versa. It is known that tumor cells undergo a metabolic switch known as the Warburg Effect, which involves a transition from an oxidative metabolism to a glycolytic metabolism. Recent studies have highlighted that this change is not exclusive of tumor cells, even cells that make up the tumor microenvironment, in particular cancer associated fibroblast, can undergo this switch with the subsequently

production of metabolites useful to tumor cells for their growth and proliferation minimizing energy expenditure for the latter. We studied the role of BPGM, the enzyme involved in the Luebering–Rapoport shunt that catalyze the conversion of 1,3 bisphosphoglycerate into 2,3 bisphosphoglycerate. The characteristic of the shunt is the skipping of one ATP-producing step, allowing an increased glucose uptake but keeping constant the ATP/ADP ratio. Particularly we analyzed the interaction between tumor cells and CAFs in coculture highlighting BPGM protein levels changes to understand its role in the tumor contest.

3. Materials and Methods

Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) was from Invitrogen™ Life Technologies. 4-20 % MP TGX Stain-Free Gel, Trans-Blot Turbo Midi PVDF transfer packs, and ECL Substrates for High-Sensitivity Western Blot Detection were purchased from Bio-Rad. Anti-β actin (C4) (sc-47778), anti MHC-I (F3) (sc-32235) were purchased from Santa Cruz Biotechnology. Anti HLA-ABC (polyclonal) (PA5115364) was obtained from Thermo Fischer Scientific. Anti β2-microglobulin (EP2978Y) (ab75853), anti-BPGM (polyclonal) (ab97497) were from Abcam. Anti α-β tubulin (#2148) was purchased from Cell Signaling Technologies. Ovalbumin peptide (257-264) was purchased from Sigma-Aldrich, and ovalbumin was purchased from InvivoGen. Thincert cell culture insert 0,4 μm and 8 μm (GR657641) were purchased from Euroclone.

3.1. Cells cultures

AGS human gastric adenocarcinoma cells were purchased from the European Collection of Cell Cultures (ECACC). CT26.WT undifferentiated colon carcinoma cells, 4T1 murine breast cancer cells and J774A.1 murine monocytes, DU145 and PC3, human prostate cancer cell lines, and C2C12 myoblast cell line were purchased from American Type Culture Collection (ATCC). The T2Kb cell line is a human T2 cell line transfected with mouse H-2Kb class I genes. T2Kb cells express empty H-2 class I on their surface and can efficiently present exogenous peptides to murine cytotoxic T lymphocytes [93]. OT-I murine CTL cell line, Kb-restricted, specific for the OVA 257-264 peptide (pOVA) of ovalbumin protein [94]. The T2Kb and OT-I cell lines were kindly gifted by Prof. C.T. Baldari, University of Siena.

CT26, 4T1, DU145, PC3 and HDF were cultured in DMEM supplemented with 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and 10 % fetal bovine serum (FBS, Euroclone). AGS cells were cultured in RPMI supplemented with 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and 10 % fetal bovine serum (FBS, Euroclone). J774A.1 cells were cultured in DMEM supplemented with 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and 10 % HyClone defined fetal bovine serum (HyClone defined FBS, Cytiva).

T2Kb cells and OT-I were cultured in RPMI 1640 complete medium (supplemented with 2 mM L-glutamine, penicillin 100 U/mL, streptomycin 100 μg/mL) with 7.5–10 % HyClone defined fetal bovine serum (HyClone defined FBS, Cytiva). Cells were incubated at 37 °C in a humidified atmosphere containing 5 % CO2. Human-derived monocytes and CD8+ T cells were obtained from a voluntary donor after tetanus-toxoid (TT) immunization. Mouse C2C12 myoblasts were cultured in DMEM (supplemented with 2 mM L-glutamine, penicillin 100 U/mL, streptomycin 100 μg/mL) with 10% fetal bovine serum (FBS). Once reached full confluency, cell differentiation was induced by switching to 2% horse serum (HS) for 5 days. Cells were routinely grown in DMEM in a humidified atmosphere with 5% CO_2 at 37°C.

3.2. Fluorescence analysis of protein transfer

The transfer of proteins from the donor to recipient cells was evaluated using CFDA-SE. The latter is a permeable fluorescent probe and when into the cells is hydrolyzed into CFSE that bind protein amine groups generating fluorescence.

Donor cells were labeled with the dye at a concentration of 10 μM in PBS buffer for 15m, then detached and plated with recipient cells both in co-culture and in Transwell® inserts. For flow cytometry analysis, cells were detached after 24h or 40h of co-culture, fixed in 3 % paraformaldehyde, and analyzed using a BDFACS Canto-II. The ability of donor cells to transfer proteins was evaluated by measuring the ratio of the fluorescence of recipient cells after co-culture with donor cells and their autofluorescence.

3.3. Conditioned media preparation

Cancer cells were grown in starvation medium and after 24h the medium was collected, centrifuged at 1500 ×g for 10 min to discard cell debris and filtered through 0.2 µm size pore filters.

3.4. In vitro monocytes activation

Activation of monocytes, both lineage (J774.A1 or T2Kb) and healthy donor-derived monocytes, was done by treating these cells with conditioned media derived from cancer cell lines (AGS or 4T1) for 24 hours.

3.5. *Purification of membrane vesicles secreted by monocytes and human dermal fibroblasts*

MVs were purified as described by Santi et al. [95]. Briefly, the cell culture supernatant recovered from activated and non-activated monocytes (40×10^6 cells) and from CAF and normal fibroblasts (5×10^5) was centrifuged at 1000 ×g for 5 min to discard the cells. The supernatant was then centrifuged at 1500 ×g for 10 min to remove cell debris. The supernatant was centrifuged at 10000 ×g for 45 min to isolate the MVs fraction.

The pellet was resuspended in PBS to be washed and then centrifugate again at 10000 ×g for 45 min and finally resuspended in PBS for further NTA analysis or in culture medium for the other experiments.

3.6. *Nanoparticle tracking analysis with Nanosight*

MVs isolated from 40×10^6 J774A1 and from 8×10^6 T2Kb as described above were diluted in PBS to a final volume of 1 ml. MVs isolated from 5×10^5 CAF or normal fibroblast as described above were diluted in PBS to a final volume of 1 ml. The samples were diluted to reach the optimal concentration of particles per frame value. Following settings were set according to the manufacturer's software manual (NTA 3.4 Build 3.4.4): camera level was increased until all particles were distinctly visible not exceeding a particle signal saturation over 20%. The ideal detection threshold was determined to include as many particles as possible with the restrictions that 10–100 red crosses were counted while only ~10% were not associated with distinct particles. Blue cross count was limited to 5. For each measurement, five 1-min videos were captured under the following conditions: cell temperature: 25°C; syringe speed: 30 µl/s; laser: green; camera: sCMOS.

3.7. *Western blot*

For SDS-PAGE and Western blot analysis, samples were lysed in Laemmli electrophoresis buffer (without β-mercaptoethanol and bromophenol blue) and assayed for protein content using the Bicinchoninic Acid protein assay (BCA). Each sample (25 µg) was supplemented with β-mercaptoethanol and bromophenol blue and

separated using SDS-PAGE. The gels were then electroblotted onto PVDF membranes for detection. Blots were incubated with:

- anti-β-actin, anti HLA, anti MHC-I, and anti-β2 microglobulin. (Dilution 1:1000)
- Anti-α/β-tubulin, anti-BPGM. (Dilution 1:1000)

After incubation with secondary antibodies, the blots were developed using the ECL plus immunodetection system and visualized using Amersham Imager 600.

3.8. In vitro cytotoxicity assay

The experiment was performed by activating T2Kb in the CT26.WT cancer cell-conditioned medium. Activated T2Kb cells were then preloaded with OVA peptide (pOVA) (10 μg/ml) and co-cultured with CT26.WT cells for 24h. OVA-specific OT-I CD8+ T cells were then added to the T2Kb - CT26.WT co-culture for an additionally 18h in the indicated rows. Thereafter, T2Kb and CT26.WT cell viability was determined by flow cytometry analysis (ViobilityTM 488/520 fixable dye, FITC) of specific cell subpopulations using anti-CD7-PE and/or anti-CD3e-Vioblue. To inhibit cytotoxic activity, cells were incubated with anti-mouse H-2Kb (BioLegend, USA), 1h before exposure to pOVA.

In a similar experiment, monocytes isolated from a healthy human donor by density gradient centrifugation, immunized against tetanus-toxoid (TT) were pulsed with TT (0.5 μg/ml) for 24h and cultured with AGS (human gastric adenocarcinoma) cells for an additional 24h. Cytotoxic TT-specific autologous CD8+ T lymphocytes were added. 18h later the cell viability was determined by flow cytometry analysis (Viobility 488/520 fixable dye, FITC) of specific cell subpopulations using anti-CD44 Antigen Presenting Cells (APC), anti-CD8 PE, and anti-CD14 PerCP. An anti HLA class I blocking antibody (AbCam, UK) was added to the control conditions 1hbefore exposure to TT to test the inhibition of the restricted cytotoxic activity.

3.9. BCA protein assay

The BCA method is used for protein quantification. The principle of this method lies in ability of proteins to chelate and to reduce Cu^{2+} to Cu^{+} in an alkaline solution. Bicinchoninic acid forms a violet-colored compound with the cuprous ion formed,

showing an absorption peak a 562 nm. Each dosing procedure involves the preparation of tests containing known quantities of bovine serum albumin (BSA: 2 µg, 5 µg, 10 µg, 15 µg) to define standard curves. The reagent is prepared at the time of use by adding bicinchoninic acid to the solution (REAGENT A: aqueous solution of 1% BCA-Na_2, 2% $Na_2C0_3 \cdot H_2O$, 0,16% Na_2 tartrate, 0,4% NaOH e 0,95% $NaHC0_3$; REAGENT B: 4% $CuSO_4$ in deionized water), in a ratio of 50:1. The reagent is added to the samples in a ratio of 8:1. After a 30 minute incubation at 37°C, spectrophotometric readings are carried out at 562 nm, using a microplate reader. Changes in absorbance at 562 nm are proportional to protein concentration of the samples, which is then determined by referring to the curve standard.

3.10. In vivo experiments

In vivo experiments were performed in accordance with national guidelines approved by the Italian ethical committee of the Animal Welfare Office of the Italian Work Ministry (aut. No. 652/2020-PR) and conformed to the legal mandates and Italian guidelines for the care and maintenance of laboratory animals.

3.11. Immunological treatment of immunodeficient mice

The animals were randomized before cancer cell injection. Twelve six- to eight-week old male severe combined immunodeficient (SCID)-bg/bg mice (Charles River Laboratories International) were subcutaneously injected with 1×106 PC3 cells. The mice were monitored daily until measurable tumors were formed. Six mice were intratumorally injected with 1×106 T2Kb cells (previously activated with PC3 cell-conditioned media for 16h) preloaded with 10 µg/ml pOVA for an additional 4h. Six control mice were injected with vehicle (PBS). The injection volume was 100 µl. After 4h from the injection, 5x106 OT-I lymphocytes were injected into the tumor mass of all mice. The treatment was repeated every four days for a total of four times. Tumors were measured with calipers and the volumes were determined using the following formula: V = (Length × Width2)/2.

3.12. Immunological treatment of immunocompetent mice

Eighteen four-week old male BALB/c mice (Charles River Laboratories International) were immunized by subcutaneous injection of 10 µg of OVA. After three weeks,

immunization was repeated. Seven days later, all mice were subcutaneously injected with $1x10^5$ syngeneic 4T1 tumor cells. When the tumors reached the minimum measurable size, mice were randomized. Six mice were intratumorally injected with $3x10^6$ syngeneic J774A.1 monocyte previously activated with 4T1 cell-derived conditioned medium for 16h and preloaded with 10 µg/ml of OVA for 4h, six mice were intratumorally injected with only 10 µg/ml OVA and six mice with PBS (control). The injection volume was 100 µl. The treatment was repeated every three days for a total of three times. Tumors were measured with calipers and the volumes were determined using the following formula: V=(Length × Width2)/2.

3.13. Spleen mononuclear cells response to OVA

BALB/c mice fresh spleens were placed in PBS, washed twice and disrupted using gentleMACS® Octo Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Then the samples were filtered through a 70µm cell strainer and erythrocytes were removed using a Red blood cell lysis solution (Miltenyi Biotec, Bergisch Gladbach, Germany). The spleen mononuclear cells were tested for their responsiveness to OVA vaccine by measuring [3H] thymidine (Perkin Elmer, Waltham, MA, US) uptake after 120 h of stimulation. In particular, 2.2x105 cell/well were seeded in triplicate with medium (RPMI 1640 complete, 10 % FBS) alone, or with pOVA (2 µg/ml) or OVA (2 µg/ml). A mitogenic index (MI) > 5 was considered positive.

3.14. Statistical analysis

Longitudinal mouse data on tumor volumes and weight loss were elaborated and prepared for further analyses by calculating the means of repeated technical measurements and standard deviations in Microsoft Excel. Experiments were modeled using a linear regression model robust against clustered data in R (version 4.0.3) using the lm cluster function of the mice adds package (version 3.16-18), considering the formula Y~Time2+Treatment:Time2 to model the nonlinear growth of tumor size and using individual mice as clustering factors. In all experiments, statistical significance was set at p<0.001. Bar plots were prepared using the GraphPad Prism software. Box plots and spaghetti plots were constructed with R.

3.15. *In vitro HDF activation*

Cancer cells (PC3 and DU145) were grown in starvation medium and after 24h the medium was collected, centrifuged at 1500 ×g for 10 min to discard cell debris, filtered through 0.2 µm size pore filters, and cultured HDF cells for 24 hours.

3.16. *Proliferation assay*

Proliferation assay was performed using CFDA-SE fluorescent probe that label the cells as described in paragraph 3.2.

$2x10^5$ cells were labeled with CFDA-SE for 15 min. After that, cells (DU15, PC3 and HDF) were washed with PBS and incubated at 37°C with complete medium for 5 hours. Then, cells were detached and counted (time zero) and 3×10^5 were incubated with serum free-medium or complete medium for other 24 or 48 hours.

After the treatment, tumor cells and healthy cells were detached, centrifuged at 1000 rpm to discard the medium and washed 2 times with PBS, followed by treatment with paraformaldehyde (PFA) 15 min in the dark at room temperature to fix the cells. PFA was removed by centrifugation at 1000 rpm and cells were resuspended in adequate volume of PBS for cytofluorometric analysis.

3.17. *Co-culture of DU145 and PC3 with HDF*

Coculture is a technique that can be used to evaluate how two cell lines can influence each other and exchange material. Fibroblasts (HDF) and tumor cells (DU145, PC3) were cocultured using filters (Transwell® Corning Costar) having pores of 0.4 µm.

The DU145 (20000 cells/well) and the PC3 (20000 cells/well) are plated in a 6 multiwell plate for the time necessary to reach a confluence of 80%. Subsequently the complete medium is replaced with the serum free medium and HDFs (100000 cells/transwell) were plated, in starvation, on 0.4 µm transwell for 48 hours.

After 48 hours, cells were lysed with Laemli Sample buffer and a volume corresponding to 25 µg of protein was loaded into electrophoresis gel and immunoblotted.

3.18. BPGM silencing in HDF cocultured with DU145 and PC3

Silencing of the BPGM enzyme is carried out in HDFs in culture with tumor cells.

HDFs (200,000 cells/well) are plated for 48h, while the DU145 (75,000 cells/well) and the PC3 (75000 cells/well) are plated in a 6 multiwell for 48h.

The silencing protocol involves:

- Dilution of Lipofectamine in Optimem medium (Invitrogen) in a 1:6 ratio (μL:μL)
- Dilution of siRNA in Optimem medium in ratio 1:50 (μg: μL)
- Mixing of the two solutions in a 1:1 ratio and keep them at room temperature 5 minutes
- Add the solution to HDF 4 hours at 37°C
- Remove the medium and replace with complete medium for at least 2 hours.

After the silencing the cells are washed in PBS and detached with Trypsin/EDTA solution, plated (100000 cells/transwell) on 0.4 μm transwell and placed in coculture with DU145 and PC3 for 48h. The evaluation of BPGM expression was obtained after SDS-page and Western Blot.

3.19. Decellularization and matrix protein transfer

Fibroblasts were plated in p35 plates, at a density of 75000 cells/plate. Some populations were treated with conditioned media from tumor cells to achieve their activation to CAFs. In parallel, the two tumor lines DU145 and PC3 were plated in plates p35, with a density of 50000 cells/plate to be subsequently used as a control of autofluorescence.

After removing the media from the plates containing HDF or CAF s, a solution of NaCl 5 M was added for 1 h at room temperature and at 4°C for the remaining 20-23 h. This treatment allows to remove the cells and maintain the matrix deposited by the CAF s or HDFs. The proteins presents in the matrix from the CAF s and HDFs , were subsequently labeled with CFDA-SE diluted in PBS 1: 500 , for 15 minutes at 37°C. After washing with PBS twice, DU145 and PC3 were plated at a density of 150000

cells/plate on the decellularized and labeled plates. After 24 h, the cells were detached, washed 2 times in PBS, and then analyzed by cytofluorimetry.

3.20. Secretome fraction preparation

HDF were cultured in 10% FBS supplemented DMEM until they reached the confluence. Then, the medium was switched with tumor conditioned medium (derived from DU145 and PC3) according to the protocol (see paragraph 3.3) for 24h to induce the differentiation into CAF. Tumor conditioned medium was then replaced by serum free medium for other 24 h to obtain CAF conditioned medium. Conditioned medium (CM) derived from CAF was harvested and centrifuged at 1500 rpm to discard cells debris and it represents the Fraction 1, complete conditioned medium (+CM). Part of Fraction 1 was centrifuged again at 1500 rpm for 10 min and for further 45 min at 10000xg obtaining the Fraction 2, conditioned medium depleted of MVs (+CM -MVs) and purified MVs that were resuspended in serum free medium (+MVs). Part of Fraction 2 was centrifuged again at 100000xg for 90 min obtaining Fraction 3, conditioned medium depleted of exosomes (+CM-exo) and purified exosomes that were resuspended in serum free medium (+exo). Part of Fraction 3 was further boiled to obtain Fraction 4 that represent the fraction of CM depleted of extracellular vesicles and cytokines (bCM). Starvation medium was used as control.

4. Results

4.1. Project 1: Targeting of tumor cells mediated by custom antigen transfer as a novel approach in immunotherapy.

4.1.1. ***MVs represent the means by which pMHC I molecules are transferred from immune cells to cancer cells.***

The intercellular exchange of proteins is a described phenomenon. Three major mechanisms are responsible for this trafficking: trogocytosis that require the cell-cell contact and provide the exchange of cell-surface molecules; nanotubes that promote contact-dependent protein traffic; extracellular vesicles that are secreted by all the cells in the body and can transfer proteins and signals to recipient cells [96]. Among the plasma membrane proteins exchanged by EVs it has been found also MHC I, underlying the importance of EVs in eliciting the immune response [97].

We evaluate whether immune cells can transfer proteins to cancer cells using two different monocytes cell lines: T2 cells engineered to express the mouse H-2Kb MHC-I molecule (T2Kb cells) [98] or J774A.1 murine macrophages as donor cells.

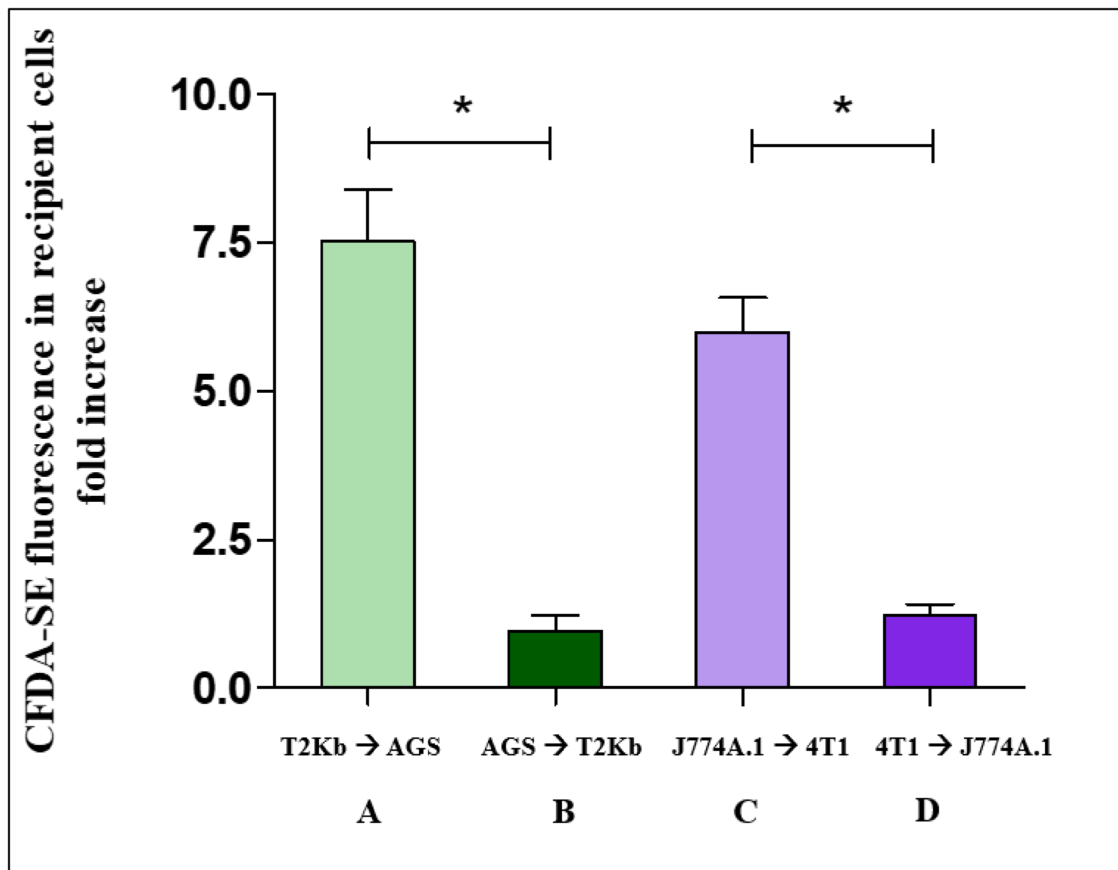

Fig 18. Protein transfer between monocytes and tumor cells*. T2Kb or J7744.1 cells were labeled with CFDA-SE and then plated in co-culture in 2:1 ratio with unlabeled AGS or 4T1 cancer cells respectively (A and C). Similarly, AGS or 4T1 cells were labeled with CFDA-SE and then plated in co-culture in 2:1 ratio with unlabeled T2Kb or J7744.1 cells respectively (B and D). After 6 hours cells were detached and analyzed by flow cytometry. Data represent the fold increase of the fluorescence intensity of recipient cells respect to their autofluorescence intensity.*

We firstly labeled the intracellular proteins of T2Kb or J774A.1 cells with CFDA-SE, a fluorescent probe that binds to the amino groups of proteins as described in Materials and Methods 3.2 [95]. Labeled T2Kb or J774A.1 cells were then co-cultured with AGS human gastric adenocarcinoma cells or 4T1 murine mammary carcinoma cells respectively. Flow cytometry analysis showed that T2Kb or J774A.1 cells transfer CFDA-SE-labeled proteins to AGS or 4T1 cancer cells after 6h of co-culture (Figure 18, columns A and C). On the contrary, CFDA-SE labeled AGS or 4T1 cells did not transfer appreciable fluorescent proteins to either T2Kb or J774A.1 cells (Figure 18, columns B and D). These results indicate that immune cells are able to transfer proteins, unidirectionally, to cancer cells similarly to CAFs [95] [53].

At this point we were interested in determining whether MVs produced by monocytic cell lines contained the MHC-I complex, and whether MHC-I could be transferred in this way to cancer cells other than immune cells.

Firstly, we have isolated MVs from the culture media of J774A.1 or T2Kb, treated or not with tumor conditioned media (t.c.m.) as described in methods. Then, we have analyzed the purified fractions by nanoparticle tracking analysis (see Materials and Methods 3.6). EVs from J774A.1 or T2Kb cells indicated a similar size distribution, ranging from 100 to 800 nm, with a predominance of 140 nm vesicles. Notably, samples derived by t.c.m activated J774A.1 or T2Kb cells contain a higher amount of MVs (Figure 19 A and B).

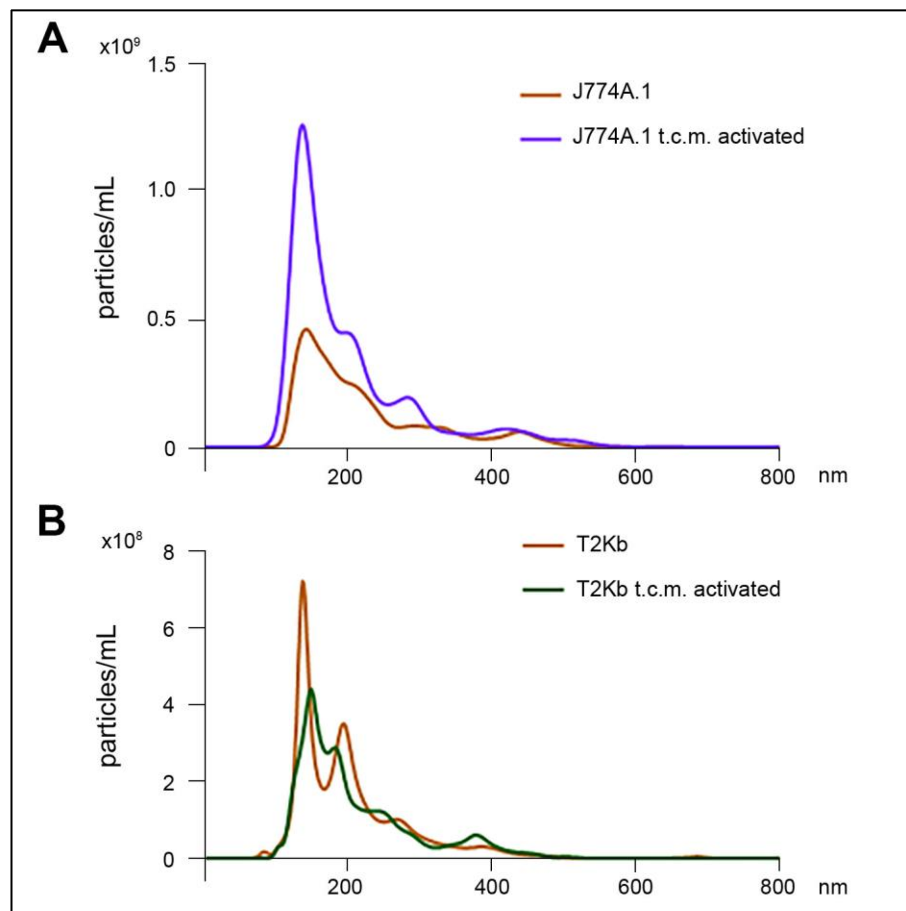

Fig 19. Nanoparticle tracking analysis of MVs isolated from J774A.1 and T2Kb cells. *MVs purified from J774A.1 or T2Kb cells, either activated or not with t.c.m. were subjected to nanoparticle tracking analysis (see Materials and Methods 3.6). Samples*

from J774A.1 or T2Kb cells show a similar size distribution, ranging from 100 to 800 nm, with a predominance of 140 nm vesicles.

Western blot analysis of MVs secreted by T2Kb or J774A.1 revealed that they contain both HLA-I and β-2-microglobulin (B2M), which are components of MHC-I complex, and that the amount of these proteins in the MVs were increased when immune cells were activated with t.c.m. (Figure 20 A and B).

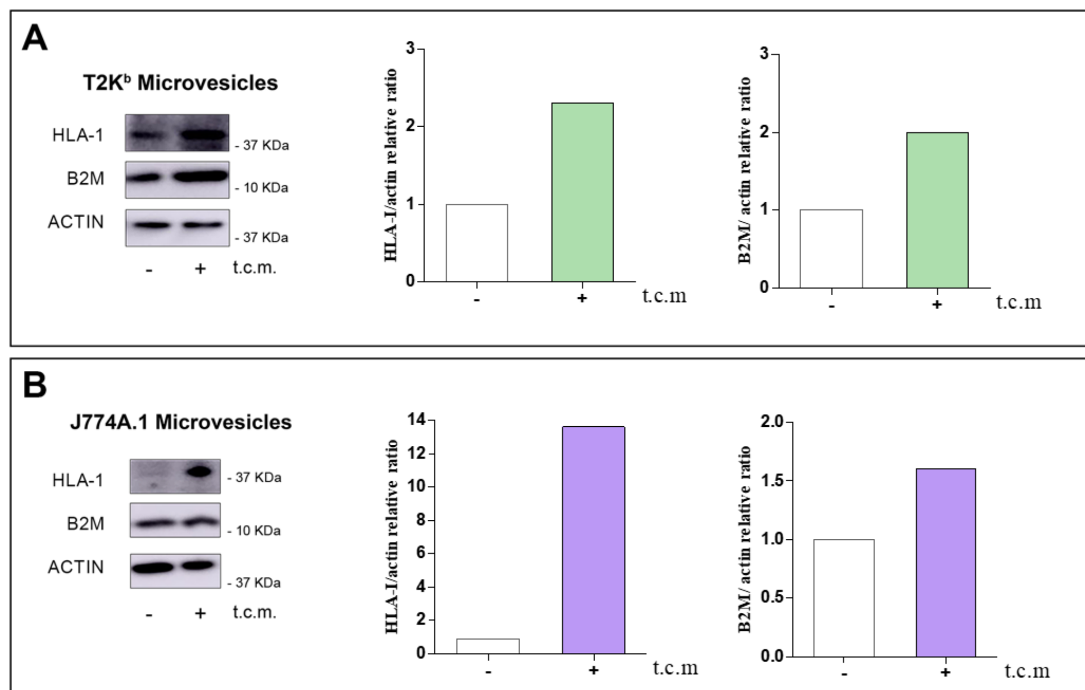

Fig 20. Microvesicles derived from activated T2Kb or J774A.1 transport MHC-I molecules*. (A) MVs purified from T2Kb cells, which were either activated or not with t.c.m. were lysed and analyzed by western blot using anti HLA-I or anti-B2M antibodies. Blots and relative diagrams showing the actin-normalized quantification are reported. (B) MVs purified from J7744.1 cells, which were either activated or not with t.c.m. were lysed and analyzed by western blot using anti HLA-I or anti-B2M antibodies. Blots and relative diagrams showing the actin-normalized quantification are reported. Data are representative of at least three independent experiments with similar results.*

4.1.2. *Monocytes derived-MVs mediated the transfer of MHC-I molecules from immune cells to cancer cells.*

To verify whether MVs mediated the transfer of MHC-I molecules from immune cells to cancer cells, we treated for 1 hour or 24 hours the AGS or 4T1 cancer cells with MVs purified from activated T2Kb or J774A.1 cells. The 1 hour treatment of cancer cells with purified MVs led to an increase in HLA-I protein levels in both types of recipient cells, while after 24 hours, the HLA-I protein levels were equal to the control. In addition, this increase was not observed when cancer cells were treated for 1 hour or 24 hours with monocyte-derived conditioned medium depleted of MVs (Figure 21 A and B).

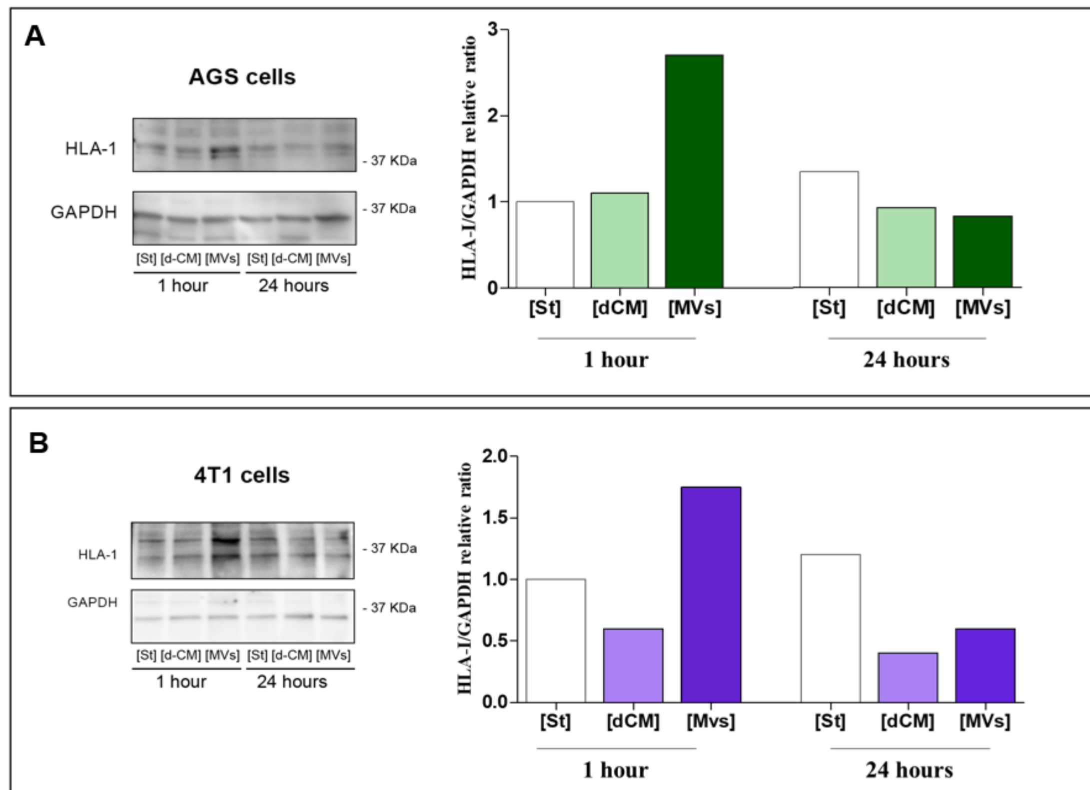

Fig 21. ***(A and B) HLA-I protein levels in AGS or 4T1 tumor cells treated with MVs isolated from activated monocytes.*** *(A) AGS tumor cells were incubated for 1 or 24 hours with MVs purified from the conditioned medium of T2Kb cells that were activated with Starvation medium (St) or t.c.m. depleted of MVs (d-CM) were used as controls. AGS cells were then lysed and analyzed by western blot using anti HLA-1 antibodies. The HLA-1 quantification, which has been normalized by GAPDH content*

of blots was reported. (B) 4T1 tumor cells were incubated for 1 or 24 hours with MVs purified from the conditioned medium of J774A.1 cells that were activated with Starvation medium (St) or t.c.m. depleted of MVs (d-CM) were used as controls. 4T1 cells were then lysed and analyzed by western blot using anti HLA-1 antibodies. The HLA-1 quantification, which has been normalized by GAPDH content of blots. Data are representative of at least three independent experiments with similar results.

These data support the evidence that MVs are involved in the transfer of MHC-I molecules from monocytes to cancer cells, whereas other components of the monocyte-derived secretome are not.

4.1.3. ***Immune cell-derived pMHC-I mediated the cytotoxic activity of $CD8^+$ T cells on cancer cells.***

MHC-I play a pivotal role in immune response. Antigen presenting cells such as dendritic cells, macrophages, neutrophils, process exogenous antigens that are then loaded onto MHC-I molecules. The pMHC-I complex transit to the Golgi apparatus and finally to plasma membrane to trigger CD8+ cells that proliferate and differentiate into CD8+ cytotoxic lymphocytes effectors. One of the mechanisms by which tumor cells can elude immune response is by downregulating MHC-I expression. Depending on the mechanism of downregulation, MHC-I expression can be restore and aid in antitumor immunity [99].

Hence, we evaluated whether the transfer of MHC-I, bound to a custom antigen, from immune cells to cancer cells, can help CD8+ T cells to direct their cytotoxic activity towards cancer cells.

We used T2Kb cells as APCs, which were activated by t.c.m. and preloaded or not with an ovalbumin-derived peptide (pOVA), and we used OT-I as effector CD8+ T cells. OT-I derived from C57BL/6 OT-I transgenic mice are engineered cells expressing a T cell receptor that recognizes the ovalbumin peptide 257-264 in the context of H2Kb MHC-I molecule. In vitro cytotoxicity tests were performed under various co-culture conditions, and for each of them, we evaluated T2Kb and CT26.WT cell viability by flow cytometry (see Materials and Methods 3.8) (Table 1).

Table 1. Cytotoxicity test on CT26.WT tumor cells mediated by the T2Kb / OT-I cell system.

Condition	Co-culture types	pOVA	Anti-mouse H-2K^b	Dead T2Kb cells (%)	Dead CT26.WT cells (%)
1	CT26.WT + OT-I	-	-	-	0.26
2	CT26.WT + OT-I	+	-	-	0.17
3	T2Kb + OT-I	-	-	5.6	-
4	T2Kb + OT-I	+	-	**65.0**	-
5	T2Kb + OT-I	+	+	**23.4**	-
6	T2Kb + CT26.WT	-	-	2.1	4.2
7	T2Kb + CT26.WT	+	-	1.9	1.8
8	T2Kb + CT26.WT + OT-I	-	-	1.5	1.4
9	T2Kb + CT26.WT + OT-I	+	-	**20.4**	**15.1**
10	T2Kb + CT26.WT + OT-I	+	+	6.2	4.1

Cells were kept in in the indicated condition for 24h in the presence or in the absence of OVA peptide (pOVA). Then, where indicated, OT-I cells were added for additional 16 hours. Cells were then detached and analyzed by flow cytometry to assay T2Kb and CT26.WT cell viability (see Materials and Methods 3.8). In each condition: CT26.WT, $1x10^6$ cell; OT-I, $4x10^6$ cell; T2Kb, $2x10^6$ cell. Data are representative of three independent experiments.

As hypothesized, OT-I cells were able to kill a higher number of T2Kb cells when pre-incubated with pOVA (Table 1; condition 4) compared to untreated cells (Table 1; condition 3). In addition, to explain the dependence of tumor cell death on MHC-I presence, we used anti-mouse H-2Kb blocking antibody. The cytotoxic effect of OT-I cells was reduced in the presence of the antibody (Table 1; condition 5). These results confirm the specificity and effectiveness of our in vitro model. When CT26.WT cells were co-cultured with T2Kb cells previously exposed to pOVA, we observed that OT-I cells exerted their cytotoxic effect not only on T2Kb cells, as described before (Table 1; condition 4), but also on CT26.WT cells (Table 1; condition 9). In contrast, the cytotoxic effect of OT-I cells on CT26.WT cells was very low when T2Kb cells and/or pOVA were not present (Table 1; conditions 1, 2, and 8), and the cancer cell death rate was similar to that observed when CT26.WT and T2Kb cells were co-cultured without

OT-I cells (Table 1; conditions 6 and 7). To test the specificity of the cytotoxic activity, co-cultured CT26.WT and T2Kb cells were incubated with the anti-mouse H-2Kb blocking antibody, which reduced the OT-I cell-mediated cytotoxic effect on both T2Kb and CT26.WT cells (Table 1; condition 10).

To further generalize and validate our method, we performed a similar experiment using a human model. We isolated monocytes and TT-specific $CD8^+$ T cells from a human donor who was previously immunized against Tetanus Toxoid (TT). Monocytes were then incubated for 24 hours with or without TT and then co-cultured with AGS cells. Subsequently, $CD8^+$ T cells were added to the co-culture and, after 18 hours, monocyte and AGS cell viability was measured by flow cytometry.

The results confirmed the specificity of this in vitro model by obtaining results similar to that previously observed. Donor-derived T cells induced a higher monocyte death rate when the cells were pre-incubated with TT (Table 2; condition 4) compared to untreated cells (Table 2; condition 3) while the use of anti HLA class I blocking antibody reversed the cytotoxic effect (Table 2; condition 5). In accordance with previous results, T cells exerted their cytotoxic effect on AGS cells when they were co-cultured with TT pulsed monocytes (Table 2; condition 9). Instead, the cytotoxic effect of T cells on AGS cells was very low when monocytes and/or TT were not present (Table 2; conditions 1, 2, and 8), and the cancer cell death rate measured under these conditions was similar to that observed when AGS cells and monocytes were co-cultured without adding T cells (Table 2; conditions 6 and 7). In addition, the T cell-mediated cytotoxic effect was reduced when AGS cells and monocytes were co-cultured in the presence of an anti HLA class I blocking antibody (Table 2; condition 10).

Table 2. Cytotoxicity test on AGS tumor cells mediated by human healthy donor-derived immune cells.

Condition	Co-culture types	Tetanus toxoid (TT)	Anti- HLA class I	Dead Monocytes (%)	Dead AGS cells (%)
1	AGS + T-cells	-	-	-	1.4
2	AGS + T-cells	+	-	-	1.6
3	Monocyte + T-cells	-	-	2.8	-
4	Monocyte + T-cells	+	-	**14.5**	-
5	Monocyte + T-cells	+	+	3.9	-
6	Monocyte + AGS	-	-	2.2	1.6
7	Monocyte + AGS	+	-	2.5	2.6
8	Monocyte + AGS + T-cells	-	-	2.8	1.4
9	Monocyte + AGS + T-cells	+	-	**17.7**	**11.8**
10	Monocyte + AGS + T-cells	+	+	3.2	2.9

Cells were kept in the indicated condition for 24 hours in the presence or in the absence of TT. Where indicated, autologous TT-specific T CD8^{+} cells were added for additional 16 hours. Cells were then detached and analyzed by flow cytometry to assay monocytes and AGS cell viability (see Materials and Methods 3.8). In each condition: AGS, 1x10^{6} cells; CD8^{+} T cells, 4x10^{6} cells; Monocytes, 2x10^{6} cells. Data are representative of three independent experiments.

These results demonstrate that the presence of monocytes is a necessary condition to allow CD8^{+}-lymphocyte cytotoxic activity on cancer cells, and that this effect is mediated by their MHC-I complex.

4.1.4. Immune microvesicles-derived pMHC-I mediated the cytotoxic activity of CD8^{+} T cells on cancer cells.

Finally, to demonstrate the involvement of MVs in the transfer of pMHC-I molecules from immune cells to cancer cells we set up a cell-free experiment.

We treated CT26.WT cells with different amounts of MVs secreted from activated T2Kb cells pre-incubated with or without pOVA. After 1 hour, OT-I cells were added,

and after an additional 16 hours, CT26.WT cells were detached and subjected to cytotoxicity tests (Table 3).

Table 3. Cytotoxicity test on CT26.WT tumor cells in a cell free system

Condition	co-culture types	pOVA	Dead CT26.WT cells (%)
1	CT26.WT + OT-I	-	5.2
2	CT26.WT + OT-I	+	4.9
3	CT26.WT + MVs*(1x) + OT-I	-	4.8
4	CT26.WT + MVs*(1x) + OT-I	+	**22.3**
5	CT26.WT + MVs*(5x) + OT-I	-	5.9
6	CT26.WT + MVs*(5x) + OT-I	+	**30.6**

CT26.WT tumor cells were treated with different concentrations of MVs purified by activated T2Kb cells. After 1 hour, OT-I cells were added and after additionally 16 hours, CT26.WT cells were detached and subjected to cytotoxicity test (see Materials and Methods 3.8). In each condition: CT26.WT, $1x10^6$; OT-I ($CD8^+$ T cells): $4x10^6$. MVs(1x): MVs from $1x10^7$ activated T2Kb cells. MVs*(5x): MVs from $5x10^7$ activated T2Kb cells. Results are representative of three independent experiments.*

OT-I cells were able to recognize and kill a higher number of cancer cells when they were treated with pMHC-I-bearing MVs (Table 3, conditions 4 and 6) than when cancer cells were treated with MVs containing an empty MHC-I (Table 3, conditions 3 and 5). In fact, the treatment of cancer cells with MVs containing empty MHC-I (Table 3, conditions 3 and 5) or with pOVA alone (Table 3, condition 2) induced cell death to the same extent as in the untreated condition (Table 3, condition 1).

4.1.5. Immunological treatment of solid tumors

We finally validate our method through experiments on immunodeficient mice or immunocompetent mice.

In the first experiment we used PC3 cancer cells that were injected subcutaneously into the flank of 6-8-week old male SCID-bg/bg mice. The T2Kb cells were activated in vitro with the tumor conditioned medium of PC3 cells and preloaded with pOVA,

after which they were injected intratumorally. After 4 hours, OT-I (lymphocytes) cells were injected into the tumor mass (Figure 22 A). Tumor growth was lower in treated mice than in control mice that were not injected with T2Kb cells or pOVA (Figure 22 B).

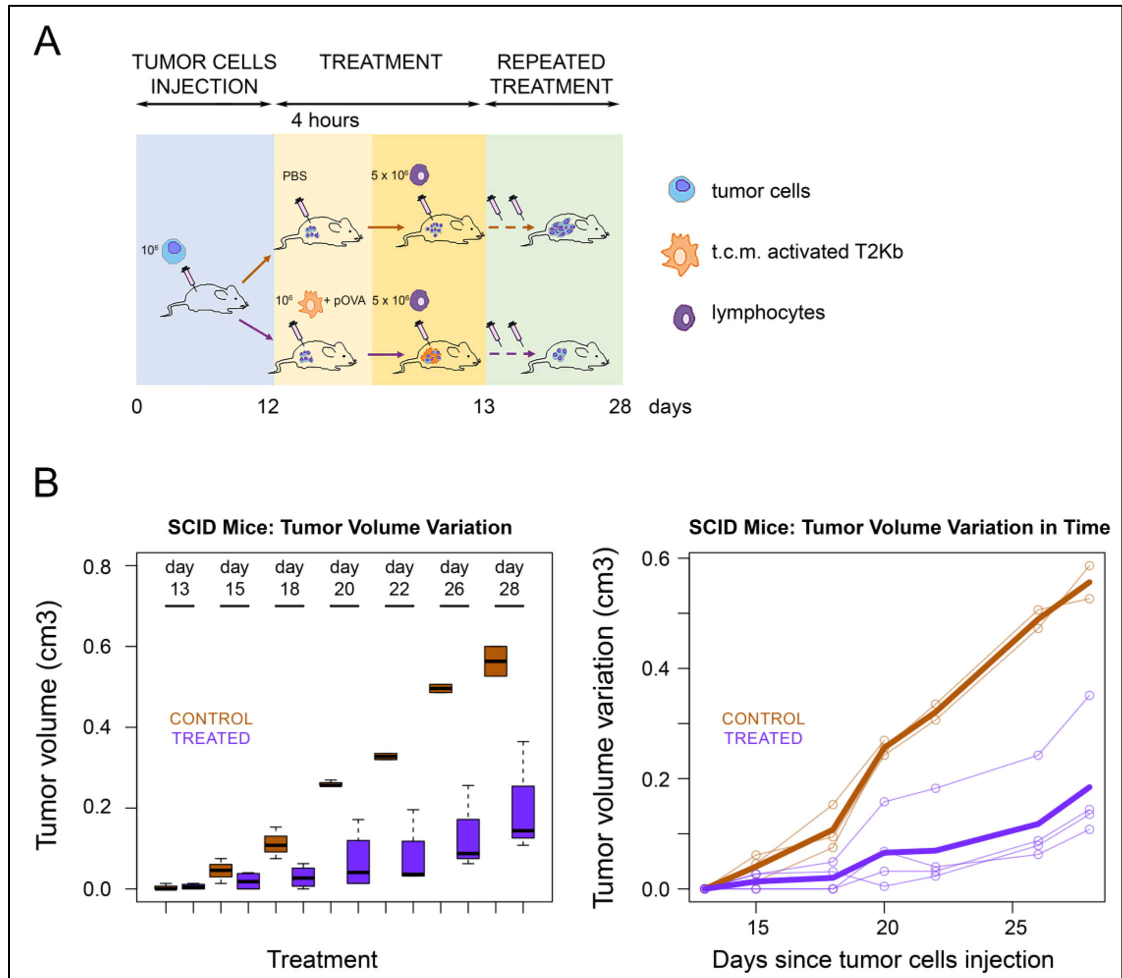

Fig 22. Immunological treatment of immunocompromised mice*. (A) Scheme of SCID mice treatment. Seven SCID-bg/bg mice were subcutaneously injected with PC3 cells. When tumors became palpable, mice were divided in two groups: four mice were injected intratumorally with monocytes plus pOVA antigen (Treated) and three mice with PBS only (Control). After 4 hours mice were injected intratumorally with anti-OVA OT-I lymphocytes. Treatment was performed, at days 13, 15, 18, 20, 22, 26 and 28 after the injection of tumor cells. (B) Tumor growth rate. Tumor growth in control (PBS) and treated (+T2Kb) SCID-bg/bg mice represented as a boxplot of tumor volume size at the different time points (left) and as a spaghetti plot of the trend of mice*

tumor volume in time, with thicker lines representing the average of all individuals (right). Data are representative of at least three independent experiments.

Similar experiments were then conducted on immunocompetent mice, demonstrating that the transfer of pMHC-I molecules from immune cells to cancer cells could represents a novel strategy for cancer treatment. We immunized immunocompetent BALB/c mice with a subcutaneous injection of ovalbumin protein (OVA) (Figure 23).

At the end of the experiment, we verified the efficacy of immunization by evaluating the response of mononuclear cells in the spleen to OVA (see Materials and Methods 3.13). Next, 4T1 cancer cells were injected subcutaneously into the flank region of BALB/c mice. When tumors had reached the minimum palpable size, mice were injected intratumorally with activated J774A.1 monocyte cells that were pre-incubated with OVA. Immunized mice injected with OVA or PBS only were used as control conditions (Figure 23 A).

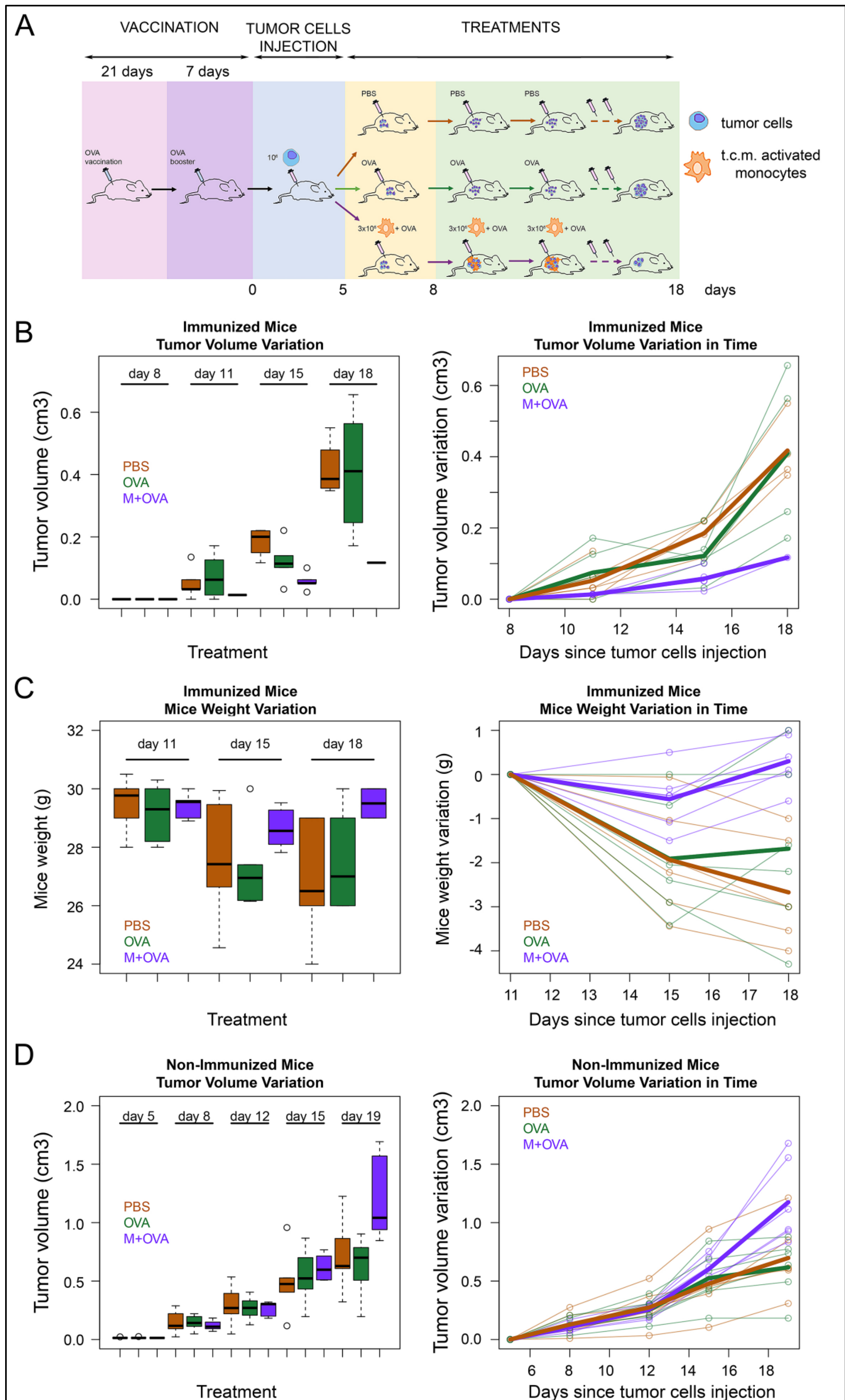
A
VACCINATION
TUMOR CELLS INJECTION
TREATMENTS
21 days
7 days
OVA vaccination
OVA booster
PBS
OVA
+ OVA
tumor cells
t.c.m. activated monocytes
0
5
8
18
days
B
Immunized Mice
Tumor Volume Variation
Tumor volume (cm3)
day 8
day 11
day 15
day 18
PBS
OVA
M+OVA
Treatment
Immunized Mice
Tumor Volume Variation in Time
Tumor volume variation (cm3)
Days since tumor cells injection
C
Immunized Mice
Mice Weight Variation
Mice weight (g)
Immunized Mice
Mice Weight Variation in Time
Mice weight variation (g)
D
Non-Immunized Mice
Tumor Volume Variation
day 5
day 12
day 19
Non-Immunized Mice
Tumor Volume Variation in Time

Fig 23. Immunological treatment of immunocompetent mice tumors. *Scheme of immunocompetent mice treatment. Eighteen immunocompetent BALB/c mice were vaccinated using an OVA protein. After three weeks the mice got a booster dose. The effectiveness of the immunization was tested as reported in the method section. Then, all mice were subcutaneously injected in the flank region with syngeneic 4T1 tumor cells. When the tumors reached the minimum palpable size (11th day after tumor cells injection), mice were divided in three groups and the treatment started: six of them were treated with activated monocytes (J774A.1 cells) plus OVA (M-OVA); six with OVA alone (OVA); and six with PBS (control). The same treatment was repeated at day 15 and 18 after tumor cells injection. (B) Tumor growth rate in immunized mice. Tumor growth from subcutaneously injected 4T1 tumor cells in the described three treatment groups of BALB/c mice represented as a boxplot of tumor volume size at the different time points (left) and as a spaghetti plot of the trend of mice tumor volume in time, with thicker lines representing the average of individuals in the three treatment groups (right). (C) Weight variation in immunized mice. Weight loss of the described three treatment groups of BALB/c mice represented as a boxplot of weights at the different time points (left) and as a spaghetti plot of the trend of mice weights in time, with thicker lines representing the average of individuals in the three treatment groups (right). (D) Tumor growth rate in non-immunized mice. Tumor growth from subcutaneously injected 4T1 tumor cells in BALB/c mice that were not pre-immunized with OVA protein. The mice were divided in three groups and treated as described above. Data are representative of at least three independent experiments.*

As shown in Fig.23 B and C mice immunization and their treatment with activated monocytes and OVA led to a growth slow down respect to that in control mice injected with OVA alone or with PBS. In fact, while treatment with OVA alone did not show significant differences with respect to PBS, the exposure of OVA through J774A.1 monocytes led to a significant reduction in tumor volume. Moreover, mice treated with activated J774A.1 monocytes plus OVA were also much less susceptible to cancer cachexia compared to those treated with OVA or PBS only. In fact, treatment with J774A.1 reduces the progressive weight loss of mice over time while treatment with OVA alone or PBS shows comparable results in terms of body weight reduction.

To obtain a better overview of our model we performed a further control experiment on non-pre-immunized mice evaluating the effects of the treatments. As expected, we

confirmed that in the absence of pre-immunization with OVA antigen, none of the treatments elicited a beneficial effect either in terms of tumor regression (Fig.23 D). Hence, the pre-immunization is a necessary condition for the efficacy of the treatment.

4.2. *Project 2: The role of BPGM in tumor cells proliferation*

4.2.1. Glycolytic enzyme 2,3-bisphosphoglycerate mutase (BPGM) is involved in cell proliferation.

BPGM is a trifunctional enzyme with a well-defined role in red blood cells where it participates to Rapoport Luebering shunt, producing 2,3-bisphospholgycerate, the metabolite that decrease the affinity of hemoglobin to oxygen but very low is known about its physiological role in the other cells of the body [92].

To better understand whether or not BPGM is involved in cell proliferation we analyzed several cell lines, culturing them in conditions that stimulated or not the proliferation.

We treated DU145 and PC3, two prostatic tumor cell lines, and HDF, that represent a healthy cell line, with 10% FBS supplemented medium or serum free medium. The presence of nutrients in FBS induce cell proliferation while in the serum free condition, fibroblasts are not able to proliferate. After 48 hours cells were lysed and analyzed in their BPGM content by western blot (Figure 24).

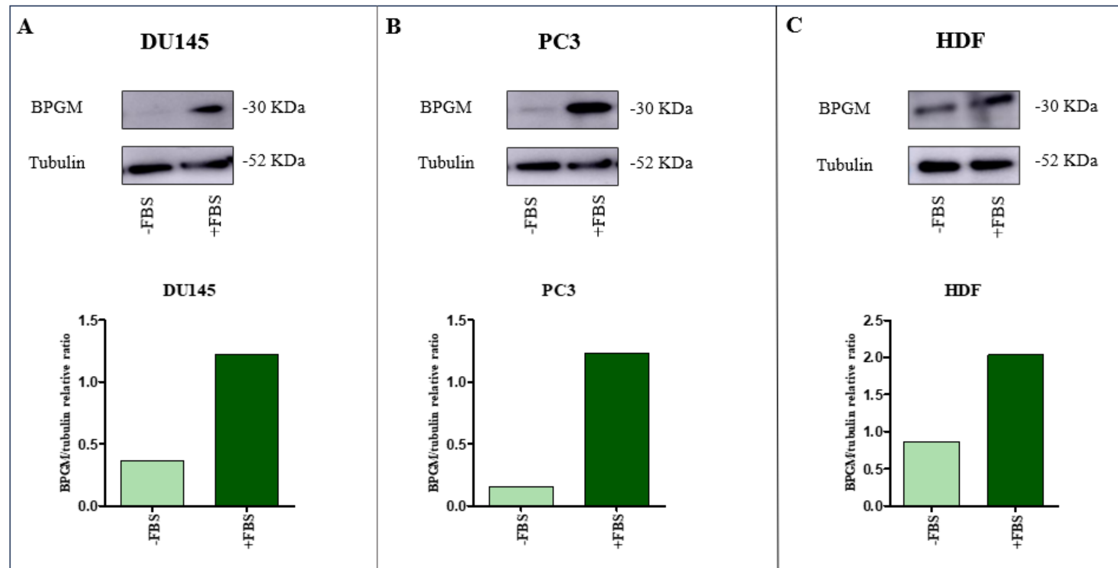

Fig 24. BPGM protein expression in tumor cells and healthy cell*s. DU145, PC3 and HDF were cultured in presence or absence of fetal bovine serum for 48 hours. Cells were then lysed and analyzed by western blot using anti-BPGM antibody. Blots and*

relative diagrams showing the tubulin-normalized quantification are reported. A) BPGM protein expression level in DU145 cells line in presence or absence of FBS; B) BPGM protein expression level in PC3 cell line in presence or absence of FBS; C) BPGM protein expression level in HDF cell line in presence or absence of FBS. Data are representative of three independent experiments.

Western blot analysis allows to state that BPGM is not an RBC-restricted enzyme, but rather it is present also in other cells of our body, regardless of their tumorigenic or healthy features. Indeed, in DU145 cells we registered a percentage increase of BPGM protein expression of 69%, in PC3 cells the percentage increase is of about 90% while HDF showed an increase BPGM protein expression of around 60% when they were cultured in presence of stimulating proliferation factors (such as nutrient present in FBS).

According to these data we hypothesized that BPGM is an enzyme correlated with proliferation of cells. To further understand its physiological role, we also analyzed C2C12 cells.

C2C12 is an immortalized mouse myoblast cell line used in in vitro models to study differentiation of myoblast into myotubes. Differentiation of myoblasts into myotubes is obtained colturing few days C2C12 cells with DMEM supplemented with horse serum, a serum poor of nutrients, in a very low percentage (2%). These two different physiological conditions can give information about the involvement of a enzyme in proliferation of cells. C2C12 cultured in a medium supplemented with 10% fetal bovine serum and C2C12 cultured in medium supplemented with 2% of horse serum were then lysed and analyzed by Western Blot (Figure 25).

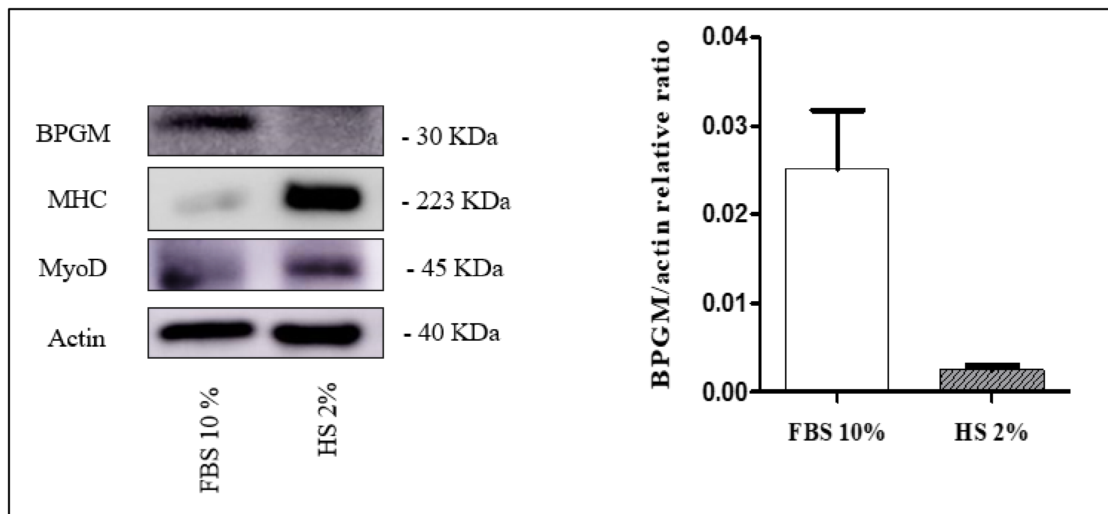

Fig 25. BPGM protein level in differentiated or not C2C12 cells. *C2C12 were cultured in 10% FBS supplemented medium or 2% HS supplemented medium to mimic respectively the condition of myoblast (not differentiated cells) or myotubes (differentiated cells). After the time needed for the differentiation process (see Materials and Methods 3.1) cells were lysed and analyzed by western blot with anti-BPGM antibody, and anti-MHC (myosin heavy chain) and anti-MyoD antibody used as marker of differentiation of the cells. anti-βactin was used to normalize the results. Blots and relative quantification diagrams are reported.*

Data show that in differentiated C2C12 BPGM expression drastically drop down. Since the condition of differentiation is correlated to decrease proliferation of cells, these data enhance the hypothesis of the close correlation between BPGM expression and proliferation of the cells.

4.2.2. *BPGM silencing decrease cells proliferation.*

To date, there is no information about the role of BPGM in cell proliferation since it is considered an erythrocytes specific enzyme. However according to our previous data, BPGM is regulated in different physiological condition.

To demonstrate the phenotypic effect on cell proliferation we treated the DU145 and PC3 in the same condition of presence or absence of 10% FBS for 24 or 48 hours.

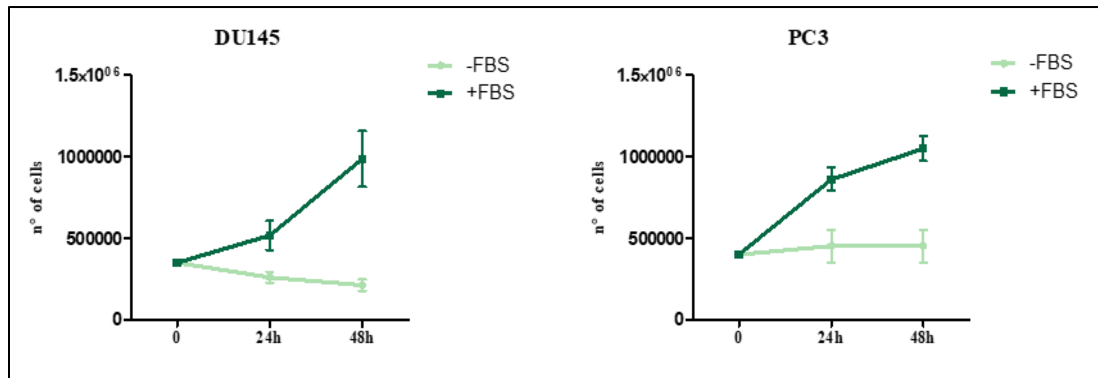

Fig 26. Evaluation of cell proliferation in presence or absence of stimulating proliferation factors. *A) DU145 cells were treated for 24h or 48 hours with 10% supplemented DMEM medium (dark green) or serum free DMEM (light green). B) PC3 cells were treated for 24h or 48 hours with 10% supplemented DMEM medium (dark green) or serum free DMEM (light green). Cells were the detached and counted by Burker Chamber.*

It is well known that the presence of FBS, containing nutrients ensure cells proliferation. Indeed, the treatment of both cells line DU145 and PC3, with 10% FBS supplemented medium induce proliferation of the cells, at 24 and 48 hours while the starvation condition led to a progressively decrease of proliferation (Fig.26).

To understand the involvement of BPGM in cancer cells proliferation we decided to silence the enzyme and analyze the proliferation of the cells, using as control cells treated with 10% FBS medium. The silencing of BPGM in DU145 and PC3 was obtained using sequence-specific siRNA (see Materials and Methods 3.18).

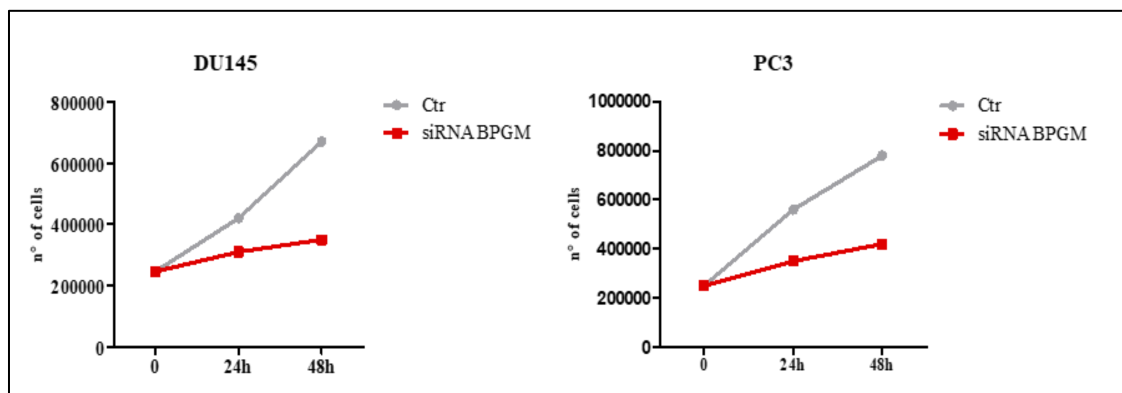

Fig 27. Effect of BPGM silencing in tumor cells proliferation. *A) DU145 were treated with 10% FBS supplemented medium as control condition (grey) and with 10%*

FBS after silencing BPGM with sequence-specific siRNA (red) for 24h or 48 hours B) PC3 were treated with 10% FBS supplemented medium as control condition (grey) and with 10% FBS after silencing BPGM with sequence-specific siRNA (red) for 24h or 48 hours. After the treatment, cells were detached and analyzed by flow cytometry.

The silencing of BPGM and the consequences of the silencing were tested also in human normal fibroblasts (HDF).

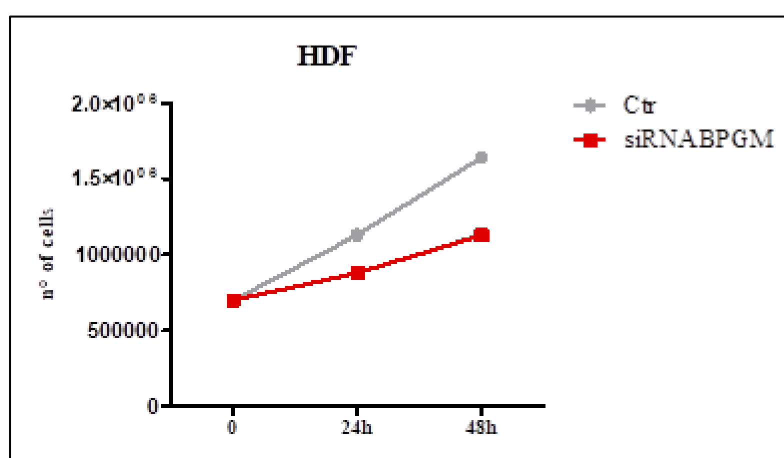

Fig 28. ***Effect of BPGM silencing in healthy cells proliferation***. *HDF were treated with 10% FBS supplemented medium as control condition (grey) and with 10% FBS after silencing BPGM with sequence-specific siRNA (red) for 24h or 48 hours. After the treatment, cells were detached and analyzed by flow cytometry.*

The experiments result on tumor or non-tumor cells, give indication of the involvement of BPGM in cells proliferation, since the silencing of the enzyme drastically decrease proliferation rate in the three analyzed cells line (Fig.27 and Fig.28).

4.2.3. ***BPGM enzyme is differentially regulated in co-culture systems of fibroblast and tumor cells.***

Co-culture system has long been studied as means to define the interaction between populations that are grown with certain degree of contact. The system is used to study the natural interactions between cells, to improve the success of growth of a specific

population or establish synthetic interactions between population. For example, some cells are difficult to be growth in monoculture but the presence of another population may improve the culturing success or cell behavior [100].

However, a system where two populations grow together is always more reliable in terms of similarity to the biological system than the monoculture.

We analyzed the behavior of HDF co-cultured with tumor cell lines DU145 and PC3. Particularly we tested the BPGM protein level change in this system. We cultured HDF in 10% FBS medium until they reached a confluence of 70% and then we plated DU145 or PC3 on a 0.4 μm transwell in serum free medium for 48 hours. Then the cells were lysed and analyzed by western blot for BPGM protein expression (Figure 29).

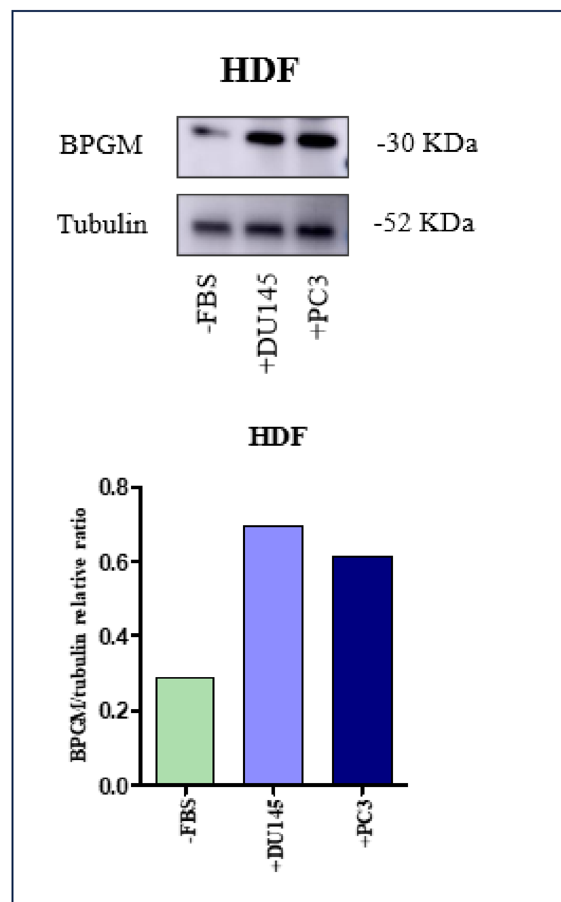

Fig 29. BPGM protein expression in HDF co-cultured with tumor cells*. HDF were cultured in serum free medium as control conditions and in co-culture with DU145 or PC3 for 48 hours. The cells were then lysed and analyzed by western blot to assess the*

content of BPGM, using anti-BPGM antibody and anti-tubulin to normalize the results. Blots and relative quantification histogram are reported.

The results on HDF showed an increment of 57% in BPGM protein expression when HDF were co-cultured with DU145 and an increment of 50% when co-cultured with PC3 respect to the control condition.

We also studied the behavior of tumor cells in term of BPGM protein expression when the cells where co-cultured with HDF (Figure 30).

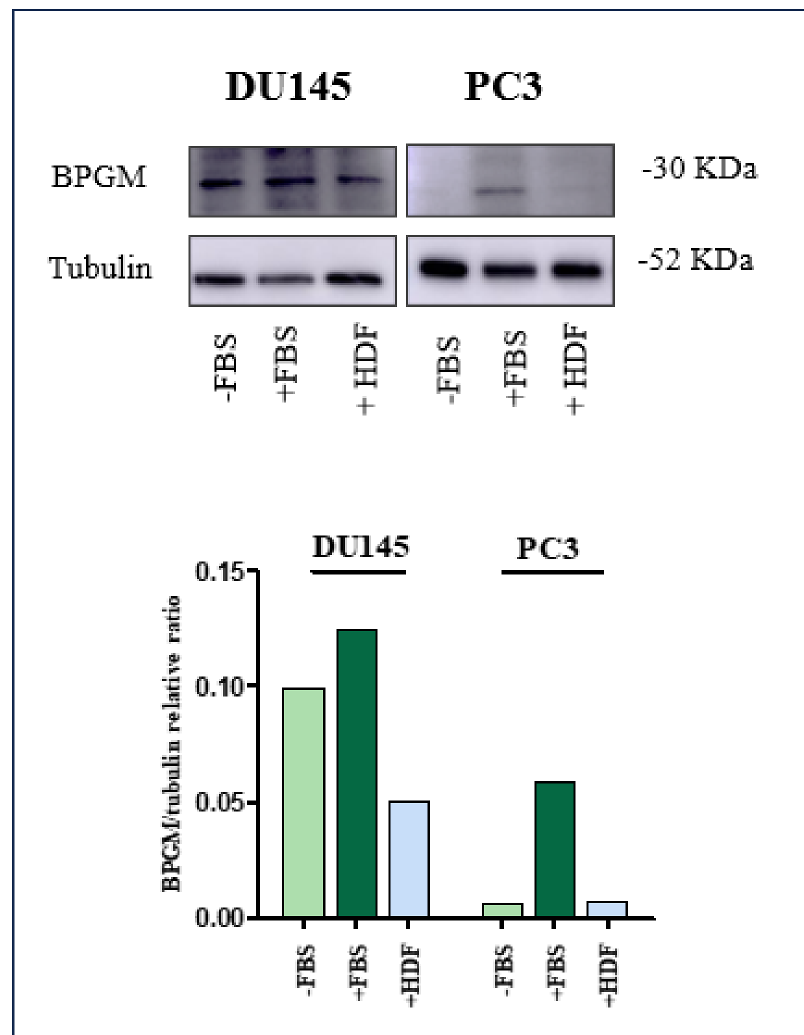

Fig 30. BPGM protein expression in tumor cell co-cultured with HDF. *DU145 and PC3 where cultured in serum free medium used as control condition, in 10% FBS medium and in co-culture with HDF in serum free medium system for 48 hours using*

0.4 µm Transwell. Cells were then lysed and analyzed by Western Blot using anti-BPGM antibody and anti-tubulin antibody to normalize results.

After culturing tumor cells with HDF for 48 hours we observed a decreased expression of BPGM protein level. In DU145 cells, the level of the protein in co-culture, decrease of about 58% respect to the level of the protein in stimulating proliferation condition (+FBS). In PC3 cells, we observed a decrease of 82% of protein level respect to the proliferating condition (+FBS), similar to the protein level expressed by the cells in serum free condition.

Surprisingly, BPGM is regulated differently in co-culture or single culture, either in tumor cells or in fibroblast, highlighting how two populations can influence each other.

4.2.4. ***BPGM silencing in HDF restore higher protein level in tumor cells.***

Fibroblasts are mesenchymal cells, playing a major role in tissue structural support participating also to wound healing and by the secretion of cytokines chemokines and grow factors[101].

Our experiment demonstrated how tumor cells showed decreased level of BPGM protein when plated in co-culture with HDF, while in HDF we observe the opposite trend. My group of research already demonstrated that BPGM upregulation in HDF and consequently the Luebering-Rapoport shunt is an indication of increased glucose uptake (data not shown). This result paved the way to hypothesized that alongside glucose uptake there would be also an increase in biomass production suggesting that also in this case, fibroblast could act as tissue supporters in order to produce substrates that can be subsequently transferred to cancer cells that, in this specific condition, do not need to upregulate BPGM.

To demonstrate the influence of fibroblast on tumor cells in term of BPGM expression we silenced the BPGM enzyme in HDF (see Materials and Methods 3.18) with siRNA and we co-cultured HDF with DU145 and PC3 for 48 hours using a Transwell system with pore of 0.4 µm (Figure 31).

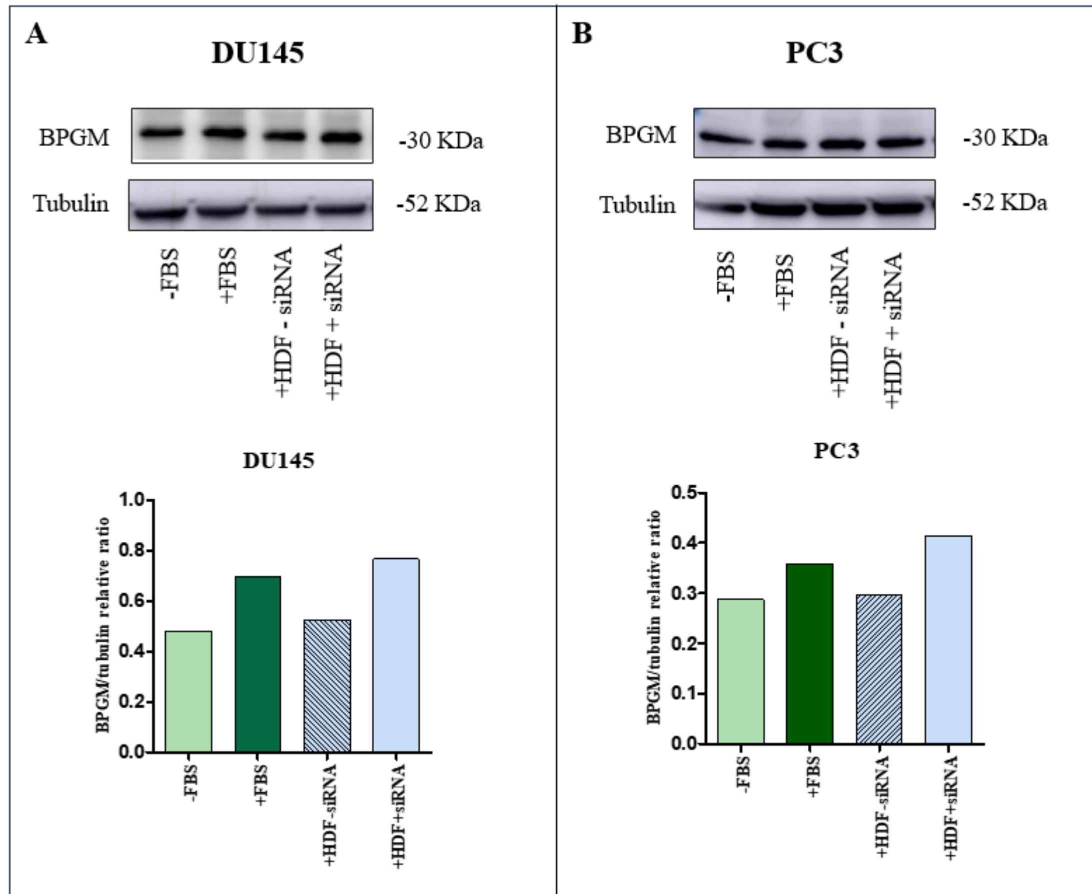

Fig 31. BPGM protein level in tumor cells co-cultured with BPGM-silenced HDF*.* *A) DU145 were co-cultured with BPGM-silenced HDF (+HDF siRNA) or not silenced (+HDF-siRNA) plating the cells on 0.4 µm pore Transwell system in serum free medium for 48 hours. Starvation (-FBS) and FBS supplemented medium (+FBS) were used as control. B) PC3 were co-cultured with BPGM-silenced HDF (+HDF+siRNA) or not silenced (+HDF-siRNA) plating the cells on 0.4 µm pore Transwell system in serum free medium for 48 hours. Starvation (-FBS) and FBS supplemented medium (+FBS) were used as control. Cells were analyzed by western blot with anti-BPGM antibody and anti-tubulin to normalize data.*

DU145 and PC3 were cultured in 10% FBS supplemented DMEM until they reached a confluence of 70%. HDF were silenced for BPGM according to the manufacturing protocol and then were plated on 0.4 µm pore Transwell. DU145 and PC3 were also incubated with serum free medium, 10% FBS supplemented DMEM and co-cultured with not BPGM-silenced HDF (+HDF-siRNA) on a 0.4 µm pore Transewell system that represented the control condition. The immunoblot analysis on DU145 show an increased BPGM expression of 42% when tumor cells were co-culture with HDF

silenced for BPGM protein (+HDF+siRNA) respect to the control condition in serum free medium, while the co-culture with not BPGM-silenced HDF confirm the results showed in the previous paragraph that is a decreased BPGM level when tumor cells are co-cultured with normal fibroblasts (Fig.31 A). Similar results were obtained for PC3 cell line, where the co-culture of tumor cells with HDF silenced for BPGM induce an increase in BPGM protein expression of 33% respect to the starvation condition while the co-culture with not silenced HDF restored the protein expression at the level of control condition (Fig.31 B).

The data indicate that co-culture effect on tumor cells BPGM protein level is correlated to BPGM protein expression in fibroblasts: enzyme silencing in HDF leads tumor cells to maintain BPGM expression levels like those observed in the presence of FBS.

*4.2.5. **HDF and CAF derived microvesicles characterization.***

According to the obtained results we hypostasized the involvement of extracellular vesicles, in particular of microvesicles, in the transfer of biomass between fibroblast and cancer cells. All the cells of our body are able to release extracellular vesicle that cover a main role in cell communication by transferring biomolecules such as, lipids, proteins and nucleic acids [102]. The interaction between tumor cells and fibroblasts induces the differentiation of the latter into cancer associated fibroblasts. Among the stromal cells in tumor microenvironment, cancer associated fibroblast play important role in cancer progression. Particularly previous study demonstrated that CAF derived EVs are internalized in tumor cells, contributing to migration, invasion, and metastasis [103]. Santi et.al demonstrated also that the transfer of protein in co-culture model is almost unidirectional from CAF to cancer cells and it is mediated by microvesicles [95].

We therefore hypothesized that the low expression of BPGM in tumors cells placed in coculture with the CAFs, could be determined by the fact that the tumor cells, by incorporating the extracellular vesicles released by the fibroblasts, do not need to increase their own biomass synthesis using the Luebering-Rapoport pathway. To develop this topic, we conducted preliminary experiments which allowed us to

highlight some of the characteristics of the microvesicles released by fibroblasts and CAFs

We isolated microvesicles derived from a similar number of HDF and HDF activated by DU145 and PC3 conditioned media. The isolation was obtained via differential centrifugation. The 10000xg pellets (microvesicles) were analyzed by nanoparticles tracking analysis (Figure 32).

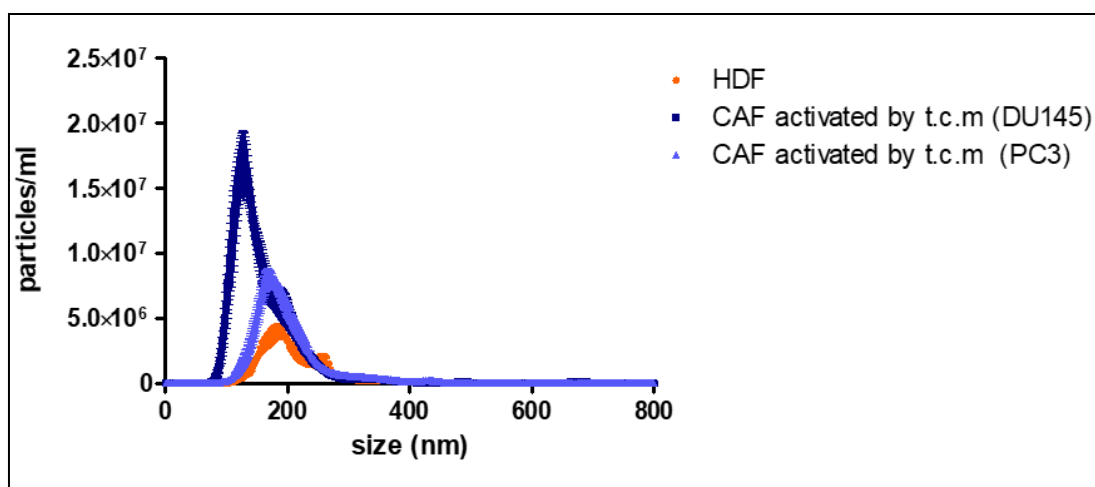

Fig 32. Nanoparticles tracking analysis of HDF and CAF derived microvesicles. *(orange) Size distribution of microvesicles derived from HDF; (dark blue) size distribution of microvesicles derived from HDF activated with DU145-conditioned medium; (light blue) size distribution of microvesicles derived from HDF activated with PC3-conditioned medium. The average size distribution of the three populations is 161,6 nm.*

The NTA analysis show that CAFs release a major number of microvesicles respect to HDF. The concentration of microvesicles derived from HDF is 3.74×10^8 particles/ml, while the concentration of microvesicles derived from CAFs activated by DU145 and PC3 conditioned medium is respectively $1{,}31 \times 10^9$ and, 6.99×10^8 particles/ml (Figure 33).

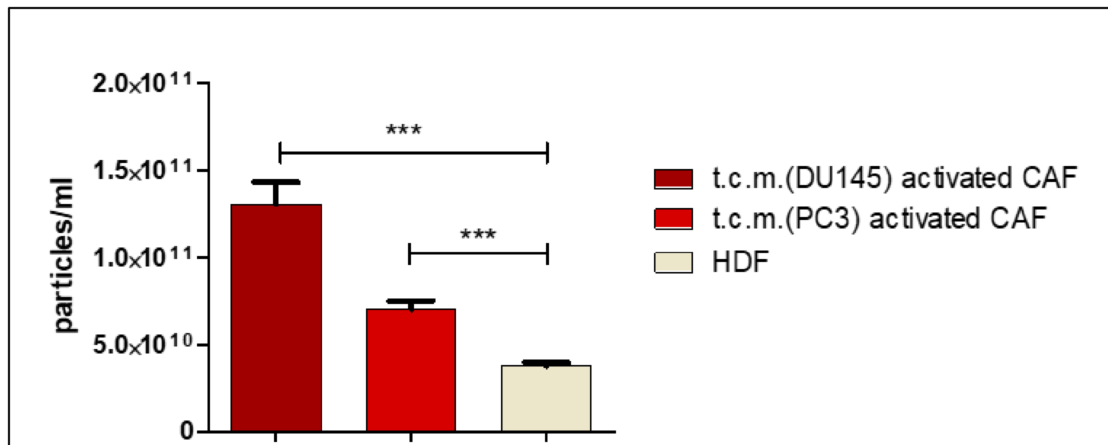

Fig 33. Concentration of microvesicles derived from HDF and CAFs. *(dark red) Concentration of microvesicles derived from HDF activated with DU145 conditioned medium; (light red) Concentration of microvesicles derived from HDF activated with PC3 conditioned medium; (beige) Concentration of microvesicles derived from HDF. Concentration of HDF- derived microvesicles is significatively lower than CAFs-derived microvesicles. The concentration of microvesicles was obtained by NTA analysis.*

Most vesicles, exosomes and microvesicles, secreted by fibroblasts, remain trapped in the extracellular matrix secreted by themselves and from the matrix they are captured by neighboring cells. To assess whether changes in BPGM protein level in tumor cells were influenced by the transferring of protein mediated by extracellular vesicles we set up a “decellularization” experiment. We cultured HDF and CAF and we let them deposit extracellular matrix for 48 hours. CAFs used in the experiment were inducted to differentiation by previously incubation with DU145 or PC3 conditioned medium for 24 hours. After matrix deposition, we treated the plates with a NaCl solution to kill and eliminate the cells and retain only the matrix and the extracellular vesicles in it.

The matrix was treated with CFDA-SE, after that DU145 and PC3 were plated on the dishes. After 48 hours the fluorescence of DU145 and PC3 cells was evaluated by cytofluorimetric analysis (Figure 34).

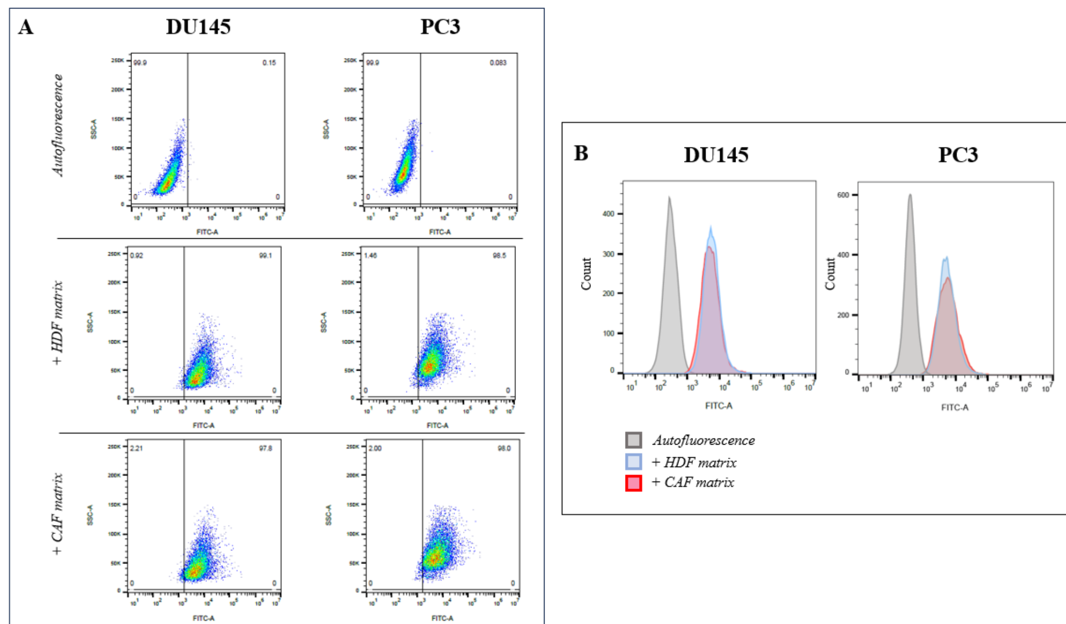

Fig 34. Cytofluorimetric analysis of DU145 and PC3 after "decellularization" experiment*. Tumor cells were gated based on size and complexity of events (FSC-A and SSC-A), and further plotted in FSC-A and FSC-H to gate single cells, excluding doublets. From the single cells gate, cells that incorporated fluorescent component of the matrix were defined as FITC positive. A) Fluorescence of DU145 and PC3 after incubation on CFSA-DE labeled extracellular matrix derived from HDF or CAF. B) Histograms are relatives to the fluorescence of the cells after the "decellularization" experiment. Data are obtained by FlowJo software.*

Tumor cells take up protein materials from the matrix where they were plated on and we didn't observe quantitative differences between the incorporation of protein present in the matrix released by HDF or CAFs.

4.2.6. ***Role of extracellular vesicles in regulating 2,3-Bisphosphoglycerate mutase (BPGM) protein level in tumor cells.***

As aforementioned, extracellular vesicles produced by HDF and CAF, can be present in solution or can be trapped in extracellular matrix. To better understand the involvement of microvesicles in the modulation of BPGM protein level of tumor cells we analyzed the effect of CAFs secretome on DU145 and PC3.

We cultured HDF in presence of tumor conditioned medium derived from DU145 and PC3 to induce the differentiation of HDF into CAF. The CAF conditioned medium was then collected and fractioned as described in Materials and Methods 3.20.

DU145 and PC3 were incubated with the different fractions of CAF conditioned medium for 24 hours and then analyzed in their BPGM content to understand which part of the secretome was involved in the modulation of BPGM protein expression (Figure 35).

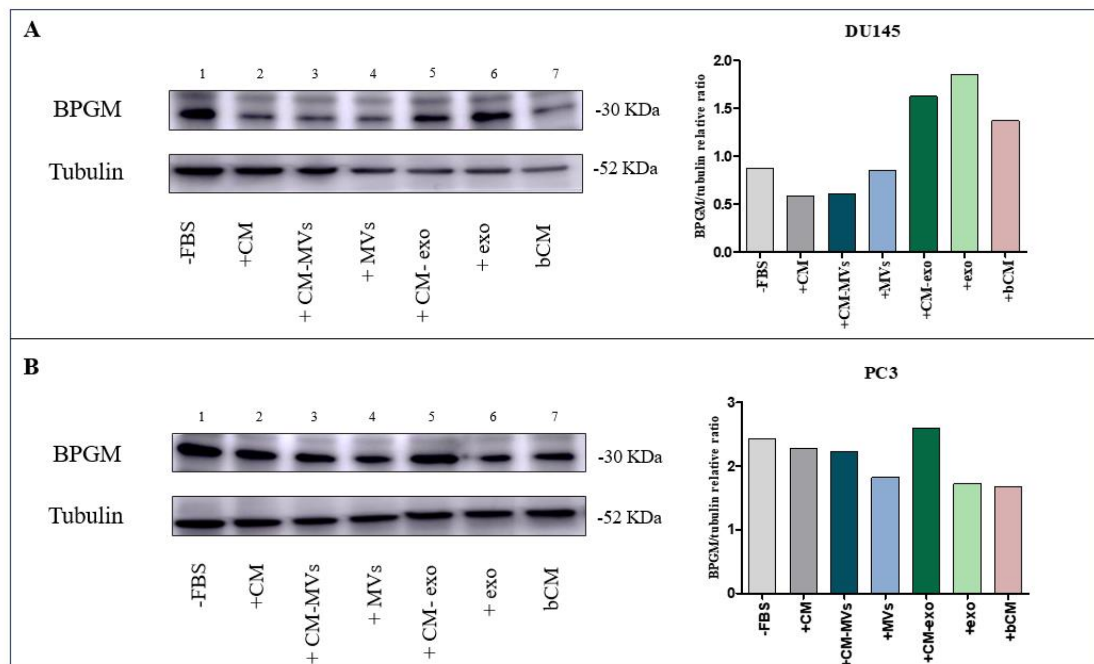

Fig 35. BPGM protein expression in tumor cells after incubation with different fraction of CAF secretome*. A) DU145 were treated with different fraction of CAF secretome. The differentiation into CAF was induced by treating HDF with DU145-coditioned medium. 1. DU145 were incubated with serum free medium (-FBS) 2. DU145 were incubated with complete CAF-conditioned medium (+CM) 3. DU145*

were incubated with CAF-conditioned medium depleted of microvesicles (+CM-MVs) 4. DU145 were incubated with purified microvesicles (+MVs) 5. DU145 were incubated with CAF-conditioned medium depleted of exosomes (+CM-exo) 6. DU145 were incubated with purified exosomes (+exo) 7. DU145 were incubated with exosomes depleted and boiled CAF-conditioned medium (bCM). B) PC3 were treated with different fraction of CAF secretome. The differentiation into CAF was induced by treating HDF with PC3-coditioned medium. 1. PC3 were incubated with serum free medium (-FBS) 2. PC3 were incubated with complete CAF-conditioned medium (+CM) 3. PC3 were incubated with CAF-conditioned medium depleted of microvesicles (+CM-MVs) 4. PC3 were incubated with purified microvesicles (+MVs) 5. PC3 were incubated with CAF-conditioned medium depleted of exosomes (+CM-exo) 6. PC3 were incubated with purified exosomes (+exo) 7. PC3 were incubated with exosomes depleted and boiled CAF-conditioned medium (bCM). After 24h of incubation cells cere lysed and analyzed in their BPGM content by western blot using anti-BPGM antibody. Anti-tubulin antibody was used to normalize the data.

Data show an increased expression of BPGM protein level when tumor cells (DU145 and PC3) were incubated with CAF conditioned medium depleted of exosomes. Differently from what we expected, the result suggests that the fraction of extracellular vesicles mostly involved in the regulation of BPGM protein expression are exosomes while microvesicles results to have a minor activity. The effect is particularly evident in DU145, where the deprivation of exosome in CAF conditioned medium determines an increased expression of BPGM of 64% in tumor cells (Fig.35 A). In this way, the deprivation of biomass present in the conditioned medium induce an upregulation of the protein to restore the level needed by the cells to proliferate.

5. *Discussion*

It is well established that the acquisition and maintenance of cancer hallmarks such as sustained proliferative signaling, resistance to apoptotic cell death, neoangiogenesis induction, invasion and metastasis, inflammation, and the escaping from system response depend on the interaction between cancer cell and the not-transformed cells of the tumor microenvironment . Hence, the cells of TME or their relative sustaining pathway represents an opportunity for therapeutic intervention. The advantage in targeting cells of tumor microenvironment is that the target does not undergo to genetic drift hence it is not prone to drug resistance. In developing new drugs what is important is to develop a specific drug to target cancer related cells but avoiding healthy cells. [9]

Thus arises the need to identify new therapies that complement the traditional ones which in many cases fail. Surgery is the most resolutive especially at early stage, radiotherapy can also damage healthy cells as well as chemotherapy which damages rapidly proliferating cells and then led to the development of resistance by tumor cells. Among the modern modalities such as hormone therapy, anti-angiogenic, stem cell therapies, immunotherapy [104] turns out to be effective and has led to the development of different types of techniques based on triggering an immune response: i) immune checkpoint inhibitors ii) T-cell transfer therapies iii) monoclonal antibodies iv) treatment vaccines v) immune system modulators vi) dendritic cell immunotherapy.

Given the well-established role of extracellular vesicles in the tumor microenvironment crosstalk and their use in cancer treatment, such as drugs delivery due to their biocompatibility [105] we carried out a pilot study proposing a new immunotherapy treatment for solid tumors.

We pursue the idea to exploit the physiological trafficking of extracellular vesicles to transfer custom-defined antigen via MVs to cancer cells making them target of a cytotoxic immune response. Based on previous studies conducted in our laboratory we found out that CAFs were able to transfer proteins to cancer cells (data not shown) and consequently we move our attention to other mesodermal-derived cell lines such as monocytes demonstrating that even in this case they were able to transfer protein in

a unidirectional manner (Fig. 18). Particularly, we observed that monocytes after their activation with tumor conditioned medium, where enriched in MHC-I content and they were able to transfer the molecules to cancer cells in a very short time (Fig. 20).

Given the presence of MHC-I on activated MVs, we hypothesized to exploit them to deliver a previous established antigen from monocytes to cancer cells in order to make them target of the cell-mediated immune response. To verify the hypothesis, we set up an in vitro cytotoxic experiment to demonstrate that monocytes preloaded with a custom antigen transfer the complex pMHC-I to cancer cells via MVs and this condition is necessary and sufficient to trigger the CD8+ cytotoxic immune response (tables 1-3). Then, we translated the experiment *in vivo* using both SCID-bg/bg (immunodeficient) and BALB-C (immunocompetent) mice. The experiments demonstrated that after the purposed treatment we obtain a greatly slowdown in tumor growth as well as of cancer cachexia (Fig. 23 B and C).

We propose this innovative treatment because it opens new perspective in solid tumor therapies. Even the recent immunotherapy treatment of solid tumor aid to the resolution of the disease there are still several pitfalls that can contribute to a not total resolution of the problem. For instance, cancer vaccine is helpful to prepare a response of the body against cancer, but it is very hard to select a single tumor antigen as good candidate due to their genetic instability and the heterogeneity of cancer cells. For these reasons even assuming to find cancer biomarkers, some clones can develop resistance to the treatment as well as common chemotherapy. The advantages of the method are: i) not specificity to a tumor antigen; ii) based on a custom-antigen to be selected among many well-established options iii) independence of solid tumor. The antigen is delivered bound to MHC-I to the surface of cancer cells leading to cytotoxic killing of the cells but not giving rise to resistant clones. The other characteristic of the method that we propose, is the delivery of pMHC-I also to the cells of tumor microenvironment (CAF, endothelial cells, and cells of the immune system) that support tumor cells in their growth and survival. This represents a further advantage in the treatment of a solid tumor since it is not selective for tumor cells but it also against the cells that contribute to is growth. Finally, this kind of treatment can be repeated indefinitely with the same antigen or other defined antigen, provided that the patient is already immunized against them.

Alongside the aforementioned project I conducted a study on the involvement of bisphosphoglycerate mutase (BPGM) in tumor cells proliferation. BPGM is an enzyme with a pivotal role in erythrocytes and for long time was considered exclusive of these cells. BPGM is responsible for the production of 2,3-bisphosphoglycerate, a substrate that bind hemoglobin allowing the release of oxygen even in low pO2 conditions. The enzyme is the main actor in the Luebering-Rapoport shut, where a step of ATP formation is bypassed and it is demonstrated that the skipping in ATP production allow to increase glucose uptake with the constant maintenance of ATP/ADP ratio. [106]

We propose a study on the role of BPGM and its regulation in co-culture conditions as consequence of evidences that BPGM is involved in changes in metabolic profile of fibroblasts and cancer cells and also its gene upregulation in CAFs respect to fibroblast [106].

We demonstrated that the overexpression of BPGM is a common feature of proliferating cells, regardless of their malignant nature. The presence of nutrients in culture medium induces the expression of the enzyme, while the nutrient restriction led to the opposite effect as well as the physiological condition of differentiated or not differentiated cells (Fig. 24 and Fig. 25). Moreover, we demonstrated the strictly correlation between BPGM expression and proliferation since we observed a decreased proliferation when BPGM enzyme was silenced (Fig. 27 and Fig. 28).

Moving from a monoculture to a co-culture condition we observed a different behavior of fibroblast and cancer cells. Of course, the crosstalk between cells is a common feature in TME and the exchange of soluble factors or corpuscle materials is well established leading to modification in the metabolic profile of cells. When in co-culture with tumor cells, fibroblast assume the phenotype of CAFs, due to the cytokines and other factors secreted by tumor cells. In this contest, CAFs showed increased level of BPGM enzyme (Fig.29), vice versa, tumor cells modify the expression of the protein (Fig. 30), with the opposite trend. These data demonstrated the regulation of BPGM expression due to the co-culture and particularly the influence of tumor cells in the metabolism of fibroblast. Indeed, when BPGM was silenced in CAFs, the expression of the enzyme in tumor cells is restored and the level is similar to what we observe in proliferating conditions.

According to our opinion, the obtained results provide concordant data with the "Reverse Warburg" theory. CAFs support tumor growth by the release of metabolites and the secretion of extracellular vesicles, particularly microvesicles. The activation of fibroblast into CAFs induces metabolic switch, from respiratory to glycolytic metabolism. The "aerobic glycolysis" that occur in CAFs led to increased production of metabolic intermediates and secretion of extracellular vesicles. Cancer cells uptake of microvesicles and metabolites derived from CAFs allow them to economize in some biosynthesis pathway and increase they proliferative capability at the same time.

Based on the results that we obtained we hypothesize that CAFs increase their BPGM enzyme expression to maintain a high glycolytic flux and high production of intermediates that will be taken up by cancer cells. Particular interest was paved on the transfer of biomass to cancer cells, that, as already described, occur via extracellular vesicles, mainly microvesicles, from CAFs.

To confirm our hypothesis and deepen our understanding, we isolated microvesicles from fibroblast and CAF, obtained by activation with tumor conditioned medium, to characterize them morphologically and quantitatively. We observed that CAFs increased their microvesicles production with respect to fibroblasts, and the average dimension, obtained by NTA analysis, is around 160 nm for both populations (Fig. 32 and 33). NTA analysis was performed using liquid samples. The EVs were visualized thanks to the light scatter obtained when the vesicles were irradiated by the laser beam and through the NTA software analysis it was possible to determine their size and concentration [107]. CAFs and fibroblast derived-extracellular vesicles uptake by cancer cells has been demonstrated by the "decellularization" experiment where fibroblasts and CAF were let to deposit extracellular matrix and after the elimination of cells, cancer cells were plated on a fluorescently labeled extracellular matrix to observe whether there was a transfer of materials or not (Fig. 34). According to our results, we observed a transfer of biomass from fibroblast to cancer cells without any evident transfer increment when fibroblasts are activated into CAFs.

To demonstrate the involvement of extracellular vesicles in BPGM enzyme regulation we analyzed the CAFs secretome to clarify which fraction was responsible for the modulation of the protein level. We observed that the regulation of BPGM is dependent on the presence of exosomes, indeed the exosome elimination from CAFs

conditioned medium induce and increased expression of the protein in both the tumor cell lines. Hence, the results confirm our hypothesis according to which in the contest of TME, CAFs produce biomass that can be taken up by cancer cells, that do not require an energy consumption increasing biosynthetic pathway themselves.

6. *Conclusions*

This thesis highlights the importance of the tumor microenvironment in cancer progression and the crosstalk that occur between the cells in TME. Studying the molecular interactions and the protein exchange are challenges with the common goals to find new possible and efficient therapies for treatment of solid tumors.

Particularly we demonstrated the role of BPGM in tumor cells proliferation. Deepen the knowledge of its involvement in cells crosstalk can led to the definition of fundamental step in tumor onset and eventually define them as therapeutic targets toward which develop specific therapies to enhance the efficacy of the treatments and improving the prognosis of patients.

Moreover, the best results achieved with my work has been the proposal of a new immunotherapeutic approach for the treatment of solid tumors that would have several advantages: i) it is direct against all the cells of TME, tumor cells and stomal cells responsible for the tumor progression ii) it is independent by the solid tumor type, since pMHC-I is transferred to different type of cells iii) any type of antigens can be used iv) it does not give rise to resistant clones v) it does not give rise to inflammation vi) it can be used for the treatment of localized metastasis.

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