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Tumor-derived exosomes favor immunosuppression and metastatic spread by acting on myeloid cells

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ABSTRACT

It is widely recognized that the immune system can be highly affected by tumors through a plethora of mechanisms that allow avoiding an efficient recognition and eradication of cancer cells. Among these mechanisms, tumor secretome, including tumor-derived soluble factors (TDSFs) and, more recently, extracellular vesicles (EVs), is currently drawing much attention in the immune-oncology field. Particularly, by interacting either with stromal or other tumor cells, tumor-derived exosomes (TEX) have been demonstrated as key regulators in cancer development, as well as on the metastatic process. Since myeloid-derived suppressor cells (MDSCs) are critical contributors to the aforementioned processes, we investigated the TEX-MDSCs interaction, highlighting the main functional consequences of this crosstalk. Indeed, we could demonstrate a TEX-mediated effect on MDSC suppressive functions, which was even more striking in the case of bone-marrow naïve monocytes. Furthermore, we demonstrated that this is mainly mediated by iNOS engagement on myeloid cells, possibly induced by molecules enriched within TEX. In addition, the injection of TEX derived from high metastatic cancer cells in naïve tumor-free mice before the tumor challenge with a low metastatic cell line induced an increased spread of cancer cells in the lungs of TEX-treated mice. In the attempt to dampen TEX detrimental effects in tumor models, we blocked exosome secretion through GW4869 drug administration, which did not ameliorate the spread of metastatic cells. On the contrary, by targeting one of the TEX-downstream mediators, i.e. a member of the S100 proteins family, tumor-bearing mice displayed a restrained suppressive tumor network and a strong reduction in the metastatic incidence. Finally, we demonstrated that S100A8/A9 sera levels negatively correlated with distant metastasis-free survival in pancreatic ductal adenocarcinoma (PDAC) patients. In conclusion, our preliminary data highlighted the urgency of developing novel and more effective therapeutic approaches based on a full characterization of TEX-

induced pathways in myeloid cells within the local tumor milieu and, moreover, at distal sites of metastasis.

SOMMARIO

E’ ampiamente riconosciuto che il sistema immunitario può essere profondamente influenzato dal tumore attraverso una serie di meccanismi che consentono di evitare un efficiente riconoscimento ed eradicazione delle cellule cancerose. Tra questi meccanismi rientra il rilascio di fattori solubili (TDSFs) e, più recentemente, di vescicole extracellulari, che attualmente stanno attirando molta attenzione nel campo dell’immuno-oncologia. In particolare, gli esosomi derivati da tumore (TEX), interagendo con cellule stromali e/o tumorali, rappresentano dei regolatori cruciali sia del processo di tumorigenesi che del processo metastatico. Poiché le cellule soppressorie di origine mieloide (MDSCs) contribuiscono in modo critico ai suddetti processi, abbiamo studiato l'interazione TEX-MDSCs, evidenziandone le principali conseguenze funzionali. Nel dettaglio, abbiamo potuto dimostrare un effetto mediato da TEX sulle funzioni soppressive delle MDSCs, ancora più evidente nel caso di monociti naïve isolati da midollo osseo, probabilmente legato all’induzione di iNOS da parte di molecole contenute all'interno di TEX. Inoltre, *in vivo*, il pre-condizionamento con TEX derivanti da una linea di tumore fortemente metastatica in animali naïve prima dell’inoculo di una linea tumorale poco metastatica ha permesso di evidenziare un incremento della disseminazione distale di cellule maligne. Successivamente, nel tentativo di contrastare gli effetti dannosi di TEX nei modelli tumorali, abbiamo bloccato la secrezione degli esosomi attraverso il farmaco GW4869, che, tuttavia, non è riuscito a contrastare la diffusione delle cellule metastatiche. Al contrario, il blocco di uno dei mediatori a valle di TEX, ovvero una proteina della famiglia S100, si è rilevato efficace nel determinare un forte miglioramento del microambiente tumorale soppressivo in un modello di tumore mammario, insieme ad una sensibile riduzione dell'incidenza metastatica. Abbiamo inoltre dimostrato che alti livelli plasmatici delle proteine S100A8/A9 correlano negativamente con la sopravvivenza libera da metastasi in pazienti affetti da adenocarcinoma duttale del pancreas (PDAC). In conclusione, i nostri dati preliminari hanno evidenziato l'urgenza di sviluppare approcci terapeutici nuovi e più efficaci basati su una piena

caratterizzazione dei processi indotti da TEX nelle cellule mieloidi sia all'interno del milieu locale del tumore che, soprattutto, nei siti distali di metastasi.

INTRODUCTION

Chapter 1: The immune system in cancer

1.1. Tumor microenvironment

The growth and the establishment of a neoplastic mass, named as tumorigenesis, can be described as a complex and multifactorial process, where many cellular and extracellular factors are playing an active role. In last decades, the importance of a deep understanding of the environment surrounding the tumor has clearly emerged, leading to a promising and dynamic field of scientific research focusing on tumor microenvironment (TME). One of the first scientists who highlighted the importance of the TME was Stephen Paget, who formulated the "seed and soil" theory back in the late 19th century. Paget's concept remained dormant until the middle seventies of the 20th century, when small groups of people revisited Paget's ideas, focusing either on the functions of cellular and humoral immune components in TME and the angiogenesis process as key components of tumorigenesis and metastatic spread [1]. From there on, the research field on the TME moved forward, expanding and enlarging its scope to new frontiers, including studies on the interactions either between the extracellular matrix (ECM) and tumor cells or between fibroblasts and tumor cells. Indeed, structural components of the TME include the tumor lymphatic and blood vessels, the ECM, where collagen and hyaluronic acid represent the most abundant factors, and the stromal cells surrounding the tumor. Among these, three main categories can be identified: angiogenic vascular cells, cancer-associated fibroblastic cells (CAFs, including activated tissue fibroblasts, activated adipocytes, a-smooth muscle actin (a-SMA) myofibroblasts and mesenchymal stem cells) and infiltrating immune cells [2]. Focusing on the cellular components of TME, it is known for example that angiogenic vascular cells represent an important tool for an optimal supply of nutrients and oxygen, which allows cancer cells to partially avoid cell death that would otherwise result from hypoxia and lack of survival factors and serum-derived nutrients. For instance, it has been

shown that the disruption of the tumor vasculature can increase the efficacy of anti-tumoral treatments in preclinical human tumor xenograft models [3]. Furthermore, angiogenic vascular cells are responsible for the production of paracrine trophic factors and for modulating cancer cell dissemination and seeding. Other crucial TME cellular components are CAFs, able to promote tumor progression in many different ways, such as by secreting matrix-metallo proteases (MMPs), multiple growth factors, stemness-factors or other molecules that induce the epithelial-to-mesenchymal transition (EMT) process [4]. The infiltrating immune cells, which can vary depending on type and location of the tumor, include T cells, B cells, macrophages, inflammatory monocytes, myeloid-derived suppressor cells (MDSCs), neutrophils, mast cells and platelets, all playing a pivotal role in almost all the hallmarks of cancer [5]. Thus, it is currently widely

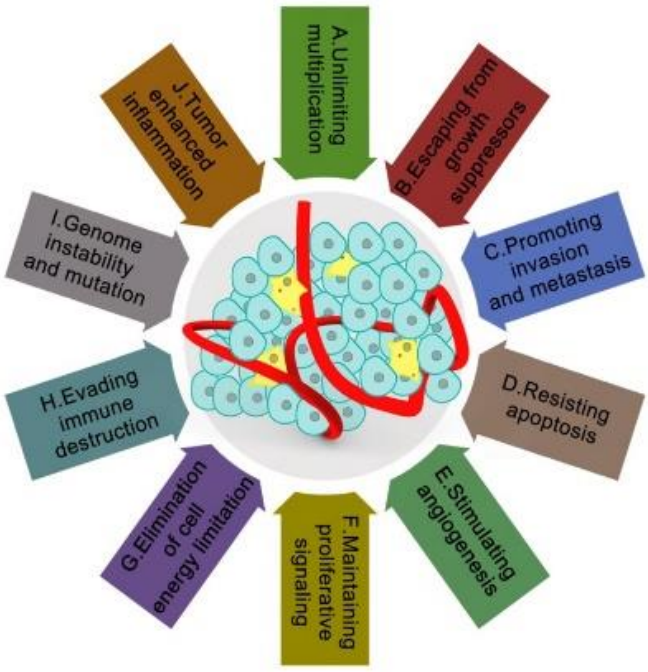

Figure 1. The ten characteristics of cancer: the interplay with the tumor microenvironment. The process of tumorigenesis is mainly based on the complex crosstalk that exists between cancer cells (light blue cells) and the surrounding stoma components. Cancer cells become more and more able to avoid their elimination by acquiring ten principle characteristics: A) unlimited multiplication; B) escaping from growth suppressors; C) promoting invasion and metastasis; D) resisting apoptosis; E) stimulating angiogenesis; F) maintaining proliferative signalling; G) elimination of cell energy limitation; H) evading immune destruction I) genome instability and mutation; J) tumor-enhanced inflammation. (Adapted from Wang M et al; *J. Cancer*, 2017)

recognized that cancers develop in complex tissue environments, which they depend upon for growing, invading and eventually reaching secondary sites of disease. The bidirectional interaction between cancer cells and the above mentioned associated stroma components represents a powerful relationship that influences either disease initiation, progression and patient prognosis and, on the other hand, the TME itself, establishing a crosstalk that can be highly detrimental for the outcome of the neoplastic disease.

Concerning one of the two sides of this crosstalk, it has been proven how tumor microenvironments are deeply influenced by the ten main characteristics of cancer (Figure 1). Among these characteristics, the ability of evading immune distraction is part of a complex process (fully described in the next paragraph), named cancer Immunoediting theory, by which tumor cells progressively acquire the ability to avoid the detection by the immune system or to limit the extent of immune-mediated killing.

1.2. Cancer Immunoediting

The cancer Immunoediting theory was formally enunciated and published by Robert Schreiber in 2002 [6], overcoming and ameliorating what was known to be the cancer immunosurveillance hypothesis of Burnet and Thomas [7]. This first hypothesis embodied the idea that the immune system could be considered as a "surveyor" that can constantly monitor the whole organism's homeostasis, recognize and destroy nascent potentially transformed cells. However, in the last decade it has emerged that the hypothesis advanced by Burnet and Thomas in 1970 represents just one phase of a more complex process that is established by the crosstalk between the emerging cancer cells and the immune components. Particularly, the cancer Immunoediting theory underlines how normal cells, subjected to common oncogenic stimuli, can eventually undergo transformation and become tumor cells, thus starting the process of cancer immunoediting, which develops in three distinct phases: elimination, equilibrium and evasion. At the early stages of tumorigenesis, tumor cells can express distinct tumor-specific antigens and generate pro-inflammatory signals that can alarm different components of the immune system. In the elimination phase the immune system is

able to recognize and eradicate cancer cells thanks to the activation of both innate and adaptive immune components, such as macrophages, natural killer cells (NKs), natural killer T cells (NKT) or γδ T cells. If the elimination phase occurs efficiently, all tumor cells are cleared and the tissue physiological homeostasis is re-established. Otherwise, if a portion of tumor cells is able to survive, the equilibrium phase arises: immune and tumor cells start a dynamic process where the first can carry out a selective pressure on the transformed cells that might lead to the survival of new cancer cell variants, which have acquired the ability to resist, suppress or avoid the immune attacks by accumulating several mutations. Eventually, these cell variants are able to step into the escape phase, where the immune system is no longer capable of controlling and/or eradicating the threat given by the neoplastic transformation, thus resulting in the growth of the tumor mass.

The ability of tumor cells to escape the immune eradication relies on many different mechanisms, which concern intrinsic characteristics of the transformed cells themselves or that can be related to their ability of altering the effector mechanisms of the immune system. Regarding the main characteristics that cancer cells may own, many evidences clearly proved that they can lose the expression of tumor-specific antigens (especially those that are more immunogenic), thus avoiding an effective recognition, or, moreover, they can negatively modulate the expression of the major complex of histocompatibility II (MHCII), resulting in a diminished capacity of antigen presentation [8]. Nevertheless, cancer cells are able to downregulate the expression of death receptors in order to increase their survival and maintaining their uncontrolled proliferative property.

Simultaneously, the growing tumor can create a suitable tollerogenic framework for its development either by the expression of molecules that affect the functionality of the effector immune cells (e.g. programmed death ligands 1/2) or by the continuous release of TDSFs, including cytokines, chemokines and metabolites [9]. All these TDSFs favor the recruitment of immunosuppressive populations, such as T regulatory cells (Tregs), which act by avoiding the generation and activation of effector cytotoxic T cells (CTLs), or NKT cells, able to inhibit the CTLs' functions. Moreover, TDSFs affect also the normal

hematopoiesis inducing the alteration of the natural commitment of other immune cells such as macrophages, neutrophils and dendritic cells (DCs) and an aberrant expansion and accumulation of immature myeloid cells with immunosuppressive features like myeloid-derived suppressor cells (MDSCs), which will be deeply described in the following paragraph.

1.3 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) represent a pool of different and heterogeneous populations of cells characterized by their myeloid origin, immature state and ability to potently suppress T lymphocytes' functions. Firstly described by Strober and colleagues as natural suppressor (NS) cells, this population has started to be identified as "MDSCs" just in 2007, when the term was coined in order to highlight its main properties [10]. In physiological conditions, these cells act in order to inhibit T cell immunity, thus avoiding aberrant continuous responses that might be detrimental for the organism. However, acute-phase conditions, such as chronic infection, sepsis or cancer lead to normal and abnormal/emergency myelopoiesis that is controlled by the production of granulocytic or monocytic growth factors (GM-CSF, G-CSF and M-CSF) leading to the production of mature and immature myeloid cells from precursors in the bone marrow (BM), including MDSCs. Their pivotal role in the establishment of a favorable microenvironment for the tumor growth has been highlighted by several evidences, which have showed their expansion occurring in different solid and hematologic neoplasms in both mouse model [11] and human patients [12]. Furthermore, the levels of circulating myeloid cells can be prognostic of a poor clinical outcome in different human cancers, such as breast cancer and non-small lung cancer [13, 14].

Although the heterogeneous nature of MDSCs, numerous studies in mice have led to the identification of two main subsets with different phenotypic and biological properties: monocytic (M)-MDSCs and polymorphonuclear (PMN)-MDSCs. Both the subpopulations share the CD11b myeloid marker but can be easily distinguished by the different expression of the two main Gr-1 epitopes, Ly6C and Ly6G. M-MDSCs (Gr-$1^{lo/int}$CD$11b^{+}$Ly$6C^{hi}$Ly$6G^{-}$) display the highest

immunosuppressive activity in an antigen-non-specific manner, whereas PMN-MDSCs (Gr-1l^{hi}CD11b^{+}Ly6C^{lo}Ly6G^{+}) are less immunosuppressive and exert their function by antigen-specific mechanisms [11, 15]. Indeed, besides the phenotypical characterization, what really defines MDSCs are their suppressive regulatory properties, which can directly cause immune dysfunction or that can be considered as "non-immune" related mechanisms [9]. Among the main direct immune-regulatory functions of MDSCs three enzymes, arginase 1 (ARG1), indoleamine-2,3-dioxygenase 1 (IDO1) and nitric oxide synthase 2 (NOS/iNOS), play an essential role (Figure 2) [16]. Indeed, these enzymes, by depleting essential aminoacids, such as L-arginine (by ARG1 and NOS2 activity) [17], L-tryptophan (by IDO1 and IDO2) and L-cysteine (whose transport is altered) [18], induce an impairment of T cell proliferation and function. Moreover, MDSCs can

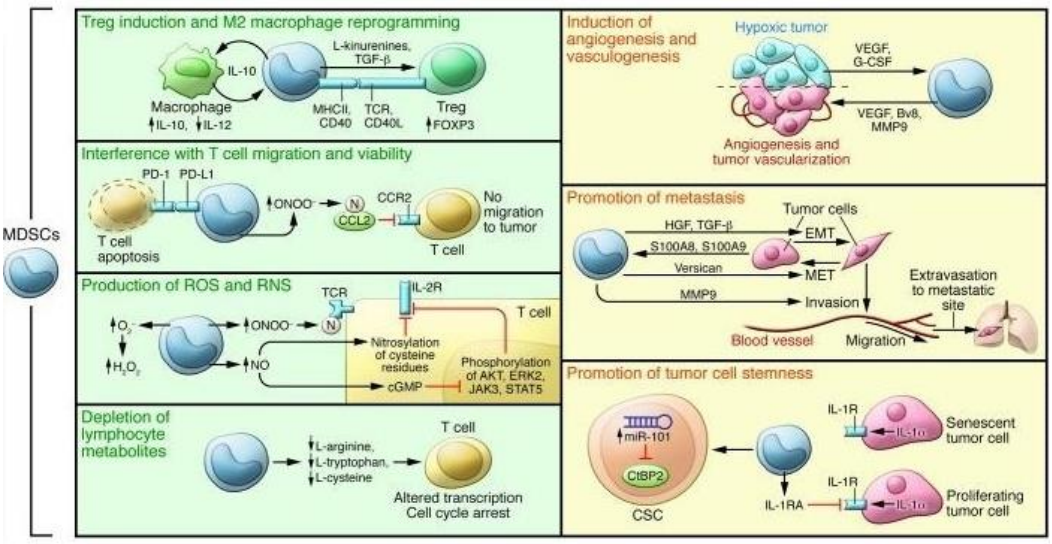

Figure 2. The MDSC regulatory functions in tumor progression. MDSCs are able to affect the tumor microenvironment via direct immune (green left panel) or non-immune mechanisms (orange right panel). In detail, MDSCs may act by inducing T regulatory cells or reprogramming macrophages towards the pro-tumorigenic M2 phenotype, or by interfering with T cell migration and viability. In addition, thanks to the activation of enzymes like iNOS, ARG1 and IDO1, they determine the depletion of essential metabolites for T cell survival and activation, and, morevor, they can secrete ROS and RNS, responsible for the alteration of important functions of T cells. Concerning the non-immune mechanisms exerted by MDSCs, it has been demonstrated their involvement in the promotion of metastases (by the release of factors that contribute to the EMT, MET, invasion and migration processes), in angiogenesis and vasculogenesis induction, as well as in tumor cell stemness promotion. (Adapted from Ugel et al. *JCI*, 2015)

inhibit T lymphocytes fitness by producing reactive oxygen species (ROS) or reactive nitrogen species (RNS) [19]. ROS, primarily produced by NADPH

oxidase 2 (NOX2), are highly released during injury or damage and, as already proved, their inactivation can revert the immunosuppressive capacity of MDSCs towards T cells. Indeed, ROS and RNS, which are produced by MDSCs, provoke the nitration of essential surface receptors of T cells: for instance, the nitration of tyrosine residues on T cell receptor (TCR), or on CD8 molecules, changes the conformation of the TCR abolishing antigen-specific recognition [20]. Furthermore, in MDSCs isolated from tumor-bearing mice, the up-regulation of NOX2 activity and ROS release lead to the enhancement of several NOX2 subunits, thus creating a detrimental feed forward loop causing high amount of ROS released. Another direct immune-regulatory mechanism is related to the MDSC expression of the T cell-inhibitory receptors ligands, such as programmed death ligands 1/2 (PD-L1/2), which bind to PD-1, B7-1/2 (binding CTL-A4) and FASL (binding FAS), all associated to the inhibition of T cell responses. More specifically, it has been demonstrated that the tolerogenic and immunosuppressive environment created by tumors critically depends on the transcription factor C/EBPβ, as demonstrated by the evidence that its lack in myeloid cells results in a full abrogation of their activity on antigen-activated $CD8^+$ T cells [21]. Furthermore, in 2013 Sonda and colleagues unveiled a direct miR-142-3p-Cebpb interaction that promotes myeloid cells differentiation toward immunosuppressive cells [22].

In addition, MDSCs are able to exert their regulatory properties by several indirect mechanisms, such as interfering with T cells migration and viability and inducing specific subpopulations of regulatory cells, like antigen-specific Tregs, and M2-macrophages by tumor-growth factor β (TGF-β) and interleukin-10 (IL-10) release respectively. Finally, MDSCs not only play a role in creating an immunosuppressive microenvironment but they can also favor tumor growth and metastatic spread by non-immune related mechanisms [23]. For instance, through the secretion of soluble mediators like vascular endothelial factor (VEGF), bombina variegata peptide 8 (Bv8), matrix metalloproteasis 9 (MMP-9) and/or TFGβ and hepatocyte growth factor (HGF), they can induce tumor neoangiogenesis and EMT, a condition in which cells acquire improved spreading features. Interestingly, MDSCs located in the pre-metastatic niche may release the

proteoglycan versican inducing also the opposite process (MET) allowing tumor cells to seed and colonize the organ [24]. At the same time, *in vitro* experiments unveiled that MDSCs are able to determine a stem-like phenotype in cancer cells based on TGFβ, EGF and/or HGF release, finely tuning tumor senescence [25]. In addition, they exert this activity either via the secretion of soluble mediators such as IL-1RA [26] or with direct effect on cancer stem cell expansion [27], as well as via miRNA-mediated gene regulation [25].

In humans, after twenty years of intensive research, scientists of the field elaborated a multiparametric flow cytometry panel for MDSCs characterization and enumeration, which allows the simultaneous detection of all the human subsets. Similarly to what previously described in mice, MDSCs can be divided in two main cell subsets: M-MDSCs ($CD11b^{+}CD14^{+}HLA\text{-}DR^{-/lo}CD15^{-}$), PMN-MDSCs ($CD11b^{+}CD14^{-}$ $CD15^{+}$) and a third group, called "early stage MDSC" (eMDSCs, $Lin^{-}HLA\text{-}DR^{-}CD33^{+}$) comprising more immature progenitors [28]. A useful marker for the identification of the most suppressive MDSCs subset is the α-chain of IL-4R; in fact, its expression on MDSCs of colon cancer and melanoma patients correlates with a more immunosuppressive phenotype [29]. Recently, MDSCs have been indicated as predictive marker of response in advanced melanoma patients treated with anti-CTLA-4 antibody ipilimumab, since clinical responders to the therapy showed significantly less $Lin^{-}CD14^{+}HLA\text{-}DR^{-}$cells [30]. However, since human MDSCs display a high plasticity and surface markers shared with common myeloid subsets, in order to state them as proper MDSCs it is necessary to integrate the phenotypic characterization demonstrating their *in vitro* suppressive ability. Giving this and other evidences, a deep investigation of which are the strategies that may be pursued in order to target MDSCs in cancer becomes crucial, in order to switch the tolerogenic and pro-tumorigenic TME to a more responsive and efficient one.

1.4 MDSC regulation and recruitment

Besides the immunoregulatory properties belonging to MDSCs, many research groups are focusing on how these cells are recruited and regulated during tumor progression and metastatic spread. As previously mentioned, TSDFs

include cytokines and chemokines that are responsible for the generation, expansion and recruitment of MDSCs at tumor site. Concerning the most relevant cytokines, tumor-derived GM-CSF has been demonstrated to play a pivotal role in the generation of MDSCs; indeed, in combination with IL-6, IL-1β, prostaglandin (PG) E2, tumor necrosis factor (TNF)-α or VEGF is able to mediate, in humans, the generation of highly suppressive MDSCs from $CD33^+$ peripheral blood mononuclear cells (PBMCs) isolated from healthy donors [31]. IL-6 is another cytokine that is critically responsible for MDSCs generation and survival, as highlighted by Wu and colleagues, who demonstrated that increased IL-6 concentrations correlated with MDSC frequencies and their immunosuppressive functions in tumor-bearing hosts [32]. VEGF and TGF-β have also been demonstrated to have a strong impact on the regulation of hematopoiesis and on the MDSC generation and expansion. For instance, the combination of the two factors is able to prevent dendritic cells (DCs) maturation, to polarize myeloid cells towards an immunosuppressive phenotype and to partecipate to the induction of tumor-associated macrophages (TAMs) [33]. Other well-known factors involved in MDSC expansion and activation, which may vary according to the tumoral context, include: granulocyte CSF (G-CSF), macrophage CSF (M-CSF), stem cell factor, TGF-β, TNF-α, prostaglandin E2, the S100 proteins S100A9 and S100A8, and interleukin (IL)-1β.

Once generated and expanded, MDSCs are recruited to the TME through specific chemokines that allow MDSC migration inside the cancer site. Chemokines are small (8–14 kDa), structurally related chemotactic molecules that regulate trafficking of various cells (including leukocytes) through interactions with specific seven-transmembrane, G protein-coupled receptors. The pattern of chemokines responsible for MDSC recruitment has been shown to be dependent on the MDSC subset and on the tumor model. One of the most well described mechanism of recruitment involves the chemokine (C-C motif) ligand (CCL) 2 and its receptors in the attraction of M-MDSCs [34]. Furthermore, chemokine (C-X-C motif) ligand (CXCL) 8 and CXCL12 can determine a dramatic MDSC accumulation in gastric and ovarian cancer microenvironment, whilst in the context of a breast cancer mouse model CXCL12 seems to exert the opposite

effect [35]. Other groups have then underlined the major role of other type of chemokines, such as CCL3, CX3CL1, and CCL5, the latter recently highlighted as strong activator of the hypoxia-inducible factor (HIF)-1α signalling cascade causing upregulation of VEGF expression, in turns responsible for MDSC generation and activation [36].

In the last few years, it has emerged that MDSCs could also be induced and expanded in response to extracellular vesicles (EVs) released from cancer cells, as tools to increase the ability of escaping from the immune control. Therefore, it becomes important to study not only how soluble inflammatory factors are able to affect the TME, but also to include in the analysis tumor-derived EVs, which are of critical importance in understanding the network between the cancer cells and the immune ones.

Chapter 2: Tumor-derived exosomes

2.1 Extracellular vesicles: features and functions

Every known organism, from the simplest to the most evolved one, requires efficient cell-to-cell communications in order to develop a complex network between the different types of cells that is essential both in physiological and pathological conditions. In order to communicate, cells must be able to send and receive signals, which in turn may trigger a variety of responses according to the type of signal and recipient cells. This communication may occur through direct interaction that requires cell-to-cell contact, as in the case of group of cells that signal across gap junctions; this mechanism allows triggering a coordinated response to a signal that only one of the cells may have received [37]. Concerning another form of direct signalling, two cells may bind to one another via interaction between proteins on the cell surface, thus starting an intracellular signalling resulting in a specific cell response. This kind of signalling is critically important for the immune system, where immune cells take advantage of cell-surface markers to recognize "self" cells, as well as, infected and transformed ones, in order to activate specific tolerogenic or immunogenic programs, respectively. Nonetheless, normal and altered cells can also communicate by the release into interstitial spaces and into body fluids of soluble factors (cytokines and chemokines) and/or of bilayered membrane-bound vesicles, both allowing the delivery of messages not only locally but also at the systemic level. In regard to extracellular vesicles (EVs), the last few years have seen a massive increase of the amount of groups who have been focusing in this promising research topic, especially in light of the strong evidences that highlight the prominent role of EVs in the context of cancer and metastasis [38]. By now, it is generally accepted that cells release several EVs populations with distinct biophysical features and biological functions. According to the most common classification, microvesicles, apoptotic bodies and exosomes are among the EVs populations most widely studied and well characterized (Figure 3).

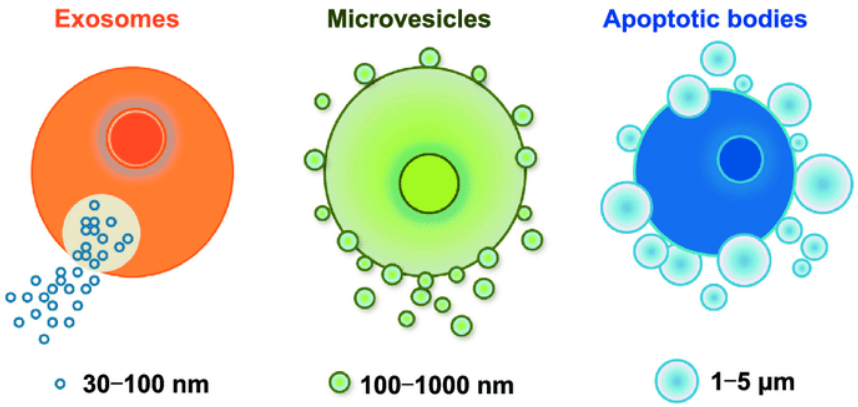

Figure 3. General classification of EVs. Extracellular vesicles are normally classified according to their size and mechanism of secretion. Briefly, exosomes (left) are defined as membrane-bound vesicles ranging from 30 to 100 nm in greatest dimension, and they originate from multivesicular bodies (MVBs) within the cell. Microvesicles (middle), ranging from 100 to 1000 nm, are released from cell membrane surfaces during activation or apoptosis of all eukaryotic cells, whilst apoptotic bodies (right), sized from 1 to 5 µm, are commonly generated during the latest stages of the apoptotic process. (Adapted from Chen et al., *Gen. Prot. Bioinformatics*, 2017)

Microvesicles

These common EVs are small membrane-enclosed bodies whose dimensions can range between 100 and 1000 nanometres (nm) and are formed by the outward budding of the cell membrane. Once generated, microvesicles can enable the horizontal transfer of cargoes that include molecules and effectors, thus affecting the extracellular milieu with consequences for the surrounding environment. For instance, it has been shown that microvesicles found in the circulatory system contribute to the coordination of the pro-coagulatory response [39] by the exposure of tissue factor, a transmembrane protein that acts as cofactor for the VIIa factor (FVIIa), the primary biological initiator of the coagulation cascade. Moreover, microvesicles play a major role in inflammation, exerting both pro-inflammatory [40] and anti-inflammatory [41] effects on their environment. In both cases, this may occur principally through the transfer of bioactive molecules, like cytokines and chemokines, to target cells. Indeed, it is already established, for example, that microparticles derived from N-formyl peptides (fMLP)-stimulated polymononuclear cells can induce the expression of IL-6 and monocyte chemotactic protein 1 (MCP-1) in endothelial cells, thus resulting in their activation [40]. If microvesicles are released by cancer cells they are commonly

named as “oncosomes”, term that highlights their origin and their ability to transfer oncogenic properties to target cells. Recently, it has been suggested a distinct role for oncosomes in tumor progression, which might play a selective metabolic function over the other EVs subclasses. Particularly, Minciacchi and colleagues have demonstrated that oncosomes are enriched in proteins able to affect glutamine metabolism in recipient cancer cells, such as HSPA5 (*Heat shock protein 70 family protein 5*) and GOT1 (*Glutamate oxaloacetate transaminase 1*), leading to metabolic derangement in tumor cells and tumor-stroma associated with cancer cell proliferation [42]. Furthermore, oncosomes are known to be able to horizontally transfer metalloproteinases, RNA, caveolin-1, and the GTPase ADP-ribosylation factor 6, being biologically active towards cancer cells, endothelial cells, and fibroblasts, thus potentiating advanced disease [43].

Apoptotic Bodies

The release of these vesicles into the extracellular milieu represents the ending phase of the apoptotic cell death, during which cells disintegrate and generate apoptotic bodies (AB) and apoptotic microvesicles, also known as ectosomes. Sized from 1 to 5 μm, apoptotic bodies contain parts of the dying cells and are generally engulfed by phagocytic cells, where their inner components may be recycled. Moreover, it has been suggested that membrane-bound fragmentation of the apoptotic cell might be important for chemotactic signalling to mononuclear phagocytes, as macrophages can be attracted by CX3CL1/fractalkine released from apoptotic lymphocytes via caspase- and Bcl-2 regulated mechanisms [44]. Interestingly, apoptotic bodies contribute to antigen presentation via direct and cross-presentation mechanisms in several disease settings, such as autoimmunity, antimicrobial immune responses and organ/transplant rejection. In this regard, it has been recently shown that apoptotic vesicles generated from dendritic cells and B16-F1 melanoma cells present on their surfaces MHC II molecules, suggesting their potential ability of activating $CD4^+$ T cells [45]. Finally, apoptotic bodies can carry a variety of biomolecules, such as vesicle-associated cytokines and/or damage-associated molecular patterns (DAMPs), which could directly modulate immune cells. This happens for example in some autoimmune diseases, where

high mobility group box protein B1 (HMGB1) derived from peripheral blood mononuclear cells and T cells can be found in apoptotic vesicles [46].

Exosomes

Among the EVs subpopulations, exosomes probably represent the most well studied and characterized one, as many research groups are putting efforts in the investigation of their regulatory properties, both in physiological and pathological conditions. Exosomes, observed via electron microscopy, can be defined as spherical-to-cup shaped, bilayered, membrane-bound nanovesicles, whose dimensions may vary from 30 to 100 nm. They are characterized by the phenotypic expression of specific surface molecules that are commonly used to identify them. The most frequent ones are the surface tetraspanins, CD9, CD63, CD81, CD82, and other markers like intracellular adhesion molecule-1, $\alpha_v\beta_3$ integrin, Alix, CD80, CD86, CD96, Rab-5b and the MHC class I and class II complexes.[47-49]. The pattern of molecules expressed on exosomes surface can be highly different depending on the origin of the secreting cell; for instance, exosomes containing waste and less-needed products are likely to have surface molecules that instruct macrophages to remove them [50]. On the contrary, exosomes that are released from cancer cells, may result enriched in some proteins, like heat shock proteins (HSPs), whose expression has been associated to tumor progression [51]. Regarding their inner content, it is widely assessed that exosomes can transfer to target cells a variety of different molecules, including either proteins (enzymes, cytoskeletal proteins, signal transduction proteins, heat shock proteins and multivesicular bodies (MVBs) biogenesis proteins), RNA molecules, such as mRNA and/or miRNA, and also lipids. The type of content boarded depends on both the secreting cell type and its status, meaning that what a cell secretes is associated to the cell function and condition. Besides their size and surface markers, what distinguishes this type of vesicles is their origin within the cells; indeed, exosomes generate from MVBs, which then fuse with the plasma membrane for the release of vesicles in the extracellular microenvironment. The regulation of this process requires multiple molecular factors, like the complex ESCRT (*Endosomal Sorting Complex Required for Transport*), which is directly

responsible for cleaving the membrane buds and allowing the vesicles release, together with the syndecan 1-syntetin and Alix proteins. Moreover, other factors including calcium, calcium ionophores, phosphatidylinositol 3-kinase, heat, ischemia, cellular stresses, pH, phorbol esters and loss of cellular attachment, are all somehow affecting the release of exosomes, suggesting how complex might be the regulation of this process. Once secreted, exosomes provide autocrine, paracrine and endocrine signals by interacting with the target cells and contributing to intercellular communication. However, how exosomes from different origins target specific cells is still to be fully elucidated. Some insights come from the evidence that exosomes, as well as the other types of vesicles, can initiate a signalling by an antigen-antibody interaction in the recipient cell and/or by the activation of a receptor on the cell membrane of the target cell. Active uptake of exosomes by dendritic cells, for example, seems to be mediated by the direct interaction with surface molecules like CD9, CD11a, CD54, CD81 and $\alpha_v\beta_3$, although the blocking of these signalling proteins with antibodies cannot completely abrogate exosomes internalization, unveiling that combinations of surface signals might be necessary for this process [52]. Nevertheless, exosomes can also be taken up via endocytosis or fuse directly with the plasma membrane, thus releasing their cargoes into the cytosol of target cells. The final aim of these ways of interactions is to vehicle signals and information critical for establishing a physiological network between cells. In this regards, as already mentioned, exosomes may serve as "cleaners" because of their ability to export waste products, no-longer useful molecules as cells differentiate and harmful molecules. More importantly, to date there are several evidences underlying their roles in the intercellular communication between "normal" immune cells. Among these roles, it has been demonstrated that exosomes are involved in the establishment of maternal-fetal tolerance, as proved by the fact that placental exosomes can carry immunosuppressive and anti-inflammatory molecules, such as FAS ligand, ULBP 1 o 5 that bind to the NKG2D receptor [53], or the chromosome 19 miRNA cluster [54]. Furthermore, exosomes can play an important role also in the processing of antigens by APCs (*Antigen Presenting Cells*). Indeed, it has been shown that exosomes can either enhance $CD4^+$ T cells activation, by expressing

MHC class II-peptide complexes, which are derived from peptide-pulsed DCs [55], or they can drive $CD8^+$ T cell responses, as demonstrated by Charlotte Admyre for monocyte-derived-DCs exosomes on autologous T cells from human pheripheral blood samples [56]. Giving this information, there is no doubt about the potent regulatory properties of exosomes; therefore, it is not surprising that also transformed cells may take advantage of these tools to shape and affect their environment in a detrimental way.

2.2 Exosomes as important drivers of tumorigenesis

Cancer cells can exploit a variety of mechanisms in order to avoid their recognition and eradication by the immune system, and the secretion of vesicles with immunomodulatory properties likely well contribute to this aim. The key role of tumor-derived exosomes (TEX) in cancer is strongly supported by the latest evidences showing the plethora of effects of TEX on different actors within the tumor microenvironment (Figure 4). Particularly, TEX have the ability to decrease the immune surveillance of tumors, and, on the other hand, they can mediate non-immune functions on primary malignant lesions. One of the main pro-tumorigenic effects of TEX concerns the induction of a decrease in the proliferation and cytotoxicity of NKs and T cells. In this regard, it has been shown that NKG2D ligand-expressing exosomes may serve as decoys with a powerful ability to down-regulate the cognate receptor and impair the cytotoxic function of NKs [57]. The impairment of the NK functions might also be due to the TEX cargo, like TGFβ1, which can post-transcriptionally up-regulate mature microRNA-1245 expression, thus leading to the down-regulation of NKG2D and impairment of the NKG2D-mediated immune responses [58]. Furthermore, TEX have been found to directly affect T cells viability and cytotoxicity. Indeed, TEX from kidney adenocarcinoma cells contain Fas ligand that allow them to trigger Jurkat T cells apoptosis *in vitro* [59], as well as TEX isolated from sera of patients with oral cancer have been shown to effectively induce activated T cells apoptosis via the expression of Fas ligand [60]. In addition, the transfer of molecules as Trail or related molecules (e.g. TNFα) can cause apoptosis of activate T lymphocytes by suppressing CD3ζ chain and Jak3 [61]. Another mechanism by which TEX may

promote tumorigenesis is by decreasing the number and/or activity of APCs, including DCs, reducing tumor-associated antigens presentation and therefore, anti-tumoral responses. In this regard, it has been demonstrated that exosomes from Lewis Lung Carcinoma (LLC)-, or 4T1 breast cancer cells, can block the differentiation of myeloid precursor cells into $CD11c^+$ DCs and induce cell apoptosis [62]. Interestingly, TEX may also induce $CD14^+$ cells to shift towards immunosuppressive $CD14^+$ $HLA\text{-}DR^{-/low}$ cells, which in turn can release TGFβ and inhibit T cells activity [63]. The establishment of an immunosuppressive microenvironment also happens through the accumulation and induction of both Tregs, via TGFβ1 and IL-10, and MDSCs populations, which have a critical role in tumorigenesis. Exosomal TGFβ, which is able to increase prostaglandin E2 (PGE2) expression, seems to have a key role in the accumulation of MDSCs in renal cell carcinoma [64], whereas pancreatic ductal adenocarcinoma (PDAC)-derived exosomes can enrich the microenvironment in MDSCs in a S*MAD4*-dependent manner [65], therefore suggesting that different mechanisms may participate in MDSCs recruitment depending on the cancer type. Besides MDSCs accumulation at tumor site, it is crucial how TEX modulate their activation: indeed, recently it has been proved that the interaction between exosomal HSP70 and MDSCs determines the suppressive activity of the latter via phosphorylation of Stat3 (p-Stat3) and this occurs in a TLR2-MyD88-dependent manner [66]. More importantly, in a human setting of chronic lymphocytic leukemia (CLL), in response to TEX monocytes and macrophages have been proved to skew towards pro-tumorigenic phenotypes, including the release of tumor-supportive cytokines and the expression of immunosuppressive molecules such as PD-L1, giving important insights regarding the therapeutic potential of a deep comprehension of TEX-mediated immunosuppressive effects [67].

As above mentioned, an important “non-immune” pathway by which TEX affect the tumor microenvironment relies on their ability to stimulate angiogenesis, thus allowing the supply of oxygen and nutrients to the developing tumor. Specifically, malignant cells in hypoxic and similar environments can secrete exosomes containing TGFβ and VEGF that are known to be involved in angiogenesis stimulation. Furthermore, TEX can directly target vascular

endothelial cells, as demonstrated in gastric cancer where exosomes deliver miR-130a from gastric cancer cells into vascular cells to promote angiogenesis and tumor growth by targeting c-MYB [68]. TEX can further sustain tumorigenesis thanks to the autocrine signalling that they are able to induce in cancer cells

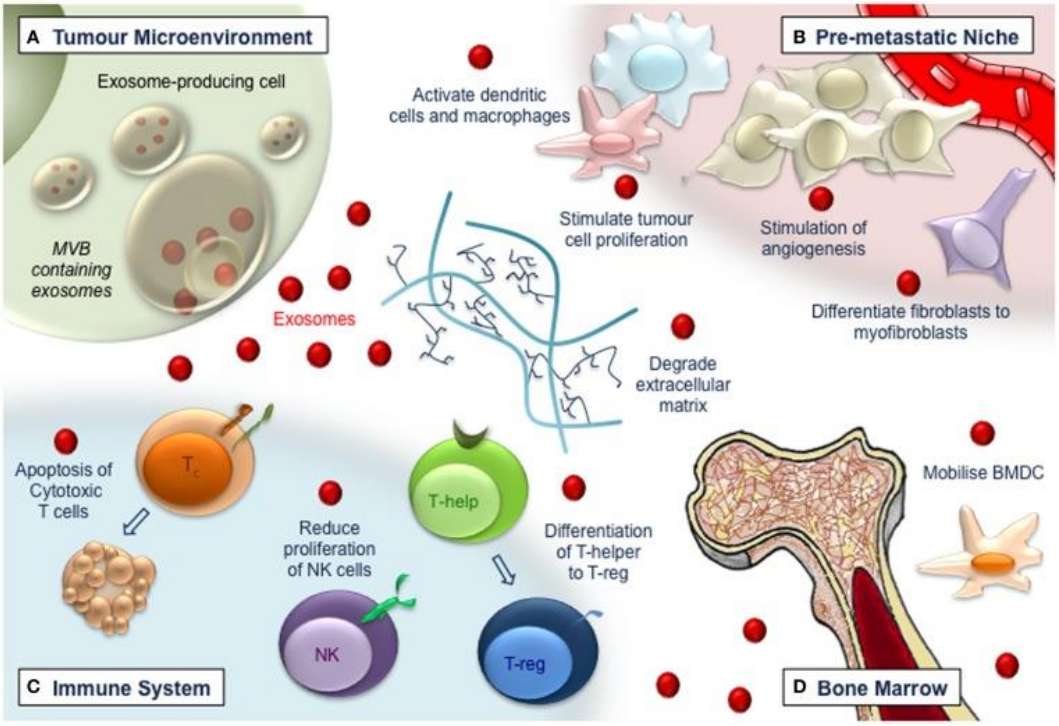

Figure 4. Multiple roles of exosomes in tumorigenesis. Exosomes released from tumor cells (red dots) can deeply contribute to the tumorigenesis process and to the dissemination of cancer cells by different mechanisms. Once secreted in the tumor microenvironment (A) exosomes (red dots) may participate to the formation of the pre-metastatic niche (B) by activating dendritic cells and macrophages, stimulating tumor cell proliferation and angiogenesis, differentiating fibroblast into myofibroblasts and degrading extracellular matrix thus favouring cell invasiveness and migration. Moreover, they can modulate the immune system towards a more favorable and immunosuppressive environment, thanks to the induction of cytotoxic T cell apoptosis, differentiation of T helper cells into T regulatory cells and reduction of NK cells proliferation (C). Finally, they may act directly on the bone marrow inducing the mobilization of bone marrow-derived cells (BMDC) that are then recruited to tumor and pre-tumor tissue where they contribute to cancer development (D). (Adapted from Tickner J et al., *Frontiers in Oncology*, 2014).

themselves, which in many cases inhibits the apoptotic process and increases cell proliferation and invasion. This has been proved for example for gastric cancer exosomes, which allow the promotion of tumor cell proliferation via PI3K/Akt and MAPK/ERK pathways [69], or for prostate cancer (PCa), where exosomes carrying Survivin, an inhibitor-of-apoptosis (IAP) protein family member, have been detected in PCa patients with disease progression, suggesting its critical role

in favouring the resistance to apoptosis [70]. Notably, TEX are also involved in inducing changes in fibroblasts towards myofibroblasts, a phenomenon that can lead to the degradation of the extracellular matrix and increased production of pericellular hyaluronic acid, thus facilitating the invasion of neoplastic cells; how TEX can thus facilitate the metastatic spread of malignant cells will be further described during this discussion. Finally, it is worth mentioning another major feature of TEX, which consists in their ability of conferring to malignant cells chemoresistance against numerous, but not all, chemotherapeutic drugs. As exosomes are physiologically programmed to get the cells rid of waste products and less-needed molecules, they can also export potentially harmful products, such as chemotherapeutic drugs like cisplatin and doxorubicin [71], but not 5-fluorouracil [72]. Moreover, in the breast cancer context, $HER2^+$ exosomes can bind to anti-HER2 antibodies (for example, Herceptin®), therefore limiting their bioavailability and therapeutic efficacy. Consequently, sustained tumor growth is permitted via interactions between HER proteins on the surface of tumor cells and growth factors/EGFR ligands in the tumor microenvironment [73]. Thus, exosome-induced chemoresistance is now emerging as a novel mechanism that still needs clarifications in order to be efficiently contrasted.

2.3. Novel opportunities regarding TEX in the clinical approaches

Having pinpointed all the principle ways by which cancer cells may modulate their microenvironments by the release of exosomes, it is worthwhile describing how the understanding of these mechanisms could be exploited in the clinical approaches (Figure 5). Some steps forward have been recently made in the identification of exosomal markers that can be used as novel biomarkers for the early detection of tumors. Interestingly, TEX mirror the molecular features of the original neoplastic lesions; for example, TEX circulating in patients with glioblastoma multiforme and high-grade gliomas contain neural markers (e.g. LI-NCAM and CD171) [74], as well as those from melanoma patients are enriched in molecules involved in melanin synthesis and other melanoma markers (e.g. Melan A/Mart1) [75]. In addition, other groups highlighted the presence of Claudin 4 in exosomes isolated from the blood of patients with ovarian carcinoma [76] and

serine 2-ETS related gene, prostate cancer antigen 3 and transmembrane protease enriched in TEX present in urine samples of patients with prostate cancer [77]. All these evidences strongly suggest that the detection of these exosomal biomarkers may aid in the therapeutic efficacy of diagnosis, in the prediction and clinical decisions, including early detection, and in determining prognosis. Nevertheless, it has been demonstrated that, when specific biomarker panels ("exosomal signatures") are identified, their measurement in the exosomal fraction may result more effective in solving clinical issues than their measurement in the whole bodily fluids [74]. In parallel, targeting TEX that are proved to be harmful to the organism by favouring tumor progression and metastatic spread, could represent another important therapeutic strategy. To this aim, at the systemic level TEX could be decreased taking advantage of an instrument, termed as hemopurifier, which allows the selective removal of TEX through immobilized antibodies that bind their surface molecules [78]. On the other hand, TEX could be specifically targeted to reduce the TEX-mediated immunosuppression of immunity in cancer patients. Although these approaches may be theoretically feasible, their complexity, together with the lack of a strong specificity in targeting TEX, have pushed the researchers to pursue other types of strategies based on exploiting exosomes as cell therapy surrogates or as drug delivery vehicles. Concerning the first strategy, TEX that are expressing specific TAA could be used directly as "vaccines" to stimulate an anti-tumoral response [79]; indeed, a number of animal studies indicate that antigen-containing exosomes can induce a specific immune response that can protect against tumor progression or various infections. Alternatively, TEX can be used to pulse DCs in order to obtain DCs-derived-exosomes capable of initiating cytotoxic T lymphocytic responses [80]. The clinical use of exosomes is also being investigated by commercial and academic organizations as vehicles to deliver chemotherapeutics, small molecules, agents of gene therapy, and/or to target cells more specifically than systemic administration. This could be feasible due to the fact that TEX can be specifically absorbed by neoplastic cells. For instance, Ohno and colleagues have pinpointed that exosomes, engineered to express the transmembrane domain of platelet-derived growth factor receptor fused to the GE11 peptide, which binds specifically to

epidermal growth factor receptor (EGFR), can efficiently deliver the tumor suppressor miRNA let-7a to EGFR-expressing breast cancer cells and, moreover, they can inhibit breast cancer development *in vivo* [81]. Similarly, it has been recently proposed a novel strategy for engineering exosomes to make them bind specifically to HER2/Neu^{+} breast cancer cells and to deliver siRNA molecules

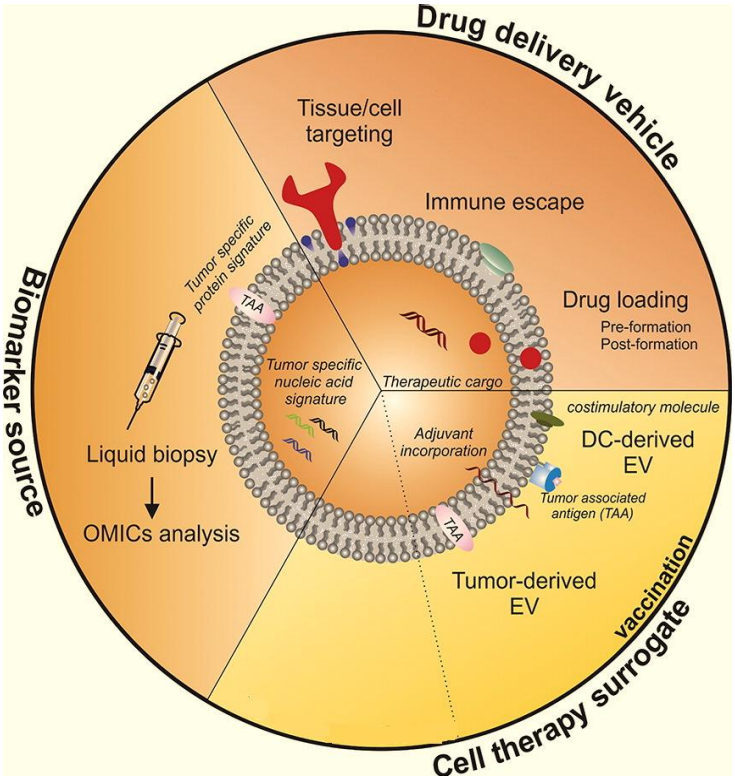

Figure 5. Exosome-based applications in clinics. In the latest years, it is becoming increasingly clear that exosomes provide opportunities for therapeutic applications. First of all, the enrichment of tumor-associated antigens expressed on tumor-derived exosomes renders these vesicles feasible for detecting them in liquid biopsies and for utilizing them as biomarkers in many types of cancers. Secondly, tumor-derived exosomes alone, or DCs-derived exosomes, might be useful as cell therapy surrogates to trigger specific anti-tumoral responses by adaptive immune cells. Third, given the natural function of carriers, exosomes could be engineered to express surface markers capable of binding to specific target cells, and loaded with a variety of possible therapeutic molecules that can be either drugs or silencing small molecules. (Adapted from Stremersch S. et al., *J. Control Release*, 2016)

against TPD52 gene, whose expression is associated with the increased anchorage-independent growth and cell proliferation [82]. The biocompatibility and toxicity profiles of exosomes, which are natural carriers of different sort of molecules *in vivo*, support their application in drug delivery systems. However, some issues remain to be solved, such as the normal clearance mechanisms that

limit a long-lasting effect, or the immunogenicity and toxicity of exosomes, which can arise depending either on the animal models used in testing or on the source and composition of exosomes themselves.

Chapter 3: The metastatic process

3.1. Invasiveness, migration and spread of malignant cells

It is widely accepted throughout the oncology field that the main threat given by cancer is often associated to the metastatic potential of the primary tumor. Nevertheless, cancer remains the leading cause of death worldwide, and it is estimated that metastases are responsible for almost 90% of cancer deaths [4]. In addition, the majority of patients with metastatic disease display just a temporary response to conventional treatments, thus highlighting that further elucidations in this field are strikingly urgent. In general terms, the metastatic process can be defined as a complex multi-step process of transformed cells spreading from the primary tumor to surrounding tissues and to distant sites. Given the numerous barriers and immune defences that cancer cells must overcome, less than 0.1% of them can successfully reach a secondary site and develop distal metastases; therefore, the metastatic process is considered as highly inefficient. In solid malignancies, this process, generally defined as "metastatic cascade", requires that cancer cells acquire the ability to invade surrounding tissues, infiltrate into the vasculature and thus colonize distal sites [83].

Tumor cells display an impressive variety of invasion strategies, which could be alternatively performed in order to adapt to changing and challenging environments. Among these strategies, two fundamentally different patterns of invasive growth can be distinguished: collective (group) cell migration and single cell migration. Collective cell migration (and subsequent invasion) is commonly found in several cancer types, such as breast cancer, epithelial prostate cancer, large cell lung cancer, melanoma, rhabdomyosarcoma, and most prominently in squamous cell carcinomas. The process involves whole groups of cells that migrate interconnected by adhesion molecules, such as cadherins, and other communication junctions [84]. Particularly, cancer cells form protrusions (pseudopodia) at the leading edge, which can interact with the cytoskeleton actin via integrins involvement, and proteolytic degradate the extracellular matrix, thus creating a space for the tumor to invade. For instance, it has been proved, using time-resolved multimodal microscopy, that HT-1080 fibrosarcoma and MDA-

MB-231 breast cancer cells coordinate mechanotransduction and fibrillar collagen remodelling by segregating the anterior leading group of cells of the tumor, containing β1 integrin, the matrix metalloproteases MT1 (MT1-MMP) and F-actin [85]. In the case of single cell invasion (also named as individual cell migration), tumor cells independently of each others invade the surrounding tissues via two different types of movements: mesenchymal (fibroblast-like) and amoeboid, which can be shifted from one type to the other to adapt to the characteristics of the microenvironment [86]. Acquiring the ability to detach from the tumor mass and invade the surrounding tissues require undergoing certain changes, among which one of the most relevant is the acquisition of the morphological and phenotypical properties of mesenchymal cells. This morphogenetic transformation, called "epithelial-to-mesenchymal transition or EMT, is characterized by deep cellular morphology changes, alteration of cell–to-cell and cell–to-matrix interactions, as well as the development of migratory behavior and invasiveness [87]. At the molecular level, it is widely demonstrated that multiple complex signalling systems are required for the induction of EMT. The diminished expression of E-cadherin, which contributes to the cell-cell adhesion, is one of the key events of EMT, and, therefore, many factors are involved in its regulation. For instance, zinc finger proteins (ZEB1, ZEB2), bHLH protein (Twist), and the Snail family of zinc finger proteins (Snail, Slug) are known to transcriptionally repress E-chaderin, thus favouring EMT. Furthermore, recent independent studies revealed that the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) and miR-205 play critical roles in regulating EMT, targeting the E-cadherin repressors ZEB1 and ZEB2 [88, 89].

Besides the intrinsic changes that cancer cells undergo to increase their potential migratory and invasive properties, a critical element that can permit or restrain the invasion of primary tumor cells is the local remodelling of the host microenvironment and in particular the ECM. Tumor-associated ECM is constantly remodelled in order to create a path for reaching distal sites; this process has been suggested to be due to the clustering of integrins and other receptors, thus downstream activating intracellular kinase signalling pathways,

which subsequently determine, among other things, EMT and cancer cell migration and invasion [90].

Although the acquisition of migratory and invasive properties of cancer cells are strictly necessary for the metastatic process, the effective spread of malignant cells requires other critical processes, such as the so called "angiogenic switch" [91], which consists in the alteration of the local balance between pro-angiogenic and anti-angiogenic factors. Indeed, cancer cells, in order to reach distal sites, need to activate cellular programs that allow them to overcome the endothelial barrier and enter the bloodstream. Tumor angiogenesis (i.e. the growth of new blood vessels around the primary tumor) is driven by the secretion of pro-angiogenic growth factors (such as VEGF), recruitment of immune cells, and alteration of the perivascular ECM either by tumor cells or by surrounding stromal cells. This process leads to the generation of leaky tumour vessels, which are thought to facilitate the dissemination of cancer cells throughout the body.

Finally, in the last years numerous efforts are being invested in the understanding of the phenotypical and molecular changes that identify the formation of the pre-metastatic niche [92]. This is because it is widely demonstrated that, for the metastatic process to be efficient, it is crucial the "assembling" of a favourable environment that allows cancer cells to take roots at distal sites, as well as it is essential for their survival and outgrowth [93]. Furthermore, the deep investigation of the early events in the development of the metastatic spread, could give important insights in the early detection and cure of aggressive diseases. Pre-metastatic niche formation is initiated with the above mentioned local changes, such as the induction of vascular leakiness and the remodelling of stroma and extracellular matrix, followed by systemic effects on immune cells. All of these processes are mainly induced by the pro-metastatic secretome, which includes soluble factors released by primary tumor cells: VEGF and placenta growth factor (PLGF), as well as pro-inflammatory cytokines and chemokines, such as TNFα and TGFβ released by melanoma B16 cells, were the first molecules discovered to support the formation of the pre-metastatic niche in the lung [94]. In addition, another important factor for the vessel barrier breakdown, essential for the extravasation of cancer cells at secondary sites, is the

MMP9 enzyme, produced at high levels by myeloid progenitor cells within the pre-metastatic lung. Indeed, it has been shown that the activation of this enzyme leads to ECM remodelling and formation of a proliferative, immunosuppressive and inflamed environment within the lung [95]. Systemically, the seeding of cancer cells in a favorable and tolerogenic environment like the pre-metastatic niche implies the recruitment of specific subsets of the immune system. Particularly, bone marrow derived cells (BMDCs) like macrophages, $CD11b^+$ myeloid cells (including MDSCs, tumor-associated neutrophils, tumor-associated macrophages and Tregs) and $VEGFR1^+$ haematopoietic progenitor cells (HPCs), are commonly recruited in the pre-metastatic niche, favoring the assessment of an immunosuppressive environment (Figure 6).

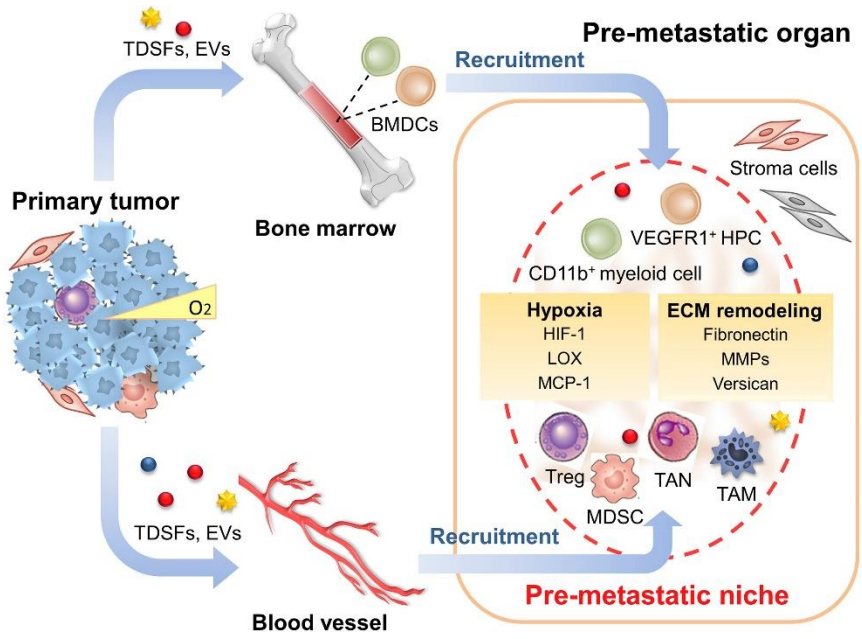

Figure 6. The pre-metastatic niche formation. Primary cancer cells produce tumor derived secreted factors (TDSFs) and extracellular vesicles (EVs) that, acting both on bone marrow and on blood vessels, can favour the formation of a tolerogenic environment in distal organs. Indeed, these factors contribute to the ECM remodelling, the establishment of a hypoxic environment and, furthermore, to the recruitment of immune cells ($VEGFR^+$ HPC, $CD11b^+$ myeloid cells from the BM, MDSCs, TANs, TAMs and Tregs from the blood circulation, thus determining favourable conditions for cancer cells seeding (Adapted from Peinado H. et al, *Nature Reviews*, 2017).

For instance, MDSCs, which can either be recruited to the niche or they may develop from tissue-resident myeloid populations, are known to support the

tolerogenic environment through different mechanisms, as by suppressing interferon-γ (IFNγ)-mediated immune responses, inducing pro-inflammatory cytokines [95] or releasing high levels of MMP9, a crucial regulator of ECM remodelling and the angiogenic switch [96]. Interestingly, many recent evidences have highlighted that also tumor-derived exosomes play a pivotal role in facilitating the spread of malignant cells by promoting the metastatic niche-formation [38]. TEX, indeed, can travel long distances and carry tumoral cargoes at distal organs, thus allowing an "undisturbed" transport of pro-tumorigenic and pro-metastatic molecules affecting the recipient environment.

3.2. TEX role in favouring the metastatic spread

Tumor-derived exosomes contribution in potentiating the aggressiveness and outgrowth of the primary tumor by autocrine and paracrine effects is combined to a major role also in facilitating the spread of malignant cells at distal organs. Nevertheless, TEX are involved in a variety of processes associated to the metastatic spread, such as the promotion of invasion and migration of tumor cells, the conditioning of lymph nodes, the generation of pre-metastatic niches, the organotropism of metastases, as well as the modulation of bone marrow and stromal components like fibroblasts, endothelial cells, myeloid- and other immune-related cells [97]. Invasion and migration of cancer cells are two main aspects that could be highly conditioned by TEX. In 2015, Harris D.A. and colleagues have shown *in vitro* that exosomes isolated from intermediate-metastatic (MCF-7 transfected with Rab27) or highly metastatic breast cancer cells (MDA-MB-231) could transfer invasion-promoting molecules to tumorigenic, but not metastatic, MCF-7 breast cancer cells [98]. According to the authors, this may occur either by the TEX-mediated horizontal transfer of specific pro-metastatic molecules, as miR-10b, which was identified as an important component promoting invasion of breast cancer cells [99], or by providing matrix attachment for migrating cells, such as fibronectin [100]. Important insights concerning the pro-metastatic functions of TEX come from studies of metastases of melanoma. Particularly, exosomes from melanoma cells were shown to induce the production of collagen 18, laminin 5, and mitogen-activated protein kinase

(p38), all matrix components that may support the growth of metastatic cells and, in addition, they can stimulate the production of $\alpha_v\beta_3$ integrin, ephrin receptor β4, and stabilin 1, which aid in the recruitment of malignant cells to the ipsilateral sentinel lymph node [101]. Importantly, TEX can also increase metastases either by inducing the expression of angiogenetic factors like VEGFβ, TNFα and hypoxia-inducible factor 1α, or by transferring miRNAs and proteins that in turn promote the metastatic spread. Exosomes derived from several types of cancer cells, indeed, can deliver EGFR to endothelial cells and may induce angiogenesis through VEGF/VEGFR2 pathway [102]. Moreover, melanoma-derived exosomes carrying miR-9, which directly targets E-cadherin (CDH1) leading to increase cancer cell motility and invasiveness, have been shown to activate JAK-STAT signaling in ECs [103], promoting migration and neovascularization. The clinical relevance of this finding is strongly evident in the context of breast cancer, where miR-9 has been defined as metastamiR (metastasis-regulatory microRNA) and it has resulted highly upregulated in breast cancer cells compared to healthy mammary tissues [104].

The generation of the pre-metastatic niche is another important step of the metastatic process in which tumor-derived exosomes are known to play a critical role. As already mentioned, the establishment of special niches in (pre)metastatic organs involves the stimulation of local stromal cells by tumor-derived factors, including vesicles, and chemokines that attract tumor cells and hematopoietic progenitors. To this aim, TEX have been shown to modulate target cells in several ways, hereafter summarized by some examples. The study of pre-metastatic niches in the lymph nodes and the lungs of rat pancreatic adenocarcinoma models has unveiled that exosomes, independently of the highly or poorly metastatic origin cells, can favor cancer cell embedding and growth in pre-metastatic niches in a CD44v6-dependent manner. CD44v6, indeed, is involved in c-MET activation and upregulation of its downstream genes, including uPAR, responsible for initiating a pro-metastatic signaling through its association with integrins, EGFR, PDGFR and vitronectin [105]. Notably, TEX can further support the process by enhancing the permeability of lung ECs, as demonstrated by the injection of B16-F10 fluorescently-labeled exosomes and

their rapid detection in the organ blood vessels and subsequently in the target organs [106]. Interestingly, different authors have demonstrated via gene expression profiling that in lung tissues B16-F10 exosomes were able to up-regulate genes involved in the pre-metastatic niche formation (e.g. S100A8 and S100A9), and TNFα as mediator of vascular permeability [107]. As part of the pre-conditioning of the metastatic organ, tumor-derived exosomes can support the formation of an immunosuppressive microenvironment also by regulating immune cells functions. In 2010, Liu Y. and colleagues have shown that melanoma B16-derived exosomes induced a switch of myeloid cells towards COX2, IL6, VEGF and arginase expressing MDSCs in the lung, in a manner that was dependent on MyD88, in turn leading to an increase of the metastatic potential [108]. Similarly, in the context of breast cancer, as previously mentioned in this introductory part, the TEX-mediated induction of PD-L1 expression in monocytes and macrophages recruited in the pre-metastatic niche may further enhance the establishment of a tolerogenic microenvironment [67].

Lastly, it is worth mentioning an important finding in this field that has pinpointed the relevance of TEX in affecting metastatic organotropism, i.e. where malignant cells deriving from a specific primary tumor preferentially take root at distal sites [109]. According to the authors, both in immune-compromised and immune-competent models, the organ specificity of exosome biodistribution is strictly related to the organotropic distribution of the cell line of origin and, moreover, this is dependent on the pattern of integrins expressed on exosomes surfaces. Particularly, they found that integrin alpha 6 (ITGα6), and its partners ITGβ4 and ITGβ1, were highly expressed in lung-tropic exosomes, whilst ITGβ5, which associates only with ITGαv, was detected primarily in liver-tropic exosomes, therefore justifying why different types of cells in the analyzed organs were responsible for the up-taking of specific types of exosomes (Figure 7). At a functional level, in order to unveil the downstream effects of exosomal interaction with target cells, they performed un unbiased analysis of gene expression by RNA sequencing in Kupffer cells educated with highly or poorly metastatic exosomes, and they found cell migration genes, among which S100A8 and S100P, as the most abundantly upregulated in the highly-metastatic exosomes educated cells.

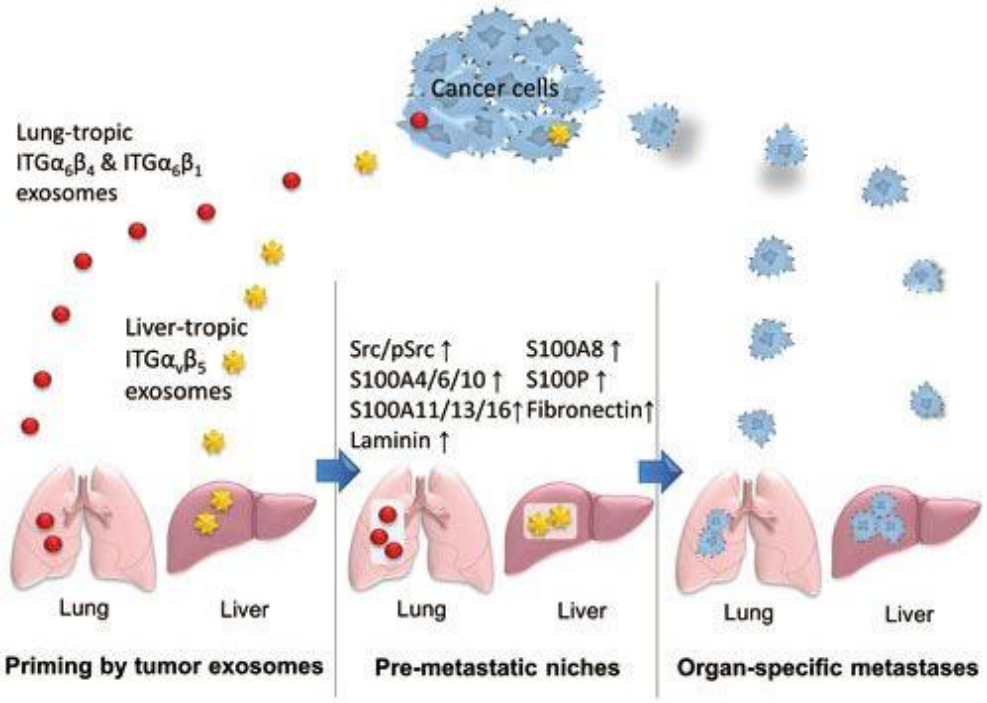

Figure 7. Metastatic organotropism mediated by TEX. The specific pattern of expression of integrins on TEX surfaces is able to prime specific distal organs before the arrival and seeding of cancer cells. This exosomes-target cells interaction causes the activation of intracellular pathways within the recipient cells leading to the upregulation of genes, such as those belonging to the S100 family, known to support cell migration and invasiveness. Eventually, these events render distal organs favorable and permissive environments for the survival and growth of malignant cells. (Adapted from Liu Y. and Cao X, *Cell Res.*, 2016)

Accordingly, tumor-derived exosomes-educated lung fibroblasts strongly upregulated S100A4, -A6, -A10, -A11, -A13 and -A16, thus confirming that TEX may trigger signalling pathways and inflammatory responses in target cells rendering the environment permissive for the growth of metastatic cells.

3.3. S100 proteins in the metastatic process

Since their first detection in 1965 by Moore and colleagues [110] as acidic cytoplasmic proteins specific for the nervous system, S100 proteins have been deeply investigated either in homeostasis or in disease conditions. The S100 protein family, which can be found only in vertebrates, represents the largest group of EF-hand signaling proteins (i.e. Ca^{2+} binding proteins) in humans, and are described as typically symmetric dimers with each S100 subunit containing four α-helices [111]. Target binding is commonly Ca^{2+} dependent and post-

translation modification can affect S100-target complex formation and intracellular localization. According to these differences, S100 proteins are functionally divided in three main subgroups: those that exert only intracellular regulatory effects, those with both intracellular and extracellular functions and those that mainly display extracellular regulatory functions. In general, S100 proteins can participate to local intercellular communication, as well as coordinate biological processes over long distances, following the interaction with a variety of target proteins including cytoskeletal subunits, enzymes, receptors (among which RAGE (*Receptor for Advanced Glycosylation End-products*), TLR4 (*Toll-like receptor 4*), G-protein-coupled receptors, and IL-10R (*Interleukin-10 Receptor*)) and transcription factors [112]. In homeostasis, S100 proteins are involved in many physiological processes, such as the regulation of proliferation, differentiation, apoptosis, Ca^{2+} homeostasis, energy metabolism, inflammation and migration/invasion, suggesting how their aberrant expression and/or regulation may represent a critical event in disease progression. Indeed, focusing on the main topic of this dissertation, recent *in vivo* evidences suggest a prominent role of most S100 proteins in the active contribution to pro-tumorigenic processes like cell proliferation, immune evasion, angiogenesis and metastatic spread. This critical role is quite evident just looking at the expression pattern of S100 proteins in cancer, where it is widely assessed that, in most of cancer types, upregulation of numerous S100 proteins may occur. A peculiar pattern of S100 proteins depending on both the stage and the subtype of cancer can be observed. For instance, there are several evidences demonstrating S100B upregulation in malignant melanoma [113], S100A4 and S100A10 for brain tumor [114] and, more importantly, S100P in all human cancers examined. Furthermore, a strong alteration of many of the S100 proteins, i.e. S100A1, S100A4, S100A6, S100A7, S100A8, S100A9, S100A11, S100A14 and S100P, is often found in breast cancer [115], where their upregulation correlate with the aggressiveness of the disease. Given these evidences, the understanding of the mechanisms behind S100 proteins expression represents a useful starting point in order to move towards new therapeutic approaches. The complex regulatory network, often cancer specific, that controls S100 proteins expression includes epigenetic mechanisms

and signal transduction pathways. Several examples of epigenetic modulation of S100 proteins expression have been described: illustrative cases can be found in the context colon cancer, where seven S100 genes are directly targeted by histone-lysine methyltransferase MLL2 [115]. Alternatively, they can be overexpressed due to the epigenetic regulation of miRNAs, as for S100A4 that has been described to be post-transcriptionally regulated by tumor suppressor miRNAs (miR-505c-5p and miR-520c-3p), which in turn may be epigenetically silenced in colorectal cancer [116]. In addition, the regulatory mechanisms modulating S100 proteins include many signaling pathways, such as the WNT-β-catenin or the cAMP/CREB pathways for regulating S1004 and S100P expression respectively in a colon cancer setting [117, 118]. Nonetheless, S100A8 and S100A9 are known to be regulated by diverse signaling pathways depending on the specific type of cancer. For instance, in liver cancer it has been demonstrated that these two S100 family members are NF-κB (*Nuclear Factor kappa-light-chain-enhancer of activated B cells*) target genes that support malignant progression of hepatocellular carcinoma (HCC) cells by activation of ROS and protection against apoptosis [119]. Alternatively, S100A8 and S100A9 could be regulated either by CEBPβ-signaling in prostate cancer [120], or they could be expressed as a downstream result of the S100 signalling cascade, entering a feedback loop that further promotes tumorigenesis.

Although it is certainly important investigating the mechanisms responsible for S100 proteins regulation, given the strikingly evidences that correlate their expression to the aggressiveness of cancers, it is evident the necessity to stress how these proteins affect the disease progress, especially in terms of promotion of the metastatic spread. To this aim, the example of breast cancer could be particularly illustrative. For instance, S100A7, apart from mediating pro-survival effects in ERα-negative breast cancer cells, it can improve their invasiveness via upregulation of MMP9 secretion and EGFR signaling [121]; moreover, it can facilitate the recruitment of TAMs, which support the metastatic process [122]. Another important player in breast cancer aggressiveness is represented by S100A4 (also known as metastatin, which clearly suggests its involvement in the metastatic process), whose overexpression has been found to

increase the migratory capacity of breast cancer cells, likely by binding to several cytoskeletal and adhesion proteins, such as F-actin and liprin β1 [123]. Interestingly, also tumor-associated stromal cells may facilitate the metastatic process, as demonstrated for $S100A4^+$ fibroblasts, which are required for the colonization of breast tumor cells to the lungs [124]. Lastly, it is worthwhile highlighting the role of S100A8/S100A9 heterodimer in contributing to the dissemination of cancer cells, as their upregulation strongly correlates with the invasiveness of breast cancer ductal carcinoma. Tumor-derived factors have been shown to upregulate S100A9 in myeloid precursors that, subsequently, promotes MDSCs accumulation to the detriment of more mature immune cells differentiation, such as macrophages and dendritic cells. Furthermore, S100A8 and S100A9 are responsible for maintaining an autocrine feed forward loop, via binding to RAGE on MDSCs surface, which is critical for MDSCs recruitment and activation, thus supporting an immunosuppressive environment. More importantly, several evidences have demonstrated that these two S100 family members are also involved in the formation of the pre-metastatic niche [125]. Back in 2006, Hiratsuka and colleagues proved that this is a multistep process in which primary tumors first stimulate the expression and accumulation of S100A8 and S100A9 in lungs (by secreting TNFα, VEFG-A and TGFβ), which can act as chemotactic factors for $CD11b^+$ myeloid cells. These, once recruited and accumulated, could further amplify the S100A8/A9 abundance in the pre-metastatic phase, thus facilitating cancer cells migration to the lungs in the proper metastatic phase [126]. More recently, it has been demonstrated that primary breast cancer cells can induce the accumulation of MDSCs within the brain to form "pre-metastatic soil" enriched in inflammation mediators, such as S100A9, that attract additional myeloid cells and metastatic tumor cells, thus confirming the critical role of these mediators in the pre-metastatic niche formation [127].

AIM OF THE STUDY

In 2011, Hanahan and Weinberg clearly assessed that evading immune distraction is a critical step in tumor progression and therefore should be considered as a new hallmark of cancer [5]. Indeed, tumor cells display a plethora of diverse mechanisms that can allow to avoid their detection and recognition from the immune system, and, moreover, the effective activation of immune cells for the eradication of the neoplastic threat. It is well established that among these cells are myeloid populations, which could be deeply affected by tumor-derived soluble factors, such as cytokines and chemokines, which might modulate their recruitment and functions. Nevertheless, cancer cells may create a favorable tolerogenic environment also by releasing exosomes, whose features of natural carriers throughout the whole organism could be exploited by the tumor to affect both the local tumor framework and future distal sites of disease. However, despite many insights are coming from the recent literature, a deep understanding of the mechanisms behind exosome-mediated effects in the tumor extracellular milieu is certainly needed. In light of this, the first part of our project aimed at assessing the ability of TEX to affect the MDSC regulatory functions *in vitro*, as these cells are strongly involved in determining an immunosuppressive TME. Therefore, we first wanted to investigate the ability of tumor-derived exosomes to interact with myeloid cells, both *in vitro* and *in vivo*, and, secondly, to look for the functional consequences of this interaction in terms of immunosuppressive ability. Moreover, we exploited possible pathways that were likely responsible for the observed effects, in order to find putative candidates to target for abrogating the detrimental TEX-mediated effects. Moreover, since our data showed that TEX were relevant also for supporting the pre-metastatic niche formation and consequent spread of malignant cells, the second aim of our project was to study their contribution in this context. Particularly, we drew our attention to the understanding of how TEX may modulate myeloid cells, both in bone marrow and lung, thus promoting their shift towards immunosuppressive phenotypes, as well as a higher recruitment at distal sites of metastases. Finally, these investigations were exploited to generate proofs of concepts concerning possible strategies

targeting the downstream mediators of TEX, which might be useful in the development of new therapeutic approaches.

MATERIAL AND METHODS

1. Cell lines.

The E0771 (H-2^b) breast cancer cell line (CH3 BioSystems), derived from C57BL/6 mice was cultured in RPMI 1640 (Life Technologies, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine (Euroclone, Milano, Italy), 10 mM HEPES (Euroclone, Milano, Italy), 20 μM β-mercaptoethanol (Sigma-Aldrich, Saint Louis, MO, USA), 150 U/ml streptomycin (Euroclone, Milano, Italy), 200 U/ml penicillin (Euroclone, Milano, Italy) and 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). The MCA-derived spontaneously metastasizing sarcoma MN-MCA1 cell line (a kind gift of Prof. Antonio Sica; Istituto Humanitas, Milan, Italy) was grown in DMEM (Euroclone, Milano, Italy) supplemented with 2 mM L-glutamine (Euroclone, Milano, Italy), 10 mM HEPES (Euroclone, Milano, Italy), 150 U/ml streptomycin, 200 U/ml penicillin (Euroclone, Milano, Italy) and 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA, USA). The murine immortalized fibroblast cell line NIH/3T3-J2 (kind gift of Prof. H. Green; Harvard Medical School, Boston, MA) immortalized mouse fibroblasts was grown in DMEM (Euroclone, Milano, Italy Lonza) supplemented with 10% heat-inactivated Bovine Calf Serum (Sigma-Aldrich, Saint Louis, MO, USA) and all the other components described above. All these cell lines were tested to be free from Mycoplasma contamination by PCR screening.

2. Mice.

C57Bl/6 mice were purchased from Charles River Laboratories Inc. (Calco, Italy). OT-1 TCR-transgenic mice (C57BL/6-Tg(TcraTcrb)1100Mjb/J), 37B7 TCR-transgenic mice and $CD45.1^+$ congenic mice (B6.SJL-PtrcaPepcb/BoyJ) were from Jackson Laboratories (Bar harbor, ME, USA). B10;B6-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Wjl} ($RAG2^{-/-}$ $\gamma c^{-/-}$) mice were obtained by Taconic (Denmark). All animal experiments were approved by Verona University Ethical Committee (http://www.medicina.univr.it/fol/main?ent=bibliocr&id=85) and conducted

according to the guidelines of Federation of European Laboratory Animal Science Associations (FELASA). All animal experiments were in accordance with the Amsterdam Protocol on animal protection and welfare.

3. Exosomes isolation and quantification.

Exosomes from E0771, MN-MCA1 and NIH/3T3-J2 cell lines were purified from culture conditioned media by a combination of consecutive ultracentrifugations as previously described [128]. Briefly, all cell lines were cultured as described above, but using an exosome-depleted FBS (i.e. FBS that underwent the consecutive ultracentrifugations procedure). After 72 hours, when cells were at 60% to 70% of confluence, conditioned medium was collected, centrifuged 10 min at 300 x g, 4°C to remove cell debris, and filtered with a 0.22 μm filter. The resulted cell-free medium was concentrated by ultracentrifugation at 100.000 x g, 4°C for 2 hours. The supernatant was then carefully removed, and, after having washed exosome-containing pellets in 1 mL of ice-cold PBS, a second ultracentrifugation (100.000 x g, 4°C for 2 hours) was performed. The isolated exosomes were then suspended in ice-cold PBS, quantified as amount of total proteins using a colorimetric assay (Bradford) and then stored at -80°C.

4. Analysis of EVs size.

The measurement of the mean size of isolated EVs was estimated through a Dynamic Light Scattering (DLS) analysis using a Zetasizer Nano ZS (standard laser beam λ = 632.8 nm; Malvern, Westborough, MA, USA). Samples were prepared diluting the exosomes solution to a final concentration in a range from 0.1 mg/mL to 1.0 mg/mL. Five different measurement were then performed for each sample.

5. Flow cytometric analysis of exosome-coated beads.

For exosomes surface antigens detection by flow cytometric analysis, exosomes needed to be previously treated to coat aldehyde/sulfate latex beads (Life

Technology, Carlsbad, CA, USA). Briefly, 15 µg of isolated exosomes were incubated with 5 µl of 4 mm diameter beads for 15 minutes at room temperature on a tube rotator wheel. After the addition of filtered PBS to a final volume of 1 ml, samples were incubated on a tube rotator wheel for other 30 minute. Samples were then saturated with 2M glycine and PBS 2% BSA for 30 minute on the rotator wheel and then washed with PBS 2% BSA for three times. For the detection of exosomal surface antigens exosome-coated beads were incubated with FITC conjugate anti-CD9 (clone M-L13, BD Bioscience), PE-conjugated anti-CD63 (clone HSC6, eBioscience) antibodies for 1 h at 20°C, stirring. After two washings steps, samples were analyzed by FACSCanto II (BD Biosciences). Each analysis included IgG-matched isotype controls. Events were gated according to light-scattering properties, selecting single-bead populations.

6. Exosomes staining with PKH26 dye.

Exosomes isolated from the conditional media of the above described cell lines were fluorescently labelled using PKH26 dye (Sigma-Aldrich, Saint Louis, MO, USA), which is able to aspecifically bind to lipidic membrane. In brief, 100 µg of exosomes were incubated with 6 µl of PKH26 dye (4 µM) in a total volume of 2 ml of Diluent C. Samples were put on a rotator wheel for 5 minutes at room temperature and afterwards the reaction was blocked adding an equal volume of filtered PBS-BSA 1%, incubating for other 5 minutes in a rotator wheel. Exosomes were then concentrated using Vivaspin® 500 Centrifugal Concentrator with a molecular cutoff of 300000 MW (Sartorius, Gottingen, Germany) at 2,000 rpm, 4°C. Unconjugated dye was removed by several washing step with PBS for 2 times. PBS without exosomes was treated with the same procedure as a negative control. The efficiency of exosome labeling was analyzed using a spectrophotometer, measuring the absorbance at 549,84 nm. PKH26-labelled exosomes were used for evaluating the uptake ability of recipient cells, either *in vitro* or *ex vivo.*

7. PKH26-labelled exosomes uptake in vitro.

MDSCs were differentiated *in vitro* from murine bone marrow cells as previously described [129]. In order to assess the ability of taking up exosomes, PKH26-labelled exosomes were used for flow cytometry analysis. Particularly, fluorescent exosomes (20 µg for each well) were added to the MDSCs culture at different time points (36, 24, 12 and 6 hours) starting from the 3rd day of differentiation of the cells. Afterwards, cells were collected and stained with the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit® (ThermoFisher, Waltham, MA, USA) for gating the viable cells, and the following antibodies to detect the subpopulations of MDSCs: PerCP-Cy5.5 conjugated anti-CD11b (clone M170), V450 conjugated anti-Ly6C (clone HK1.4) and APC-Cy7 conjugated anti Ly6G (clone RB6-8C5).

8. Ex vivo PKH26-labeled exosomes tracking.

PKH26-labelled exosomes were retro-orbitally injected into C57BL/6 (20 µg of exosomes/mouse). At 12, 24 and 36 hours after injection, mice were sacrificed and various tissues were harvested (lung, spleen, liver, blood and BM) for *ex vivo* fluorescence quantification using flow cytometry. Additionally, immune populations in the lung, spleen, and BM that had taken up PKH26-labelled exosomes were assessed.

9. Confocal analysis.

MDSCs, previously incubated with PKH26-labelled exosomes for 24 h, were let adhere on 14-mm round Menzel-Glaser glass for 2h then fixed with 4% paraformaldehyde for 10 minutes at room temperature. After extensive wash with PBS, the cells were incubated for 1 hour at room temperature with PBS containing FcR blocking reagent (Miltenyi) diluted 1:25. Cells were then stained with anti-Ly6C FITC (HK1.4 clone) in PBS for 2h at room temperature, in the dark. Slides were then washed with PBS 0.05% Tween- 20 and cells were then stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) diluted 1:500 in PBS for 10 minutes at room temperature, in the dark. After extensive washes with PBS, coverslips were mounted with ProLong Gold antifade Mounting media

(ThermoFisher Scientific) in Superfrost Plus adhesion microscope slides (ThemoFisher Scientific) and acquired by confocal microscopy (TCS SP5, Leica Microsystems CMS GmbH, Wetzlar, Germany). Cells were located and positioned using bright field illumination (BF). Fluorescence images were captured sequentially, using a 405-nm laser line for DAPI, a 488-nm laser line for FITC and 543-nm laser line for PKH26. Images were analyzed by LAS AF Lite 2.0.2 (Leica Microsystems CMS GmbH) and NIH-Image J programs (Bethesda, USA). Images (512x512 pixels in TCS SP5 system) were acquired with a 63x oil immersion objective. 10 different fields of each coverslip were taken randomly. Exposure times of each channel were kept constant over the whole series after calibrating on a bright representative sample to avoid saturated pixels.

10. Mouse proliferation assay.

To analyze cell proliferation, an *in vitro* labelling system was used to trace multiple cell divisions using dye dilution by flow cytometry. The immunosuppressive activity was evaluated plating *in vitro* differentiated MDSCs, or freshly isolated myeloid cells from the bone marrow or the tumor of C57BL/6 mice, in 96 wells plate at a final concentration of 24% of total cells in culture in presence of splenocytes from 37B7 or OT-1 transgenic mice, labelled with 1 μM CellTrace (Thermo Fisher Scientific, Waltham, MA, USA) and diluted 1:10 with $CD45.1^+$ splenocytes, in the presence of TRP-$2_{180\text{-}188}$ peptide (1 μg/ml final concentration). After 3 days of co-culture, cells were stained with APC-Cy7 conjugated anti-CD45.2 (clone 104, eBioscience, Thermo Fisher Scientific, Waltham, MA, USA) and PerCP-Cy5.5 conjugated anti-CD8 (clone SK1, eBioscience, Thermo Fisher Scientific, Waltham, MA, USA), and CellTrace signal of gated lymphocytes was analyzed. We performed FACS evaluation with a FACS-Canto II (BD, Franklin Lakes**,** NJ, U.S.A.) to determine the percentage of division of $CD8^+$ cells, thus calculating the percentage of suppression of MDSCs or myeloid cells (FlowJo software, Tree Star, Inc. Ashland, OR, USA).

11. Real-time PCR.

Total RNA from MDSCs or murine myeloid cells was isolated by TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). For exosomal RNA, Norgen's Exosome RNA Isolation Kits was used according to manufacturer's instructions. The amount and purity of isolated RNA was then analyzed by ND-1000 Spectrophotometer (NanoDrop Technologies). cDNA was prepared using the Euroscript M-MLV reverse transcriptase kit (Euroclone, Milano, Italia) and Real Time PCRs were run using 2x SYBR Green master mix (ABI, Thermo Fisher Scientific, MA, USA). All samples were normalized using GAPDH endogenous control primers. Post qRT-PCR analysis to quantify relative gene expression was performed by the comparative Ct method ($2^{-\Delta\Delta Ct}$).

12. ELISA for S100A8/A9 detection.

Human or murine sera were collected and kept at -80°C after having removed cell debris and erythrocitic contaminants. ELISA for detecting the heterodimer S100A8/A9 (R&D, Minneapolis, MN, USA) were performed following manufacturer's instructions.

13. Exosome education.

In order to investigate the role of exosome in the pre-metastatic niche formation, 8-week-old C57BL/6 female mice were "educated" with the retro-orbital injection of 5 µg/mouse of exosomes isolated from a highly metastatic tumor cell line (MN-MCA1), every 2 days for 3 weeks, whilst control mice received an equal volume of filtered PBS. After exosomes conditioning, mice were challenged with a tumor cell line with a low metastatic potential. Specifically, pre-conditioned mice were orthotopically injected in the mammary fat pad with $0.5x10^6$ E0771 cells. To favor distal dissemination, primary tumors were removed at a volume of 600 mm^3 and mice were sacrificed after 2 weeks after the surgery.

14. In vivo blocking of exosome secretion.

C57BL/6 mice were challenged with the highly metastatic cell line MN-MCA1 ($0.1x10^6$/mice) in order to evaluate the effect of blocking exosome secretion on the metastatic spread. GW4869 inhibitor of nSMase2-dependent exosome secretion (Sigma-Aldrich, Saint Louis, MO, USA) was given at 2.5 mg/kg intraperitoneally every two days. When tumors reached a volume of 600 mm^3 mice were sacrificed and lungs were collected for further analyses.

15. In vivo blocking of S100A9.

C57BL/6 mice were injected in the mammary fat pad with $0.5x10^6$ E0771 cells. The S100 inhibitor Tasquinimod (Cayman Chemicals, Ann Arbor, MI, USA), was given orally every two days (5 mg/kg in a final volume of 100 µl). Primary tumors were removed when reached a volume of 600 mm^3 to favor distal dissemination, while continuing with the Tasquinimod treatment until sacrifice, when various tissues were collected for performing phenotypical and functional evaluations.

16. Lung metastases count.

For the *in vivo* experiments, lungs were harvested at sacrifice and fixed in 10% neutral buffered formalin (BioOptica) for metastases detection and quantification. The number of lung metastases was determined by two pathologists, independently and in a blind fashion, using a Leica DMRD optical microscope (Leica).

17. Patients.

57 treatment-naïve resectable patients with histologically proven non-metastatic PDAC and 9 healthy donors were included in the study. Peripheral blood samples were prospectively collected from all patients before surgical resection. Clinico-pathologic features of patients included age, gender, tumor location, tumor size, differentiation status, lymph node involvement and TNM stage, patterns of

resection margins. Distant metastasis free survival (dmFS) was determined from the time of surgery until metastatic PDAC tumor recurrence.

18. Statistical Analysis.

All data are presented as mean ± standard error (SE) of the mean. Statistical analyses were carried out using SigmaPlot (Systat Software, San Jose, CA), or GraphPad Prism software program (GraphPad Software, San Diego, CA) and the statistical language R for human data. For statistical comparison of two groups, non-parametric Mann-Whitney Wilcoxon test was used. For the comparison of more than two group ANOVA test was used. A value of $p < 0.05$ was considered significant. Survival curves were drawn by Kaplan-Meier estimates and compared by log rank test. The optimal cutoff thresholds of biomarkers were obtained based on the maximisation of the Youden's statistics J=sensitivity+specificity+1 [130] using an R-based software as described in Budczies et al [131].

RESULTS

1. Isolation and characterization of exosomes from immortalized cell lines

In order to investigate *in vitro* the tumor-derived exosome immunoregulatory properties, we applied a well-characterized size-based isolation technique, the ultracentrifugation, to purify exosomes from two murine tumor cell lines, MN-MCA1 (fibrosarcoma) and E0771 (breast cancer) and from NIH/3T3-J2 (murine fibroblasts, hereafter named 3T3-J2) as healthy control. We collected conditioned media of cell lines after 72 h of culture and performed several centrifugation steps to discard cell debris and bigger vesicles, as reported in Figure 8A. We could classify the isolated extracellular vesicles as exosomes thanks to the use of a Zetasizer Nano ZS instrument: indeed, the isolated vesicles sized between 10 and 100 nm; particularly, MN-MCA1-derived exosomes mean diameter was 56.94 nm ± 46.34, whilst E0771 and 3T3-J2 ones were 58.71 nm ± 39.28 and 50.70 nm ± 39.58 respectively (Figure 8B-C-D, upper panels). Moreover, we confirmed that we effectively isolated exosomes by looking for the

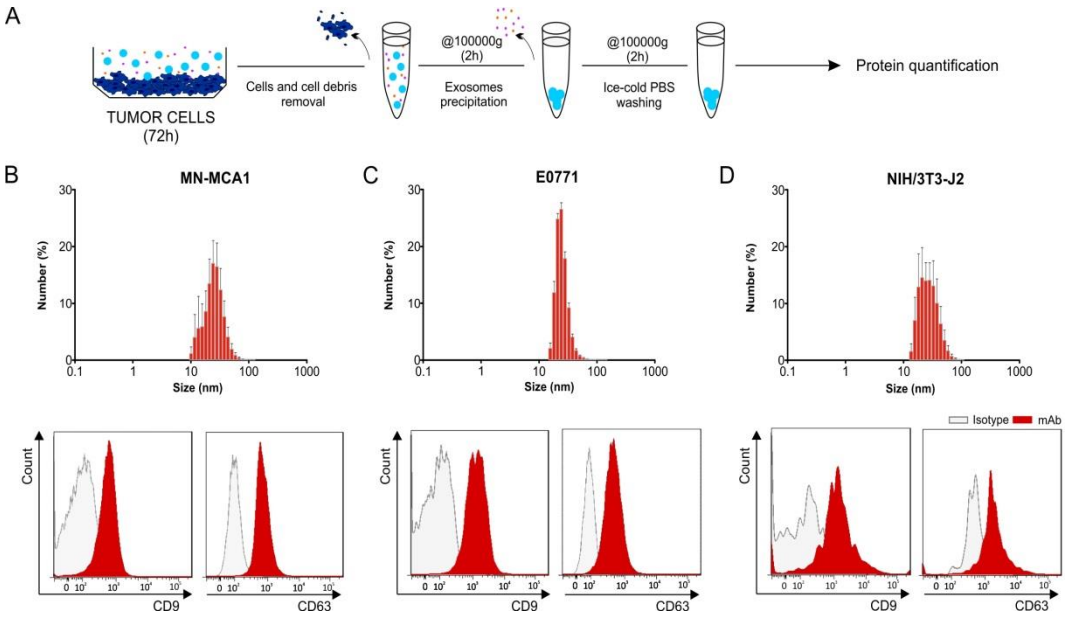

Figure 8. Isolation and characterization of exosomes. A) Experimental schedule for isolating exosomes from conditioned media of the immortalized cell lines MN-MCA1, E0771, NIH/3T3-J2. B-C-D) In the upper panels the measurements of exosomes size by dynamic light scattering are shown (mean ± SD of five measurements). Bottom panels show the mean fluorescent intensity for the exosomal markers CD9 and CD63. Isotype antibodies (grey) were used as negative controls.

presence of surface markers that are commonly used to identify them, i.e. CD9 and CD63. For this purpose, exosomes coupled to 4-mm diameter aldehyde/sulfate latex beads were analyzed by flow cytometer using anti-mouse CD9 and CD63 antibodies and matched isotype antibodies as controls. All exosomes isolated expressed both markers (Figure 8B-C-D, bottom panels) on their surfaces, confirming that the purification procedure was correctly performed and, moreover, that these markers are exposed independently of the exosome nature, either cancerous or normal.

2. Exosomes are effectively taken up by myeloid cells

It is well established that exosomes can mediate intercellular communication by different mechanisms that include direct interactions with receptors on target cells surfaces, thus activating intracellular pathways, or fusing their membranes with the recipient cells and transferring their cargoes. Particularly, EVs released from tumor cells generally target myeloid cells, such as MDSCs, whose modulation may aid cancer cells to grow and disseminate. Briefly, we differentiated MDSCs starting from bone marrow cells isolated from femurs of C57BL/6 mice for 4 days with a combination of GM-CSF and IL-6 (Figure 9A), as previously described by our group [129]. To verify whether MDSCs could effectively take up exosomes, we previously labelled them with the common fluorescent red-emitting dye PKH26, which aspecifically binds to lipid membranes. The protocol for exosomes staining was also applied to an equal volume of PBS, which was used to exclude from the analysis the signal given by clusters of dye which can eventually be formed. The uptake experiments were performed by incubating MDSCs with 20 μg of PKH26-labelled exosomes or PBS for each well at 36, 24 and 12 h before the flow cytometry analysis, which allowed us to evaluate the percentage of cells positive for the dye, therefore identifying those interacting with exosomes. Particularly, our data indicated that among the two main subsets of mouse MDSCs, M-MDSCs ($CD11b^{+}Ly6C^{high}Ly6G^{-}$cells) and PMN-MDSCs ($CD11b^{+}Ly6C^{low}Ly6G^{+}$ cells) (Figure 9B), the majority of $PKH26^{+}$ cells were found within the M-MDSC subset, both when incubated with tumor-derived exosomes (TEX) and fibroblasts-derived exosomes (“healthy” exosomes, named HEX) at each of the time point considered (Figure 9C).

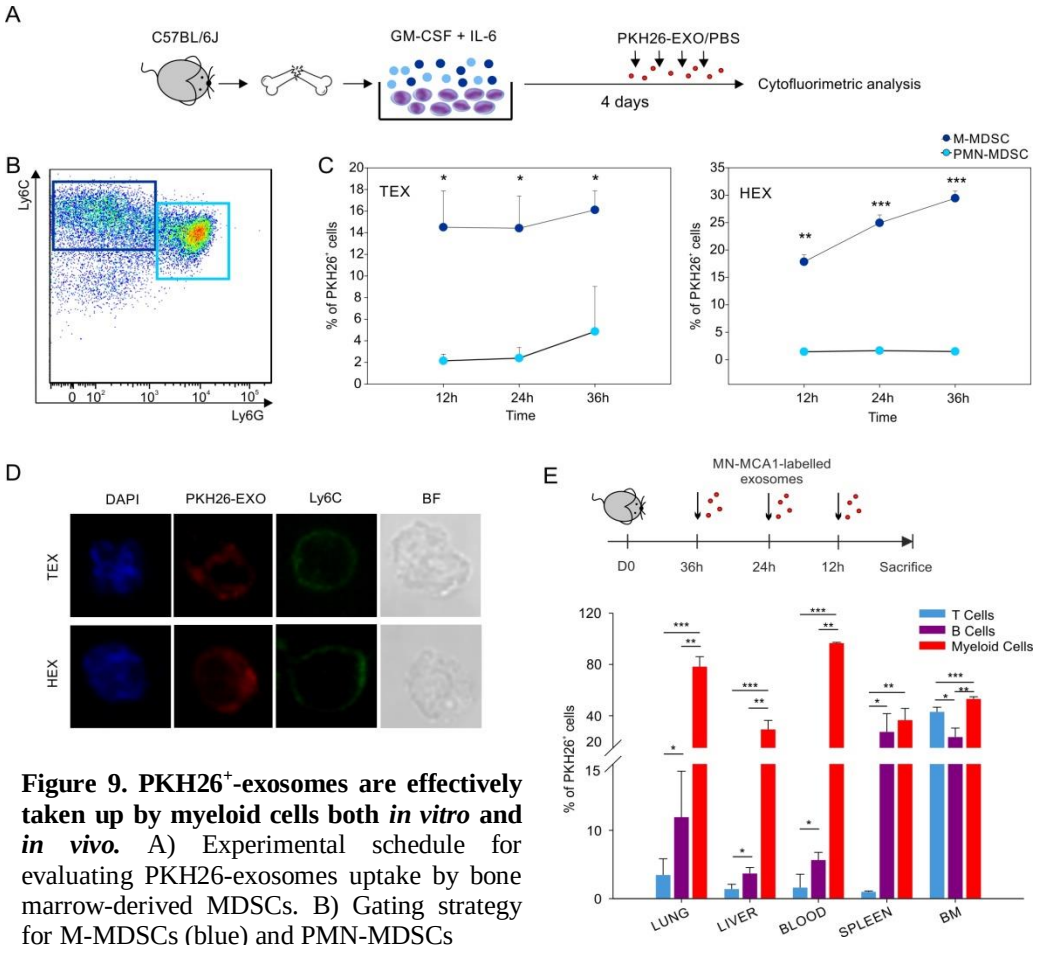

Figure 9. PKH26$^+$-exosomes are effectively taken up by myeloid cells both *in vitro* and *in vivo*. A) Experimental schedule for evaluating PKH26-exosomes uptake by bone marrow-derived MDSCs. B) Gating strategy for M-MDSCs (blue) and PMN-MDSCs (light blue). C) PKH26$^+$ cells gated on single cells after 12, 24 or 36h of incubation. Values were normalized excluding PKH26$^+$ cells resulting from exosomes remaining on cell surfaces and subtracting the fluorescence value of the PBS control. D) Confocal analysis representing M-MDSCs after 24h of incubation with labelled TEX or HEX. E) *In vivo* tracking of MN-MCA1-labelled exosomes. The histograms refer to the 24h time point and represent the percentage of immune cells that took up labelled exosomes. Statistical analysis was performed by ANOVA test; *p<0.05, ** p<0.01, *** p<0.001.

Moreover, we could confirm the effective interaction of labelled TEX or HEX by M-MDSCs also by confocal analysis (Figure 9D). Finally, we wanted to prove that also *in vivo* exosomes are able to interact with immune cells, and especially with the myeloid compartment. As showed in Figure 9E, MN-MCA-derived-labelled exosomes systemically injected in C57BL/6 mice could be easily detected in immune cells after 24h, in almost all organs analyzed through *ex vivo* fluorescence quantification performed by flow cytometry. Interestingly, myeloid cells (red bars) resulted as the main CD45$^+$ cell subset to be involved in the interaction with TEX, with peaks around 80-90% in lung and blood, whilst CD3$^+$ T cells and B220$^+$ B cells were just partially interested.

3. TEX but not HEX can enforce immunosuppressive functions in MDSCs

Since we demonstrated that exosomes can be efficiently engulfed by MDSCs, we moved forward to explore the main effects resulting from this interaction. There is already evidence that highlights the critical impact of TEX in modulating TME and especially immune cells, by interacting with receptors and/or via the release of specific cargoes. In this context, we planned to mimic *in vitro* the interaction between TEX and MDSCs, which is likely to happen during tumorigenesis, in order to have insights about the mechanisms by which these vesicles can modulate MDSC functions. Briefly, we took advantage of the above described setting, treating MDSCs for 24h with TEX or HEX and then collecting them for performing the functional assay (Figure 10A). Our results clearly demonstrated that MDSCs treated with either MN-MCA1-or E0771-derived exosomes, were able to increase their suppressive properties when co-cultured at different ratios

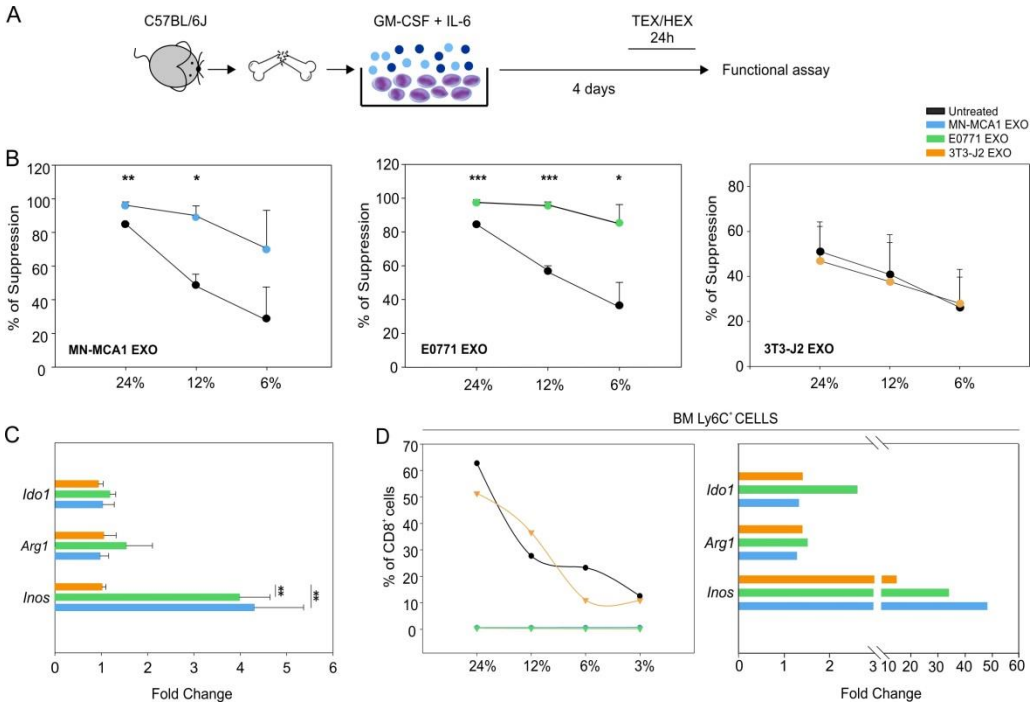

Figure 10. TEX can skew MDSCs towards a more immunosuppressive phenotype. A) Representative scheme of the functional *in vitro* experiments with MDSCs. B) Functional suppression assays performed on MDSCs treated with MN-MCA1-, E0771- or 3T3-J2-derived exosomes. Suppression percentages were calculated based on proliferating activated CellTrace^{+}CD8^{+} cells at the end of a co-colture with different amount of TEX/HEX-treated MDSCs (24%, 12% and 6% of the final co-culture). C) Real Time PCRs on MDSCs. Fold changes were calculated on the untreated samples. D) Representative functional assay (left panel) and Real Time PCR (right panel) performed on FACS-sorted Ly6C^{+} cells isolated from WT bone marrow and treated 24h with TEX or HEX. The TruCountTM tubes were used to determine the absolute cell number of CD8^{+}cells in the samples. Statistical analysis was performed by ANOVA test; *p<0.05, ** p<0.01, *** p<0.001.

for 3 days with antigen-specific activated $CD8^+$ T cells (Figure 10B), suggesting a modulation induced by vesicles released from cancer cells. Moreover, we did not observe any impact on immunosuppressive function on MDSCs treated with exosomes isolated from normal 3T3-J2 cells. We then looked for the possible mechanisms that could explain the increase in immunosuppression. Not surprisingly, we found an increased PD-L1 expression in MDSCs (data not shown), which is in line with data recently published for human $CD14^+$ monocytes [67]. More interestingly, performing a Real-Time PCR on candidate genes involved in immunosuppression, we could appreciate a significant increase in *Inos* mRNA levels in TEX-treated MDSCs that was not observed for the HEX-treated ones, whilst *Ido1* and *Arg1* levels were not affected (Figure 10C). These findings were then confirmed by performing the same kind of experiment on FACS-sorted $Ly6C^+$ cells isolated from the bone marrow of C57BL/6 mice, in order to investigate whether monocytes, which are not commonly immunosuppressive under tumor-free conditions, could acquire a different phenotype in presence of TEX. Indeed, we observed that activated $CD8^+$ T cells lost almost all their proliferative properties in presence of TEX-, but not HEX-, treated monocytes (Figure 10D, left panel). Furthermore, *Inos*, *Arg1* and *Ido1* mRNA levels were modulated similarly to what was previously demonstrated for *in vitro* differentiated MDSCs (Figure 10D, right panel), providing a proof of concept about the influence of TEX treatment.

4. *Inos* is likely to be involved in TEX-mediated modulatory functions

We the explored whether TEX were able to increase MDSC suppressive functions *in vitro* by upregulating *Inos* expression rather than other immunomodulatory enzymes. In order to assess this aspect, first we differentiated MDSCs from bone marrow cells of different mouse strains, i.e. C57BL/6 (WT), as well as mice genetically deficient for either *Inos*, *Ido1* or *Arg1* genes. Interestingly, when incubated for 24h with either MN-MCA1-derived or E0771-derived exosomes, only iNOS KO MDSCs did not display an increased suppression of T cell proliferation, as it occurred for WT, IDO1 KO or ARG1 KO MDSCs (Figure 11A).

Notably, in three independent experiments we could confirm a significant difference in the immunosuppressive functions of WT MDSCs compared to iNOS KO MDSCs, whose immune modulatory properties were not even slightly affected by the treatment with TEX (Figure 11B), highlighting a possible role of the iNOS enzyme as downstream regulator induced by TEX in MDSCs. These data made us pointing our attention to potential upstream mediators leading to iNOS activation, in turns eventually contributing to the *in vitro* increased immunosuppression. Therefore, a further investigation on TEX cargoes was strictly necessary. Particularly, we focused on the transcriptome contained in TEX and HEX, since it is known that the transfer of miRNAs and mRNAs mediates many of the regulatory functions of TEX. To this aim, total RNA from both TEX

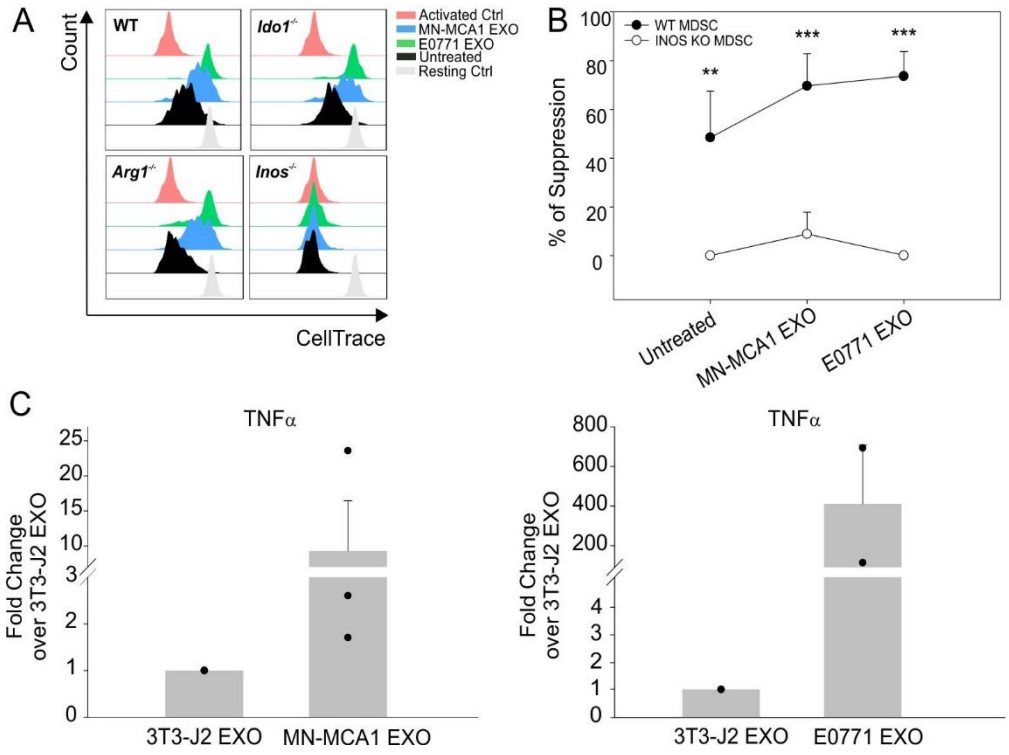

Figure 11. TEX-mediated effects on MDSCs occur in an *Inos*-dependent manner. A) Representative plot of the functional assay performed either on either WT MDSCs or MDSCs deficient for *Ido1*, *Arg1* or *Inos* respectively, treated 24h with TEX. B) Functional assays comparing WT MDSCs and iNOS KO MDSCs suppressive properties when pre-treated 24h with TEX. C) RT-PCRs on exosomes'mRNA contents. Fold Change values were calculated on the 3T3-J2 exosomes values and GAPDH used as endogenous control. Statistical analysis was performed by ANOVA test; ** $p<0.01$, *** $p<0.001$.

and HEX was isolated, cDNA retro-transcribed and RT-PCRs performed on candidate genes. It is already known that different molecules, such as LPS, IFN-γ, ROS and TNFα, are able to induce iNOS by activating several intercellular

pathways that converge on NF-κB activation and *Inos* transcription. Interestingly, among all the possible mediators able to upregulate *Inos*, TNFα mRNA resulted one of the most abundant in TEX compared to HEX, whose values were used as controls (Figure 11C). Although just two batches of E0771-derived exosomes could be tested, it is important to underline that in both cases TNFα levels were strikingly higher than the control, which led us to speculate that TNFα could represent a possible upstream mediator of the TEX-mediated *Inos* up-regulation and functions.

5. TEX contribute to the pre-metastatic process by acting on myeloid cells

The critical role of tumor-derived exosomes in immunosuppression by modulating myeloid cells has clearly emerged from recent evidences and highlighted by our described data. Nevertheless, we wanted to verify whether a similar mechanism could be exploited by neoplastic cells also for the pre-metastatic niche formation, which represents one of the crucial step in favoring the spread of cancer cells. In order to elucidate this aspect, we took advantage of an experimental protocol, which we defined as "exosome education", which allowed us to examine the ability of exosomes isolated from highly metastatic cells to influence infiltrating myeloid cells of tumor-free C57BL/6 mice, to increase the metastatic spread of a low metastatic cell line. Briefly, MN-MCA1-derived exosomes, or an equal volume of vehicle for the control group, were retro-orbitally injected, every 2 days for 3 weeks, to "educate" tumor-free mice (Figure 12A). Subsequent characterization of infiltrating immune cells in the lung confirmed no significant changes in immune composition and, in particular, in myeloid cells frequency compared to mice of control group (data not shown). After 3 weeks of pre-conditioning, educated mice (n=24) and control mice (n=25) were challenged with $5x10^5$ E0771 breast cancer cells, a poorly metastatic cell line, in the mammary fat pad. When the tumor reached a volume of 600 mm^3, the primary mass was surgically removed to avoid the death of the hosts and therefore allow detection of tumor cell dissemination. At sacrifice, myeloid cells were isolated from lungs and bone marrows and examined by RT-PCR (Figure 12B). Interestingly, as further confirmation of *in vitro* evidence, we could observe both in lung and bone marrow a significant upregulation of *Inos*, previously hypothesized as important

player in the TEX-mediated immunosuppression, together with a strong downregulation of *Ido1*, suggesting the existence of a dichotomy between the two enzymes in this setting. Furthermore, in lung myeloid cells we found a slight increase in the immunosuppressive cytokine *Il-10*, whereas *Tnfα* was strongly induced in both organs of educated mice compared to the control group,

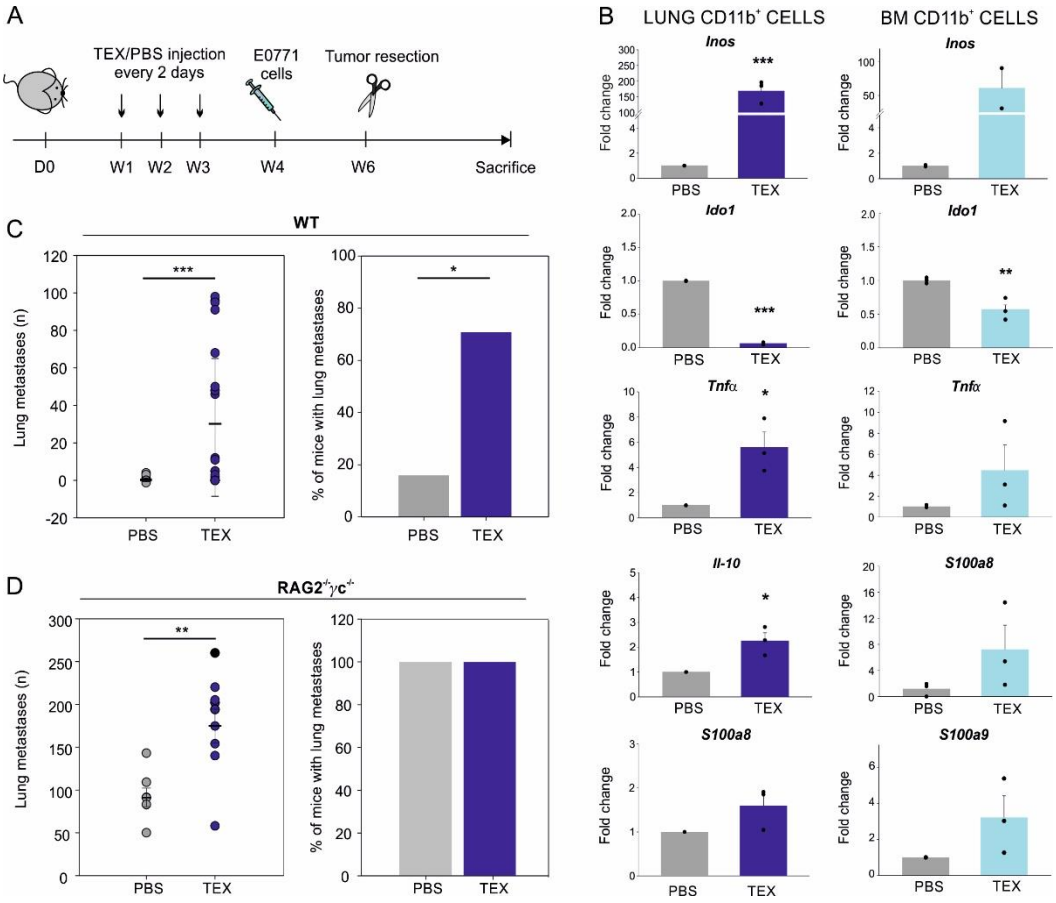

Figure 12. Mice educated with highly metastatic TEX displayed a strong increase in the dissemination of poorly metastatic cancer cells. A) Experimental schedule of «exosome education». B) Real Time PCRs on selected genes, involved in immunosuppression and metastatic spread, of CD11b^{+}cells isolated from lung and bone marrow of either PBS- or TEX-treated mice (3 mice for each group). Fold Changes were calculated over the PBS controls and GAPDH used as normalizer gene. C) Lung micrometastases count (left panel) and metastasis incidence (right panel) at sacrifice of WT mice (PBS n=25, EXO n=24 pooled from 2 independent experiments). D) Lung micrometastases count (left panel) and metastasis incidence (right panel) at sacrifice of RAG2$^{-/-}$γc$^{-/-}$ mice (PBS n=5, EXO n=8). Statistical analyses were performed by Mann-Whitney Wilcoxon test, *p<0.5; **p <0.01; ***p <0.001.

supporting our hypothesis of a TNFα-iNOS axis triggered by TEX in myeloid cells. Moreover, in the lung of educated mice, we could appreciate an trend towards upregulation of the S100 gene member *S100a8*, as well as both *S100a8* and *S100a9* in the bone marrow. These two important members of the S100 proteins family are known to be important chemotactic factors for the recruitment of myeloid cells favoring the metastatic spread [132]. Notably, these data agree

with recent evidences that have underlined how exosomes can both modulate myeloid precursors towards immunosuppressive populations and, furthermore, they can act as chemotactic factors in the lungs leading to higher recruitment and activation of suppressive myeloid subsets [133]. Thus, we speculated that the TEX-mediated establishment of this immunosuppressive and pro-metastatic phenotype in myeloid cells could be relevant in favoring the pre-metastatic niche formation and thus seeding of cancer cells. Indeed, in two independent experiments, TEX-conditioned mice displayed a significant increment in lung metastases number, as well as in tumor metastases incidence, compared to PBS-treated control mice (Figure 12C). To further confirm the prominent role of the myeloid compartment in this process, we then performed the same experiments in $RAG2^{-/-}\gamma c^{-/-}$ mice, which lack mature T and B cells as well as NK cells. Although all mice of both groups displayed a numerous amount of lung metastases due to the lack of a fully competent immune system, the increase in lung metastases number mediated by TEX-education was still detectable, therefore indicating a role of the innate immunity in the interaction with exosomes, in turns favoring the spread of cancer cells (Figure 12D). It is worth mentioning, however, that immune competent mice could partially control metastatic spread unless exposed to TEX, differently from the immune deficient ones.

6. Blocking exosomes secretion does not decrease the spread of cancer cells to the lungs

After having proved that TEX may be pivotal in the formation of an immunosuppressive and pro-metastatic environment within the pre-metastatic niche, we then wondered whether blocking exosomes secretion could represent a useful strategy to impair TEX-mediated detrimental effects. To this aim, we used a neutral sphingomyelinase inhibitor, named GW4869, which is the most widely used pharmacological agent for blocking exosome generation [134]. In details, GW4869 is able to block the enzymatic activity of the sphinghomyelinase N-SMase2, therefore inhibiting the ceramide-mediated inward formation of multivesicular bodies (MVBs) and release of mature exosomes from MVBs (Figure 13A). For the purpose of our project, we set up an *in vivo* protocol in which C57BL/6 mice were ortothopically injected in the femoral quadriceps with

the highly metastatic cell line MN-MCA1 ($1x10^5$ cells/mouse) and treated with the exosome blocking agent GW4869 (2.5 mg/kg i.p) every two days (Figure 13B). Tumors were left growing for 25 days while continuing with the treatments

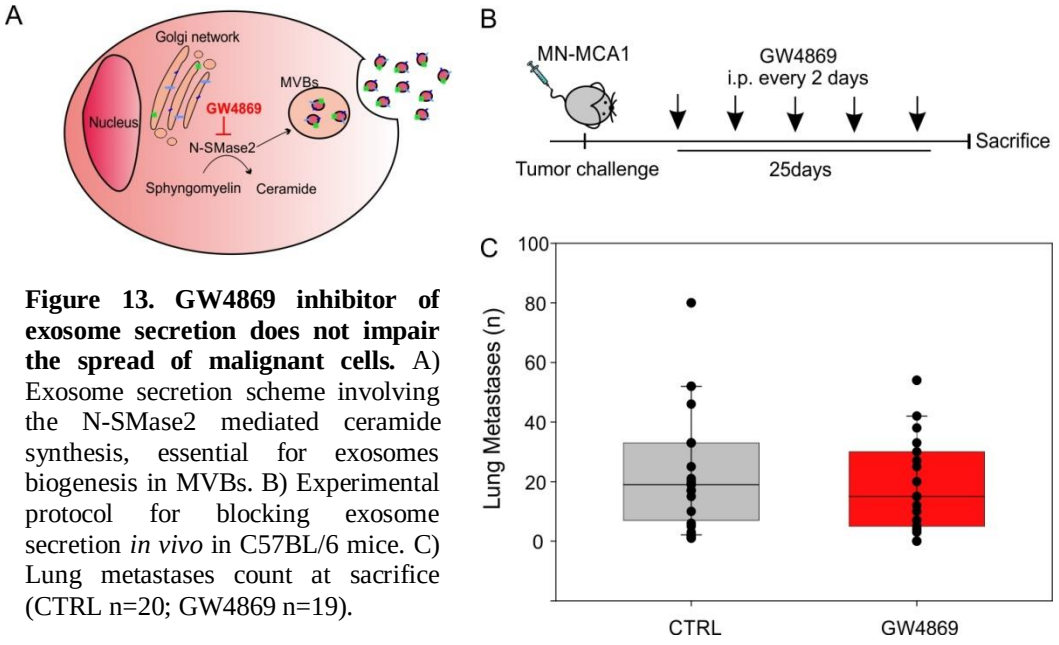

Figure 13. GW4869 inhibitor of exosome secretion does not impair the spread of malignant cells. A) Exosome secretion scheme involving the N-SMase2 mediated ceramide synthesis, essential for exosomes biogenesis in MVBs. B) Experimental protocol for blocking exosome secretion *in vivo* in C57BL/6 mice. C) Lung metastases count at sacrifice (CTRL n=20; GW4869 n=19).

and, when primary mass reached 600 mm^3, lungs were collected for evaluating the number of micrometastases. Surprisingly, in two independent experiments (CTRL n=20; GW4869 n=19), the treatment with GW4869 did not impair the metastatic spread to the lungs, neither in terms of number of metastases (Figure 13C) or of metastases incidence (data not shown), therefore suggesting that more specific approaches in blocking TEX-mediated effects were needed in order to obtain a therapeutic benefit.

7. The *in vivo* blocking of S100A9 can modulate myeloid cells affecting both immunosuppression and metastatic spread

The lack of efficacy of blocking exosome secretion led us to speculate that, although TEX are increased in some types of cancer and are likely the most abundant vesicles released, presumably the GW4869 nonspecific inhibitory functions are also impairing important physiological processes in cells other than the transformed ones. Therefore, we moved to investigate other possible strategies that could be pursued in order to abrogate the detrimental effect of tumor-derived exosomes, both in the tumor microenvironment as well as at distal sites. Among

all possible targets, we focused on S100 family members S100A8 and S100A9, whose expression was previously shown to be increased in myeloid cells of exosomes-educated mice (see page 58). More importantly, S100A8 and S100A9

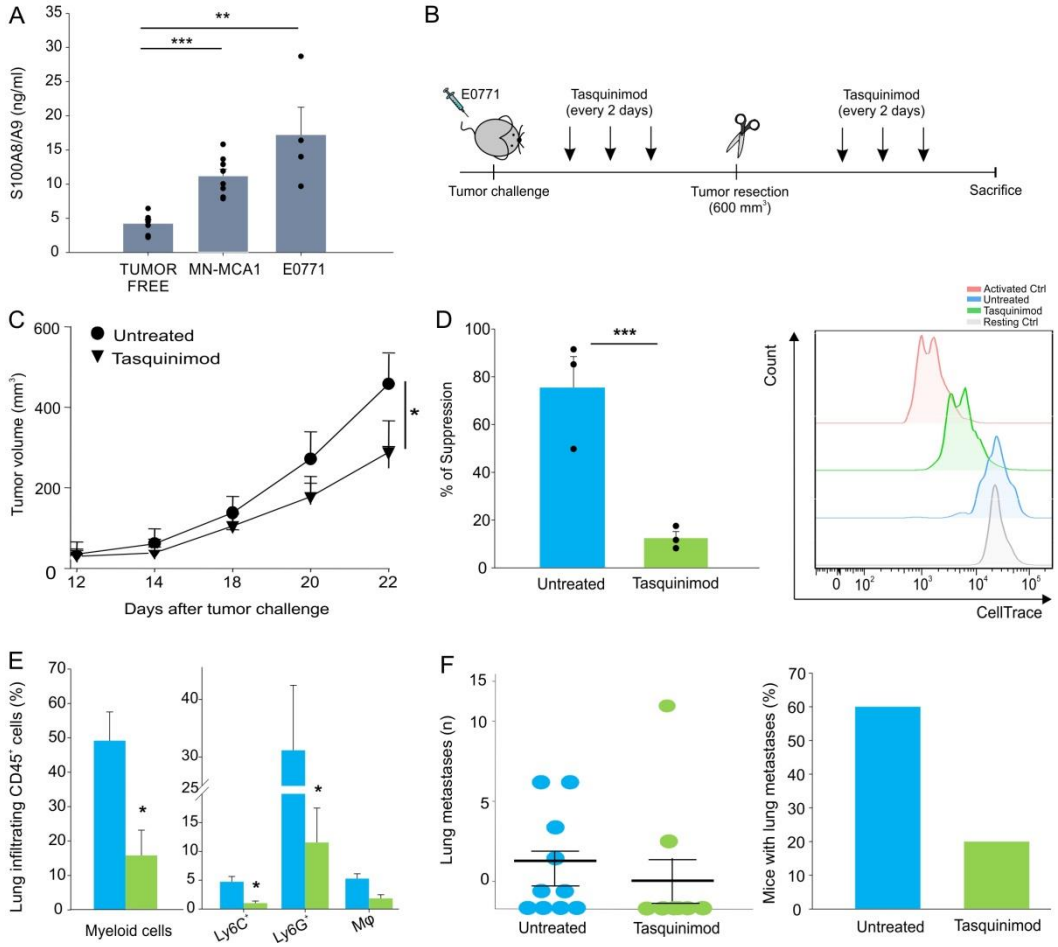

Figure 14. Blocking S100A9 *in vivo* can have beneficial effects on tumor growth and metastatic spread. A) S100A8/A9 heterodimer quantification on plasma of tumor-bearing mice (injected with MN-MCA1 or E0771 cell lines) compared to tumor-free mice. B) Experimental setting for the *in vivo* blocking of S100A9. C) Average of primary tumor growth over time in control or Tasquinimod-treated mice (Untreated n=10; Tasquinimod n=10). D) Functional suppressive assay performed on $CD11b^+$ cells isolated from tumors of control (n=3) or treated mice (n=3). The results show the percentage of suppression (left panel) and a representative proliferation plot (right panel). E) Flow cytometry analysis of lung infiltrating total myeloid cells, $Ly6C^+$ or $Ly6G^+$ cells and macrophages (Mφ). All percentages were expressed on total $CD45^+$ cells. F) Lung micrometastases count (left panel) and metastases incidence (right panel). Statistical analysis was performed by ANOVA test; *p<0.05, ** p<0.01, *** p<0.001.

are widely considered as key modulators of myeloid cells (particularly of MDSCs), both by contributing to their recruitment in pre-metastatic organs and, furthermore, by directly regulating their functions [135]. Giving these premises, we decided to interfere with S100A9 by using Tasquinimod, a small molecule oral inhibitor that has shown anti-angiogenic, antitumor and immune-modulatory

properties in preclinical models of prostate cancer and other solid tumors. First, we verified that circulating S100A8/A9 heterodimer was significantly increased in cancer, as demonstrated by two different models of tumor-bearing mice (Figure 14A). Importantly, S100A8/A9 could not be detected in tumor cells conditioned media (data not shown), suggesting that stromal cells are the main responsible for their secretion. As E0771-bearing mice showed the highest levels of S100A8/A9, C57BL/6 mice were challenged with E0771 cancer cells and treated every two days by oral gavage with the S100A9 inhibitor Tasquinimod (n=10) or an equal volume of sterile water (n=10). The treatments were continued after the primary mass removal until sacrifice, when tumors and lungs were collected (Figure 14B) and analyzed. Interestingly, Tasquinimod-treated mice displayed a significant reduction in growth of the primary tumor (Figure 14C), which could be associated with a strong and significant decrease in the *ex vivo* suppressive functions of $CD11b^+$ cells isolated from tumors of treated mice compared to controls (Figure 14D). These data highlighted that blocking S100A9 protein may result in the impairment of the myeloid-mediated immunosuppressive milieu thus rescuing a functional immune response that can effectively control tumor growth. Furthermore, we further analyzed the recruitment of myeloid cells in lung by flow cytometry. Indeed, Tasquinimod-treated mice showed a significant decrease in $CD11b^+$ infiltrating the lung, which is also reflected in the lower percentages of $Ly6C^+Ly6G^-$ cells (M-MDSCs) and $Ly6C^{low}Ly6G^+$ cells (PMN-MDSCs), whilst macrophages were just partially affected (Figure 14E), leading us to speculate that also the favorable suppressive environment for cancer cells dissemination within the lungs could be compromised. Notably, we could indeed observe a strong decrease both in the number of lung metastases and metastasis incidence in treated mice compared to controls (Figure 14F, left and right panels respectively), which, although not statistically significant, gave us important insights about the possible efficacy of the drug in affecting the metastatic spread.

8. High levels of circulating S100A8/A9 in cancer patients are predictive of poor distant metastasis-free survival

After having proven the *in vivo* beneficial effects by interfering with S100A9 signaling, we wondered whether we could translate our findings in a human

cancer setting. Many of the S100 proteins are already known to be highly overexpressed in different types of human cancer, and, particularly, S100A8 and S100A9 are abundant in colorectal, breast, prostate and lung cancer, among the others. However, few data are available about one of the most aggressive and metastatic cancers, i.e. pancreatic cancer. In this regard, we recently demonstrated that pancreatic ductal adenocarcinoma (PDAC) is not an "immune desert" as previously thought, and a high number of tumor-infiltrating $CD45^+$ cells can be found in patients' biopsies (data in publication). Thus, we wondered whether the aggressiveness and invasive potential of PDAC cells could be associated to high amount of circulating S100A8/A9. To elucidate this aspect, we enrolled a cohort of 57 PDAC patients and 9 age- and sex-matched healthy donors (HD). Clinico-

A

Patients Characteristics	N°	%
Age (years)		
Median	66	
Range	37-77	
Gender		
Female, n (%)	28	49.12
Male, n (%)	29	50.88
Tumor Stage		
T1, n (%)	1	1.75
T2, n (%)	2	3.51
T3, n (%)	53	92.98
T4, n (%)	1	1.75
Nodal stage		
N0, n (%)	8	14.04
N+, n (%)	49	85.96
Metastasis stage		
M0, n (%)	57	100
M+, n (%)	0	0
Location		
head, n (%)	43	75.44
body/tail, n (%)	14	24.56
Resection margins		
R0, n (%)	30	52.63
R1, n (%)	27	47.37
Radiotherapy		
no, n (%)	40	70.18
yes, n (%)	17	29.82
Tumor grade		
G1, n (%)	4	7.02
G2, n (%)	38	66.67
G3, n (%)	15	26.32

R1 resection denotes a microscopically positive margin;
T Tumor; N node; G grade.

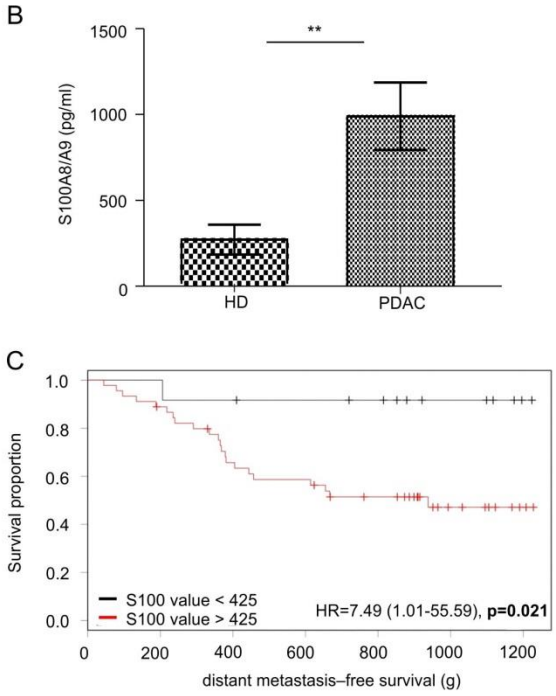

Figure 15. PDAC patients display high levels of circulating S100A8/A9, which negatively correlates with distant metastasis-free-survival. A) Clinical characteristics of the study population. B) S100A8/A9 protein levels in sera of PDAC patients (n=57) compared to healthy donors (n=9). C) Correlation between S100A8/A9 sera concentration in PDAC patients and the distant metastasis-free-survival (patients with S00A8/A9 values above or below the cutoff value are represented in red and black respectively). Statistical analyses were performed by Mann-Whitney Wilcoxon test, **p<0.01. Survival curves were drawn by Kaplan-Meier estimates and compared by log rank test.

pathologic features of patients were reported in Figure 15A and include age, gender, tumor location, tumor size, differentiation status, lymph node involvement and TNM stage, patterns of resection margins. PDAC and HD sera were analyzed by ELISA for the quantification of S100A8/A9 heterodimer. A significant increase in patients' samples compared to the control group was evidenced

(Figure 15B), confirming the possibility to exploit S100A8/A9 as diagnostic biomarker in PDAC patients. More importantly, sera concentration of these proteins negatively correlated with the distant metastasis-free-survival (dmFS): particularly, when S100A8/A9 levels were found above the cutoff value of 425 pg/ml, which allowed the division of PDAC patients in two different clusters, the dmFS at 3 years was almost halved (Figure 15C). In conclusion, these data highlighted that S100A8/A9 concentration could represent a useful tool also as prognostic factor for the metastatic potential of the disease, therefore possibly allowing more prompt and efficacious therapeutic approaches.

DISCUSSION

The complexity and multi-step process of tumor development and progression has been extensively studied over the past 50 years and it is now widely recognized. Particularly, it has clearly emerged that cancer cells, despite all accumulated mutations, do not act alone in cancer progression but instead they can employ and modulate normal cell types that may serve as active collaborators toward a neoplastic phenotype. A striking evidence is represented by the fact that cancer cells are not capable of forming tumors when injected into a nonmalignant environment, such as developing embryos [136], which clearly justify why cancer is now considerable as an "ecological disease", modulated by many components of TME. For instance, tumor cells have been shown to direct contrast T cells fitness by expressing immune checkpoint molecules, such as PD-L1/PD-L2, as demonstrated by the efficacy and enthusiastic outbreak concerning immune checkpoint inhibitors strategies. Secondly, tumors can indirectly influence the framework of microenvironment by modulating both stromal and immune cells through their secretome, which includes TDSFs and EVs. As previously reported, among extracellular vesicles TEX are the most well-studied and characterized: indeed, their abundance in cancer patients and evidence of their modulatory properties are pushing researchers to investigate their role in pathogenesis. Hence, for the aims of this project, we specifically focused on the crosstalk and interactions between TEX and the supporting myeloid cells composing TME in mice, since they are crucial in the aforementioned neoplastic processes. First, we took advantage of mouse immortalized cell lines to isolate exosomes from their conditioned media. Among the available isolation methods, we chose the ultracentrifugation protocol because of its simplicity, little technical expertise requirement and relatively low time consumed. Indeed, we could efficiently isolate exosomes, as confirmed both by the diameter mean size and by the surface expression of CD9 and CD63 markers, which are commonly used to mark exosomes. However, a more complex panel of surface markers could allow a better characterization of exosomes of different origins; in fact, several companies are now developing flow cytometry strategies for performing an *in vitro* broad

characterization of extracellular vesicles, which may unveil useful tumor-specific surface markers.

Subsequently, being interested in the TEX-myeloid cells interaction, we moved to evaluate the ability of *in vitro* differentiated MDSCs to uptake exosomes, mimicking what it is likely to happen in TME, where MDSCs constitute a major population involved in the establishment of a permissive milieu. Interestingly, our *in vitro* data demonstrated that MDSCs can interact with exosomes independently of their tumor or normal origin and, furthermore, that M-MDSCs, whose ability of engulf labelled exosomes was also shown by confocal analysis, represent the major subset involved in this process. This could be explained by the fact that monocytic cells are particularly prone to detect, recognize and eventually engulf particles, including vesicles, in turn activating intracellular pathways. Although recent evidences suggested a temperature-dependent and actin-dependent uptake mechanism by *ex vivo* MDSCs [137], the exact mechanism by which this interaction occurs is still to be clarified. More importantly, by *ex vivo* flow cytometry analysis we could also observe that PKH26-labelled-TEX were preferentially taken up by myeloid cells, rather than $B220^+$ B cells and $CD3^+$ T cells, in almost all organs analyzed, thus highlighting the central role of the innate immune system in the interaction with TEX. Therefore, we plan to characterize deeper this process by dissecting which are the specific myeloid cell subsets mainly involved in TEX given *ex vivo*, and, moreover, we could study this interaction directly *in vivo* through the injection of trackable engineered tumor cell lines (e.g. carrying GFP-linked CD9) [138].

Since we were interested in how TEX could modulate myeloid cells, we took advantage of the MDSC setting to test the functional consequences of TEX interaction. As recently reported by our group [139], to assess the extent of the MDSC suppressive ability it is mandatory to perform functional *in vitro* assays that give a quantitative and reliable proof of their functions. Notably, TEX, but not HEX, could enforce the immunosuppressive ability in MDSCs, and, more importantly, they conferred potent suppressive properties to $Ly6C^+$ monocytes isolated from the bone marrow of tumor-free mice. These data are consistent with previous findings regarding TEX contribution to immunosuppression [140], which can be either direct (such as by expressing PD-L1 themselves) or indirect through the modulation of suppressive cell types. In our setting, we discovered

that TEX-treated MDSCs displayed a significant increase in *Inos* expression, rather than other known MDSC-related immunosuppressive enzymes (i.e. *Ido1* and *Arg1)*, which was partially mirrored by BM-Ly6C$^+$ cells, leading us to speculate of a possible indirect immunosuppressive TEX-mediated process. To better unveil the specific downstream mediators of the enhanced suppressive ability of MDSCs after TEX treatment, we exploited MDSCs differentiated from the bone marrow of mice lacking *Inos, Ido1* or *Arg1*. The functional suppressive assay revealed that only iNOS KO MDSCs did not enhance their suppressive functions when incubated with TEX, whereas all the other KO MDSCs behaved similarly to the WT control. Given this proof of concept, we confirmed the involvement of iNOS by three independent functional assays where we could observe the same lack of TEX-mediated suppressive increase. It is important to emphasize that iNOS is a key mediator of M-MDSCs suppressive function, which were above described as the main subset involved in the interaction with TEX, thus suggesting a possible association between TEX uptake and iNOS upregulation. Although the expression regulation of iNOS has been extensively studied in various types of cells, including myeloid cells *in vitro* [141] the molecular mechanism underlying iNOS expression regulation in MDSCs in cancer is for the most part unknown. For this reason, we looked to the transcriptome within TEX and HEX to investigate whether differences in candidate molecules, known to upregulate *Inos,* could be detected. Among them, TNFα mRNA levels were increased both in MN-MCA1-derived exosomes and, particularly, in E0771-derived exosomes, compared to 3T3-J2-derived controls. Although not statistically significant, this enrichment in TNFα, which was particularly evident in the case of the breast tumor model, made us hypothesize that the horizontal transfer of this cytokine to MDSCs may trigger an intracellular pathway leading to NF-κB activation, in turns responsible for the up-regulation of *Inos* [142].

We next moved to an *in vivo* setting where we could investigate TEX involvement in the pre-metastatic niche formation, where the MDSC recruitment and activation have been shown to be crucial. First, we demonstrated that the continuous exposure of C57BL/6 mice to MN-MCA1-derived exosomes before the tumor challenge was sufficient to modulate broadly CD11b$^+$ cells both in lung and in bone marrow. Particularly, as a further confirm to our *in vitro* data,

myeloid cells isolated form TEX-conditioned mice, showed a significant up-regulation of *Inos* compared to control mice, likely contributing to the establishment of a suppressive environment. Moreover, *Tnfα* was also strongly upregulated by TEX-conditioning in myeloid cells within both organs analyzed, supporting the hypothesis about TNFα-iNOS axis, whereas, surprisingly, *Ido1* was significantly downregulated, thereby suggesting a dichotomy between iNOS and IDO1, as proven for iNOS and ARG1 in the context of infections [143]. To investigate this aspect, we plan to use the CRISPR/CAS9 technology in order to delete the *Tnfα* gene in tumor cell lines, isolate TEX and investigate whether iNOS up-regulation is eventually abrogated. In addition, although not striking, the slight increase in the S100 family member *S100a8* in the lung, as well as both *S100a8* and *S100a9* in the bone marrow of TEX-conditioned mice, emphasized the hypothesis of TEX as promoter not only of a more suppressive phenotype in myeloid cells but also of pro-metastatic properties. It was previously demonstrated that the up-regulation of S100A9 in myeloid precursors inhibits DC and macrophage differentiation, inducing the MDSC accumulation [144]; in addition, TDSFs including VEGF-A, TGFβ and TNFα can stimulate S100A8/A9 in pre-metastatic lung, which are able to act as chemoattractant for $CD11b^+$ cells [126], thus creating a favorable microenvironment promoting tumor spread. Indeed, our data clearly demonstrated that TEX-education could significantly increase the spread of a poorly metastatic cell line (i.e. E0771), either in C57BL/6 mice or in mice lacking T, B and NK cells, enforcing the hypothesis of a critical role of myeloid cells in this process.

Having assessed the detrimental effects of TEX both in immunosuppression and in favoring the metastatic spread, next step of our project concerned the attempt to block TEX secretion. In order to do that, we exploited GW4869, a molecule that can block the ESCR-independent exosome biogenesis/release by inhibiting the nSMase-2 enzyme. Its beneficial effects have been previously demonstrated in Lewis lung carcinoma- (LCC-) bearing mice, which displayed a lower number of lung multiplicities when treated with GW4869 compared to controls [145]. To appreciate better a possible decrease in the spread of cancer cells, we injected a highly metastatic cell line (MN-MCA1) in C57BL/6 mice, which received GW4869 treatment every two days until sacrifice.

Surprisingly enough, blocking exosomes secretion did not provide any beneficial effect, as demonstrated by comparing the number of lung metastases of mice in both experimental arms. We hypothesized that this inefficacy could be due to the nonspecific activity of the inhibitor, which may compromise also the physiological exosomes-mediated network between normal cells. Alternatively, GW4869, although widely used to block exosomes release, has been recently shown to induce a significant increase in the secretion of microvesicles in human tumor cell lines, an activity that may mediate other important functions in TME [146]. All things considered, we plan to block selectively exosomes release by deleting RAB35 in tumor cell lines, inject them in mice and look for the metastatic spread, thus avoiding the aforementioned limitations of using GW4869.

Subsequently, we wondered whether blocking a downstream mediator of TEX-modulation in myeloid cells could instead ameliorate the neoplastic disease both at primary tumor as well as at future distal sites of metastases. Therefore, we pointed our attention to S100A8 and S100A9 proteins, since we found them increased in infiltrating myeloid cells after TEX-education and, furthermore, they were identified as potent amplifiers of inflammation, tumor invasion and metastases in cancer [147]. Thereby, we attempted to validate our hypothesis in E0771-bearing mice through the oral administration of Tasquinimod, which inhibits S100A9. Primary tumor growth was significantly decreased in treated mice compared to those of the control group. We speculate that this could be due to the impairment of MDSC suppressive functions caused by the drug, as demonstrated through the functional assay performed on $CD11b^+$ cells isolated from tumors. The most relevant finding was obtained after looking at drug effects in the lungs of Tasquinimod-treated mice compared to controls: indeed, we could demonstrate a significant decrease in lung-infiltrating, total myeloid cells, together with a strong reduction in MDSC subsets, whilst macrophages were not particularly affected. These data gave us a confirmation of the efficacy of the drug, as S100A9 participates to the recruitment of myeloid cells into the pre-metastatic niche, in turns favoring the seeding of migrating cancer cells. Nonetheless, as expected, either lung metastases number and metastatic incidence in the lung were strongly reduced by blocking S100A9 engagement. Therefore, although Tasquinimod is currently under investigation mainly in the context of prostate cancer [148], our preliminary data suggest that a possible application in

other types of solid cancer might be pursued. Particularly, S100A8/A9 heterodimer expression has been found increased in many tumors, including gastric, colon, bladder, ovarian, thyroid, breast, skin and pancreatic [149], thus being considered as a potential therapeutic target. We assessed the possible role of S100A8/A8 in PDAC progression by measuring its concentration in PDAC patients' sera (n=57) compared to healthy donors (n=9). More importantly, we demonstrated its relevance in metastatic spread by the providing evidence of a negative correlation between circulating levels of S100A8/A9 and the dmFS, thus paving the way for the therapeutic targeting of S100 proteins in patients, in combination with different anti-cancer immunotherapies, as an approach to control metastases dissemination.

In conclusion, we believe that a combination of a specific TEX-targeting strategy coupled with the block of S100A8/A9 signaling in myeloid cells, for instance using Tasquinimod-carrying liposomes specific for monocytes, may represent a novel strategy to ameliorate the favorable immunosuppressive environment established by primary tumors through exosomes, eventually leading to beneficial effects either locally and/or in terms of control of distal metastases spread.

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