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Identification of potential myeloid cell-related biomarkers of early stage of metastatic process in pancreatic tumor

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ABSTRACT

During cancer progression, tumor cells acquire a high number of genetic and epigenetic alterations, which promote a more aggressive phenotype with the ability to escape from immune surveillance. One of the most efficient mechanism of evasion is the capacity of tumor cells to alter the normal hematopoiesis by the continuous release of tumor-derived secreted factors (TDSFs) including cytokines, chemokines and metabolites. Indeed all these metabolites induce an abnormal expansion and accumulation of immature myeloid cells with immunosuppressive features like myeloid-derived suppressor cells (MDSC) and tumor associated macrophages (TAM), which can promote tumor growth and distal dissemination inducing the formation of a suitably conducive microenvironment at secondary sites (pre-metastatic niche). The pre-metastatic niche formation has been well characterized in cancer mouse model but its presence in human patients has to be confirmed yet. Using a humanized immune reconstituted (HIR) mice, engrafted with human hematopoietic stem cells (HSC), we demonstrated that the presence of a human immune system increased the metastatic potential of a human pancreatic tumor cell line compared to tumor-bearing immunodeficient mice. Human leukocytes that infiltrated the lung pre-metastatic niche in HIR pancreatic tumor-bearing mice were characterized by a gene signature strongly related to the myeloid cell compartment allowing us to identify potential molecular targets playing a role in the control of tumor cells aggressiveness and metastatic properties. Indeed, we detected a myeloid cell population, characterized as $CD14^{+}CD206^{+}$ cells, that were exclusively present in bone marrow (BM), lung, blood and spleen of tumor-bearing HIR mice. A peculiar profile of human cytokines (CCL2, IL6, CXCL8, IL1A) and chemotactic factors (S100 proteins), which are involved in the recruitment of tumor cells toward the metastatic niche, was also found. Surprisingly several interferon-stimulated genes (ISGs), typical of the innate immune response against pathogens, resulted upregulated in both BM and in lung of tumor-bearing HIR mice. Finally, we demonstrated that tumor-released exosomes drive the activation of ISG-related

molecular signature in primary myeloid cells; moreover, their injection in naïve, tumor-free mice favors the distal dissemination of poorly metastatic tumor cells.

SOMMARIO

Durante la progressione tumorale le cellule neoplastiche acquisiscono alterazioni genetiche ed epigenetiche in grado di indurre un fenotipo più aggressivo capace di sfuggire alla sorveglianza messa in atto dal sistema immunitario. Uno dei più efficienti meccanismi di "evasione" è la capacità delle cellule tumorali di alterare la normale ematopoiesi grazie al continuo rilascio di fattori solubili tra cui citochine, chemochine e metaboliti. Questi fattori hanno la capacità di indurre l'espansione di cellule del sistema immunitario con caratteristiche immunoregolatorie come le cellule soppressorie di origine mieloide (MDSC) e i macrofagi associati al tumore (TAM). Queste popolazioni oltre a favorire la crescita tumorale possono promuovere la formazioni di metastasi inducendo la creazione di un microambiente favorevole nella futura sede di metastatizzazione generalmente definito con il termine di nicchia pre-metastatica. La nicchia pre-metastatica è stata ben caratterizzata nei modelli murini; tuttavia la sua presenza nell'uomo deve essere ancora confermata. Utilizzando come modello preclinico dei topi umanizzati immunoricostituiti (HIR), abbiamo dimostrato che la presenza di un sistema immunitario umano nell'ospite aumenta la capacità metastatica di una linea tumorale pancreatica umana. Infatti, la frequenza di metastasi polmonari risulta maggiore nei topi umanizzati portatori di tumore rispetto ai topi immunodeficienti inoculati con lo stesso tumore. I leucociti umani infiltranti il polmone metastatico sono caratterizzati da una specifica "signature" molecolare fortemente collegata con le cellule mieloidi. Abbiamo identificato una popolazione di origine mieloide, doppia positiva per i marcatori CD14 e CD206, presente esclusivamente nel midollo osseo, nei polmoni, nel sangue e nella milza di topi umanizzati col tumore, insieme ad alcune citochine (CCL2, IL6, CXCL8, IL1A) e fattori chemiotattici (proteine S100) coinvolti nel reclutamento delle cellule tumorali nella nicchia pre-metastatica. Inaspettatamente alcuni geni attivati in risposta agli interferoni di tipo 1 (ISGs), tipici della risposta immune innata contro i patogeni, risultano iper espressi nel midollo osseo e nel polmone dei topi umanizzati col tumore. Infine in questo studio abbiamo dimostrato il ruolo degli esosomi rilasciati dalle cellule tumorali sia nell'attivare nelle cellule primarie

mieloidi la "signature" molecolare correlata ai geni ISG sia nel promuovere il processo metastatico *in vivo* favorendo la disseminazione distale di cellule tumorali poco metastatiche.

INTRODUCTION

Chapter 1:TUMOR MICROENVIRONMENT

1.1 Immune surveillance of tumors and cancer immunoediting theories

The concept that the immune system can recognize and eliminate primary developing tumors in the absence of external therapeutic intervention has existed for nearly 100 years. Initially proposed by Paul Ehrilch in 1909, the idea of immune control of neoplastic disease was not vigorously pursued until the midpoint of the twentieth century when the concept of tumor-specific antigen (TSA) was introduced. In fact, the genetic instability of cancer cells leads to the generation of a large variety of antigens (Ag) present on tumor cells. These antigens could be new proteins exclusively expressed on transformed cells (TSA) or be native proteins that are expressed also in normal tissue even if at lower levels (tumor-associated antigen or TAA). The immune system, and in particular T lymphocytes, can recognize these Ags and destroy the newborn transformed cells long before they become clinically detectable. But the clear evidence of the prominent role of immune system in primary tumors eradications was obtained with the introduction of immunodeficient mice. In particular, it was discovered that in mice deficient for recombination activating gene 2 (RAG-2) and thus lacking T, B, and NKT cells as well as in mice deficient in interferon (IFN)-γ signaling the frequency of sarcoma, adenoma and adenocarcinoma were increased in comparison to immune-competent hosts (1). Moreover, a high infiltration of T lymphocytes in tumor correlated with improved patients prognosis and increased therapeutic success (2). To explain the presence of solid tumors that are clinically manifested, by avoiding detection by the immune system or limiting the extent of immunological killing, the formal articulation of the Cancer Immunoediting theory by Robert Schreiber was published in 2002 (1). The main concept of this theory predicts that the dynamic interaction between immune system and neoplastic cells evolves through three phases: elimination, equilibrium and

escape. Elimination incorporates the classical idea of cancer immunosurveillance in which the immune system is able to recognize and eliminate tumor cells.

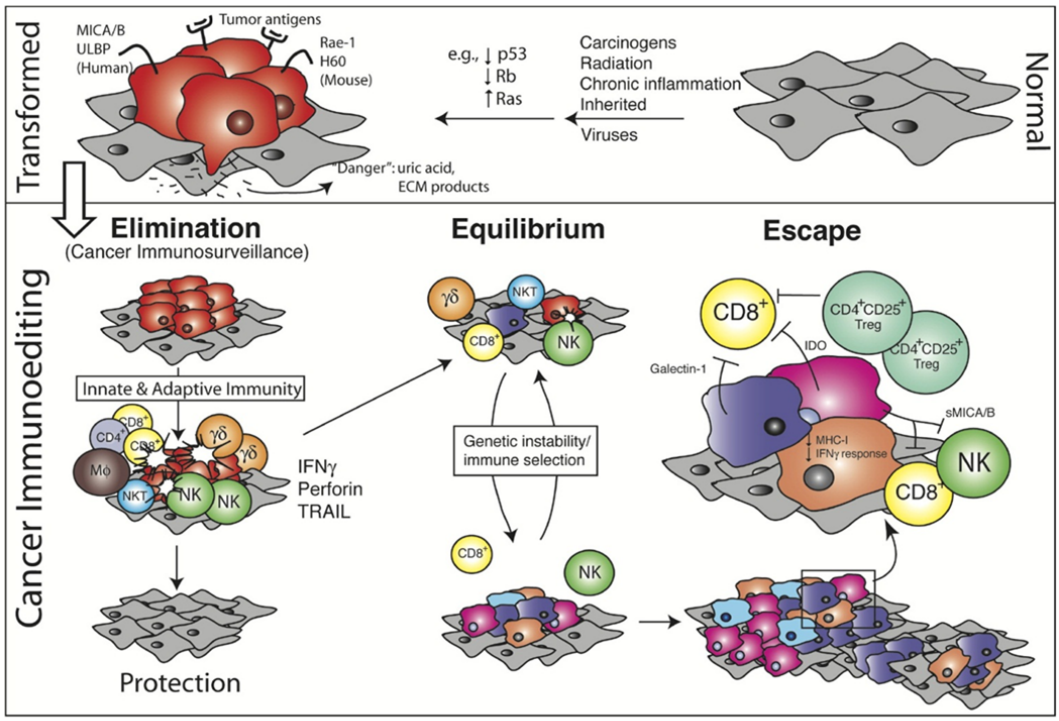

Figure 1. The Three Phases of the Cancer Immunoediting Process: Normal cells (gray) subject to common oncogenic stimuli ultimately undergo transformation and become tumor cells (red). Even at early stages of tumorigenesis, these cells may express distinct tumor-specific markers and generate proinflammatory "danger" signals that initiate the cancer immunoediting process (bottom). In the first phase of elimination, cells and molecules of innate and adaptive immunity, which comprise the cancer immunosurveillance network, may eradicate the developing tumor and protect the host from tumor formation. However, if this process is not successful, the tumor cells may enter the equilibrium phase where they may be either maintained chronically or immunologically sculpted by immune "editors" to produce new populations of tumor variants. These variants may eventually evade the immune system by a variety of mechanisms and become clinically detectable in the escape phase. Adapted from Dunn P et al.; Immunity, 2004.

The elimination phase can be complete, when all tumor cells are cleared, or incomplete, when only a portion of tumor cells are eliminated. In the case of partial tumor elimination, the equilibrium phase arises: immune cells and tumor cells enter a dynamic process where new tumor variants with increased resistance to the immune attack may survive. This process results in the selection of tumor cell variants that are able to resist, avoid, or suppress the antitumor immune response, leading to the escape phase. During the escape phase, the immune system is no longer able to contain tumor growth, and a progressively growing tumor results. One of the most efficient mechanisms of evasion is the ability of

tumor cells to create a suitable framework, called tumor microenvironment (TME), through the continuous released of tumor-derived secreted factors (TDSFs) including cytokines, chemokines and metabolites. All these factors, by acting on stromal cells, can favor tumor cells spreading inducing extracellular matrix (ECM) remodeling, formation of new blood vessels and the suppression of host's anti-tumoral immune response. In fact, TDSFs alter the normal hematopoiesis inducing an abnormal expansion and accumulation of immature myeloid cells with immunosuppressive features like myeloid-derived suppressor cells (MDSC) and the alteration of the natural commitment of other immune cells such as macrophages, neutrophils and dendritic cells (DC). The establishment of a tolerant microenvironment is recognized as a very critical point in cancer progression, because it is responsible of the impairment in host's antitumor response; therefore, it is not surprising that cancer-related inflammation has become one of the hallmarks of cancer (3).

1.2 Metastatic process

The last phase of cancer-immune evasion is characterized by the development of a metastatic disease in which new tumors appears at secondary sites. Although metastasis represent the major cause of cancer-related death (4), the metastatic process is considered largely inefficient due to the many obstacles tumor cells must overcome to produce a secondary tumor; in fact, less than 0.1% of disseminated cancer cells successfully develop distal metastasis (5). In solid malignancy, metastasis formation represents a multi-step process, generally defined as "metastatic cascade" that includes the local tumor cell invasion, the tumor cell infiltration into the vasculature followed by the tumor cell colonization at the distal sites (6). This process has been described as a natural selection in which tumor cells acquire new proprieties, mediated by genetic and epigenetic alterations, such as the ability to invade surrounding tissues reducing cell-to-cell adhesion and degrading the ECM. However, is still not clear why some types of cancer cells are prone to metastasize to specific organs. In 1989 Stephen Paget

and colleagues formulated a first hypothesis about an organ-specific pattern of metastasis (7) named "seed and soil" theory, in which the authors proposed that secondary growth of cancer cells (the "seed") is dependent on the competence of the distal organ (the "soil"). This theory has been supported by recent publications that underline the ability of selective cancer cells to colonize at defined tissues (8). For example, the major sites of metastasis of breast carcinoma are the bone, the lung and the brain (9). Colorectal and pancreatic carcinoma show preferential sites of distal colonization in the liver and lung. Moreover, even though are highly invasive, some cancer cells rarely seed other organs (8). All these findings highlight the essential role plays by the generation of a supportive and receptive tissue microenvironment at the metastatic-designed site, defined as "pre-metastatic niche", ready before the arrival of tumor cells, which enhance metastatic efficiency and determine metastatic organotropism.

1.3 The pre-metastatic niche promotes metastasis in a spatiotemporal manner

The pre-metastatic niche is extensively involved in the metastatic process in sequential and distinct phases (Figure 2).

Priming: In the priming phase, primary tumors undergo uncontrolled proliferation and become hypoxic and inflammatory. These processes result in the production of various TDSFs, extracellular vesicles (EVs), and other molecular components, which might induce the mobilization of bone marrow derived cells (BMDCs) and regulatory/suppressive immune cells to the future metastatic organ.

Licensing: In this phase, BMDCs and regulatory/suppressive immune cells are continuously mobilized and recruited into the secondary sites. The strength and duration of the interplay between TDSFs and recruited cells induce significant modifications at the metastatic site such as ECM remodeling, integrin activation, cytokines and chemokines release, which create a tumor-promoting and fertile milieu for the incoming circulating tumor cells (CTCs).

Initiation: Mature pre-metastatic niche educated by primary tumor, favor CTCs extravasation from vasculature and their seeding, colonization and survival at the metastatic organ, resulting in the initiation of micrometastasis formation.

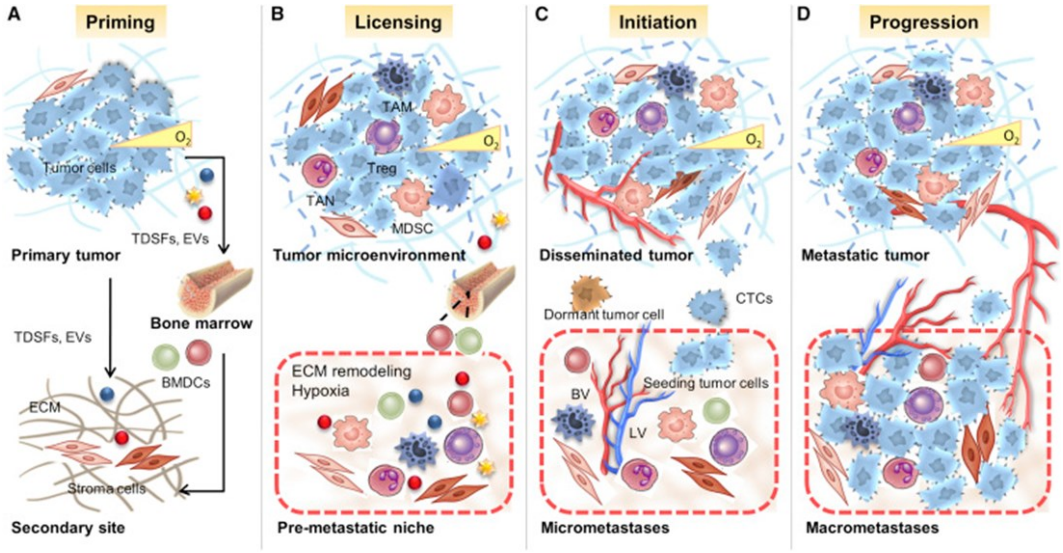

Figure 2. Role of the Pre-metastatic Niche in the Promotion of Tumor Metastasis. Adapted from Liu Y. et al.; Cancer Cell, 2016.

Progression: In the last phase an increasing numbers of tumor cells continue to leave the primary site and colonize the mature pre-metastatic niche at secondary site. Components of the pre-metastatic niche directly promote tumor cell growth and expand tumor mass in the metastatic organ, leading to the pathological progression from micrometastases to significant macrometastases.

1.4 Mechanisms of invasion

Cellular invasiveness is required at different steps of the metastatic cascade, and it is also needed for regional invasion. Several cellular mechanisms are associated with cancer cell invasion, including epithelial-to-mesenchymal transition (EMT) and the formation of new blood vessels.

Epithelial-mesenchymal transition: The induction of the morphogenetic process of EMT is a pivotal mechanism in metastasis promotion. During EMT, tumor cells lose epithelial characteristics and acquire mesenchymal properties

characterized by deep changes in cellular morphology, altered cell–cell and cell–matrix adhesion and the development of migratory behavior and invasiveness (10,11). Moreover, it has been already proposed that metastases are seeded by cancer stem cells (CSCs) that undergo EMT, allowing them to invade from the primary tumor site to distal tissues (12). In addition, the metastatic niche cellular organization release some compounds such as lysil oxidase (LOX), matrix metalloproteinases (MMPs) and tumor-growth factor beta (TGFβ) that sustain the EMT process (13,14). The EMT process is characterized by the initiation of a new transcriptional program in which the activation of the super-family of zinc-finger (Snail), Slug and SIP-1 (ZEB-2) transcriptional factors repress E-cadherin expression and instead promote the expression of mesenchymal markers like N-cadherin and vimentin (15). However, when CSCs reach the secondary site the reverse process named mesenchymal to epithelial transition (MET) occurs allowing tumor cells seeding. A recent study has shown that the metastatic niche ECM component versican can stimulate MET in tumor cells by reducing phospho-Smad2 levels and resulting in enhanced proliferation and faster metastasis formation (16). Thus, it is conceivable that the metastatic niche can both stimulate and reverse EMT, possibly depending on its state of maturation (Figure 3).

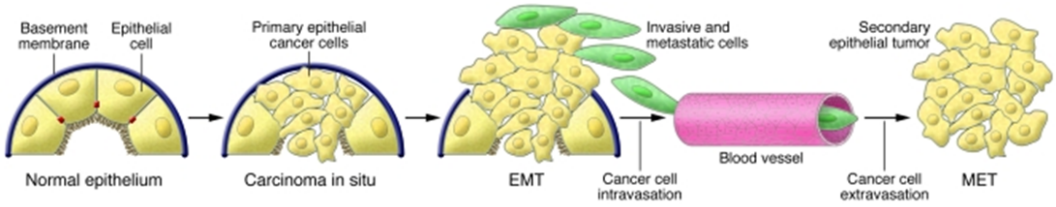

Figure 3. EMT and MET transitions during tumor progression. Progression from normal epithelium to invasive carcinoma goes through several stages. The invasive carcinoma stage involves epithelial cells losing their polarity and detaching from the basement membrane. The composition of the basement membrane also changes, altering cell-ECM interactions and signaling networks. The next step involves EMT and an angiogenic switch, facilitating the malignant phase of tumor growth. Progression from this stage to metastatic cancer also involves EMTs, enabling cancer cells to enter the circulation and exit the blood stream at a remote site, where they may form micro- and macro-metastases, which may involve METs and thus a reversion to an epithelial phenotype. Adapted from Kalluri R. et al.; J Clin Invest., 2009.

Tumor angiogenesis: Angiogenesis, the physiological process through which new blood vessels assemble from pre-existing vessels, is a fundamental prerequisite for the solid tumor growth and metastasis. Tumors with a diameter less than 2 mm are quiescent and they can obtain nutrients by diffusion. When the tumor size

reaches several millimeters in diameter, the tumor will be deprived of oxygen and nutrition since molecules cannot reach to the center of tumor; at this stage, the tumor cells need to generate a new vascular network. Moreover, tumor-promoted vessels present specific properties compared to normal vessels that facilitate both cancer progression and tumor cell intravasation, as well as the development of resistance to anti-angiogenic treatment (17,18).

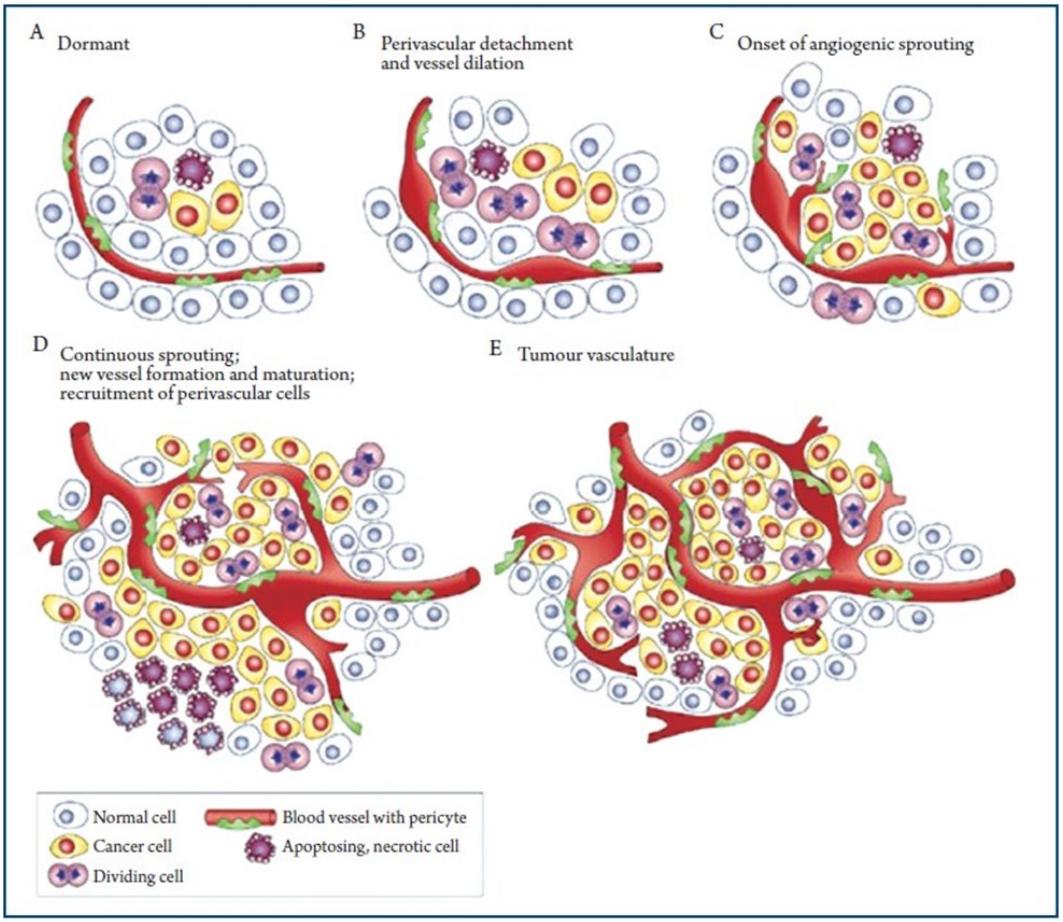

Figure 4. Tumor-promoted angiogenesis. **The angiogenic switch during tumor development occurs at different stages in the tumor-progression pathway, depending on the nature of the tumor and its microenvironment. Most tumors start growing as dormant nodules (A) until they reach a continual level of proliferating and dying cells. The initiation of angiogenesis, or the 'angiogenic switch', must occur to enable exponential tumor growth. The switch begins with perivascular detachment and vessel dilation (B), followed by angiogenic sprouting (C), new vessel formation and maturation, and the recruitment of perivascular cells (D). Blood-vessel formation will continue as long as the tumor grows, and the blood vessels specifically feed hypoxic and necrotic areas of the tumor to provide it with essential nutrients and oxygen (E). Adapted from Bohannan C. et al.; Transl Gastrointest Cancer, 2012.**

Spontaneous tumor-developing mouse models clearly demonstrate that tumor cells present an intrinsic tumorigenic potential that is not sufficient for their

unlimited and restless growth. In fact tumors without a functional vascular network are severely restricted in their growth potential. Therefore, it is mandatory for tumors that progressively develop in size to activate an "angiogenic switch" by perturbing the local balance of proangiogenic and antiangiogenic factors. Among the many pro-angiogenic factors, vascular endothelial growth factor (VEGF) family members play a central roles in vasculogenesis, under physiological condition as well as in tumor angiogenesis. VEGF affects tumor angiogenesis through a multiplicity of actions: inducing endothelial cell proliferation and survival, favoring vessel permeability, impairing vascular function, inhibiting DC differentiation, maintaining cancer cell proliferation and supporting the recruitment of different BM-derived populations (19). Indeed, VEGF is up-regulated in a vast group of human cancers, as unveiled by both experimental and clinical studies (20).

1.5 The role of the immune system on metastasis promotion: the generation of the pre-metastatic niche

In the recent past, cancer research preferentially focused on the primary tumor, exploring adhesion, migration and metastasis as intrinsic properties of the tumor cells (21); today, the metastatic process is under new intense investigation. It is now clear that pre-metastatic niche formation is the result of a systemic process involving a complex network in which tumor cells, BMDCs and stroma components are tightly connected and interplay each other. In fact, primary tumor cells, through the secretion of TDSFs, orchestrate pre-metastatic niche formation by mobilizing and recruiting BMDCs directly from the BM to the metastatic site (22). These cells within the pre-metastatic niche remodel the local microenvironment by secreting inflammatory cytokines, growth factors, and proangiogenic molecules, thus supporting tumor cell colonization, proliferation, and promoting tumor metastasis (23). Among these immune cells, $CD11b^{+}$ myeloid cells are certainly the most interesting and expansion of TAMs, tumor-associated neutrophils (TANs) and MDSCs in response to different TDSFs has been extensively investigated. All these myeloid cells at primary site induce the

establishment of a immune suppressive milieu blocking natural killer (NK) cells and cytotoxic $CD8^+$ T lymphocytes (CTLs), thus editing tumor cells to select escape variants and, at the same time, increasing the density of blood vessels that promote tumor cell dissemination. Moreover, TAMs can induce regulatory T cells (Treg) accumulation trough the secretion of CC-chemokine ligand 22 (CCL22) (24) whereas MDSCs, releasing interleukin-6 (IL-6), IL-23 and transforming growth factor β (TGF-β), promote the recruitment of T helper 17 (T_H17) which support MDSC immune regulatory functions (25). Kaplan and colleagues demonstrated, for the first time, the existence of a pre-metastatic niche composed by BMDCs in the lungs of mice engrafted with the subcutaneous injection of Lewis lung carcinoma (LLC) and melanoma (B16) tumor cells before metastatic tumor cells are detected. These BMDCs, including $CD11b^+$ myeloid cells, express very late antigen 4 (VLA4; also known as integrin $\alpha_4\beta_1$) and are recruited by fibronectin (the selective ligand of VLA4), which is deposited at pre-metastatic sites in response to tumor-derived VEGF-A and placental growth factor (PIGF) (26). Furthermore, TDSFs such as tumor necrosis factor alpha (TNFα) and TGF-β, along with VEGF-A, favor the release of specific chemoattractants such as S100A8 and S100A9 that, via serum amyloid A3 (SAA3) secretion, recruit TLR4-expressing $CD11b^+$ myeloid cells to the metastatic site. The accumulation of myeloid cells at pre-metastatic niche promotes local inflammation that support the migration of primary tumor cells to the secondary distal sites (27). Pre-metastatic niche formation has been reported also in immunodeficient mice engrafted with human breast cancer cell line (MDA-MB-231): in this tumor xenograft model, an intense accumulation of mouse myeloid $CD11b^+$ cells was detected in the pre-metastatic lungs through the secretion of LOX, which increases the adherence of $CD11b^+$ cells by crosslinking collagen IV (28).

1.5.1 MDSCs

Myeloid cells act as a first line against infections but are also crucial in initiating, sustaining or inhibiting T cell immunity. Nevertheless, the expansion of myeloid cells with immunoregulatory features has been reported in different solid and

hematologic malignancy in both mouse model (29) and human patients (30). Moreover, the levels of circulating myeloid cells correlate with the clinical outcome in different human cancers (31,32). In 2007 these cells were tentatively named myeloid-derived suppressor cells (MDSCs), which highlights their myeloid origin and their immune-suppressive function on T lymphocytes in a tumor-driven context (33). MDSCs in mouse model can be defined on the basis of the co-expression of the surface markers $CD11b^+$ and $Gr\text{-}1^+$; however they cannot be consider as an homogeneous cell population. Recent studies in mice led to separate at least two main subsets with different phenotypic and biological properties: monocytic M-MDSCs ($Gr\text{-}1^{lo/int}CD11b^+Ly6C^{hi}Ly6G^-$) with high immunosuppressive activity exerted in an antigen-nonspecific manner, and polymorphomuclear/granulocytic PMN-MDSCs ($Gr\text{-}1^{hi}CD11b^+Ly6C^{lo}Ly6G^+$), which are the most represented population but result less immunosuppressive on a cell per cell basis (34,35). Besides the phenotypical characterization, the main MDSC-related property is their immune regulatory function: in fact, mouse M-MDSCs and PMN-MDSCs express surface markers typical of inflammatory monocytes and neutrophils, respectively, but normal counterparts lack the immunosuppressive functions. MDSCs inhibit T cell recruitment, activation and proliferation with both direct and indirect mechanisms (36). One of the well-described MDSC function is the deprivation of essential amino acids in tumor microenvironment, like L-arginine, L-cysteine and L-tryptophan, which are necessary for T cell metabolism. Through the activity of enzymes such as arginase (ARG1), inducible nitric oxide synthase (iNOS, NOS2) and indoleamine 2,3-dioxygenase (IDO), MDSCs can promote T cell proliferation arrest and functional inhibition, in part by downregulating the $CD3\zeta$ chain in T cell receptor (TCR) complex (37). Moreover, MDSCs can inhibit T lymphocyte functions by producing reactive oxygen species (ROS) or reactive nitrogen species (RNS) (38), which are able to bind proteins, DNA and lipids altering their activity. In addition, MDSCs can promote the induction of specific subpopulations of regulatory cells like $CD4^+$ Treg and M2-polarized macrophages by TGF-β and IL-10 release, respectively (39,40). Recent studies demonstrated that MDSCs accumulated in the pre-metastatic niche also inhibit anti-tumor T cells through ARG1 activity and

ROS production (41). The immunosuppressive and pro-metastatic functions of MDSCs may be further enforced by cancer-induced regulatory B cells (Breg) via TgfbR1/TgfbR2 signaling. In the presence of Breg cells, MDSCs produce more ROS and nitric oxide (NO) and become more suppressive towards $CD8^+$ T cells (42). Moreover, PMN-MDSCs can be recruited to the lung pre-metastatic niche via monocyte chemotactic protein 1 (MCP-1/CCL2), suppressing the IFN-γ production and cytotoxicity of NK cells located within the niche (43). All together these data suggest that regulatory immune cells inhibit local tumor immunity and contribute to the formation of immunosuppressive pre-metastatic niche. In addition, MDSCs can also exert non immunosuppressive actions promoting tumor progression, invasion and metastatization (44). Indeed, MDSCs control cancer stemness through tumor senescence inhibition. They accomplish this activity either with soluble mediators such as IL-1RA (45) or with direct effect on cancer stem cell expansion (46), as well as via miRNA-mediated gene regulation (47). MDSCs play also a crucial role in promoting the angiogenic switch in response to the hypoxic microenvironment. In fact, it was demonstrated that tumor hypoxia trigger local immune suppression inducing the up-regulation of both ARG1 and iNOS in MDSCs (48). The released NO induce the activation of hypoxia inducible factor (HIF)-1α promoting the generation of new vessels via VEGF synthesis. MDSCs directly reinforce this loop by secreting matrix metalloprotease 9 (MMP9), which induces VEGF release from the matrix (49). Furthermore, the recruitment of MDSC mediates resistance to anti-VEGF antibody therapy (50) as well as to the tyrosine kinase inhibitor such as Sunitinib, an antiangiogenic agent, in both preclinical tumor models and patients with renal cell carcinoma (51). Hypoxia can also affect MDSC-dependent immune dysfunctions within the pre-metastatic niche. The injection of breast cancer-preconditioned hypoxic media promotes CCL2-mediated homing of PMN-MDSCs and NK cells to the lungs and, after tumor injection, increases lung colonization (43). Indeed, after being recruited to the pre-metastatic niche, MDSCs contribute to generate an immune tolerant environment by releasing IL-6 (52) or triggering tumor cell migration (i.e. through TNFα release) (53). Another important proangiogenic factor secreted by MDSC at the tumor site is bombina variegata peptide 8 (Bv8), which is

upregulated via the activation of the signal transducer and activator of transcription 3 (STAT3) (54). Bv8 production by PMN-MDSC has been shown to induce lung pre-metastatic niche formation and promote lung metastasis (55). In fact, the blockade of Bv8 in combination with anti-VEGF antibody showed an additive effect in halting angiogenesis and tumor growth (56). MDSCs can assist metastatic process also by inducing EMT of tumor cells, a condition in which cells acquire improved spreading skills. This ability is accomplished by MDSC-driven hepatocyte growth factor (HGF) and TGF-β release in melanoma cancer cells (57) and by high mobility group binding (HMGB)1 secretion in colorectal carcinoma (58). Interestingly, MDSCs placed in pre-metastatic niche may induce also the opposite differentiation process, MET through the release of the proteoglycan versican, favoring tumor cells ability to seed and colonize the organ (16).

Human MDSCs do not express restricted markers, such as Gr-1, therefore in the last 20 years multiple human MDSC subsets with different phenotypes have been described in several types of tumors. Similarly to the mouse system human MDSC can be classified in two main subsets M-MDSC ($CD11b^{+}CD14^{+}IL4R\alpha^{+}HLA\text{-}DR^{-/lo}CD15^{-}$) and PMN-MDSC ($CD11b^{+}CD14^{-}CD15^{+}$) with the addition of a third group comprising cells with more immature features, called "early stage MDSC" or eMDSC ($Lin^{-}HLA\text{-}DR^{-}CD33^{+}$). Each class contains more than one MDSC subset (59). Several studies in human patients unveiled that MDSC accumulation is strictly associated with the clinical stage in many solid tumors, such as pancreatic adenocarcinoma (60), hepatocellular carcinoma (61), non-small cell lung cancer (62) as well as in hematological malignancies like non-Hodgkin lymphoma (63). Moreover, the frequency of MDSC in blood is associated with metastatic disease. In NSCLC patients, circulating M-MDSCs correlated with extrathoracic metastases (62). Furthermore, in patients with melanoma, the development of metastases and poor survival were associated with increases in both circulating PMN-MDSCs (64) and M-MDSCs (31).

1.5.2 TAMs

Macrophages can originate from circulating monocytes and can be recruited to the tumor site through tumor or stroma-derived chemotactic factors such as CCL2, or can be tissue-resident macrophages that are completely maintained by self-renewing during the adult life (65,66). Circulating monocytes poorly contribute to the maintenance of tissue-resident macrophages under steady state conditions, while they dramatically expand during inflammation and tumor progression. Macrophages are phenotypically identified as $CD11b^{+}F4/80^{+}CD115^{+}$ cells in mice and as $CD68^{+}$ expressing cells in humans (67). However, different subsets of macrophages exist, whit various surface markers distribution and functions (68). One of the typical characteristic of macrophages is their plasticity. In fact, these cells present a high ability to integrate distinct signals from the microenvironment and to acquire distinct phenotypes (69). In a simplistic point of view, macrophages may result in two opposite polarization states. Typical Th1 cytokines (e.g. IFN-γ) alone or combined with microbial components (e.g. LPS) give rise to "classical-activated" M1 macrophages, which promote Th1 response and mediate anti-microbial and anti-tumor actions through the production of reactive oxygen and nitrogen intermediates and inflammatory cytokines (e.g. IL-1β, TNF, IL-6) (69). On the other hand, IL-4 and IL-13 inhibit this classical activation and induce the alternative M2 form of macrophage polarization (70). M2 macrophages are characterized by a high production of IL-10, low expression of IL-12 and a poor antigen presenting capacity (71). M2 macrophages produce chemokines involved in Treg cell, Th2 cell and basophil recruitment (72,73) and suppress Th1 adaptive immunity. This "alternatively-activated" macrophages participate in the resolution of inflammation, in the protection against parasites, promote wound healing, angiogenesis and tissue remodeling (74). Interestingly, M1 and M2 phenotypes should be viewed as the extremes of a continuum; there are various intermediate state that can be achieved through different signals (75). Macrophages infiltrating the tumor may display dual functions. TAMs can exert anti-tumor activities through a direct cytotoxic activity against tumor cells, as well as through the release of a wide range of soluble mediators able to recruit and activate both the innate and adaptive immune system (76). On the other hand,

several evidence indicates that TAMs displaying a M2-like phenotype can induce extracellular matrix remodeling, promotion of tumor cell invasion and metastasis, angiogenesis, lymphangiogenesis and immune suppression (68) exerting a pro-tumoral function. TAMs can support tumor dissemination through neoangiogenesis promotion. Indeed, TAMs express mediators such as TGF-β, VEGF-A, VEGF-C, platelet-derived growth factor (PDGF) and MMP-9, which are directly or indirectly involved in new vessel formation and sprouting (77,78,79,80,81). For instance, TAM-derived MMP-9 induces the release of heparin-bound growth factors, particularly VEGF-A, crucial for the angiogenic switch (82). The angiogenic potential of TAM has been further proven by depletion studies demonstrating a reduced blood vessel density in tumor environment (83). TAMs also directly help invasion of the surrounding tissue and intravasation of tumor cells. Intra-vital imaging captured tumor cells in MMTV-PyMT cancer model (in which females develop mammary tumors that metastasize to the lung), invading surrounding tissues together with TAMs (84); in particular, tumor cell intravasation occurs in association with perivascular TAMs (85). In these processes, cancer cells secrete colony-stimulating factor 1 (CSF1) to promote macrophage mobility and their secretion of epidermal growth factor (EGF). TAM-derived EGF, in turn, activates the EGF receptor in cancer cells, which enhances their invasion capability and motility by increasing invadopodium formation and matrix degradation, thereby accelerating the invasion and the intravasation of cancer cells (86). TAMs also promote tumor egress and metastasis through the secretion of CCL18 and osteonectin (also known as SPARC), which modulates the extracellular matrix adhesive properties of cancer cells (87,88). In a pancreatic tumor model, TAMs were shown to produce cathepsin B and cathepsin S, which promote cancer cell egress and angiogenesis (89). Thus, TAMs help tumor cells to enter the blood vessels by cytokine secretion and extracellular matrix remodeling. TAMs exert also an immunosuppressive activity, through the expression of a wide range of molecules, such as TGF-β, iNOS, arginase-1, IDO and IL-10 (90,91,92). Furthermore, recent studies have reported that TAMs in primary tumors can suppress $CD8^+$ T cell activities through their expression of inhibitor ligands such a programmed cell

death ligand 1 (PDL1) (93) and B7-H4 (94) or indirectly via CCL22-mediated recruitment of Treg cells (24). Accumulating evidence indicated that TAMs, as well as MDSCs, contribute to these early steps of metastasis. The pre-metastatic niche might host a peculiar population of pro-metastatic macrophages named metastasis-associated macrophages (MAMs). These cells are characterized by the expression of CD11b, VEGF receptor 1 (VEGFR1), CXC-chemokine receptor 3 (CXCR3) and CC-chemokine receptor 2 (CCR2) and by the absence of CD11c, Gr-1 and angiopoietin receptor (TIE2) markers. MAMs can be considered as a distinct subpopulation of macrophages that differentiate in the metastatic environment from inflammatory monocytes, recruited through the CCL2-CCR2 axis, and promote extravasation and survival of tumor cells. *Ex vivo* imaging of the metastatic lung has revealed that macrophages directly contact the extravasating cancer cells, and loss of these macrophages dramatically reduces the number of cancer cells that migrate from the blood vessels (95). Moreover, MAMs increase the survival of disseminated human breast cancer cells through engagement of VCAM1 on tumor cells, which results in signaling for cell survival through the activation of protein kinase B (96).

1.5.3 TANs

Neutrophils are the predominant leukocyte subset in human peripheral blood, with a well-established role in the first line of defense against microbial pathogens. Because of their short life span and fully differentiated phenotype, their role in cancer-related inflammation has long been considered negligible. In analogy with macrophages, tumor-associated neutrophils (TAN) can exert pro-tumor as well as anti-tumor functions and evidence from animal models suggests that neutrophils can be polarized toward distinct phenotypes in response to distinct tumor-derived signals (97,98,99). Neutrophils are involved in the process of carcinogenesis through the release of nitric oxide derivatives and ROS (100,3,101). Observations in cancer patients have linked elevated neutrophil counts in blood with increased risk for metastasis (102). In a transgenic mouse models that mimic human breast cancer, neutrophils localized to the lung, where they produced leukotrienes that

facilitated colonization by selectively propagating cancer cells with higher tumorigenic potential (103). Moreover, in another breast cancer mouse model, neutrophils promoted lung metastasis by dampening antitumor T cell immunity (104). These data support the idea that neutrophils are one of the major cellular components and drivers of lung pre-metastatic niche formation, since they support more efficient tumor cell extravasation and proliferation in this organ, leading to more lung metastasis (105).

1.5.4 Dendritic cells

DCs are the most potent antigen-presenting cells (APCs) with the unique ability to activate naïve T lymphocytes (106). DCs also have a tolerogenic function, participating in the enforcement of central and peripheral tolerance and the resolution of ongoing immune responses. Most DCs are of myeloid origin and derive from myeloid-committed bone marrow precursors or from circulating monocytes, especially during inflammation (107,108). DCs play a pivotal role in the tumor microenvironment. Tumor-infiltration by mature activated DCs confers an increase immune activation and recruitment of cancer-fighting immune effector cells. However, as with TAMs and MDSCs, tumor cells can inhibit DC differentiation and favor the recruitment of tolerogenic DCs (DCtol). It was previously demonstrated that TDSFs including VEGF, GM-CSF, M-CSF, IL-6, IL-10 as well as tumor microenvironmental factors like hypoxia can impair terminal DC differentiation, inducing the expansion of immature DCs characterized by low costimulatory molecule expression and poor activating abilities (109). T cells that encounter their antigen on a DC in the absence of appropriate co-stimulatory signals become tolerized and do not respond on subsequent encounters with the antigen. These immature, tumor-conditioned DCs may also exert an active suppression on T cells immunity through the expression of IDO (110,111). Another catabolizing enzyme, arginase, which depletes L-arginine, has been found to be expressed in DCs in a breast cancer model and mediate suppression of CTL function (112). In this regard, DCs with decreased

stimulatory abilities have been identified in patients suffering from melanoma, breast cancer (113) and prostate cancer (114).

1.5.6 Treg lymphocytes

Tumor microenvironment, as well as pre-metastatic niche, are characterized by the presence of regulatory cells of lymphoid origin. Treg lymphocytes are a particular population of T lymphocytes, expanded in peripheral sites, able to constrain the immune response. Treg lymphocytes constitute about 10% of $CD4^+$ T cells in normal humans and are required for maintenance of immune homeostasis, self tolerance and prevention of autoimmune disease. At least two subsets of Treg exist, natural Treg (nTreg), generated in the thymus during the normal development of the immune system, and iTreg that are $CD4^+$ T cells induced in the periphery by several stimuli, the most common being TGFβ. These cells are characterized by a high level expression of the IL-2 receptor α chain (CD25) and the transcription factor forkhead box protein 3 (FOXP3) that plays a critical role in Treg differentiation and maintenance (115). Interestingly, increased numbers of Treg have been observed in the blood and at tumor sites of patients with cancers of various origins (116) and they have been associated with the failure of naturally occurring antitumor immunity. In fact, Treg depletion by anti-CD25 antibodies was associated with reduced tumor growth and improved anti-tumor immune response in different mouse tumor models (116). Moreover, Treg might take part in metastasis promotion; for example, $CD4^+$ Tregs accumulate in lymph node metastases (117) and that they are also recruited to and required for lung metastases in experimental breast tumors (118).

1.6 Extracellular vesicles (EVs) as emerging coordinators of pre-metastatic niche

Interactions with and between cells in the pre-metastatic niche have generally been assumed to occur either through cell–cell contact or release of TDSFs. However, recent studies put in light an involvement of other mechanisms of cell-

to-cell communication such as tumor-derived EVs, which act as potential mediators for educating the pre-metastatic niche. EVs can be grouped into three major categories: exosomes (30–100 nm in diameter), microvesicles (100–1000 nm in diameter), and a newly identified cancer-derived EV population termed ‘‘large oncosomes’’ (1–10 μm in diameter) (119).

Exosomes. Exosomes are small membrane-bound vesicles, 30 to 100 nm in size, capable of mediating communication with surrounding cells or ECM components through cell surface receptor interactions or the horizontal transfer of their contents into recipient cells. Exosomes originate in the late endosomes compartment form multivesicular bodies (MVBs), which contain molecules from the Golgi (e.g., MHC class II molecules) or the cell surface (e.g., growth factor receptors). Moreover, exosomes contain cytosolic materials and are enriched in endosome-associated protein markers such as the Rab proteins, ALIX, TSG101, endosome-specific tetraspanins (CD9, CD63, CD81, CD82), or endocytic proteins, such as transferrin receptors and clathrins (120,121). Beyond their characteristic collection of membrane markers, the molecular content of exosomes can vary significantly based on the physiological conditions and the original cell type (122). Exosomes are vehicles to deliver mRNA, small RNAs, microRNAs, and proteins under normal and pathological conditions; they have been purified from *in vitro* cultures of multiple cell types including tumor cell lines (123). Tumors release exosomes into the surrounding microenvironment as well as into the bloodstream. Exosomes have thus been suspected as potential mediators of tumor invasion and pre-metastatic niche formation (22). Recent studies have demonstrated that tumor-derived exosomes can mediate ECM remodeling through the expression on their surface of fibronectin, promoting nascent adhesion assembly and increase cell motility (124). Moreover, exosome release from malignant cells can induce angiogenesis by stimulating cytokines and growth factors secretion by endothelial cells, (125) and participate in EMT-like process (126) thus promoting tumor cells spreading. Tumor exosomes exert complex effects on neighboring stromal cells such as endothelia cells, fibroblast and immune cells at secondary sites.

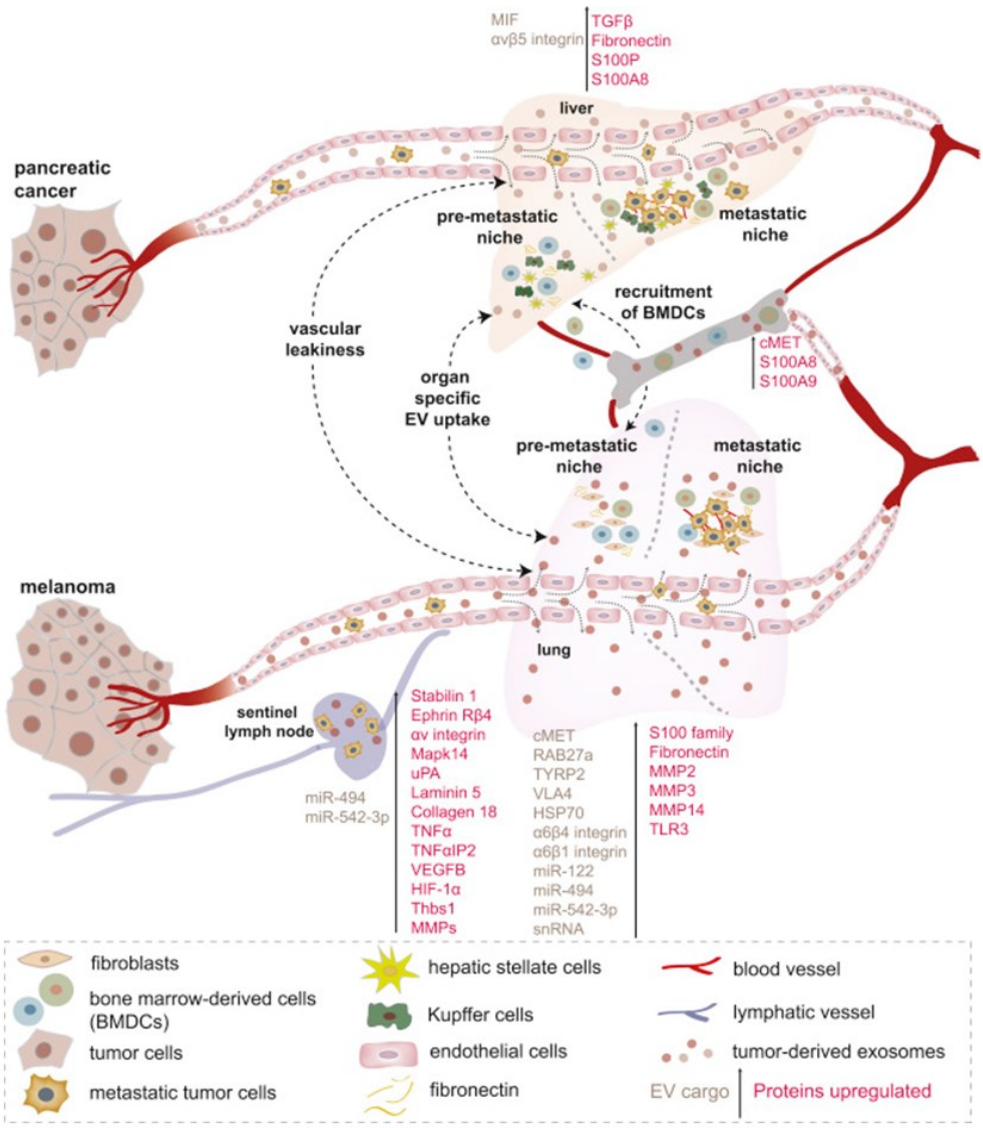

Figure 5. Tumor-Derived EVs Promote Pre-metastatic Niche Formation and Metastasis. EVs play a distinct role at multiple steps in pre-metastatic niche formation at distant sites of future metastasis. Through an unknown mechanism, tumor EVs can induce vascular leakiness and interact with the resident cells of distant organs. Depending on their membrane composition, such as specific exosomal integrin combinations (exosomal $\alpha_6\beta_4$ and $\alpha_6\beta_1$ integrins associated with lung metastasis/exosomal $\alpha_v\beta_5$ integrin with liver metastasis), EVs are targeted to particular resident cell types within a particular organ. Upon their uptake by the recipient cells, EVs can induce the expression of several inflammatory mediators (e.g., S100 family proteins, TGF-β, IL-6, IL-8, and TNF-α), resulting in the activation and remodeling of stromal cells and the recruitment of BMDCs to the pre-metastatic niche , which are critical for tumor progression. Adapted from Becker A. et al.; Cancer Cell, 2016.

Exosomes from melanoma cells have recently been described to reprogram BM progenitors through the Met tyrosine kinase receptor, inducing a pro-vasculogenic cellular phenotype promoting vascular leakiness at pre-metastatic sites (127). Moreover, pancreatic cancer-derived exosomes that are enriched in macrophage migration inhibitory factor (MIF), recruit the resident macrophages to the liver (Kupffer cells) inducing pre-metastatic niche formation and a subsequent increase in liver metastatic burden (128). Furthermore, it was demonstrated that microRNAs (such as miR-100-5p, miR-21-5p, and miR-139-5p) contained in prostate cancer-derived exosomes cooperatively modify the pre-metastatic niche for tumor metastasis (129). Interestingly, a recent study showed that exosomes can direct the organ specific metastatization through a unique pattern of exosomal integrins. For instance, $\alpha_6\beta_4$ and $\alpha_6\beta_1$ exosomal integrins are associated with lung metastasis, $\alpha_v\beta_5$ exosomal integrin with liver metastasis, and $\alpha_v\beta_3$ exosomal integrin with brain metastasis models. After fusing with resident cells, these vesicles activate Src phosphorylation and pro-inflammatory S100 expression in the future metastatic organ (130). These new findings suggest that primary tumor-derived exosomes can educate the secondary site into a tumor-promoting microenvironment and also direct BMDCs to form the pre-metastatic niche to promote metastasis, using mechanisms that depend on tumor types and secondary sites.

Tumor-derived microvesicles. Microvesicles tend to be larger in size (100–1000 nm) relative to exosomes but the primary distinction between the two type of vesicles is the mechanism of biogenesis. In fact, microvesicles arise through outward budding and fission of plasma membrane and is the result of dynamic interplay between phospholipid redistribution and cytoskeletal protein contraction. Like exosomes, the content of microvesicles appears highly enriched for a subset of proteins. So it is not surprising that also these structures may be involved in pre-metastatic niche formation. Osteopontin in tumor-derived microvesicles has been found to contribute to BMDC mobilization and colonization of tumor cells (131). Furthermore, microvesicles from mesenchymal-like cells may promote endothelial cell activation through Akt phosphorylation, aiding the creation of a

pro-metastatic vascular niche to support tumor cell invasion and chemo-resistance during metastasis (132).

Tumor-derived oncosomes. Oncosomes are atypically large (1-10 μm diameter) cancer-derived extracellular vesicles (EVs), originating from the shedding of membrane blebs and associated with advanced disease. They harbor bioactive molecules such as metalloproteinases, RNA, caveolin-1, and the GTPase ARF6, involved in local invasion and their abundance correlate with tumor progression (133). In particular, caveolin-1-positive large oncosomes can mediate intercellular transfer of miR-1227 from tumorigenic prostate cells to cancer-associated fibroblasts (CAFs), enhancing the migration of CAFs to secondary sites for pre-metastatic niche formation (134).

All these findings suggest that circulating tumor-derived EVs could be promising as biomarkers to monitor cancer progression and metastatization. In fact, in plasma of melanoma patients exosomes are enriched in caveolin-1 or S100B compared with the healthy controls, rendering these positive exosomes as another potential melanoma biomarker (135,136). Furthermore, exosomal glypican-1 has also been proposed as a diagnostic and prognostic marker for pancreatic cancer disease (137).

Chapter 2: PANCREATIC DUCTAL ADENOCARCINOMA (PDAC)

2.1 Pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) accounts for more than 90% of all pancreatic cancers and originate in cells that make the digestive fluids. Statistics on the most recent available data indicate that pancreatic cancer is the only major neoplasm presenting unfavorable trends in both sexes over the last 15 years (138). In the US, pancreatic cancer ranks the fourth leading cause of cancer death (139). With a 5-year survival rate of only 3% and a median survival of less than 6 months, a diagnosis of pancreatic adenocarcinoma carries one of the worst prognoses of all medicine (140). For the 15–20% of patients who undergo potentially curative resection, the 5-year survival is only 20% (141). Some improvements in surgical outcome occur in patients who also receive chemotherapy and/or radiotherapy, although the impact on long-term survival has been minimal, owing to the intense resistance of pancreatic adenocarcinoma to all extant treatments. So, management of most patients focuses on palliation.

2.2 Epidemiology of pancreatic cancer

The etiology of pancreatic adenocarcinoma has been extensively studied. Pancreatic adenocarcinoma is a disease that is associated with advancing age, rare before the age of 40, it culminates in a 40-fold increased risk by the age of 80. Besides age, other risk factors include habits such as smoking, elevated alcohol consumption, obesity and diets high in meat and low in vegetables and folate; most of these risk factors are suspected etiological factors for cancer in general (142). Past medical history, such as chronic pancreatitis, diabetes mellitus (long-term diabetes is associated with at 50% increased risk) and possibly *Helicobacter pylori* infection, as well as male sex, non-O blood group (143) and occupational exposures (e.g., to chlorinated hydrocarbon solvents and nickel) (144) are also listed among pancreatic cancer risk factors. On the genetic level, numerous

studies have documented an increased risk in relatives of pancreatic adenocarcinoma patients (approximately threefold), and it is estimated that 10% of pancreatic cancers are due to an inherited predisposition (145).

2.3 Pathophysiology and genetics of pancreatic adenocarcinoma

Ductal adenocarcinomas are most likely to develop from ductal proliferative lesions arising in the pancreatic duct system. Three main pre-invasive lesions have been identified in the pancreas, namely pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystadenomas (MCNs) (146).

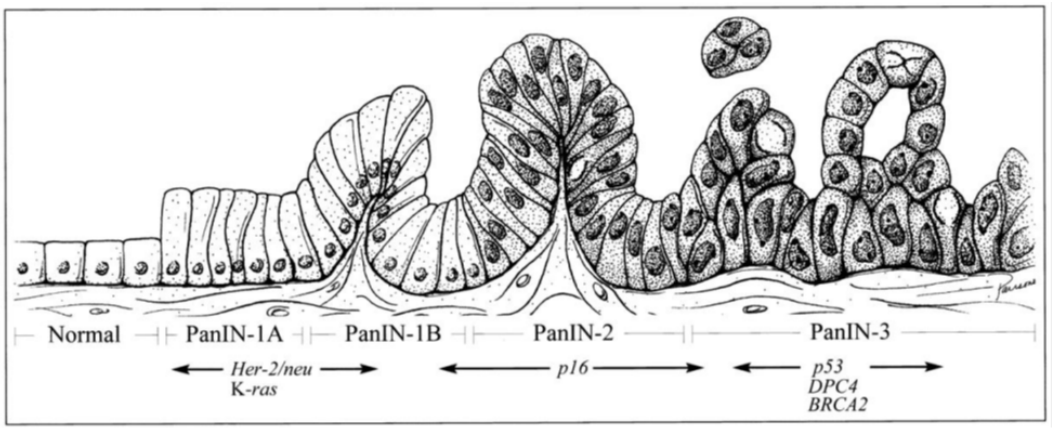

Figure 6. PanIN progression model, showing genetic alteration. It is hypothesized that a duct lesion can progress from a histologically normal duct to flat duct lesion (PanIN-1A) to papillary duct lesion (PanIN-1B) to atypical papillary duct lesion (PanIN-2) to severely atypical duct lesion/carcinoma in situ (PanIN-3). The approximate timing of alterations in the K-ras, HER-2/neu, p16, p53, BRCA2, and DPC4 genes is indicated on the model. PanIN=pancreatic intraepithelial neoplasia. Adapted from Wilentz RE et al.; Cancer Res, 2000.

However, the majority of PDACs arise from PanINs, whereas IPMN- or MCN-induced PDAC only occurs sporadically. PanIN has three different stages of progression before it gives rise to PDAC (147,148), (PanIN-1, PanIN-2 and PanIN-3. Figure 6), depending on the degree of cytological atypia of the duct lining cells. These lesions are microscopic (<5 mm diameter) and are not directly visible by pancreatic imaging. Pancreatic cancer, like many other malignant diseases, results from the accumulation of clonally selected genetic and epigenetic

alterations throughout the stages of PDAC development starting from PanIN lesions. The most frequent genetic abnormalities in invasive pancreatic adenocarcinomas are mutational activation of the KRAS oncogene (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, detectable in the 95% of patients with PDAC) (149), inactivation of tumor-suppressor genes including CDKN2A (cyclin-dependent kinase inhibitor 2A, detectable in the 90% of patients with PDAC) (150), TP53 (tumor protein p53, detectable in the 50%–75% of patients with PDAC) (151), SMAD4 (mothers against decapentaplegic homolog 4; its loss correlated with distant metastases, indicating that this marker could be potentially used to stratify patients for more aggressive local or systemic treatment) (152), BRCA2 (breast cancer 2 early onset gene, which occurs in 1–2% of patients with PDAC) (153), widespread chromosomal losses, gene amplifications (154), and telomere shortening (155). KRAS mutations (156) and telomere shortening are the earliest known genetic abnormalities recorded, even in low-grade pancreatic intraepithelial neoplasia (157), and telomere shortening is believed to contribute to chromosomal instability, whereas inactivation of TP53 (151), SMAD4, and BRCA2 happens in advanced pancreatic intraepithelial neoplasia and invasive carcinomas (148,158). Importantly, the disease phenotype can be closely recapitulated using genetically-engineered mouse models with conditional modification of the expression of genes involved in PDAC formation and progression (159). These data support the notion that PDAC has a genetic etiology.

2.4 Diagnosis, staging and current treatment options

Early-stage pancreatic cancer is usually clinically silent, and disease only becomes apparent after the tumor invades surrounding tissues or metastasizes to distant organs. Survival rates for patients with PDAC are extremely poor, primarily due to the majority of tumors being at an advanced stage at diagnosis (160). Indeed, only the 10% of cases are resectable at presentation and more than 90% of patients undergoing potentially curative resection still die of the disease due to local recurrence and/or distant metastases in the absence of adjuvant therapy.

Unlike many other malignant diseases, the metastatic spread of PDAC is thought to begin when the primary tumor is very small and is hard to detect through CT imaging scans or abdominal ultrasounds (161). For early stage PDAC, adjuvant therapy follows surgical resection in an effort to prolong survival. The early metastasizing nature of pancreatic cancer, along with the large proportion of patients presenting with locally advanced disease and the high frequency of microscopic incomplete resections, provides a strong rationale for neoadjuvant systemic therapy. Nevertheless, neoadjuvant approaches employing increasingly active chemotherapeutic regimens in metastatic PDAC (e.g. FOLFIRINOX [folinic acid, 5-FU, irinotecan and oxaliplatin], gemcitabine plus nab-paclitaxel and/or chemioradiotherapy) may allow down staging of borderline resectable disease and some locally advanced cases, improving R0 resection and survival. For those with unresectable locally advanced disease, treatment options include FOLFIRINOX, gemcitabine-based chemotherapy or chemo-radiotherapy. While chemioradiotherapy results in longer survival in patients with locally advanced PDAC compared with radiation alone or no treatment, it does not provide longer survival than systemic chemotherapy alone and increases toxicity (162).

Early diagnosis of PDAC seems the best option to improve patients survival. Unfortunately, early detection is still limited because specific biomarkers able to identify the early phase of tumor progression are not available. Currently, the best tumor marker for pancreatic cancer is carbohydrate antigen 19-9 (CA19-9), which provides suspicion of cancer or disease, but is not sensitive or specific enough for diagnosis (163). In fact, CA19-9 has been detected in chronic pancreatitis, and may be normally expressed in some patients with early stage PDAC, as well as other cancers (164,165). The lack of diagnostic tools makes this disease very deadly and the need for more research to improve detection will benefit patient outcomes. Interestingly, in the last few years tumor-released exosomes positive for Glypican1 were proposed as a potential PDAC biomarker able to detect also the early phases of pancreatic cancer (137). This point the attention on exosomes as an innovative and attractive new classes of circulating biomarkers.

Chapter 3: HUMANIZED MICE

3.1 Humanized immune-reconstituted (HIR) mice

Knowledge in biomedical research is largely gained by observation, by *in vitro* assay and animal experiments, and by safety-driven clinical trials. Despite the broad range of approaches, progress in medicine is mostly slow and many pathophysiological processes continue to be major challenges for humans. There is therefore a need to provide a preclinical platform where human-specific physiology and disease are recapitulated, which allows deeper understanding and preclinical testing of physiology, pathology, and therapy. Humanized immune-reconstituted (HIR) mouse models represent one promising approach in this direction and they are generated with human-to-mouse xenotransplantation of functional elements of the human hematolymphoid system. These models represent a bridge between experimentation on animal models and studies on human subjects, since they combine some peculiar human features with the experimental convenience to test them in small models.

3.2 Requirements for human hematolymphoid system development, maintenance, and function in mice

Different sources of human hematopoietic cells can be used for transplantation into mice. Engraftment of human hematopoietic stem cells (HSCs, positive for the CD34 marker) represents a better approach for the reconstitution and maintenance of a full hematopoietic system. $CD34^+$ cells can be isolated from human fetal liver, cord blood, adult BM, or peripheral blood after specific mobilization with GCSF. $CD34^+$ cells isolated from fetal or neonatal origin tissues display higher frequency and efficacy of engraftment, nevertheless adult-derived cells are also used successfully. However, the presence of cells of a different species in the mouse context generates an immediate rejection. In order to make the human

tissue accepted by the host immune system, several immune genetic deficiencies have been introduced in the recipient. Independent models have been generated and three main mouse strains are the most commonly used today: NSG mice (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}), NOG strain (NODShi.Cg-Prkdc^{scid}Il2rg^{tm1Sug}) and BRG strains based on C;129S4-Rag2^{tm1Flv}Il2rg^{tm1Flv} stocks of mice. These models contain genetic deficiencies that impair cell types responsible for xenograft rejection. Target mutations in key enzymes involved in the mechanism of V(D)J recombination prevent T and B lymphocytes maturation, thus the mediators of the adaptive response are both absent: *scid* mutation impairs a protein kinase involved in DNA repair (166), while genetic alteration in one of the *Rag* genes prevents DNA recombination (167,168). Moreover target mutation in IL-2Rγ induce NK clearance and the alteration of the *IL2rg* gene is responsible for the alteration in cytokines signaling including IL-15 that is critical for NK development and maintenance (169). Tolerance of human immune cells to the mouse is also required to maintain health and survival of the host. This problem is particularly evident when human differentiated cells, such as T cells, are transplanted, as they can be activated in the xenogeneic environment by mouse MHC, causing graft-versus-host disease (GVHD) (170). However, current transplantation of human hematopoietic stem cells (HSCs) allows human CD45$^+$ cells maintenance in the mouse for periods exceeding 6–9 months. An important requirement for the successful xenotransplantation of a human hematolymphoid system is the development, maintenance and functionality of human cells in the mouse host. For all these processes, cross-reactivity of cytokines, chemokines, and adhesion molecules is necessary for the optimal role of human cells. Multiple factors are involved in the tight regulation of hematopoiesis, from the maintenance of self-renewing HSCs in the BM niche to their differentiation into multiple cell lineages, their localization and maintenance in peripheral tissues, and their activation under conditions of infection or other challenges. Although some of these human soluble factors are secreted by hematopoietic cells, many of them are generated by the mouse host: murine chemokines/interleukins are not able to sustain and promote human cell differentiation. Early work by Shultz and colleagues with NSGs showed that the administration of IL-7 enhanced the production of mature T cells

in HIR mice (171). Moreover, it was demonstrated that supplemental administration of IL-15 and Flt-3/Flk-2 cytokines (encoded on plasmids expressed in hepatocytes) resulted in elevated levels of NK cells, while granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 were able to increase the DC population, and macrophage colony-stimulating factor (M-CSF) was able to increase the number of monocytes and macrophages detectable in the blood stream (172). Although their addition improves immune cell differentiation and expansion, non-physiologic concentration of these cytokines within the HIR can misdirect immune cell development and trafficking. Cytokines have been delivered exogenously into HIR mice by injection of recombinant cytokines, hydrodynamic injection of plasmid DNA, or lentiviral or adenovirus (173) delivery *in vivo*. Another effective approach employs genetic engeneering of human cytokines in the genome of recipient mice. This approach is based on either bacterial artificial chromosome (BAC) transgenesis or knock-in replacement of the mouse gene with the specific human counterpart promoting a more physiological expression of the encoded protein (174,175,176). Finally, transgenic expression of human MHC molecules in the mouse host is essential for the optimal function of T and NK cells and, consequently, for appropriate innate and adaptive immune responses.

3.3 HIR Mice for cancer research

Using currently available immunodeficient mice, it is now possible to grow almost all types of primary human tumors *in vivo*, including most solid tumors and hematological malignancies (177). The transplanted tumors are maintained at low passage so that fidelity of patient tumor heterogeneity and histology are maintained. Indeed, the genomic characterization of patient-derived tumors in immunodeficient mice has shown that the patient's primary tumor genomic profile is recapitulated in the tumor graft derived from the explants (178). Xenograft models can be selected for biomarker-driven translational medicine studies and to test therapeutic options in order to determine the best approach available for treating individual patients (179). Xenograft models have also proved valuable

role in recent studies of metastasis. Engraftment of primary human stage III cutaneous melanomas into NSG mice has shown that metastasis in the recipient mice correlates with patient-specific clinical outcomes. Human melanomas that metastasized efficiently in the mice eventually progressed to advanced stage-IV disease in the patients (180). These findings suggest that the use of immunodeficient mice for the study of patient tumors could indeed provide rationale for therapeutic decisions and cancer treatment in the clinic. Furthermore, the use of humanized mice engrafted with patient-derived tumor xenografts allow the evaluation of immunotherapeutic approaches *in vivo*. It must be pointed out that xenograft model present a relevant limit due to the absence of a complete human immune system. In fact, in this model is not possible study the complex interaction present in tumor microenvironment, which plays a pivotal role in tumor progression and metastatization. To overcome this limit, recipient mice must be engrafted with HSCs that can generate a functional human immune system while at the same time supporting the growth of a human tumor. Thus, HIR mouse models bear a great potentials for the future studies in cancer research for the identification of immune biomarkers and for the analysis of human pathologies in time window that cannot be investigated in humans due to the lack of symptoms and to difficult diagnosis. Novel mouse strains are under development to express human HLA class I and class II molecules and microenvironmental factors, such as species-specific growth factors required for certain human tumors graft (e.g., IL6-dependent myelomas), to lack mouse MHC class I and II molecules, and to have additional deficits in innate immunity. These models are required to assure future challenges and opportunities of humanized mice.

AIM OF THE STUDY

It is well established that the immune system plays a central role in controlling cancer progression by killing transforming cells. However, cancer cells may evade the immune system control by promoting the generation of suitable microenvironments both at primary site and in the future metastatic site. Interestingly, BM derived-cells play a dominant role in the pre-metastatic niche formation, which favors tumor cells seeding and proliferation at secondary sites. The study and the definition of the pre-metastatic niche patients are still limited since the time line of metastatic spread is indefinite. However, by the generation of a mouse preclinical model, which can be repopulated with human hematopoietic cells and injected with human cell lines of pancreatic cancer, the study the early phases of metastatic process is made possible. The first aim of our project was to identify human myeloid cells essential to form the metastatic niche using pancreatic tumor-bearing HIR mice. We demonstrated that lungs of tumor-bearing mice displayed a higher infiltration of human myeloid cells near the tumor cells, supporting the idea of a early-metastatic niche formation. Moreover, the presence of a human immune system in tumor-bearing HIR mice induces a significant increase in the number of lung metastases in comparison to unreconsituted, immune deficient mice bearing the same tumor. Given the important role of human myeloid cells in metastasis promotion we performed a microarray analysis to evaulate the gene profile of metastasis-infiltrating human myeloid cells. In addition to provide important informations about the biology, the differentially expressed genes, indentified in these studies, may be used as potential biomarkers for better clustering tumor-bearing hosts with a metastatic disease. In particular, our data showed an activation of a type 1 interferons induced signature (ISG), typical of innate immune response against pathogens, in human leukocytes of both BM and lung of tumor-bearing HIR mice. Therefore, the second aim of our work was to characterize the exosome role in activating the ISG transcription on myeloid cells. These data allowed us to define a critical network in which tumor-released exosomes can be taken by myeloid cells and trigger the gene signature identified on metastases-infiltrating myeloid cells.

Finally, we provided evidence that the *in vivo* administration of tumor-derived exosomes promoted an increased aggressiveness of a poorly metastatic cell line.

MATERIALS AND METHODS

1. Mice

NOD.Cg-Prkdc^{scid} Il2^{rgtm1Sug}/JicTac (NOG) mice and C57BL/6 mice were purchased from Taconic Biosciences and Charles rivers, respectively, and maintained under specific pathogen-free conditions in the animal facilities at Verona University. Animal experiments were performed according to national (protocol number 12722 approved by Ministerial Decree Number 14/2012-B of Jenuary 18, 2012) and European laws and regulations. All animal experiments were approved by Verona University Ethical Committee and conducted according to guidelines formulated by the council of Europe for experimental animal use. All animal experiments were in accordance with the Amsterdam Protocol on animal protection and welfare. Additionally, all animal experiments were conducted according to the guidelines of Federation of European Laboratory Animal Science Association (FELASA).

2. HIR mice generation

For HIR mice generation, NOG mice of 6 weeks of age received 1.2 Gy total body irradiation (TBI) using ^{137}Cs-source irradiator. Four hours after pre-conditioning, mice were intravenously injected with 1x10^5 human CD34$^+$ cells isolated from healthy donor apheresis after G-CSF mobilization.

3. Cell lines

The human PT-45 cell line, derived from a primary pancreatic adenocarcinoma (ATCC), the human MDA-MB 231 cell line (ATCC), a breast cancer adenocarcinoma and the murine breast cancer cell line E0771 (CH3 BioSystems) were used for orthotopic tumor mouse models generation in vivo. Cells were cultured at 37°C and 5% CO2 atmosphere, in RPMI supplemented with 10% FBS

(Biochrom), 2mM L-glutamine (Lonza) and 100U/ml penicillin/streptomycin (Lonza). The FC1199P3, a KPC-derived PDAC cell line (kindly provided by Prof. David Tuveson,; Cold spring Harbor laboratory, USA) and the MCA-derived spontaneously metastasizing sarcoma MN-MCA1 (a kind gift of Prof. Antonio Sica; Istituto Humanitas, Milan, Italy) were grown in DMEM (Lonza) supplemented with 2 mM L-glutamine (Lonza), 10 mM HEPES (Lonza), 150 U/ml streptomycin, 200 U/ml penicillin (Lonza) and 10% heat-inactivated FBS (Biochrom).

4. Orthotopic tumor mouse models.

For the pancreatic model, anesthetized HIR mice were orthotopically injected into the exposed pancreas with $1x10^6$ PT-45 tumor cells suspended in Matrigel (BD biosciences) using a 29 gauge needle, after a small 0.5 cm incision in the left abdomen was made. The peritoneum was then closed with dissolvable suture and the skin incision closed with wound clips. For the generation of the breast cancer model eleven-week-old female HIR mice were injected unilaterally with $1x10^6$ MDA-MB 231 tumor cells into the fourth abdominal fat pad by subcutaneous injection at the base of the nipple. Tumor growth was monitored externally using a calipers and once average tumor volumes reached of 600 mm^3 the primary mass were removed to favor distal dissemination under anesthesia.

5. Histology

For metastases detection and quantification lungs were harvested and fixed in 10% neutral buffered formalin (BioOptica). To optimize the detection of microscopic metastases and ensure systematic uniform and random sampling, lungs were cut transversally into 2 mm thick parallel slabs with a random position of the first cut in first 2 mm of the lung, resulting in 5-8 slabs for lungs. The slabs were then embedded cut surface down and sections were stained with Hematoxylin and Eosin (BioOptica) for detecting lung metastases. The number of

lung metastases was determined by two pathologists, independently and in a blind fashion, using a Leica DMRD optical microscope (Leica). The percentage of mice bearing metastases was also recorded. Some of the lung sections were also stained with anti-human CD33 (clone PWS44, Novocastra) antibodies to detect human leukocytes and in particular myeloid cells.

6. Phenotypic analyses of human cells in HIR mice by flow cytometry

Peripheral blood, spleen, lung and BM of HIR mice were processed to single cell suspension and stained with the following anti-human mAb: FITC conjugated anti-CD19 (clone HIB19, BD Biosciences), PE conjugated antiCD14 (clone M5E2, Biolegend), PERCP/Cy5.5 conjugated anti-CD45 (clone 2D1, eBioscience), PE-Cy7 conjugated anti-CD3 (clone UCHT1, eBioscience), APC-conjugated anti-human CD206 (clone 19.2, BD Biosciences), BV421 conjugated anti-CD33 (clone WM53, Biolegend) and APC-Cy7 conjugated anti-mouse CD45.1 (clone A20, eBioscience) for leukocytes detection. All samples were acquired with a FACSCanto II (BD Biosciences) and analyzed with FlowJo software (Treestar Inc.).

7. Isolation of human and murine leukocytes

Human and murine leukocytes were separated through cell sorting. Briefly, BM was flushed out from posterior limbs and red blood cells lysed with 5 minutes incubation in ACK at 4°C (Lonza); lung tissue were disaggregated to obtain single-cell suspensions with 45 minutes incubation in digestive cocktail (181) at 37°C with shaking. Single cell suspensions from BM and lung were then stained with PerPC-Cy5.5 conjugated anti-human CD45 (clone 2D1, eBioscience), APC-Cy7 conjugated anti-mouse CD45.1 (clone A20, eBioscience), and fixable violet dead cell stain (Life technology). Human and mouse leukocytes were sorted on a FACS Aria II (Becton Dickinson) on the basis of speciespecific CD45 expression. For all samples the positive fractions was obtained with a purity of ≥ 95%. Cells

were pelleted and frozen in Trizol (Life technology) at –80°C for subsequent gene expression microarray.

8. Gene expression

Total RNA was isolated using TRIzol reagent (Life technology) and RNA integrity assessed using Agilent-2100-Bioanalyzer (Agilent Technologies). cDNA from human leukocytes was synthesized and amplified from total purified RNA with Ovation Pico WTA System V2 (NuGEN). All the samples were hybridized to Affymetrix U133 PLUS 2.0 arrays and scanned with an Affymetrix GCS 3000 7G scanner. Sample and genes were clustered using Pearson correlation coefficient and average as distance metric and linkage, respectively.

9. Functional assay

Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of healthy donors by density gradient centrifugation on Ficoll-Paque PLUS (GE Healthcare-Amersham). PBMCs were stained with 0.5 μM CellTrace™ Violet Cell Proliferation Kit (Invitrogen, Molecular Probes), according to manufacturer's instructions. CellTrace-labelled PBMCs were activated with coated 0,6 μg/ml anti-CD3 (clone OKT-3) and 5 μg/mL soluble anti-CD28 (clone CD28.2, eBioscience) for four days and co-cultured in 384 flat bottom well plates (BD Biosciences) with "effectors" $CD11b^-$ BM-MDSC obtained with a 4 days culture of Lin^- cells, sorted from BM of HIR mice with G-CSF and GM-CSF. Cell cultures were incubated at 37°C and 8% CO_2 in arginine-free-RPMI (Biological Industries), supplemented with 150 μM arginine, 10% FBS, 10 U/ml penicillin and streptomycin, and HEPES. At the end of the culture, cells were stained with PE-Cy7 conjugated anti-CD3 (UCHT1, eBioscience), and CellTrace signal of gated lymphocytes was analyzed. The extent of cell proliferation was quantified by FlowJo, analyzing the number of proliferating cells.

10. Exosome isolation from culture conditioned media (CCM)

Exosomes from PT-45 and MDA-MB 231 cell lines were purified from CCM by a combination of ultracentrifugation as previously described (182). Briefly, PT-45 and MDA-MB 231 cell lines were cultured in RPMI supplemented with 10% of exosome-depleted FBS (EuroClone), 2mM L-glutamine (Lonza) and 100U/ml penicillin/streptomycin (Lonza). After 3 days, when cells are at 60% to 70% of confluence, conditioned medium was collected, centrifuge 10 min at 300 x g, 4°C, and filter with a 0.22 μm filter. The resulted cell-free medium was concentrated by ultracentrifugation at 100.000 x g, 6°C for 2 hours. The supernatant was carefully removed, and exosome-containing pellets were suspended in 1 mL of ice-cold PBS and washed with a second round of ultracentrifugation (100.000 x g, 6°C for 2 hours). Exosomes were suspended in ice-cold PBS, quantify as amount of total proteins using Bradford assay and then store at -20°C.

11. Western blot analysis

Isolated exosomes or whole cell lysate (WCL) were lysed in non-reducing sample buffer (Leammli 4X without 2-mercaptoethanol) and boiled for 5 minutes at 80°C. Proteins were resolved by SDS-PAGE (SDS-polyacrylamide gel electrophoresis), transferred to polyvinylidene fluoride membranes (PVDF), blocked in 5% non-fat powdered milk in PBS-T (0.5% Tween-20) and probed with antibodies anti-CD9 (Abcam AB92726) and anti-CD63 (Abcam AB193349). Protein bands were detected using ImageQuant LAS 4000 instrument (GE Healthcare) and enhanced chemiluminescence reagent (Thermo Fischer ECL).

12. Flow cytometric analyses of exosome-coated beads

15μg of isolated exosomes were incubated with 5 μl of 4-mm diameter aldehyde/sulfate latex beads (Life Technology Molecular Probes) for 15 minute at room temperature on a test tube rotator wheel. After the addition of filter PBS to a final volume of 1 ml, samples were incubated on a test tube rotator wheel for 30

minute. Samples were saturated with 2 M 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) or PE-conjugated anti-CD63 (clone HSC6, eBioscience) antibodies for 1 h at 20°C. After two washings, samples were analyzed by FACSCanto II (BD Biosciences). Each analysis included IgG-matched isotype controls. Events were gated according to light-scatter properties, selecting single-bead populations.

13. Analysis of EVs size

The 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.

14. Monocytes and macrophages treatments

Human monocytes were obtained from normal blood donor buffy coats by two-step gradient centrifugation, first by Ficoll-Hypaque (GE Healthcare) to isolate peripheral blood mononuclear cells (PBMCs) and then by Percoll (GE Healthcare) to enrich monocytic fraction. $CD14^+$ monocytes were then separated using magnetic beads (Miltenyi Biotec). For Macrophages differentiation $1.5x10^6$ $CD14^+$ cells were cultured in RPMI 5% FBS supplemented with 25ng/mL of human M-CSF for 7 days. Monocytes and macrophages were treated for 6 hours with 25µg of tumor-derived exosomes or 10 pg/mL of hIFNα as positive control.

15. Real Time PCR

Cells were pelleted and RNA was extracted using Trizol Reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. The amount and purity of isolated RNA was analyzed by the Nanodrop spectrophotometer. cDNA was prepared using the SuperScript® VILO cDNA Synthesis Kit according to the manufacturer's instruction. Real Time PCR were run using 2x SYBR Green master mix (ABI). All samples were normalized using actin endogenous control primers.

16. *Ex vivo* fluorescently labeled exosomes tracking

Exosomes isolated from the conditional media of a highly metastatic tumor cell line, MN-MCA1, were fluorescently labeled using PKH26 dye (Sigma-Aldrich). Briefly, exosomes were incubated for 5 minutes with PKH26 dye (4μM) in a rotator wheel. The reaction was blocked adding an equal volume of filter PBS-BSA 1% and incubating for 5 minutes in a rotator wheel. Exosomes were then concentrated using Vivaspin® 500 Centrifugal Concentrator (Cutoff: 300000 MW; Viva products) at 2,000 rpm, 4°C. Unconjugated dye was removed by several washing step with PBS for 2 times. PBS without exosomes was treat with the same procedure as a negative control. The efficiency of exosome labeling was analyzed using a spectrophotometer. PKH26-labeled exosomes were retro-orbitally injected into C57BL/6 (20μg of exosomes/mouse). At 12, 24 and 36 hours after injection, 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-labeled exosomes were assessed.

17. Exosome education

To analyze the role of exosome education in tumor metastases, 8-week-old C57Bl/6 female mice were pre-educated with the retro-orbital injection of 5μg of

exosomes purified from a highly metastatic tumor cell line (MN-MCA1), every 2 days for 3 weeks. Control mice received an equivalent volume of filter PBS. After exosome conditioning mice received a tumor challenge with a cell line with a low metastatic potential. Pre-conditioned mice were orthotopically injected in the exposed pancreas with $1x10^6$ FC1199P3 cells or in the mammary fat pad with $0.5x10^6$ E0771 cells. In the breast cancer model primary tumor were removed at a volume of 600 mm^3 to favor distal dissemination.

18. Statistical analyses

All data are presented as mean ± standard deviation (SD) of the mean. Statistical analyses were carried out using SigmaPlot (Systat Software). 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.

RESULTS

1. Humanized immune-reconstituted (HIR) mouse model

A better understanding of the initial events in PDAC development is needed to improve early detection and therapy. Since the diagnosis of human pancreatic cancer is often possible only at late stage, when the symptoms of the disease finally emerge, the study of early tumor onset and biomarkers is limited to preclinical models. One of the most promising cancer preclinical models is the HIR mouse model that represents a bridge between experimentation on animal models and studies on human subjects. Human tumor tissue or cell lines can be co-engrafted into these mouse models, providing a powerful tool for studying the interactions between human immune cells and human cancers. In the last few years, our research group optimized a humanization protocol in which 6 weeks old NOG mice (NODShi.Cg-Prkdc^{scid}Il2rg^{tm1Sug}) were sub lethally irradiated (120 cGy) and transplanted with 1x10^5 human CD34$^+$ hematopoietic stem cells (HSC), purified from peripheral blood of adult donors after mobilization by G-CSF (Figure 7A).

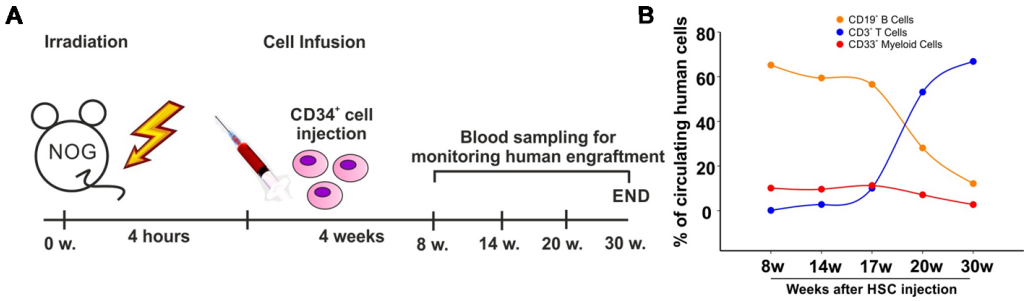

Figure 7. Human leukocytes reconstitution in NOG mice. A) Experimental schedule of the humanization protocol for HIR mice. B) Kinetics of human CD19$^+$ B Cells, CD3$^+$ T Cells and CD33$^+$ myeloid cells in circulating human leukocytes over time. Data from 2 independent experiments for a total of 11 mice per group.

Monitoring blood engraftment (percentage of human CD45$^+$ cells among all CD45$^+$ cells including human and mouse CD45$^+$) from the 8 weeks after HSC injection till 30 weeks, it was possible to notice that the human cells of

hematopoietic origin emerge with distinct kinetics. The first human cell populations identified in the blood are, in fact, $CD19^+$ B lymphocytes and $CD33^+$ myeloid cells; later on, B cells decrease in parallel with an increased frequency of $CD3^+$ T-cells, which become the most represented population in the latest phases of human repopulation. Further studies on this model demonstrated that, among the engrafted human leukocytes present in the BM of HIR mice, there are cells that display totipotency, i.e. ability to give rise to other cell subsets. Human Lin^- cells (Lin Ab cocktail includes CD3/CD19/CD56), isolated from HIR mouse and human healthy donor BM and cultured for 4 days with hG-CSF plus hGM-CSF cytokine, were able to generate MDSCs *in vitro*. In fact, the $CD11b^-$ cells purified from the *in vitro* cultures blocked the proliferation of allogenic $CellTrace^+$ PBMCs activated with anti-hCD3 and anti-hCD28 antibodies (Figure 8 A-B).

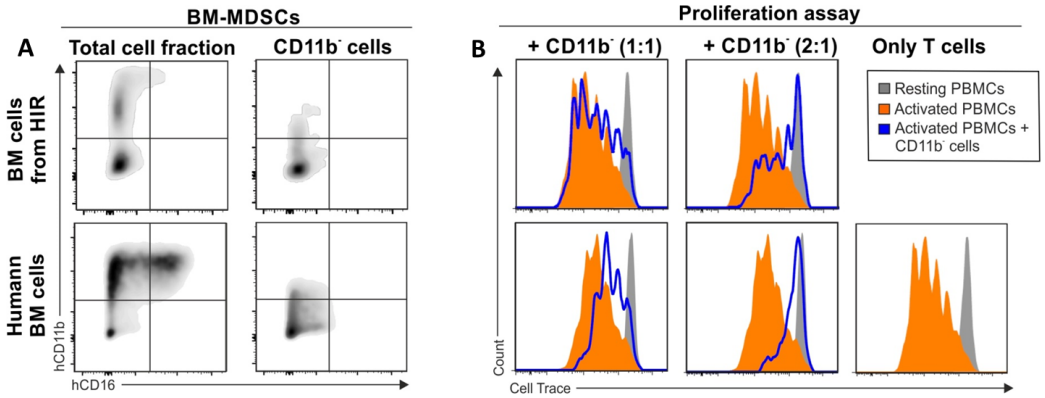

Figure 8. Presence of MDSC precursors among engrafted human leukocytes in HIR mice. A) representative flow cytometric plots evaluate CD11b and CD16 markers in BM-MDSCs or sorted $CD11b^-$ fraction in human and HIR bone marrow. B) CellTrace dilution proliferation assays show immune suppression exerted by both human and HIR $CD11b^-$ BM-MDSC against activated human allogenic PBMCs at 1:1 or 2:1 effector to target ratio.

2. HIR model of pancreatic cancer

The presence of an active and functional immune system in HIR mice is an important tool for the study of human leukocyte influence on tumors and their microenvironment. For these reason, we set up an *in vivo* pancreatic cancer model in HIR mice using an immortalized pancreatic tumor cell line (PT-45) that give rise to an adenocarcinoma with limited capability to metastasize to the lung. The experimental schedule (Figure 9A) was chosen based on previous data on human cell repopulation kinetic in mice: since we wanted to investigate the crosstalk

existing between myeloid and cancer cells, we orthotopically injected $1x10^6$ PT-45 cells at 11 weeks after HSC transplantation. After tumor challenge, we monitored the human engraftment by flow cytometry analysis of blood samples, six times until sacrifice; as showed in figure 9B, PDAC tumor did not change the percentage of circulating human leukocytes compared to tumor-free HIR mice.

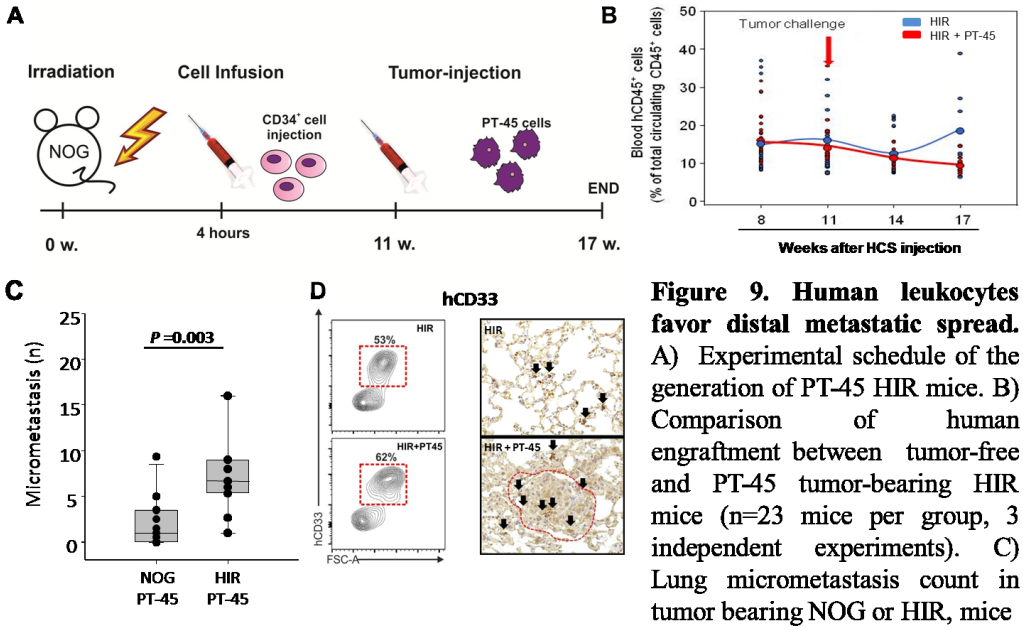

Figure 9. Human leukocytes favor distal metastatic spread. A) Experimental schedule of the generation of PT-45 HIR mice. B) Comparison of human engraftment between tumor-free and PT-45 tumor-bearing HIR mice (n=23 mice per group, 3 independent experiments). C) Lung micrometastasis count in tumor bearing NOG or HIR, mice (n=10; 15 respectively, 3 different experiments). Statistical analysis was performed by Mann-Whitney Wilcoxon test. D) Representative FACS analysis and IHC staining with anti-hCD33 antibody of lung of tumor free and tumor-bearing HIR mice.

Remarkably, the orthotopic injection of PT-45 cells in HIR mice resulted in a significant metastatic spread of cancer cells to the lung in the presence of a human hemopoietic system compared to NOG bearing the same tumor (Figure 9C): the count of lung metastases of tumor bearing HIR mice (8.2 ± 7.3; n=21) resulted significantly higher compared to either the tumor bearing NOG animals (2.1 ± 1.7; n=10. Figure 9C). In the lung of PT-45-bearing mice, we could observe an increased infiltration of human myeloid cells expressing the CD33 marker (Figure 9D). Notably, these myeloid cells were near the nests of cancer cells, supporting the idea of being part of the early "metastatic niche" (Figure 9D).

3. Expression profile of human leukocytes isolated from lung and bone marrow of tumor-bearing or tumor-free HIR mice.

The pancreatic model of HIR mice indicates a pro-metastatic activity of human leukocytes. To characterize better the immune cells important in metastasis promotion, we isolated BM resident and lung-infiltrating human $CD45^+$ cells from both tumor-bearing and tumor-free mice to unveil possible gene signatures of early metastatic niche in hosts bearing human PDAC (Figure 10A). Single cell suspensions from BM and lung were stained with anti-human CD45 and anti-mouse CD45.1 antibodies and human and mouse leukocytes were sorted on a FACS Aria II (Becton Dickinson) on the basis of speciespecific CD45 expression. Human $CD45^+$ cells, were used for total RNA extraction and cDNA amplification. All the samples were hybridized to Affymetrix U133 PLUS 2.0 arrays and scanned with an Affymetrix GCS 3000 7G scanner. In four independent experiments, we identified the most perturbed genes in BM and lung of tumor-bearing mice compared to tumor-free mice. By ordering the genes according to their expression levels, hierarchical clustering revealed different patterns of expression between tumor-free and tumor-bearing samples in both BM and lung (Figure 10B). The Significance Analysis of Microarrays (SAM) identified the differentially expressed genes, i.e genes changed at least 1.5-fold with an estimated false discovery rate (FDR) of 5%. The highest number of differentially express genes was found in lung-infiltrating human $CD45^+$ cells [782 upregulated (fold change > 1.5) and 302 down-regulated genes (fold change <-1.5)] whereas, in BM resident cells, the differences between tumor-bearing and tumor-free mice were less evident [187 up-regulated (fold change > 1.5) and 28 downregulated genes (fold change <-1.5)] (Figure 10C). Differentially expressed genes were analyzed for categories with significant enrichment ($p<0.05$) in Gene Ontology (GO) biological process using DAVID tools to identify specific pathways or biological function perturbed during metastatic process. Among down regulated genes, in both BM and lung of tumor-bearing HIR mice compared to tumor-free HIR mice, we found a substantial enrichment in categories involved in the activation of a T cell-mediated immune response like T-cell activation, T-cell differentiation and positive regulation of lymphocyte activation.

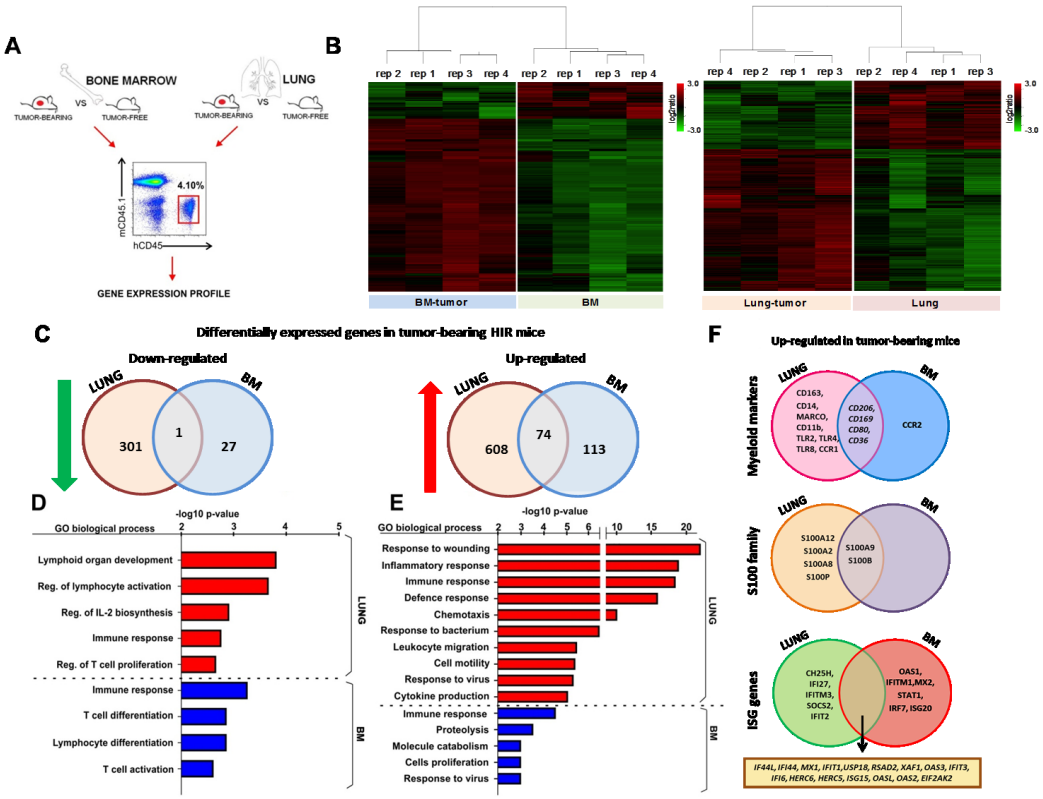

Figure 10. Gene expression profile of human leukocytes. A) Bone marrow resident and lung-infiltrating hCD45$^+$ cells were isolated from both tumor-bearing and tumor-free HIR mice, RNA was obtained and a gene expression profiling was performed. Samples were collected from 4 independent experiments (HIR=34; HIR+ PT-45=37). B) Hierarchical clustering using Pearson correlation revealed a different expression profile between bone marrow and lung tissue. Heatmap colors represent the relative expression of each sample. C) Differentially expressed genes in bone marrow and lung of tumor-bearing HIR mice compared to tumor-free humanized mice. D and E) Gene onthology enrichment analysis of biological process using DAVID bioinformatics database. F) Selection of differentially expressed genes detected in lung-infiltrating hCD45$^+$ cells or BM resident hCD45$^+$ cells from PT-45 bearing mice and tumor free mice.

On the other hand, among up-regulated genes we found the over-representation of categories that are related with the immune response activation, and in particular with the defense response against bacteria and virus (Figure 10D-E). The analysis of differentially expressed genes unveiled a pro-metastatic profile of human leukocytes infiltrating the lung of tumor-bearing HIR mice. First of all, the gene profile suggests that these human leukocytes were mainly composed by myeloid cells with macrophage-like features, since we found highly up-regulated myeloid markers such as MCR-1 (mannose receptor C, type1), CD169 (Siglec1) and CD14 (Figure 10F) together with cytokines (CCL2, IL6, IL1A, TGFA) and chemotactic factors (S100 proteins, CXCL1, CXCL2, CXCL11) involved in the recruitment of tumor cells to the metastatic site. Among these chemotactic factors, it is important

to emphasize the presence of some members of S100 family, in particular S100A8, S100A12, S100A9, S100B, S100P, which actively contribute to tumorigenic processes such as cell proliferation, immune evasion and recruitment of $CD11b^+$ myeloid cells to the metastatic site via TLR4 (27). Moreover, other metabolites involved in various process associated with metastatization results over expressed in tumor-bearing HIR mice: proteases like cathepsins (A, B, C, D) and MMPs that can degrade components of the ECM; clusterin important in TGF-β-induced EMT; and the proteoglycan versican that favor MET and thus the soil of tumor cells at the metastatic organ. Surprisingly, the analysis of the up-regulated genes revealed an unexpected IFNα/β-related gene signature, in both BM and lung, characterized by the expression of interferon stimulated genes (ISGs) (Figure 10F). These genes are typical of the innate immune response against pathogens but, in the last few years, their involvement in some cancer-induced programs has been demonstrated. The IFN-mediated pathway involved in the innate antiviral response includes IFN-stimulated gene 15 ubiquitin-like modifier (ISG15), Mx GTPase, protein kinase R, and 2′-5′oligoadenylate-synthetase-directed ribonuclease L. Recently, the overexpression of ISG15 and ISG15-mediated conjugation has been identified in a wide range of human tumors such as colon rectal cancer (183), breast cancer (184) and pancreatic cancer (185). Moreover, the up-regulation of IFIT3 in pancreatic cancer cells promote tumor-angiogenesis and chemo resistance (186).

4.Generation of a second model of tumor-bearing HIR mice

Recent studies demonstrated that tumor microenvironment as well as the pre-metastatic niche present different features in various cancer settings. We thus wondered whether the malignant cells spreading at a secondary site supported by immune cells, as well as the above described pro-metastatic signature was a peculiar feature of pancreatic cancer. For this purpose, we set up a second model of tumor-bearing HIR mice based on the injection of a human triple negative breast cancer cell line (MDA-MB 231) able to produce a high number of lung metastases. 10^6 MDA-MB 231 cells were ortotopically injected in the mammary

fat pad of female HIR mice, 11 weeks after HSC administration and when the tumor reached a volume of 600 mm^3 the primary mass was removed to favor tumor cells dissemination (Figure 11A).

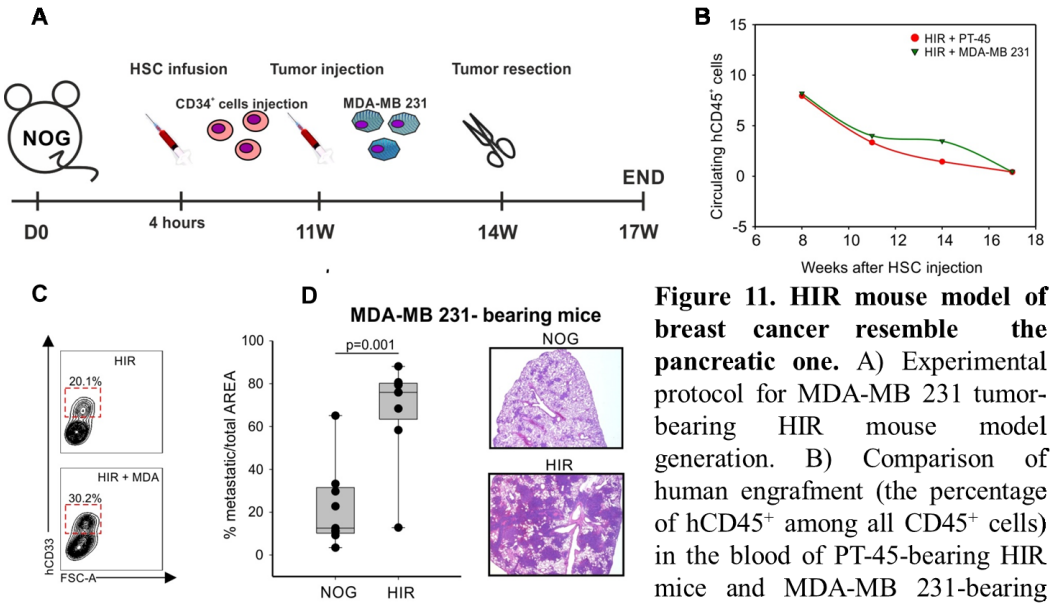

Figure 11. HIR mouse model of breast cancer resemble the pancreatic one. A) Experimental protocol for MDA-MB 231 tumor-bearing HIR mouse model generation. B) Comparison of human engrafment (the percentage of $hCD45^+$ among all $CD45^+$ cells) in the blood of PT-45-bearing HIR mice and MDA-MB 231-bearing HIR mice. C) Representative FACS analysis of lung-infiltrating $hCD33^+$ cells. D) lung metastatic AREA in MDA-MB 231 bearing NOG (n=9) and HIR (n=9) mice. Statistical analysis was performed by Mann-Whitney Wilcoxon test.

Analyzing the percentage of circulating $hCD45^+$ we could observe that HIR mice injected with either tumors presented a similar kinetic of human engraftment (Figure 11B). Interestingly, we demonstrated, also in this experimental tumor model, an increased number of human myeloid cells ($CD33^+$) infiltrating the lung of tumor-bearing mice, and their frequency correlated with an enhanced number of lung metastases compared to immune compromised tumor-bearing mice. Only the 20% of lung surface in tumor-bearing NOG mice resulted covered of metastases; this percentage drastically increased (80%) in presence of a human immune system (Figure 11C-D).

5.Validation of microarray results

The transcriptome analysis of immune cells in BM and lung unveiled a specific gene signature related to metastatic process. We decided to use both models of

tumor-bearing HIR mice to validate some of the upregulated genes included in the gene signature, in order to characterize better the myeloid population recruited to metastatic lungs. First of all, by flow cytometry we analyzed the expression of some the upregulated markers on $CD33^{+}CD14^{+}$ human monocytes (Figure 12A). We could confirm a tumor-induced expression of CD206 and CD169 markers in both lung and BM monocytes, a slight upregulation of CD68 marker in the BM of tumor-bearing mice but no significant difference in CCR2 expression (Figure 12B-C). Interestingly, CD206 resulted to be the most expressed marker in both models of tumor-bearing HIR mice, confirming the finding that it was also among the most perturbed in the gene signature in both BM (fold change = 5) and lung (fold change = 12.5).

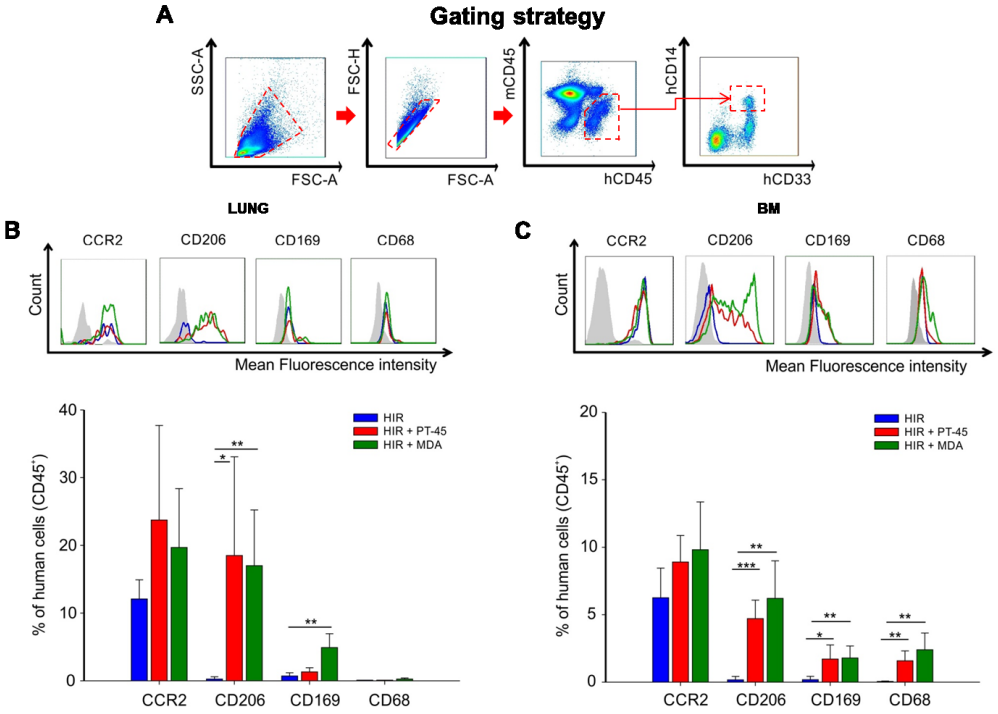

Figure 12. Validation of myeloid-related upregulated markers in PT-45 and MDA-MB 231 bearing mice. A) Gating strategy. B-C) Mean fluorescence intensity and percentage of human cells positive for CCR2, CD206, CD169 and CD68 markers in both lung and BM. Statistical analysis was performed by Mann-Whitney Wilcoxon test.

We thus identified a population bearing CD14 and CD206 markers that was almost exclusively present in both lung and BM of tumor-bearing HIR mice. Interestingly, this population was also over-represented in peripheral tissues; about the 2% of $hCD45^{+}$ cells in the spleen displayed a $CD14^{+}CD206^{+}$ phenotype whereas an higher percentage of these cells was found in the blood of tumor-

bearing HIR mice, suggesting a potential use of this population as circulating biomarker for the metastatic process (Figure 13D-E).

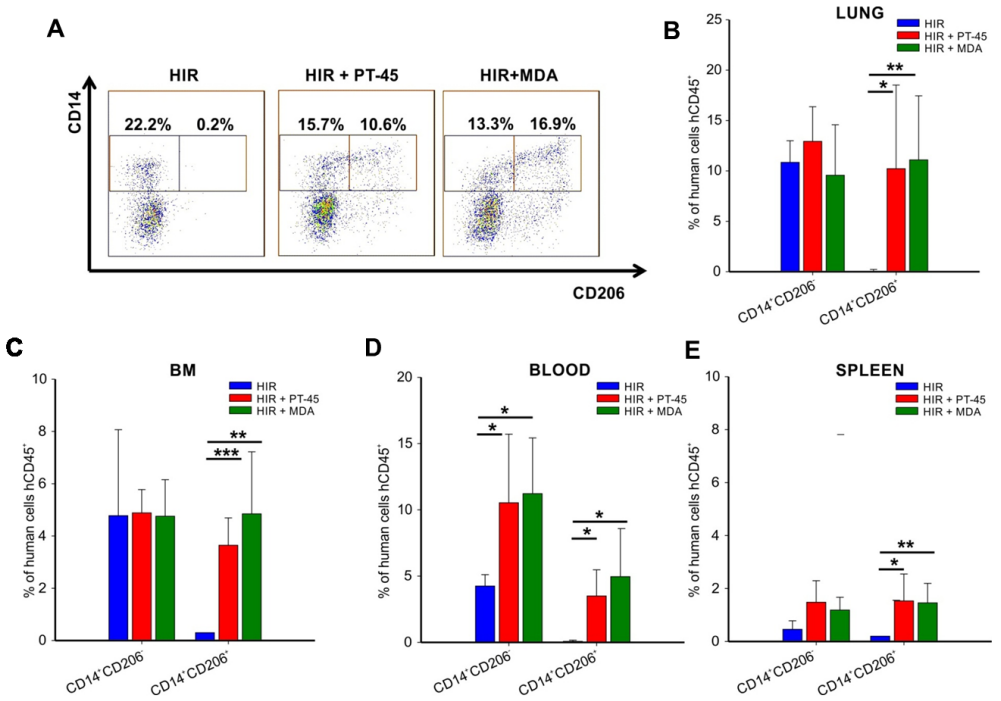

Figure 13. Identification of a CD14$^+$CD206$^+$ pupulation exclusively present in tumor bearing HIR mice. A) Representative flow cytometry of lungs of tumor-free and tumor-bearing HIR mice stained with anti-hCD14 and anti-hCD206 antibodies. B-C-D) Percentage of CD14$^+$CD206$^-$ and CD14$^+$CD206$^+$ populations among human leukocytes in lung, BM, blood, spleen. Statistical analysis was performed by Mann-Whitney Wilcoxon test.

Through a quantitative real-time PCR (qPCR), we then validated the expression on human leukocytes (hCD45$^+$ cells) isolated from the BM of tumor-free and tumor-bearing HIR mice. Two main molecular signatures were considered, i.e. S100 and ISG gene families. As showed in Figure 14A, almost all S100 proteins tested, except for S100P, were found upregulated in both models of tumor-bearing HIR mice (Figure 14A). Furthermore, the real time PCR confirmed the over expression of some of the interferon induced genes such as IFI44, MX1, USP18, IFIT3, ISG15 and STAT1 in both PT45-bearing HIR mice and MDA-MB-231-bearing HIR mice compared to tumor-free HIR mice (Figure 14B). Notably, both molecular signatures were enhanced in the breast cancer model, which is the highest metastatic among the two analyzed in our experiments.

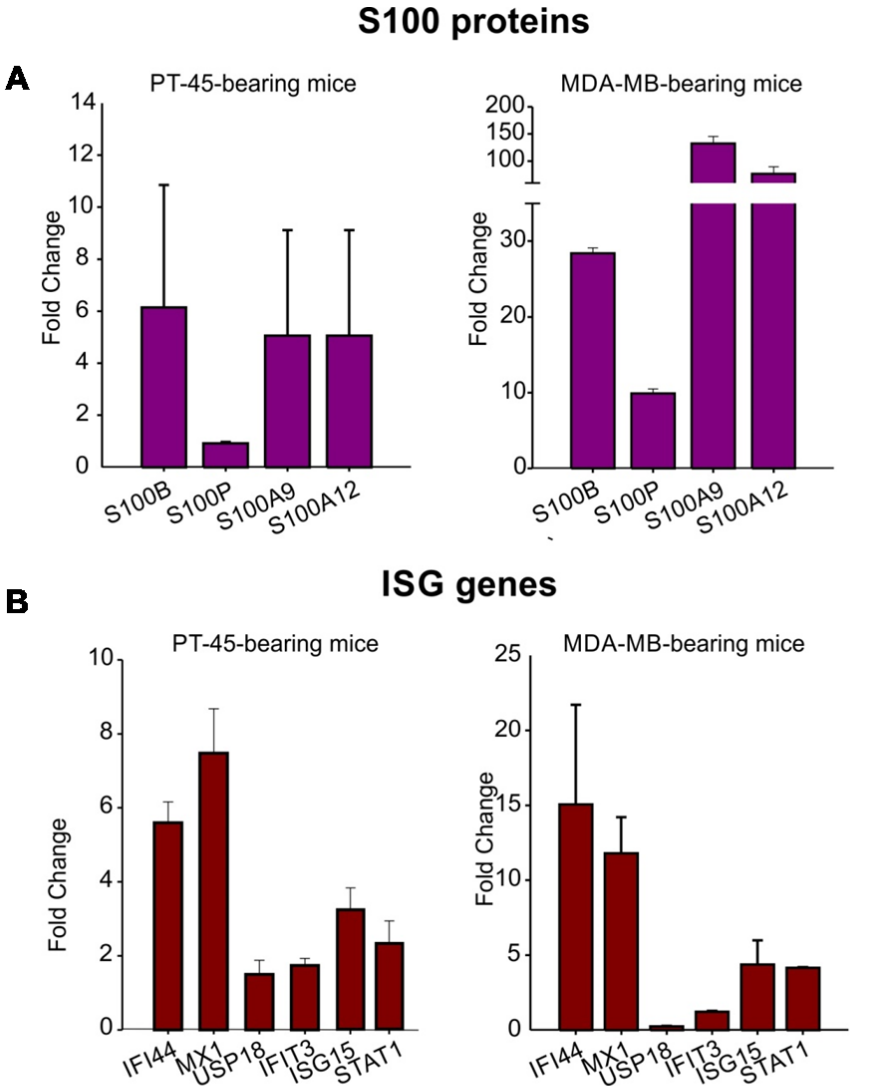

Figure 14. Validation of the pro-metastatic signature in PT-45 and MDA-MB 231 bearing mice. A-B) Validation of S100 and ISG signature in BM of tumor-bearing HIR mice by Real Time PCR. Relative target gene expression was calculated on tumor-free mice using $2^{-\Delta\Delta CT}$ method.

6.Exosomes as possible inducer of ISGs expression in primary myeloid cells.

We then decide to point our attention to IFNα/β-related gene signature. We could not detect any differences in the IFNα serum levels of tumor-bearing and tumor-free HIR mice (data not shown). Therefore, we speculated that the IFNα/β-related gene signature might be related to an increased level of circulating exosomes able to activate innate immune cells, possibly trough pattern recognition receptors (PRRs). Therefore, we isolated tumor-released extracellular vesicles (EVs) from

the conditional media of PT-45 and MDA-MB-231 cell lines through several centrifugation steps (Figure 15A). We classified these EVs as exosomes since that they displayed a small size (PT-45= 84 ± 0.4 nm and MDA-MB 231= 62 ± 0.3 nm), measured using a Zetasizer Nano ZS instrument (Figure 15B) and present typical exosome surface markers, such as CD63, by both western blot and flow cytometry (Figure 15C-D).

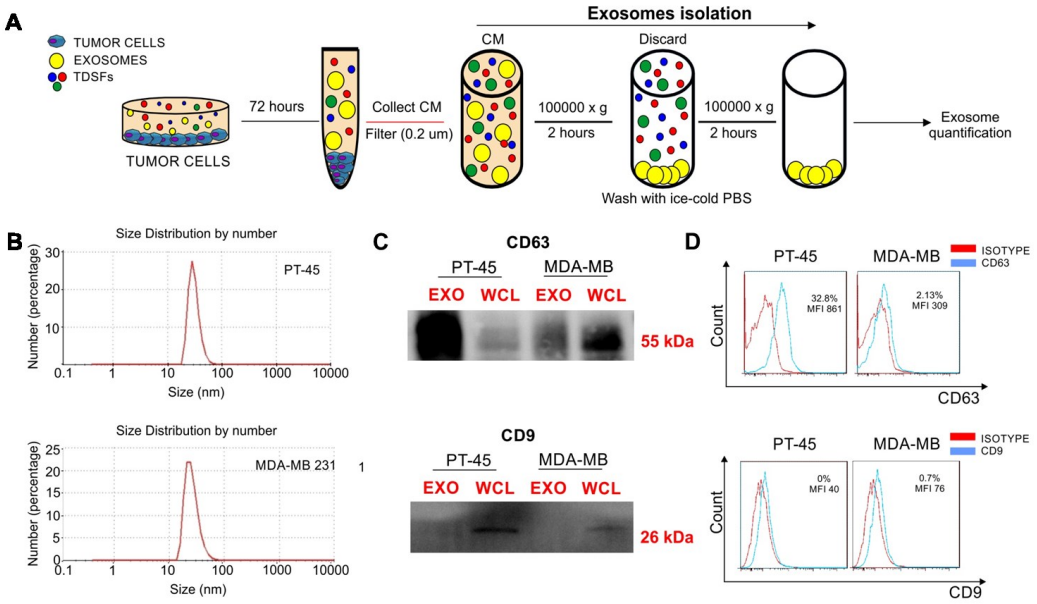

Figure 15. Characterization of tumor exosomes. A) Experimental schedule for tumor-released exosomes purification. B) Exosomes isolated from PT-45 and MDA-MB 231 cell culture supernatants, measured by nanoparticle tracking analysis (NTA) showed a peak at 84 ± 0.4 nm and 62 ± 0.3 nm respectively. C) Western Blot analysis for exosomes markers CD63 and CD9 in exosomes and whole cell lysate (WCL) samples. D) Exosomes coupled to 4-mm diameter aldehyde/sulfate latex beads were analyzed by flow cytometry for CD63 and CD9 expression.

We then verified whether these exosomes can deliver signals able to activate a type I interferon response in primary human myeloid cells. We isolated human $CD14^+$ monocytes from buffy coat and treated them with 25 µg of PT-45-released exosomes, 25 µg of MDA-released exosomes or human IFNα as positive control. After a six hour treatment, we noticed an exosome-induced upregulation of ISG gene expression (Figure 16B). Furthermore, when we differentiated $hCD14^+$ cells to human macrophages, after a seven day culture with human M-CSF, and then threated these cells with the same stimuli we detected the same gene signature,

even if at lower intensity (Figure 16C). We thus conclude that exosomes are able to interact with primary myeloid cells and activate ISG transcription.

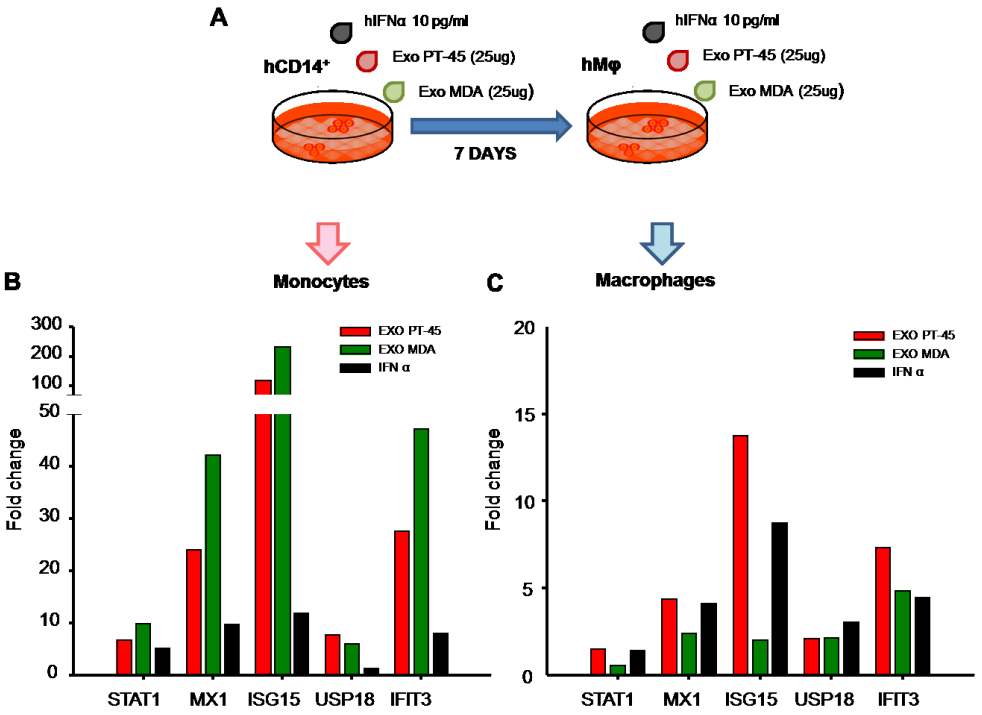

Figure 16. Exosomes activate ISG signature in human primary myeloid cells. Human CD14^{+} monocyte isolated from buffy coat of healthy volunteers were cultured in exosome free media and treated for 6 hours in presence of either PT-45-purified exosome (25µg), MDA-MB 231-purified exosome (25µg) or 10 pg/ml of IFNα as positive control. B) Relative target gene expression by RT-PCR was calculated above untreated condition using $2^{-\Delta\Delta CT}$ method.

7. Spatial distribution of highly metastatic cancer–derived exosomes.

To elucidate the role of exosomes in intercellular communication and its target effects, it was important to first determine their *in vivo* distribution. Therefore, we studied the bio-distribution of fluorescently-labeled exosomes in tumor-free mice after systemic delivery. In particular, we examined the tissue distribution of exosomes derived from a highly metastatic cell line, the mouse fibrosarcoma MN-MCA1. We labelled the exosomes with a fluorescent dye, PKH26 and administrated them in C57BL/6 mice by retro-orbital injection 36, 24 and 12 hours before the sacrifice.

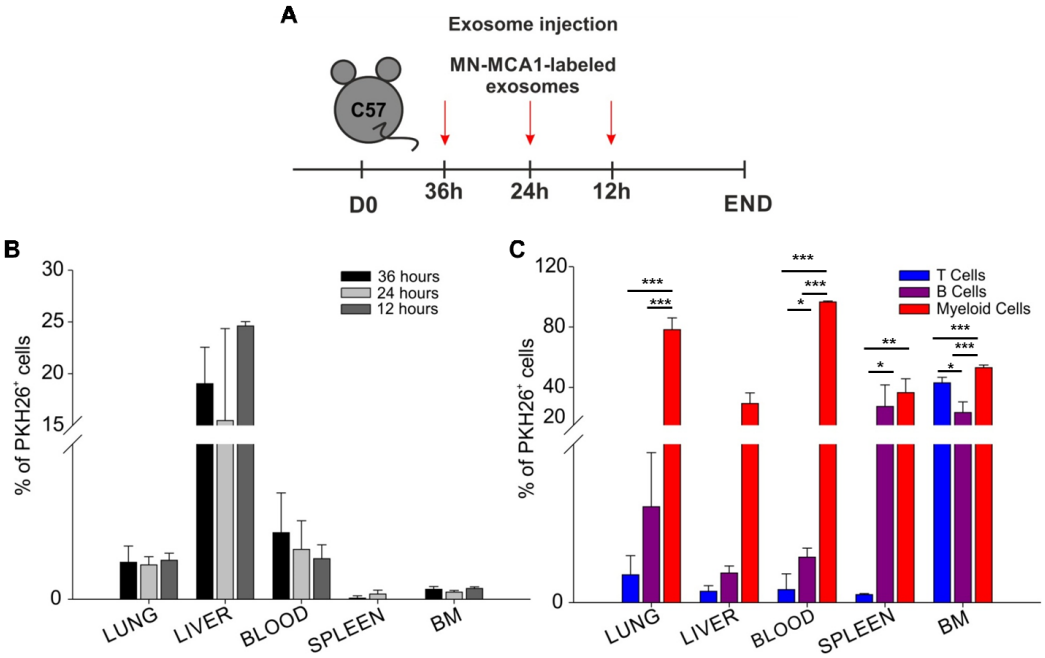

Figure 17. Ex vivo fluorescently labeled exosomes tracking. A) MN-MCA1-derived exosomes were labeled with the PKH26 fluorescent dye and retro-orbitally injected in C57BL/6 naïve mice. Mice were sacrificed 36, 24 and 12 hours after the treatment. B) *ex vivo* quantification of labeled-exosomes in different tissue by flow cytometry. C) Percentage of immune cells that took up labeled-exosomes. Statistical analysis was performed by ANOVA test; *p<0.05, **p<0.01, ***p<0.001.

The *ex vivo* fluorescence quantification performed by flow cytometry showed, in all the analyzed time points, an accumulation of labelled exosomes in the liver, lung and peripheral blood of treated mice. A limited to absent accumulation was instead detected in BM and spleen (Figure 17B). Furthermore, we demonstrated that labelled exosomes were mainly engulfed by $CD11b^+$ myeloid cells, in all the tissues analyzed, whereas very low signal was detected in $CD3^+$ T-cells and $B220^+$ B-cells (Figure 17C).

8. C57BL/6 mice preconditioned with MN-MCA1-derived exosomes present an increased number in lung metastases.

Since we observed a preferential exosome uptake by myeloid cells, we examined the ability of exosomes from highly metastatic cells to influence the lung of tumor-free C57BL/6 mice, potentially creating a pre-metastatic niche. MN-MCA-derived exosomes were retro-orbitally injected, every 2 days for 3 weeks, in a process called “exosome education”. Subsequent characterization of infiltrating

immune cells in the lung confirmed no significant changes in immune composition and in particular in myeloid cells frequency.

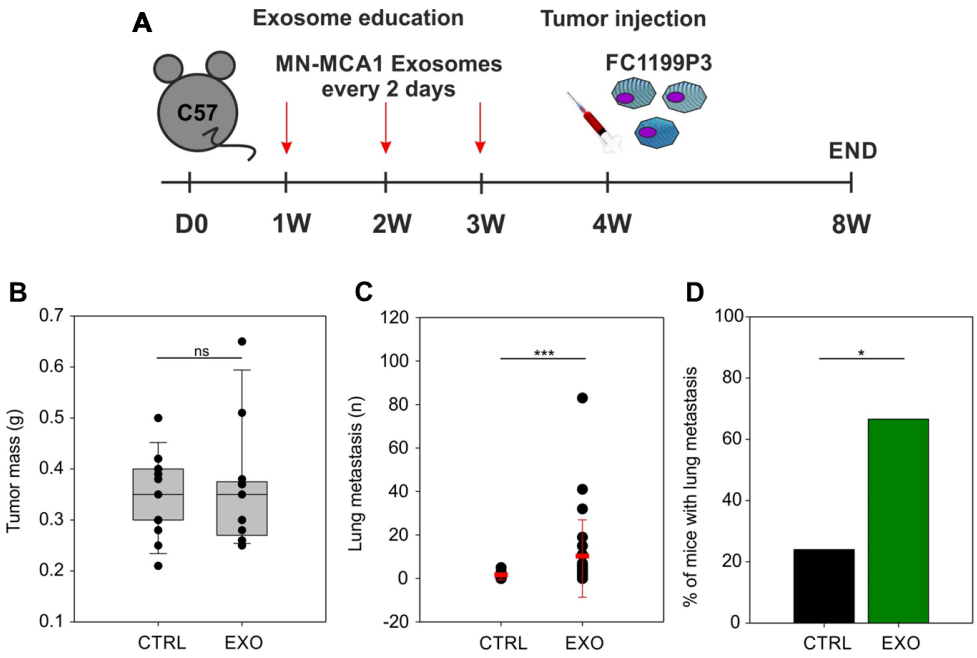

Figure 18. MN-MCA1-derived exosomes potentially create a pre-metastatic niche in the lung. A) C57BL/6 mice received 5µg of MN-MCA1-derived exosomes or PBS as control every 2 days for 3 weeks. After the pre-conditioning mice were challenged with 10^5 FC1199P3, a KPC-derived pancreatic tumor cell line. B) Primary tumor mass at sacrifice. C-D) Lung micrometastases count and metastasis incidence in PBS or Exosomes exposed mice (CTRL n=29; EXO n=27 pooled from 2 indipendent experiments). Statistical analysis was performed by Mann-Whitney Wilcoxon test; *p<0.05, ***p<0.001.

We then challenged these mice with the KPC-derived pancreatic cell line FC1199P3, which possesses a very limited metastatic potential (Figure 18A). Exosome treatment did not affect primary tumor dimension (Figure 18B); however, mice pre-conditioned with MN-MCA1 derived exosomes displayed a significant increment in the number of lung metastases compared to PBS-treated control mice ($p<0.001$). We also found an increment of mice that presented at least one metastasis in the exosomes-educated group compared to the control one ($p<0.05$) (Figure 18C). We confirmed this pro-metastatic role of the highly metastatic exosome pre-conditioning also in a second setting. After 3 weeks of pre-conditioning, educated mice (n=24) and control mice (n=25) were challenged with $5x10^5$ E0771 breast cancer cells in the mammary fat pad. When the tumor reached a volume of 600 mm^3 the primary mass was removed to allow detection

of tumor cell dissemination (primary tumor would have killed the host if not removed).

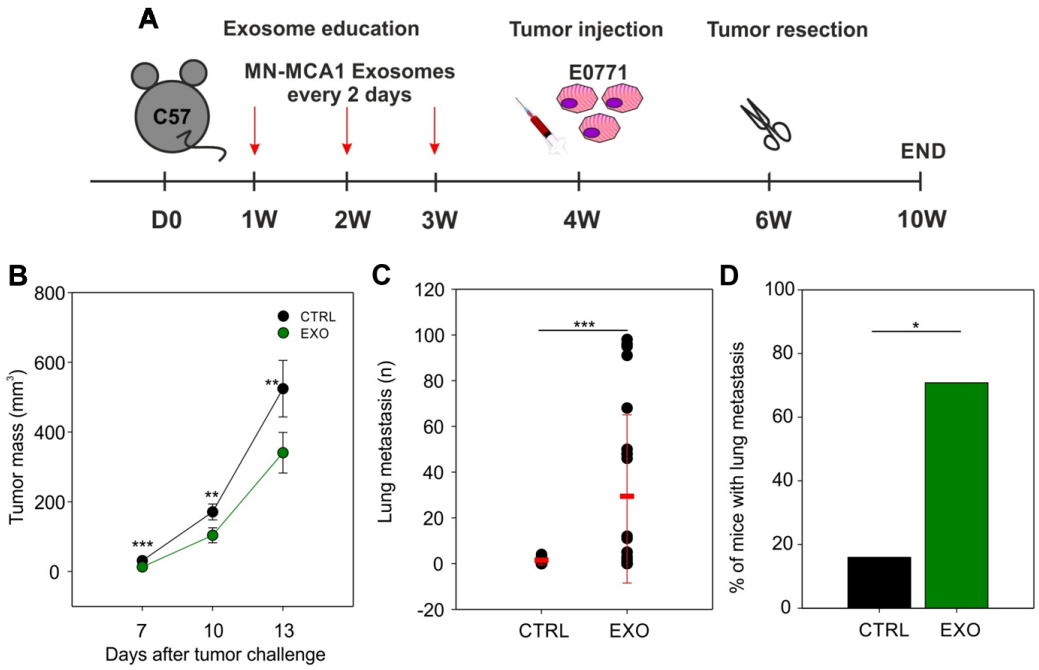

Figure 19. MN-MCA1-derived exosomes potentially create a pre-metastatic niche in the lung. A) C57BL/6 mice received 5µg of MN-MCA1-derived exosomes every 2 days for 3 weeks. Control mice received an equivalent volume of filtered PBS. After the pre-conditioning, mice were challenged with $5x10^5$ E0771, a breast cancer cell line, in the mammary fat pad. B) Avarage of primary tumor growth over time in PBS or exosomes exposed mice (CTRL n=25; EXO n=24 pooled from 2 indipendent experiments). C-D) Lung micrometastases count and metastasis incidence Statistical analysis was performed by Mann-Whitney Wilcoxon test; *p<0.05, **p<0.01, ***p<0.001.

Although we could notice a slightly reduction in primary tumor growth in exosome-educated mice, a significant increment in lung metastases number, as well as in tumor metastases incidence, was clearly evidenced in mice treated with MN-MCA1 exosomes compared to PBS-treated control mice.

DISCUSSION

Metastasis represents the end product of a multi-step biological process, which involves dissemination of cancer cells to anatomically distant organ sites and their subsequent adaptation to foreign tissue microenvironments. In several tumors, accumulating evidence hints to the existence of metastasis-supportive microenvironments termed pre-metastatic niche, in which some immune cell populations promote colonization and growth of disseminated tumor cells (23). Our data, generated using HIR mice as preclinical model, gave us a first idea about the composition of the human early metastatic niche in which the myeloid compartment of the immune system exert a metastasis-favoring activity in response to tumor-secreted factors. In fact, tumor-bearing HIR mice engrafted with both PT-45 human pancreatic adenocarcinoma cell line or MDA-MB-231 human breast carcinoma cell line displayed a higher number of lung metastases compared to immunodeficient tumor-bearing mice not reconstituted with human cells. Moreover, lungs of tumor-bearing HIR mice were characterized by a high rate of human infiltrating $CD33^{+}$ myeloid cells associated with tumor cells, probably supporting metastasis formation. It is important to emphasize that humanized mouse model is an useful tool for the studies of human physiology and pathology but it does not completely mimic the broad and complex human immune system; the development and function of several human immune cell types, such as neutrophils, NK cells and macrophages is partially defective, similarly to what described in currently available models of humanized mice (187). These limitations are linked to the imperfect binding of mouse cytokines to the corresponding human cytokine receptors. We have already improved NOG mice repopulation upon HSC transplantation by the administration of adeno-associated virus serotype 9 (AAV9) as vectors to mediate gene delivery of essential human cytokines (IL-3, G-CSF and GM-CSF) for a prolonged period of time. Notably, AAV-treatment did not affect metastatic process: in fact, AAV-treated PT45-tumor bearing HIR mice did not present any increase of lung metastases, compared to AAV-untreated PT45-tumor bearing mice, suggesting that this model allow to evaluate the impact of human tumor-derived factors (data

not shown). The AAV9 system for cytokines delivery could be suitable to set up a patient-personalized, tumor-bearing HIR mouse model in order to recapitulate the interaction between tumor cells and the immune system of the same patients at early stages of tumor progression, a time window that is still not possible to analyze in patient.

The analysis of the expression profile in BM resident and lung-infiltrating human $CD45^+$ cells from both tumor-bearing and tumor-free HIR mice unveiled some molecular pathways relevant for tumor invasion and metastatization. By flow cytometry, we validated some of the myeloid-related markers up-regulated in tumor-bearing mice. This allowed us to identify a double positive population for CD14 and CD206 markers, exclusively present in BM and lung of tumor-bearing HIR mice. We also found an increased frequency of circulating $CD206^+$ monocytes in the blood of tumor-bearing HIR mice, suggesting a possible exploitation of this cell population as circulating biomarker for the metastatic process. However, the existence of circulating $CD14^+$ cells expressing a typical M2 macrophages marker could be misleading; up to now, we cannot exclude that a limitation of our model such as the absence of some important human cytokines could be responsible for an incomplete macrophage differentiation. However, monocytes expressing M2 markers have been recently described in the blood of chronic HCV-infected patients and correlated with liver fibrosis (188). Therefore, in the next future we plan to evaluate the presence of $CD14^+CD206^+$ cells in the blood of patients with a metastatic disease in order to validate this cell population as a potential biomarker. Circulating human monocytes characterized by a distinct gene expression profile have been already indicated as candidate biomarkers for diagnosis and follow-up of colorectal cancer patients (189).

We then pointed our attention to other differentially expressed genes of our gene profile on metastasis-infiltrating leukocytes. We confirmed the over-expression of S100 protein family members and ISGs in the BM of tumor-bearing HIR mice. Notably, these gene signatures were shared by different tumor settings and their expression correlated with the metastatic potential. S100 proteins resulted highly expressed in the lungs of tumor-bearing animals. S100A8/A9 can recruit myeloid

cells, such as MDSCs, in the pre-metastatic lung through the SAA3/TLR4 axis (27). The expression of other S100 proteins, such as S100A4 and S100B, correlated with cancer metastasis and poor prognosis in patients with different cancer types (190). Interestingly, S100A4 is not only present on tumor cells but also in stromal cells, such as fibroblast and BMDCs, which actively contributes to metastatic dissemination (191). Tumor and serum levels of S100B have been used as diagnostic marker for melanoma for many years; since *in vivo* studies have confirmed its role in melanoma progression, S100B may also be considered as a potential therapeutic target (192). We have already validated the role of S100B in PDAC progression measuring the amount of auto-antibodies in the serum of PDAC patients (n=29) compared to healthy donors (n=10) or chronic pancreatitis (n=19) patients: the level of anti-S100B in PDAC patients is significantly decreased, probably due to the antigen sequestration in immune complexes (Figure 20A), suggesting S100B involvement in PDAC tumor progression. To confirm the causative role of these proteins in promoting the metastatic process, we will downregulate their expression taking advantage of the CRISPR (clustered regularly interspaced short palindromic repeats) technology to silence different S100 members in human $CD34^+$ cells before HIR mouse reconstitution. After PT-45 tumor challenge we will be able to evaluate whether the lack of such genes may have any impact on the metastatization process. Considering all these data, the therapeutic targeting of S100 proteins in patients might be a approach to control metastasis dissemination in combination with different anti-cancer immunotherapies.

The most surprising finding about human metastases-infiltrating leukocytes from tumor-bearing HIR mice was the IFNα/β-related gene signature. Among the different activators of these molecular pathways, tumor-derived exosomes can deliver nucleic acid able to mimic virus infections. This is consistent with the previous finding that RNA within exosomes, which are largely noncoding transcripts and transposable elements, stimulates pattern recognition receptors like RIG-I activating ISGs transcription. In addition, ISG expression in breast cancer cells identifies patients resistant to chemo and radio therapy .Our preliminary data showed that PT-45 and MDA-MB 231 cells released a high number of exosomes

able to activate the type I IFN downstream signaling in primary myeloid cells, such as $CD14^{+}$ monocytes and monocyte-derived macrophages. However, the importance of ISG expression in myeloid cells to promote metastatic spread has to be investigated, yet. Recently, our collaborator Prof. Peter Bailey identified four different classes of PDAC tumors, i.e. squamous, pancreatic progenitor, immunogenic, and ADEX (Aberrantly Differentiated Endocrine eXocrine), by using a combination of whole-genome and deep-exome sequencing, with gene copy number analysis to determine the mutational mechanisms and candidate genomic events important in pancreatic carcinogenesis. This work on PDAC primary tumors and patient-derived cell lines (PDCLs) revealed four subtypes associated with distinct histopathological characteristics and differential survival (193). The list of genes differentially expressed in the lung of PDAC-bearing HIR mice was mostly enriched in squamous subtype, the one with poorer prognosis.

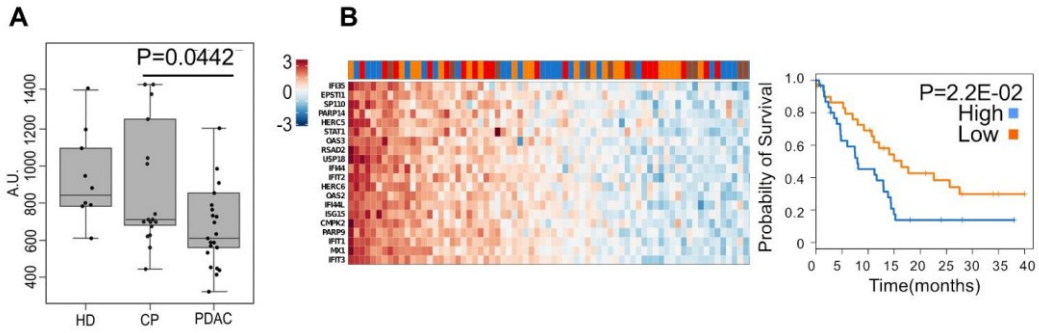

Figure 20. Potential molecular pathways involved in tumor progression and distal metastatic spread A) Plasma level autoantibody anti-S100B protein in HDs, CP and PDAC patients. B) High level of expression of IFNα-related gene correlates with poor survival rate in PDAC patients characterized by metastatic disease.

Moreover, a deeper analysis on the molecular profile demonstrated that the ISG gene signature expression in PDCLs can discriminate PDAC patients with a worse overall survival (Figure 20B). Therefore, the detection of circulating myeloid cells that express an activated ISG gene signature could be considered a potential biomarker to be tested in the next future.

Finally, we demonstrated that exosomes derived from a highly metastatic fibrosarcoma cell line (MN-MCA1), when administrated *in vivo* though a retro-orbital injection, actively interact with myeloid cells but not with T or B lymphocytes. These results support the idea that tumor-derived exosomes may

take an active part in the remodeling of the microenvironment through a close intercommunication with myeloid cells. In fact, we demonstrated that the continuous exposure of C57BL/6 mice to MN-MCA1-derived exosomes did not change the frequency of lung-infiltrating myeloid cells but create a favorable microenvironment promoting tumor spreading of a poorly metastatic cell line. Our results are consistent with the previous findings that melanoma-derived exosomes can educate BM progenitor cells toward a pro-metastatic phenotype (127) and that PDAC derived exosomes can induce the formation of liver pre-metastatic niche that foster the development of metastatic disease (128). Therefore, the detection of circulating exosome could be a promising strategy to predict tumor progression, as recently suggested by Melo and colleagues who suggested Glypican-1–expressing exosomes for early detection of pancreatic cancer (137). Our data highlight the importance of exploring the therapeutic potential of targeting tumor-derived exosomes to improve PDAC patient treatment. A first proof of concept in this field was recently suggested, purporting new treatment strategy to suppress cancer metastasis by inhibition of the pro-metastatic functions of cancer-derived EVs with antibodies (194). This strategy can be applied to prevent metastasis formation in a wide variety of tumors.

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