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REVIEW

Tumor extracellular vesicles drive metastasis (it's a long way from home)

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Abstract

Among a plethora of functions, extracellular vesicles released by primary tumors spread in the organism and reach distant organs where they can induce the formation of a premetastatic niche. This constitutes a favorable microenvironment for circulating tumor cells which facilitates their seeding and colonization. In this review, we describe the journey of extracellular vesicles (EVs) from the primary tumor to the future metastatic organ, with a focus on the mechanisms used by EVs to target organs with a specific tropism (i.e., organotropism). We then highlight important tumor EV cargos in the context of premetastatic niche formation and summarize their known effects on extracellular matrix remodeling, angiogenesis, vessel permeabilization, resident cell activation, recruitment of foreign cells, and ultimately the formation of a pro-inflammatory and immuno-tolerant microenvironment. Finally, we discuss current experimental limitations and remaining opened questions in light of metastatic diagnosis and potential therapies targeting PMN formation.

KEYWORDS

extracellular vesicles, metastasis, microenvironment, premetastatic niche

1 | INTRODUCTION

Cancer is among the most common causes of morbidity and mortality worldwide, and the vast majority of cancer-related death is due to metastasis rather than primary tumors.[1] Thus, the limitations of anti-metastasic treatments require a deeper understanding of the complex stepwise process of tumor cell dissemination toward target organs in order to design innovative therapies.[2] Metastasis is a highly inefficient process as only a very

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small proportion of tumor cells escaping primary tumors are able to successfully form micrometastatic foci in distant organs.[3,4] As they leave the primary tumor, tumor cells face hostile environments with specific and distinct properties: they need to resist harsh forces of blood or lymph shear stress, cross endothelial barriers, evade immune surveillance, settle, and finally proliferate in territories where micro-environmental properties are often distinct from their site of origin.[2,5] It is now well established that metastatic success relies on the capacity of tumor cells to adapt to these variations through cellular and metabolic plasticity. Over the past decade, however, this paradigm evolved with the identification of tumor-released factors able to modify the microenvironment at future metastatic sites before tumor cell arrival. These novel tumor-induced microenvironments are referred to as premetastatic niches (PMNs) and defined by their capacity to facilitate metastasis of circulating tumor cells (CTCs) arriving subsequently.[6] The discovery of PMNs refreshed the "seed and soil" theory established by Stephen Paget in 1889, who proposed that metastasis succeeds in organs where the local microenvironment (the soil) is favorable for tumor cells seeding and colonization (the seed).[7] It appears now that the soil can be fertilized by various types of tumor-secreted factors (reviewed in Refs [6,8]), such as growth factors,[9] cytokines,[10] and extracellular vesicles (EVs), which constitute the focus of this review.

Over the past 10 years, several studies demonstrated that tumor EVs have the capacity to spread away from the primary tumor though body fluids and reach distant organs where they can induce the formation of PMNs. EVs regroup a heterogenous collection of secreted vesicles with diameters ranging from a few nm to several μm, containing various cargos (RNAs, lipids, and proteins) and responding to a plethora of names (exosomes, microvesicles, oncosomes, and much more).[11–13] Conceptually, EVs present the advantage of harboring combinations of molecules with potential signaling properties protected or inserted within a resistant lipid bilayer.[14] Multiple evidences now show that EVs can carry functional cargo and modify the microenvironment by affecting the phenotype of their receiving cells or by altering the organization of the extracellular matrix (ECM).[14–17] Importantly, recent studies reported the capacity of EVs to mediate the communication between distant organs in several physiologic and pathologic contexts.[18–21] This raises an exciting functional potential for the high amounts of EVs present in all body fluids (average concentration 10^9 EVs/ml in human blood with important variations[22]). However, it is important to acknowledge that at this stage, the fate and function of most EVs naturally present in body fluids are far from being understood.

It is now firmly established that tumor-secreted EVs can impact multiple aspects of tumor progression such as proliferation, invasion, drug resistance, endothelial permeability, or immune response.[17,23,24] Their high heterogeneity is likely to explain the diversity of their function, their range of action (local or distant), and ultimately their impact on tumor progression (pro- or anti-tumoral). In this review, we will describe the common features of PMNs and explain how tumor EVs, and their cargo, contribute to their formation. We will discuss the diagnostic and therapeutic consequences of EVs function in PMN formation and highlight the important remaining questions (see Table 1, outstanding questions).

2 | GLOBAL FEATURES OF PREMETASTATIC NICHES

PMNs are characterized by a number of key modifications of the tissue architecture, composition, and metabolism, which facilitate CTCs arrival and expansion. So far, PMNs have been essentially described in rodent models and direct evidences of the PMNs existence in human are rare and mostly observed in sentinel lymph nodes and lungs.[6,25–27] This can be explained by the difficulty to obtain patients tissue samples from future metastatic sites. Nevertheless, PMNs have been observed in future metastatic organs of mice bearing orthotopic primary

TABLE 1 Outstanding questions

Do tumor EV subtypes and EV content evolve as tumor grows?
What is the frequency of EV release from primary tumors during tumor progression and what is the proportion of secreted EVs able to reach PMN?
Are intratumoral regions/clones identical in secreting EVs (levels and cargo)?
What is the dynamic of EVs and CTCs arrival on metastatic sites?
What is the relative contribution of EVs and other tumor-derived secreted factors to PMN?
What are the tissue-specific ligands driving EV organotropism and how can we identify them?
Once metastasis has formed, is there a permanent bi-directional exchange of EVs between primary, secondary, or tertiary tumor sites?
To what extent, do the stromal/non-tumor EVs contribute to the formation of PMN and eventually metastasis?
Are the tumor EV-induced re-programming of stromal cells a transient feature in the PMN or stable over time?
What is the balance between pro- and anti-metastatic EVs secreted by tumor cells and how can it be tuned?
What is the best strategy to target blood-borne EVs when treating metastasis?

tumors.[28–31] For instance, bone marrow lesions were observed in mice bearing mammary breast tumors, before the arrival of tumor cells.[28] However, most of our knowledge on PMN formation emerged from mouse models where PMN is induced by injection of tumor-secreted factors. Such experimental approaches provide direct evidence for the function of PMN promoting factors and opportunities to dissect the first steps of PMN formation. However, these approaches also contain inherent limits when compared to the real pathophysiologic situation, since they often rely on the repeated bolus injection of high amounts of tumor-derived factors, which unlikely mimic their natural release.

PMNs have been described in different organs such as lungs, liver, brain, lymph nodes, and bone marrow, with various associated primary tumor types (breast, pancreatic, colorectal cancer, and melanoma...).[6] The initial alteration of PMNs is believed to take place at the entry gates of the target organ, the blood vessels, which is the most efficient route for long distance communication. Several studies report the disruption of endothelial junctions, breakdown of vascular basement membranes, and ultimately permeabilization of the endothelium before the arrival of CTCs.[32,33] It is tempting to speculate that initial permeabilization of the endothelium by tumor-secreted factors triggers a positive feedback loop promoting the increased accumulation of such factors in the target organ and finally facilitating CTC extravasation. Other key features of the PMN are the activation of resident stromal cells, (such as fibroblasts or myeloid cells) and the recruitment of new cells (such as bone marrow-derived cells (BMDCs) or neutrophils) from other organs, by tumor-secreted factors.[6,34] These changes in cell phenotypes and populations will alter the homeostasis of the tissue on multiple levels: promotion of ECM remodeling, alteration of cell metabolism,[29] and triggering of a pro-inflammatory[33,35,36] and immunosuppressive environment.[33,37] ECM remodeling can be orchestrated by resident cells as fibroblasts or macrophages or by newly recruited myeloid cells.[9,31,38,39] It occurs either through the deposition of new ECM components or through the alteration of pre-existing ones (such as fibronectin, periostin, or versican among others).[9,30,31,40,41] Altered ECM composition and organization can then promote the recruitment of BMDCs as well as the homing of CTCs to the PMN.[33,34,38,42] These events are likely to constitute a second positive feedback loop contributing to the reinforcement of PMNs, as recruited BMDCs will contribute to ECM remodeling which will further promote BMDC recruitment. Finally, the activation of resident cells and recruitment of novel cells will induce the formation of a pro-inflammatory and immunosuppressive microenvironment, which will actively contribute to efficient PMN formation.[35,38,43–47]

Formation of this complex pre-metastatic environment results from the interplay between various types of tumor-secreted soluble molecules and heterogenous tumor-derived EVs. Importantly, additional external factors, such as aging, infection, cancer treatment, or surgery could directly contribute to PMN evolution. Our review is focused on the role of tumor EVs in PMN formation, but they likely function in close relationship with tumor-derived and tumor-independent factors. The journey of EVs toward the PMN is a multistep process, involving their secretion from tumor cells, their travel in blood and lymphatic circulation, their accumulation in distant organs, usually following a non-random pattern (organotropism), their exit from circulation, and their uptake by recipient cells where they prime the PMN formation (Figure 1).

3 | LEAVING THE PRIMARY TUMOR

The capacity of tumor cells to secrete high levels of pro-metastatic EVs clearly correlates with their ability to metastasize from a primary tumor.[34,48,49] For instance, depletion of genes involved in EV secretion, such as Rab27a, nSMase2, RalA, or RalB in aggressive tumor cells leads to a decrease in both the levels of secreted EVs in vitro and metastasis in vivo.[34,50–53] Importantly, the content of released EVs might even be more relevant for PMN formation than their actual number. Indeed, several studies showed that injection of an equal number of tumor EVs with different contents has different impact on PMN formation.[35,38,52] However, the heterogeneity of tumor EVs composition, in addition to the variety of documented EVs sub-populations, is far from being fully elucidated (see Table 1, outstanding questions). Therefore, it will be essential to characterize precisely the content and the amount of released EVs along tumor progression in order to define the identity of EV subtypes that directly contribute to PMN formation (see Table 1, outstanding questions). The secretion of pro-metastatic EVs is likely to vary as tumor progresses, depending on the primary tumor microenvironment. For instance, EVs secreted by tumor cells cultured in hypoxic conditions have enhanced capacities to promote PMN formation.[54,55] Importantly, the secretion of pro-metastatic EVs is enhanced when tumor cells are exposed to chemotherapeutic treatments, revealing that attempts to inhibit primary tumor can actually result in PMN priming and increased metastasis.[56,57]

Independently of their heterogeneity, the dynamics of EV release by tumors have been poorly described in vivo so far (see Table 1, outstanding questions), as it remains technically challenging to track EVs from their secretion to their uptake. It is likely that tumor EVs are

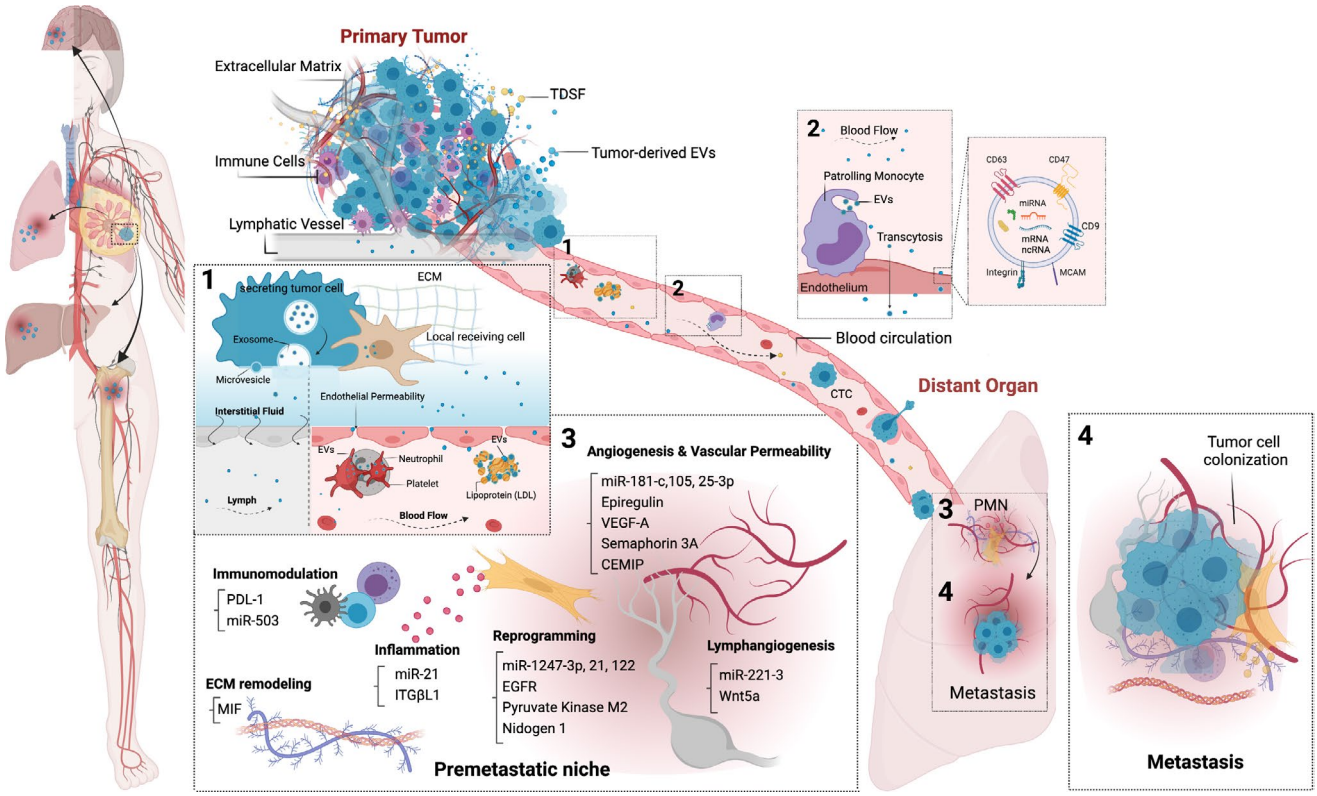

FIGURE 1 Tumor extracellular vesicles prime premetastatic niches. The journey of EVs from the primary tumor to the future metastatic organ is a multistep process initiated with the secretion of tumor-derived EVs and other tumor-derived soluble factors (TDSF) from the primary tumor. 1. Upon secretion, tumor-derived EVs leave the primary tumor and travel through the blood and lymphatic circulation, where they interact with blood components like neutrophils, endothelial cells, platelets, low-density lipoproteins (LDL), and other immune cells. These interactions affect blood homeostasis, enhance the uptake of tumor EVs by distinct recipient cells, and could induce endothelial permeabilization, thereby promoting the formation of premetastatic niche (PMN). 2. Tumor EVs are further taken up by patrolling monocytes and endothelial cells and some of the tumor EVs pass through the impermeable endothelial cells within the tissue by transcytosis. Uptake of tumor EVs by these recipient cells can directly impact the PMN formation. Inset shows a magnified tumor EV, that are encapsulated by a lipid bilayer, containing various biomolecules such as DNA, RNA, proteins as well glycans, specialized receptors at their surface (CD47, CD9, and CD63), and several adhesion proteins such as integrins and MCAM. 3. *Key features of the PMN*. Highlighted are the key features of the PMN and their associated tumor EV cargos that actively contribute to efficient PMN formation. Upon internalization by distinct recipient cells, tumor EVs deliver their cargo, induce phenotypic changes in them, thereby promoting ECM remodeling, reprogramming cell metabolism, inducing immunomodulation, angiogenesis and vascular permeability, lymphangiogenesis, and also triggering pro-inflammatory molecules. All these salient features eventually promote PMN formation. 4. Following the PMN formation, circulating tumor cells (CTCs) eventually reach the PMN and colonize in the new tissue, leading to metastasis. Highlighted in the far left, is the human women model demonstrating organotrophic metastasis, where primary breast tumor-secreted EVs prime PMN at distant organs such as lungs, brain, liver, and bone. Created with BioRender.com

secreted very early, akin to metastatic tumor cells,[58] and thereby prime PMNs before tumors can be diagnosed. Key experiments performed in mice using the Cre-lox system revealed that tumor EV transfer occurs not only at short distance between neighboring cells within the primary tumor mass but also with cells located in distant organs.[59,60] Release of EVs from the primary tumor must account for random movements in interstitial fluids, interactions with the ECM, and uptake by neighbor cells. Indeed, EVs, which often express ECM adhesion and degradation proteins at their surface, were shown to interact with distinct types of matrix and eventually remodel their organization.[40,61,62] Therefore, it is possible that only a small proportion of secreted tumor EVs reach the circulation and spread in the organism. The retention of some EVs within the primary tumors might select a sub-population of spreading EVs with specific adhesive properties. Tumor EVs can be found in blood and lymphatic circulation[63,64] (Figure 1). How they safely reach circulation has not been firmly demonstrated, but it can be speculated that they are transported by interstitial fluids to reach lymphatic vessels. Indeed, in tumors, high interstitial fluid pressure induces a convective flow from blood vessels toward the lymphatic vessels.[65] Besides,

tumor EVs could benefit from abnormally permeabilized blood vessels characteristic of tumors to reach the blood circulation.[66] Interestingly, tumor EVs bearing PMN markers are more concentrated in lymph than in blood from melanoma patients.[63,64] Besides, mice experiments revealed that lymphatic vessels are essential for tumor EVs spreading.[64] Finally, adenocarcinoma, melanoma, or gastric cancer EVs can induce PMN formation in lymph nodes.[67–69] These data suggest that tumor EVs could exploit different routes to reach distant organs and initiate PMN formation. Similarly, tumor cells can in some cases first reach the lymph node, form a first metastatic foci and then transfer to the blood circulation to seed secondary metastasis in more distant organs.[70–72] Whether tumor EVs can follow similar routes ahead of tumor cells and induce a first PMN in lymph nodes and a second one in more distant organs remains to be properly demonstrated. Therefore, in the future, a proper description of the temporal and spatial dynamics of tumor EV spreading away from primary tumors will be instrumental to properly understand the initial steps of PMN formation (see Table 1, outstanding questions).

4 | BEHAVIOR IN CIRCULATION

It is now established that tumor EVs circulate in blood and lymph vessels of cancer patients, alongside non-tumor EVs.[63,73,74] Regardless of their origin, an increase in the levels of circulating EVs or in the amount of protein per EV was reported in lymph and blood circulation of cancer patients.[34,63,75–77] Part of this increase could be directly attributed to the presence of a primary tumor rather than an indirect systemic effect, since surgical removal of the tumor tends to decrease the global levels of circulating EVs as shown for glioblastoma.[75] However, the precise proportion of tumor EVs in the circulation, and even more importantly, the proportion of tumor EVs able to induce or contribute to PMN formation are unknown. As tumor-derived EVs are 20 times more abundant than CTCs in the circulation of metastatic patients[78] a hunt for EV-associated cancer biomarkers was launched over the past years. It allowed the identification of tens of novel potential diagnosis targets, which can either be single RNAs or proteins or more complex molecular signatures.[48,79–81] Even if the clinical validation of most of these findings is still awaited, the molecular signatures carried by circulating EVs could eventually provide identification of specific cancer types, progression stages, or predict therapeutic response. In addition, the molecular study of circulating EVs in patients body fluids, if correlated with metastasis formation could contribute to a better understanding of PMNs in humans.

Despite being stable for days in serum, EVs' half-life in the circulation remain low.[82,83] Indeed, reports in mice and zebrafish show that exogenous EVs have a very short half-life (2–10 min) in the blood circulation.[82,84–86] This short circulating time is mostly explained by the rapid uptake of circulating EVs by patrolling monocytes and endothelial cells.[83,84,87] In circulation, EVs are subjected to a highly dynamic environment, defined by important biomechanical forces with unknown consequences on their biology.[5] Recently, the use of zebrafish embryo, an emerging model in cancer biology,[88–91] allowed the first in vivo description of circulating endogenous and exogenous EVs with high spatio-temporal resolution.[21,84] The distribution of circulating EVs in blood vessels follow the Poiseuille law: they circulate faster in the center of the vessel than on its margins, where they can eventually be seen rolling on the surface of the endothelium. This reduced velocity at the margin of the vessel likely drives their uptake by endothelial cells.

5 | INTERACTION WITH BLOOD COMPONENTS

Circulating tumor EVs can also interact with several blood components, such as circulating immune cells, lipoproteins, platelets, or endothelial cells, but probably not with circulating red blood cells[84,92,93] (Figure 1). These interactions can have direct consequences on blood homeostasis. For instance, several reports show that tumor EVs transport pro-coagulant factors such as tissue factor, PSGL-1, or podoplanin and promote thrombosis through interactions with platelets or with neutrophils.[94–97] The pro-thrombotic activity of tumor EVs appears to vary depending on the subtype of EV and the stage of the secreting tumor cell.[96,98] While platelet aggregation correlates with PMN formation,[99] the role of tumor EVs in this process has not yet been investigated. In addition to platelets, EVs from brain metastasis (originating from breast cancer and melanoma cells), were shown to interact with blood low-density lipoproteins and to trigger their aggregation.[92] This interaction enhances the uptake of EVs by monocytes and could, therefore, potentially affect PMN formation.

The uptake of circulating tumor EVs by endothelial cells and patrolling monocytes can directly impact PMN formation (Figure 1). Indeed, several studies report that tumor EVs induce permeabilization of the endothelium,[93,100,101] which could constitute a first step in PMN formation.[32,33] Patrolling monocytes are mostly considered anti-metastatic through their capacity to take up tumor-derived material and promote the recruitment and activation of natural killer cells.[102] Indeed, the uptake of EVs from non-metastatic tumor cells by patrolling

monocytes prevents the establishment of a PMN in the lung.[103] Similarly, in lymph nodes, sub-capsular macrophages block tumor EVs dissemination and limit tumor progression.[104] Accordingly, anti-tumor EVs induced the accumulation of patrolling monocytes to the lungs, thereby inhibiting metastasis.[105] Therefore, while the uptake of circulating tumor EVs by endothelial cells seems to mostly promote PMN formation, their uptake by patrolling monocytes prevents it. Along this line, EVs which are the most efficient at inducing PMN formation could have the capacity to escape patrolling monocytes surveillance. This could be achieved by specialized receptors at the surface of EVs, as for instance, the glycoprotein CD47 limits their uptake by patrolling monocytes.[87] Alternatively, PMN-efficient EVs could be taken up by patrolling monocyte and modify their phenotype to the benefit of PMN formation, for instance, by promoting TNF-α expression and inducing a pro-inflammatory environment.[84]

Altogether, these studies suggest that the interactions of tumor EVs with various circulating factors have direct consequences on PMN formation.

6 | ORGAN TARGETING

Deciphering the mechanisms controlling the biodistribution of tumor EVs is essential to understand the early steps of PMN formation. To date, this question has been mostly tackled by tracking pre-labeled exogenous EVs injected as a bolus in mouse circulation. This approach has some limitations since the injection site and the labeling method of EVs can impact their biodistribution.[106,107] Nevertheless, a recent study showed that prostate cancer EVs injected in the circulation reach the bone marrow similar to CD63-GFP EVs secreted by orthotopic grafted tumor cells.[108] Importantly, EVs from different cell types tend to accumulate in different organs and in general injected EVs do not arrest at the first capillary bed they encounter, suggesting the existence of specific targeting or retention mechanisms.[106] Indeed, an increasing number of studies demonstrated the existence of tumor EVs organotropism by showing that they accumulate preferentially in the organs where their secreting cells mostly form metastasis.[35,46,52,109,110] Similar to tumor cell organotropism, tumor EV organotropism could be dictated by a balance between hemodynamics, vascular patterns, and intrinsic adhesive properties.[5,111] Accordingly, circulating EVs were shown to accumulate mostly in vascular regions with a low blood flow speed in zebrafish embryo.[21,84] However, the precise contribution of hemodynamics in EVs biodistribution has not been elucidated yet. In contrast, several adhesion proteins, such as integrins, MCAM/CD146, and tetraspanins Tspan8 and CD151 were shown to mediate EV biodistribution and PMN fo rmation.[35,52,110,112–114] Depletion of these receptors or inhibition of their adhesive properties alters EVs biodistribution and their capacity to form PMNs in mice models. For example, the presence of integrin β_4 on breast tumor EVs is necessary for their lung accumulation.[35] Strikingly, forced expression of integrin β_4 on tumor EVs which normally accumulate in bones is sufficient to promote their lung tropism.[35] In addition to the identity of these adhesion proteins, their posttranslational modifications could contribute to EV organotropism, since the global levels of glycosylation on EVs were recently shown to impact their biodistribution.[115] Although this has not been formally demonstrated yet, the combination of adhesion molecules present at the surface of tumor EVs may define a zip-code for EV organotropism. Supporting this hypothesis, it was shown that the co-expression of a tetraspanin (Tspan8) with an integrin (ITGα_4) defines the novel biodistribution of pancreatic adenocarcinoma EVs in rats.[116] Importantly, although ligands of integrins, tetraspanins, or CD146 have been characterized in various contexts, their identity in EV organotropism and PMN formation has not been revealed. This is a crucial question since the receptor-mediated EV organotropism hypothesis implies the existence of organ-specific differentially enriched ligands (see Table 1, outstanding questions). Finally, while CTCs and immune cells often exploit low and high affinity receptors to engage and subsequently stabilize their adhesion,[117,118] more work is needed to identify whether such scenario is at play for EVs.

7 | MECHANISMS OF PMN PRIMING BY TUMOR EVS

Once they have reached their target organ, tumor EVs initiate most of the microenvironmental changes observed in PMNs and described earlier (Figure 1). In this section, we will review the mechanisms triggered by tumor EVs, identify the major EV cargos, and describe the subsequent chain of events leading to PMN formation.

7.1 | Vascular permeability and angiogenesis

Tumor EVs internalized by endothelial cells were reported to promote endothelial permeability through different molecular pathways triggered by their miRNAs or protein cargos. For instance, miRNAs miR-105 and miR-25-3p, respectively, present in EVs from breast or colorectal cancer cells, induce a direct or indirect decrease in the expression of tight junction components, which

leads to endothelial permeability, PMN formation, and ultimately to increased metastasis in the liver, lung, and brain of mice.[101,119] Another miRNA, miR-181c, present in EVs from breast cancer metastatic cells, downregulates the actin regulator PDPK1 and disrupts endothelial cell–cell junctions in the blood–brain barrier, thereby leading to an increased brain metastatic.[93] Besides, several tumor EV protein cargos, such as semaphorin3A, epiregulin, or VEGF-A are responsible for blood vessel permeability in distant organs.[100,120,121]

In addition, tumor-derived EVs facilitate PMN formation by promoting angiogenesis in distant organs in the absence of tumor cells.[121–123] For instance, several EV cargos, such as CEMIP, epiregulin, or VEGF-A have the capacity to induce vascular remodeling in brain or lung PMNs.[120,121,123] CEMIP-induced angiogenesis leads to the formation of pro-inflammatory peri-vascular niches where colonizing tumor cells accumulate.[123] Similar to their action on blood vessels, tumor EVs can affect lymphatic vessels and promote lymphangiogenesis in lymph nodes.[124–126] For instance, miR-221-3p enriched in EVs derived from cervical squamous carcinoma cells, induces the downregulation of the lymphangiogenesis inhibitor vasohibin-1 in lymphatic endothelial cells, thereby promoting lymph PMN and metastasis.[125] A more indirect role was described for EVs from colorectal cancer cells, which can induce the expression of VEGF-C by macrophages in an IRF-2-dependent manner. In turn VEGF-C promotes remodeling of the lymphatic network and subsequently facilitates lymph node metastasis.[124] Wnt5a present in EVs from gastric cancer activates the YAP transcription factor in bone marrow-derived mesenchymal stem cells, leading to enhanced lymphangiogenesis and PMN formation.[126] Altogether, these studies show that various cargos present in EVs from different tumor origins tune the vascular and lymphatic systems at multiple future metastatic sites.

7.2 | Matrix remodeling

Increased endothelial permeability would allow circulating EVs to cross more easily the endothelial barrier and accumulate in the target organ. In addition, tumor EVs can be transported throughout impermeable endothelial vessels by transcytosis and be released within the tissue.[127] Tumor EVs contain multiple adhesion receptors and matrix metalloproteases which allow them to bind different types of ECMs and directly alter their composition and their organization in PMNs.[40,112] In addition, tumor EVs indirectly induce matrix remodeling by activating resident cells. For instance, breast cancer or pancreatic ductal adenocarcinoma EVs activate lung fibroblasts and promote fibronectin secretion and reorganization.[31,35] Similarly, tumor EVs RNA cargos promote Toll-like receptor3 (TLR3)-dependent secretion of fibronectin by lung alveolar epithelial cells.[43] Costa-Silva and colleagues described a complete cascade of events starting with pancreatic cancer EVs and ending in the reorganization of three ECM components (vitronectin, Tenascin C, and fibronectin) and the formation of a pro-inflammatory microenvironment.[38] In brief, macrophage migration inhibitory factor (MIF) contained in tumor EVs induces the secretion of TGF-β by Kupffer cells, which in turn promotes the production of fibronectin by hepatic stellate cells and ultimately the recruitment of bone marrow-derived macrophages to the liver.[38] Moreover, osteosarcoma EVs-associated TGFβ1, upon internalization by lung fibroblasts-induced pulmonary fibroblast differentiation and upregulated variety of ECM components that promoted invasive competence of these cells and tumor progression in distant PMN.[128] Interestingly, depending on the tumor subtype they come from, breast cancer EVs can induce different compositions of ECM in lung PMNs.[30] Therefore, tumor EVs are clear regulators of ECM remodeling in PMNs. This can directly affect the cell composition of PMNs by contributing to the recruitment of various immune cells[42] and favoring tumor cell colonization.

7.3 | Activation of tissue-resident cells

As described above, activation of fibroblasts or macrophages by tumor EVs can lead to ECM modification remodeling. In addition, tumor EVs can modify the cytokine and growth factor secretion pattern of resident stromal cells. EVs from hepatocellular carcinoma, for instance, can induce the activation of cancer-associated fibroblasts (CAFs) in the lung PMN through two different mechanisms: via miR-1247-3p and the activation of NF-κB signaling pathway[129] or by Nidogen 1 and TNFR1 secretion.[130] CAF activation results in the secretion of pro-inflammatory cytokines which contribute to PMN establishment.[129,131] Activation of resident fibroblasts by tumor EVs could induce a positive feedback loop, as CAFs EVs can further promote PMN formation in lungs.[132] Other resident cells can be activated by tumor EVs, depending on the organ. In bone marrow, for instance, the transfer of pyruvate kinase M2 from prostate cancer EVs to bone marrow stromal cells leads to an increased secretion of CXCL12, which sustains prostate cancer cell growth and metastasis.[108] Likewise, the transfer of miR-21 from breast cancer EVs to osteoclasts triggers their differentiation and activation, favoring the establishment of bone metastasis in breast cancer model.[133] In the liver, EGFR-loaded EVs drive the expression of hepatocyte growth factor (HGF) in stromal cells, which further promotes liver metastasis.[134]

In addition, recent studies showed that tumor EVs contain regulators of metabolism and have the capacity to modulate the metabolism in PMNs.[29] For instance, miR-122 downregulates the glycolytic enzyme pyruvate kinase in lung stromal cells during breast cancer metastasis. The decrease of glucose uptake by stromal cells results in an increase in nutrient availability for tumor cells, thereby promoting metastasis.[29]

7.4 | Pro-inflammatory environment

EV-dependent activation of resident cells (and recruited immune cells) induces the formation of a pro-inflammatory microenvironment in various PMNs[34,35,38,135] For example, the arrival of melanoma EVs to the lungs favors the expression of pro-inflammatory molecules TNF, S100A8 and S100A9, which lead to BMDCs recruitment to the lung PMNs.[34] A similar increase of S100 proteins was observed in lung and liver PMNs.[35] In addition, secretion of pro-inflammatory cytokines can be induced by tumor EVs.[131,135,136] For instance, secretion of the IL6 by resident macrophages is increased by miR-21 containing tEVs from colorectal cancer cells in liver PMNs.[136] IL6 and IL8 can also be secreted by fibroblasts activated by integrin beta-like 1 enriched EVs from colorectal cancer cells through a TNFAIP3-mediated NF-κβ signaling pathway.[131] Altogether, EVs orchestrate the formation of an inflammatory environment that is a hallmark of PMN.

7.5 | Cells recruitment to PMN

Another important hallmark of PMNs consists in the recruitment of cells from other organs. Originally, pioneer work from the group of D. Lyden demonstrated that inflammation in PMNs leads to the recruitment of BMDCs.[34] Since then, different types of immune cells were shown to be recruited to PMNs. For instance, monocytes can be recruited to PMNs by tumor EV-induced upregulation of CCL2 in resident macrophages or endothelial cells.[57,137] Additionally, activation of alveolar epithelial cells by small nuclear RNA melanoma EVs leads to an enhanced secretion of cytokines which promotes neutrophil recruitment to the lung PMN.[43] Neutrophils can suppress anti-tumor immunity, create an inflammatory microenvironment, retain CTCs in the organ vasculature, and promote their colonization.[138–141]

7.6 | Immunomodulation

Tumor EVs have antagonist effects on the immune system, as they can both deliver tumor antigens to antigen presenting cells, thereby activating the immune system, but also suppress the anti-tumor immune response by targeting various immune cells.[24] While most studies focused on primary tumors, some evidences show that tumor EVs modulate both innate and adaptative immunity in PMN.[34,46,104,142] For instance, breast cancer EVs promote the accumulation of BMDCs, directly inhibit T-cell growth, and decrease Natural killer (NK) cell cytotoxicity leading to the formation of an immunosuppressive environment in lung PMN.[46] Interestingly, intravital imaging revealed that extravasating tumor cells release large EVs which are taken up by different myeloid cells arriving sequentially at the metastatic site. This EV uptake induces phenotypic changes in receiving immune cells and promotes metastasis.[143] Besides tumor EVs have the capacity to mediate immune suppression, notably through the PD-1–PD-L1 axis.[24] Indeed, metastatic melanoma EVs carrying programmed death-ligand 1 (PD-L1) on their surface have the capacity to inhibit anti-tumoral CD8 T-cell function and promote tumor progression.[144] A recent study suggests that breast cancer EVs carrying miR-503 promote the M1–M2 conversion of microglia, which results in enhanced PD-L1 expression and suppression of local immunity in brain metastasis.[145] Overall, the balance between pro- and anti-tumor roles of EVs on distant immune cell populations remains to be fully investigated as it could open novel therapeutic avenues.

8 | CONCLUSIONS

Although the understanding of PMNs considerably progressed since their initial description in 2005, a large number of fundamental questions remain opened (see Table 1, outstanding questions).

First of all, the existence of PMNs implies that tumor-secreted factors, including EVs, reach distant organs before the arrival of CTCs. Although the exact timing and dynamics have not been solved, some experiments using orthotopic primary tumors show the localization of tumor EVs and/or distant microenvironmental changes happening before tumor cells could be detected.[28,29,31,108] However, this sequence of events has not been firmly proven in a relevant orthotopic spontaneous tumor model. This is important in particular because tumor cell spreading to future metastatic sites was shown to be an early event in several types of cancer.[146–148] While early disseminating CTCs mostly enter dormancy,[149] it could be speculated that disseminating tumor EVs instead or in addition to altering the distant microenvironment before tumor cell arrival, are also able to help awakening rare dormant tumor cells already present on site. This is appealing since dormant cell often reside in perivascular niches,[150] where

they would be in good position to receive circulating EVs and soluble factors. Interestingly, recent studies showed that EVs from stromal cells have the capacity to mediate tumor cell dormancy.[151–155] Whether EVs shed by primary tumors can also perturb tumor cell dormancy remains to be explored, yet such discoveries would provide exciting treatment options for counteracting the major issue of tumor cell dormancy. More generally, determining the relative dynamics of EVs and cell release from primary tumors is essential for the definition and the understanding of PMN formation, but also to design adapted therapeutic strategies.[156]

Along the same line, it will be essential to describe the dynamics of EVs release during tumor progression, and its impact on driving efficient PMNs. Notably, whether tumor EVs continue to land to metastatic sites once metastasis has started is not known, yet this is likely to happen. While metastatic growth surely benefits from permanent feeding by EVs released from the primary tumor, metastatic outgrowth might feedback on the primary tumor, akin to metastatic cells. Indeed, the communication between primary and secondary tumor sites is not unidirectional as tumor cells from metastasis can recolonize primary tumors, in a process called tumor self-seeding.[157] In addition, tumor cells were shown to re-disseminate from metastatic to tertiary sites[158] raising the possibility that EVs from metastatic foci can prime additional PMNs. Interestingly, studies report that tumor EVs, either injected in the circulation or co-incubated with tumor cells before injection, promote tumor self-seeding in mice.[159,160] If it is not known yet whether EVs from metastatic sites can target primary tumors, Zomer and colleagues made elegant use of in vivo imaging to show that two distinct primary tumor sites can exchange EVs.[60] Such intravital imaging of EVs shuttling in relevant metastasis models would undoubtedly help addressing these issues and increase our understanding of the (bio)genesis of PMNs.[60,104,161,162]

PMN formation is induced by a complex interplay of soluble molecules and EVs, whose precise orchestration remains to be understood. For this, it will be essential to characterize the heterogeneity of EVs released by primary tumors, as they have antagonist effects on PMN formation, notably by inducing differential immune responses. It would be particularly interesting to link EVs heterogeneity to intratumor heterogeneity which is a key driver of therapy resistance and metastasis[163] and to document the impact of EVs released in this difficult context of therapy resistance. In addition, while significant progress has been made in understanding the identity and position of cells that have metastatic potential within tumors, whether similar regions and cellular identity correlated with EV secretion potential and function would be an exciting area of research (see Table 1, outstanding questions). It will be equally important to fully characterize EVs secreted by non-tumoral cells, which populate, react, and participate to tumor growth, as they also play a significant role in PMN formation.[132,164] Additionally, exogenous EVs, such as bovine milk-derived EVs, could directly impact metastasis.[165] Finally, tumor-secreted EVs are not always sufficient to induce PMN formation and require the additional contribution of tumor-secreted factors.[128] Therefore, the relative contribution of tumor released soluble factors and EVs and their potential cooperation will have to be studied in detail (see Table 1, outstanding questions). Interestingly, the interaction between tumor EVs and cytokines, in particular CCL2, was recently shown to modify their organotropism, the formation of PMNs, and finally lung metastasis.[142]

While several clinical trials aiming to block PMN formation are already undergoing,[6] a fine understanding of the contribution of tumor EVs to PMN formation could pave the way for novel therapeutic approaches. For instance, it could be possible to inhibit EV secretion from primary tumors, since this approach decreases metastasis in mice.[34,50–53] Alternatively, tuning the balance of pro- versus anti-tumoral EVs released by tumor cells could improve the anti-tumoral immune response (see Table 1, outstanding questions). Another exciting, yet tricky, possibility would be to target and stop tumor EVs in the circulation. In a recent study, Nishida-Aoki and colleagues showed that intravenous injection of anti-humanCD9 or anti-humanCD63 antibodies decreases lung metastasis in mice bearing orthotopic breast xenografts.[166] Therefore, the identification of tumor EVs-specific surface proteins would allow to distinguish them from non-tumoral ones and would constitute ideal candidates for such therapeutic approaches. Finally, targeting the mechanisms of pro-metastatic tumor EVs uptake by resident stromal cells constitutes a promising possibility to prevent metastasis, as shown in mice where reserpine suppresses tumor EV uptake and disrupts PMN formation.[167]

Altogether, tumor EVs are central players in PMN formation and constitute diagnostic and therapeutic targets to detect and treat metastasis progression.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

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