Pre-metastatic cancer exosomes induce immune surveillance by patrolling monocytes at the metastatic niche

Michael P. Plebanek¹,², Nicholas L. Angeloni¹,², Elena Vinokour¹, Jia Li¹, Anna Henkin³, Dalia Martinez-Marin⁴, Stephanie Filleur⁴, Reshma Bhowmick⁵, Jack Henkin⁶, Stephen D. Miller⁷,⁸, Igal Ifergan⁷,⁸, Yesung Lee⁹,¹⁰, Iman Osman⁹,¹⁰, C. Shad Thaxton¹,²,¹¹ & Olga V. Volpert⁵

Metastatic cancers produce exosomes that condition pre-metastatic niches in remote microenvironments to favor metastasis. In contrast, here we show that exosomes from poorly metastatic melanoma cells can potently inhibit metastasis to the lung. These “non-metastatic” exosomes stimulate an innate immune response through the expansion of Ly6C<sub>low</sub> patrolling monocytes (PMo) in the bone marrow, which then cause cancer cell clearance at the pre-metastatic niche, via the recruitment of NK cells and TRAIL-dependent killing of melanoma cells by macrophages. These events require the induction of the Nr4a1 transcription factor and are dependent on pigment epithelium-derived factor (PEDF) on the outer surface of exosomes. Importantly, exosomes isolated from patients with non-metastatic primary melanomas have a similar ability to suppress lung metastasis. This study thus demonstrates that pre-metastatic tumors produce exosomes, which elicit a broad range of PMo-reliant innate immune responses via trigger(s) of immune surveillance, causing cancer cell clearance at the pre-metastatic niche.
Exosomes are 30–150 nm membranous extracellular vesicles (EVs) released by most cells, which are found in biological fluids and play pivotal roles in long-distance intercellular communications. Exosomes are derived from the multi-vesicular endosome pathway, through reverse inward budding; however, the term is generally applied to the small EVs and does not discriminate between endosome and plasma membrane derived EVs. Exosomes contain and transfer multiple bioactive molecules including nucleic acids (DNA, mRNA, non-coding RNAs), proteins, and lipids. Typically exosomal membranes are enriched in tetraspanins, such as CD9, CD63, and CD81, and the proteins involved in endocytosis and cargo sorting, such as flotillin and TSG101. By transferring bioactive molecules exosomes alter the function of recipient cells; in particular, cancer cell-derived exosomes have been shown to transfer oncogetic traits from aggressive to indolent cancer cells and to normal cells through the delivery of oncogenic proteins, mRNAs, and miRNAs that inhibit tumor-suppressive factors, accelerate tumorigenesis, and enable tumor formation. Cancer-derived exosomes also support tumor progression by facilitating angiogenesis, modulating the immune system, and remodeling tumor parenchyma. Clinically, circulating EVs isolated from cancer patients have been associated with metastasis or relapse, and therefore could serve as important diagnostic and prognostic markers as well as therapeutic targets. The reverse is also true: exosome-assisted transfer of unshielded non-coding RNA from cancer-associated fibroblasts to the cancer cells stimulates pattern recognition response and subsequently tumor progression and therapy resistance. Among exosome-mediated effects, which contribute to metastatic dissemination is proteolysis-dependent matrix remodeling and epithelial-to-mesenchymal transition.

Intercellular communications via exosomes are particularly important for the formation of the metastatic niche where exosomes alter the behavior of diverse cell types including the cells of immune system. Exosomes are found in most bodily fluids including blood, urine, and saliva. Recently, it has been established that exosomes released into circulation from the primary tumor generate suitable microenvironments in secondary organs prior to the dissemination of metastases. Despite the clear importance of exosomes to cancer progression, mechanisms by which they promote the metastatic niche are extremely complex and not fully understood, with multiple factors at play. Exosome release from hypoxic tumors results in elevated angiogenesis and vascular leakage. Exosome also promote coagulation and thus increase adherence of circulating tumor cells. Cancer-derived exosomes are also thought to be involved in the suppression of innate immune responses through mobilization of the myeloid-derived suppressor cells, activation of the tumor-associated macrophages, and neutrophils. In addition, cancer exosomes can cause NK cell dysfunction by exposing NKGD ligands and hamper adaptive immune responses by repressing antigen-presenting cells and cytotoxic T cells (blocking T cell activation, proliferation, and enhancement of T cell apoptosis).

Monocytes and macrophages are essential constituents of the metastatic microenvironments, where they play either tumor-promoting or tumor-suppressive roles, depending on their activation state (polarization). Non-classical or patrolling Ly6C low monocytes (PMo) (CD14dim in humans) were initially identified for their ability to remove damaged cells/tissues and resolve the vascular inflammatory response. For their survival, PMo require the orphan nuclear receptor Nr4a1 (Nur77). Recently, Nr4a1-positive PMo have been shown to scavenge tumor cells and thus reduce metastasis in the lungs. However, the events that regulate the number of PMo at the metastatic niche remain unclear. Here, we show that exosomes released from non-metastatic melanoma cells (ExoNM) are taken up by CD11b+ myeloid cells in the bone marrow (BM) and cause a Nr4a1-driven expansion of Ly6C low monocytes, which display elevated levels of integrin-b2 (ITGB2) and CX3CR1 (fractalkine receptor), and Nr4a1 orphan nuclear receptor, which together define PMo. Pigment epithelium-derived factor (PEDF) is known for its potent anti-angiogenic and anti-cancer effects. In melanoma, the loss of PEDF promotes early invasive melanoma growth, ameboid motility, and metastasis. PEDF is also implicated in the control of inflammation and macrophage polarization; however, the underlying molecular mechanisms are unknown. Here, we demonstrate that PEDF is present at high levels on the surface of exosomes from non-metastatic melanoma cells and its presence is critical for the activation of an innate immune response and elimination of melanoma metastasis. The events triggered by exosomes involve Nr4a1 induction in BM monocytes precursors, leading to PMo expansion, recruitment, and differentiation of TRAIL-positive tumor-reactive macrophages, which kill and phagocytize the tumor cells. PMo, together with NK cells, are responsible for the diminished metastasis as is shown by immune cell depletion experiments. Our results suggest that pre-metastatic tumors generate triggers of innate immune response(s) such as PEDF, which are delivered to the cells of the immune system by exosomes; the loss of these triggers enables immune-suppression and abrogates the immune clearance of cancer cells leading to metastasis.

Results
Non-metastatic exosomes block experimental lung metastasis. Exosomes produced by metastatic melanoma cells are known to contribute to the formation of the pre-metastatic niche, which leads to colonization by circulating tumor cells. We sought to determine whether exosomes from non-aggressive, poorly metastatic melanomas (“non-metastatic” exosomes) could influence the pre-metastatic niche by comparing exosomes from metastatic and non-metastatic variants of the mouse (B16F10) and human (A375) melanoma cell lines (ExoM and ExoNM, respectively). Metastatic and non-metastatic variants of mouse (B16) and human (A375) melanoma cell lines were generated by expression of a type 2 tumor suppressor PEDF46,47 (see Methods). ExoM and ExoNM display similar size distribution, morphology, and molecular composition, as is shown by transmission electron microscopy (TEM, Fig. 1a), dynamic light scattering, nanotracking analysis (Supplementary Fig. 1a–d), and by Western blotting (Fig. 1b and Supplementary Fig. 2). ExoM and ExoNM contain similar amounts of exosomal markers CD63, CD81, and TSG101, while lacking markers that are typically not found in exosomes (Golgi marker GM130 and the nuclear marker TATA binding protein, TBP), confirming the purity of the isolates. However, these exosomes have dramatically different functional effects on melanoma metastasis. C57BL/6 mice preconditioned with ExoM from B16F10 cells and inoculated with syngeneic B16F10 pigmented melanoma cells (Supplementary Fig. 3a) present with a significant increase in the metastatic burden over the baseline (no pre-treatment). In contrast, pre-conditioning with ExoNM significantly reduces metastasis (Fig. 1c, d). In agreement, athymic nude mice preconditioned with intravenous ExoM injections prior to the tail vein inoculation of fluorophore-tagged A375 melanoma cells display significant metastatic burden after 9 weeks and identical pre-treatment with ExoNM decreases metastasis by ~10-fold (Supplementary Fig. 3b–d). These data demonstrate that “non-metastatic” exosomes have a capacity to inhibit lung metastasis. Lung colonization by metastasizing cancer cells is influenced by multiple factors, including early events (extravasation, innate
ExoNM inhibit lung colonization by metastatic melanoma. a TEM of exosomes isolated by differential ultracentrifugation from metastatic and non-metastatic subcultures of mouse B16F10 melanoma cells. Note similar size and morphology of the ExoM and ExoNM preparations. Scale bar, 50 nm. b Western blot of cell lysates and exosomes (Exo) from metastatic (M) and non-metastatic (NM) melanoma cells, probed for ESCRT protein TSG101 and tetraspanins CD63 and CD81, to verify for enrichment in exosome preparation and GM130 and TBP (negative markers). c Lung colonization assay: C57BL/6 mice pre-conditioned with ExoM or ExoNM or untreated controls were inoculated intravenously with B16F10 melanoma cells (n = 4). Gross images of the tumor burden 2-week post inoculation are shown. d Quantification of the lung colonies in e. *P < 0.05 and ***P < 0.001 by pairwise two-tailed Student’s t-test (n = 4). Mean and s.d. values are shown. e C57BL/6 mice preconditioned with ExoM, ExoNM, or untreated controls were inoculated with fluorescently tagged B16F10 cells. Fluorescence images of the lungs were taken 3 and 24 h after inoculation to assess perfusion and extravasation, respectively. Scale bar, 100 μm. f Quantification of extravasated fluorescent B16F10 cells in e. *P < 0.05, **P < 0.01, and ***P < 0.001 calculated by pairwise two-tailed t-test; n = 4 mice per group, with a minimum of five random images per lung evaluated. Mean and s.d. values are shown.

ExoNM increase the PMo population in the lungs. Previous studies show that exosomes home to specific metastatic niches, e.g., the lungs. We evaluated the biodistribution of ExoNM and ExoM labeled with fluorescent lipophilic dyes (DiI, DiD). In agreement with previous studies, exosomes home to the lungs and select lymph nodes in tumor-free and in tumor-bearing mice; in mice bearing subcutaneous B16F10 tumors, exosomes are also found in the liver and spleen (Supplementary Fig. 5a, b). Importantly, a large proportion of exosomes homes to the BM (Fig. 2a and Supplementary Fig. 5c). We have noted no significant differences between the distribution patterns of ExoM and ExoNM.

To identify target BM cell population(s), C57BL/6 mice were injected intravenously with DiD-labeled exosomes, and BM cells were isolated and stained for the myeloid markers CD11b and Gr1. FACS analysis shows enrichment in DiD-labeled exosomes in the CD11b+Gr1+ cell population (Fig. 2a). Noteworthy, exosome treatment results in a pronounced shift in the population distribution of the BM: in the total myeloid population, ExoNM cause a significant increase of the Ly6C<sup>low</sup> subpopulation, and a coordinate decrease in Ly6C<sup>high</sup> cells (Fig. 2b). Among Ly6C<sup>low</sup> cells are non-classical (patrolling) monocytes (PMo), which are critical for the resolution of inflammation and tissue damage and whose anti-metastatic function has been recently discovered. PMo have been shown to rely on the orphan nuclear receptor Nr4a1 for survival. In agreement, ExoNM but not ExoM cause greater than 3-fold increase in Nr4a1 expression in the cultured primary mouse BM monocytes (Fig. 2c, d and Supplementary Fig. 6) compared to the baseline levels.

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Furthermore, PMo express high levels of CX3CR1, which mediates their interactions with the vasculature\textsuperscript{36,39} and anti-metastatic activity\textsuperscript{37}. This led us to assess the CX3CR1\textsuperscript{+} monocytes in the lungs of mice after pre-treatment with Exo\textsuperscript{NM} or Exo\textsuperscript{M}. Flow cytometry analysis reveals low numbers of CD45\textsuperscript{+}CX3CR1\textsuperscript{+} cells in the lungs of mice treated with control vehicle or Exo\textsuperscript{M}. In contrast, there is a significant increase in CX3CR1\textsuperscript{+}CD45\textsuperscript{+} monocytes in the lungs of mice treated with Exo\textsuperscript{NM} (Fig. 2e, f). The results are confirmed by immunofluorescence (IF) staining of the lung sections of mice treated with control vehicle, Exo\textsuperscript{M} or Exo\textsuperscript{NM} for PMo marker Nr4a1 (Fig. 2g, h).

PMo are characterized by their ability to “crawl” along the vasculature in a process which requires the LFA-1 antigen, a complex of CD11a and integrin β2 (ITGB2). Consistent with the shift toward PMo phenotype, Exo\textsuperscript{NM} upregulates ITGB2 in THP-1 monocytes (Supplementary Fig. 7).

Monocytes are necessary for anti-metastatic action of Exo\textsuperscript{NM}. Because melanoma exosomes target cells of the monocytic lineage in the BM (Fig. 2a, Supplementary Fig. 5c), we assessed the contribution of monocytes and/or macrophages to the anti-metastatic effect of Exo\textsuperscript{NM} by measuring tumor cell extravasation in the lungs of mice subjected to monocyte depletion using liposome-encapsulated clodronate\textsuperscript{53, 54}. The depletion is confirmed by staining of the lung tissue for the pan-macrophage (F4/80) and myeloid (CD11b) markers (Fig. 3a and Supplementary Fig. 8a, b, respectively). After monocyte depletion, animals were pre-conditioned with Exo\textsuperscript{NM} for 72 h prior to sacrifice.

Fig. 2 Exo\textsuperscript{NM} increase PMo population. C57BL/6 mice were treated with Exo\textsuperscript{M} or Exo\textsuperscript{NM}, prior to BM isolation. a Flow cytometry shows the uptake of fluoroephore-tagged exosomes by the myeloid cells in the BM. Left: Flow plots of the total CD11b\textsuperscript{+}Gr1\textsuperscript{+} BM populations. Right: Plots show a higher percentage of CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells among the BM cells positive for exosome uptake. As a representative of three independent experiments is shown. b Treatment with Exo\textsuperscript{NM} leads to an increase in Ly6C\textsuperscript{med}CD11b\textsuperscript{+} cells compared to Exo\textsuperscript{M} in the total myeloid population. c qRT-PCR of mRNA encoding Nr4a1. Mouse BM monocytes were treated 24 h with exosomes (10 μg ml\textsuperscript{-1}). The experiment was performed in biological triplicates and technical replicates of two. *P < 0.05 by t-test in pairwise comparisons. Averages and s.d. values shown. d Western blot shows an increase in Nr4a1 in mouse BM monocytes treated with Exo\textsuperscript{NM}. Quantification was performed using ImageJ (National Institutes of Health), utilizing plot profile (areas under the peaks) and integrated density measures. A representative of three experiments is shown. e-h Exosomes increase the presence of PMo in the lung. C57BL/6 mice were treated with 10 μg Exo\textsuperscript{M} or Exo\textsuperscript{NM} 24 h prior to sacrifice. CX3CR1\textsuperscript{+} cells present in the lung were detected by FACS or in situ IF. e Mouse lungs were lavaged, washed through a strainer and single-cell suspension stained for CD11b, CD45, and CX3CR1. FACS plots were gated for CD11b\textsuperscript{+}CD45\textsuperscript{+} cells and CX3CR1\textsuperscript{+} cells quantified. f Quantification of the data shown in a (percent CX3CR1\textsuperscript{+}of total CD45\textsuperscript{+} cells). *P < 0.05 calculated as above. Mean and s.d. values are shown. g IF analysis shows the increased abundance of Nr4a1\textsuperscript{+} cells in the lungs of mice treated with Exo\textsuperscript{NM}. Scale bar, 100 μm. h Quantification of the data in g, expressed as number of Nr4a1\textsuperscript{+} cells field\textsuperscript{-1}. Scale bar: 50 μm. n = 3 mice per group, five images per section. *P < 0.05, **P < 0.001, and ***P < 0.0005 by pairwise two-tailed t-test; n = 5 mice per group, >5 random images per lung. Mean and s.d. values are shown.
ExoM or ExoNM (Fluorescence and Nomarski image overlays). Representative frames are shown. depletions. Representative lung images (5× magnification) are shown.

ExoNM elicit immune response in part via the NK cells and play a critical role in preventing metastasis by recognizing tumor cell ligands resulting in the clearance of circulating tumor cells. Since PMo in some instances differentiate into phagocytic macrophages, we tested whether ExoNM are also capable of inducing macrophage differentiation and tumor cell engulfment. Mouse macrophages (RAW 264.7) were treated with exosomes and their differentiation was assessed as the cumulative macrophage area and length of the processes. Indeed, ExoNM but not ExoM elicits pronounced macrophage differentiation manifested by cell spreading and formation of multiple dendrite-like processes. A recent study showed that PMo can control metastasis in part by engulfing cancer cells. In agreement, the exposure of RAW 264.7 macrophages to ExoNM but not ExoM markedly increases their ability to engulf fluorescence-tagged melanoma cells.

**ExoNM elicit immune response in part via the NK cells.** NK cells play a critical role in preventing metastasis by recognizing tumor cell ligands resulting in the clearance of circulating tumor cells and PMo have been previously shown to recruit NK cells (by producing CCL3/4/5) to the sites of metastasis where they eliminate cancer cells. To test whether ExoNM elicit similar events, we depleted NK cells using pre-treatment with an anti-Asialo-GM1 antibody prior to exosome treatment and tumor cell inoculation. NK cell depletion significantly reduces the propensity of ExoNM to inhibit lung colonization in the extravasation assay and similar to monocyte depletion experiments, the reversal of ExoNM effects by NK cell depletion is not absolute, suggesting other mechanisms in addition to NK recruitment causing metastasis prevention by ExoNM.

**ExoNM contain surface PEDF.** In previous studies by our laboratory as well as other groups, it was established that PEDF renders melanoma cells non-metastatic. Importantly, PEDF is also expressed by most non-metastatic cell lines and its expression in patient samples correlates with metastatic dissemination. To determine if PEDF also plays a role in the exosome-mediated inhibition of metastasis, we assessed the PEDF levels in exosomes harvested from melanoma lines with forced expression in exosomes correlates with cytoplasmic levels in the source cells (Supplementary Fig. 11a, c). To further confirm that PEDF localizes specifically to exosomes, exosomes expressing A375 melanoma cells were isolated using sucrose gradient ultracentrifugation. PEDF is detected at densities ranging from 1.08 to 1.17 g ml⁻¹, where exosomes are concentrated as is reproducible decrease in lung colonization. Of note, the decrease in microscopic colonies due to ExoNM is relieved by the clodronate but not by control PBS liposomes (Fig. 3b, c). In contrast, the increased colonization in response to ExoM remains unaffected (Fig. 3b, c). These results suggest a critical role for the cells of monocytic lineage specifically in the anti-metastatic function of ExoNM.

**ExoNM induce macrophage differentiation and phagocytosis.** Since PMo in some instances differentiate into phagocytic macrophages, we tested whether ExoNM are also capable of inducing macrophage differentiation and tumor cell engulfment. Mouse macrophages (RAW 264.7) were treated with exosomes and their differentiation was assessed as the cumulative macrophage area and length of the processes. Indeed, ExoNM but not ExoM elicits pronounced macrophage differentiation manifested by cell spreading and formation of multiple dendrite-like processes. A recent study showed that PMo can control metastasis in part by engulfing cancer cells. In agreement, the exposure of RAW 264.7 macrophages to ExoNM but not ExoM markedly increases their ability to engulf fluorescence-tagged melanoma cells.
confirmed by the presence of exosome marker CD81 (Supplementary Fig. 11b, d). Since PEDF acts via cell surface receptors, its localization within exosomes is critically important. Consistent with surface localization, limited trypsin digestion eliminates most of the exosomal PEDF but not β-actin, which is localized in the exosome lumen (Fig. 5b and Supplementary Fig. 12), suggesting PEDF to be tethered to the outer leaflet of exosomal membrane. The molecular weight of residual PEDF-reactive band on the western blot is reduced (Supplementary Fig. 12) consistent with the cleavage of membrane-tethered protein. To corroborate this finding, immunogold labeling/electron microscopy (EM) was used to directly visualize PEDF localization (Fig. 5c).

In poorly aggressive melanoma cell line C81-61, PEDF is endogenously expressed at high levels in the cytoplasm and in exosomes (Fig. 5a and Supplementary Fig. 13). PEDF knockdown using shRNA significantly reduces exosomal PEDF content (Supplementary Fig. 13) and abolishes their anti-metastatic effect (Fig. 5d). In agreement, treatment with PEDF peptide mimetic attenuates metastatic ability of B16F10 cells (Supplementary Fig. 14).

**Exosomal PEDF causes macrophage polarization/cell killing.**

Tumor-associated macrophages receive cues from the microenvironment, which determine their tumor-promoting or tumor-suppressive state (polarization) 32. Tumor-promoting macrophages express higher IL-10 levels, while tumor-reactive macrophages produce predominantly IL-12. We have discovered that ExoNM dramatically increases IL-12 mRNA in cultured macrophages; in contrast IL-12 mRNA is decreased by ExoM treatment (Fig. 5e). In agreement, ExoNM reduces IL-10 mRNA in macrophages (Fig. 5f). Importantly, a PEDF neutralizing antibody reverses the effects of ExoNM, while there is no change in ExoM function (Fig. 5e, f). Similar to ExoNM, recombinant human PEDF (rPEDF, 1–20 nM) is sufficient to promote macrophage differentiation (Supplementary Fig. 9a–d).

PEDF was also known to upregulate tumor necrosis factor-related apoptosis inducing ligand (TRAIL) 43. In agreement, macrophage exposure to ExoNM but not to ExoM causes a significant increase of TRAIL mRNA, which is abolished by PEDF neutralizing antibody suggesting PEDF dependence (Fig. 5g). In agreement, rPEDF also alters IL-10, IL-12, and TRAIL expression in RAW 264.7 macrophages in a manner similar to ExoNM. Overnight treatment with 20 nM rPEDF led to increases in TRAIL and IL-12 expression and a decrease in IL-10 expression as evidenced by the mRNA levels (Fig. 5h). Moreover, in co-cultures of RAW 264.7 macrophages with A375 melanoma cells, ExoNM potently induce apoptosis of the melanoma cells, but not of macrophages as is ascertained by TUNEL and Annexin V staining (Fig. 5i, j and Supplementary Fig. 15a). In agreement, ExoNM induce apoptosis only in co-cultures of A375 and RAW 264.7 macrophages but not in respective monocultures (Supplementary Fig. 15b, c). Together, our results indicate that exosomal PEDF can promote PMo differentiation to macrophages, as well as macrophage M1 polarization associated with killing and subsequent phagocytosis of melanoma cells.

**Exosomes from patient sera protect against metastasis.**

The experiments above demonstrate that exosomes from non-metastatic melanoma cell lines can block metastasis by activating PMo. However, it was unclear whether exosomes from patients have similar properties. To determine if exosomes produced by non-metastatic primary melanoma could curtail metastatic spread, we used archival serum samples collected at the time of surgery from patients with primary melanoma.

Fig. 4 NK cells contribute to exosome-driven tumor cell clearance. a, b C57BL/6 mice were subjected to NK cell depletion with an anti-asialo GM1 antibody and NK cell depletion assessed by flow cytometry with NK1.1 antibody. a Flow plots showing percent NK cells in the spleens of mice 96 h after treatment with isotype control or anti-asialo GM1 antibody. b Quantification of the analysis shown in a. *P < 0.05 by two-sided t-test (n = 3). Mean and s.d. values are shown. c, d Following NK cell depletion, mice were treated with the indicated exosomes and used in an extravasation assay with CFSE-tagged B16F10 cells as described above. c Extravasation images taken 24 h after melanoma cell inoculation. Scale bar: 100 μm. d Quantification of experiment in c. Data are expressed as numbers of extravasated cells per high-powered field (n = 4, five random images per animal analyzed for each condition). *P < 0.05 and **P < 0.01 calculated by Tukey’s multiple comparison with (Bonferroni post-test); n.s., non-significant. Median and s.d. values are shown.
The patients were subsequently stratified on the basis of recurrence after 5-year follow-up (recurrent/metastatic and non-recurrent/non-metastatic). Exosomes were isolated from sera by ExoQuick precipitation followed by affinity spin columns and the preparation quality was assessed by TEM (Fig. 6a) and by Western blot for exosomal markers (Supplementary Fig. 16a–c).

To test the effect of the patients’ exosomes on lung metastasis, nude mice were pre-treated with human exosomes as described above and subsequently received tail vein injections of CFSE-labeled A375 melanoma cells. Of note, serum exosomes from recurrent patients cause a significant increase in the number of lung colonies compared to the “neutral” exosomes from healthy volunteers or exosome-depleted patient serum (Fig. 6b, c and Supplementary Table 1). More importantly, exosomes from the patients with non-recurrent tumors cause a statistically significant decrease in lung colonization compared to control exosomes (Fig. 6b, c and Supplementary Table 1), suggesting that our findings using ExoNM from cultured melanoma cell lines reflect the properties of exosomes generated by human melanoma. Of note, we have assessed the correlation between exosomal PEDF levels and survival. Patients were separated into survivors and non-survivors cohorts (n = 41 and 79, respectively) with recurrence established in a more than 5-year follow-up. Although there is significant variability in PEDF contents, exosomes from non-survivors present with significantly lower PEDF contents (Fig. 6d). Together, our results indicate that exosomes from the sera of non-metastatic melanoma patients suppress metastatic colonization and this effect is associated with higher PEDF content in serum exosomes.

Discussion

Over the past few years, there has been an explosion of research focused on exosomes, which led to the discovery that exosomes...
released from tumor cells interact with a broad range of cell types in distant organ environments allowing for the formation of tumor-promoting pre-metastatic niches\textsuperscript{22,23}. Given the importance of exosomes to the interaction between cancer cells and the cells of the microenvironment, we reasoned that exosomes from melanoma cells with varying propensities to metastasize would have contrasting effects on pre-metastatic niche formation. Indeed, while previous data established that exosomes from highly aggressive melanoma cells promote metastasis, in this study, we provide data that demonstrates the anti-metastatic functions of exosomes released from non-metastatic melanoma cells. Moreover, these findings likely reproduce the processes that take place in patients with non-recurrent melanoma and possibly other cancers, because exosomes from archival serum samples taken at the time of surgery from melanoma patients whose tumor did not recur, retained the ability to suppress lung colonization in mouse model.

In a recent study, Hanna et al. discovered the key role of non-classical PMo in the clearance of cancer cells at the site of metastasis, whereby they recruit cytotoxic NK cells leading to eradication of metastasizing cancer cells\textsuperscript{37}. We have independently discovered that the clearance of metastasizing cancer cells driven by the “non-metastatic” exosomes stemmed from the expansion of the Ly6C\textsuperscript{low} PMo population. We have traced these exosomes to the CD11b\textsuperscript{+}Gr-1\textsuperscript{+} cells in the BM of recipient animals and demonstrated that they induce the Nr4a1 nuclear receptor in BM monocytes causing PMo population expansion and increased presence at the pre-metastatic niche. In agreement with findings by Hanna et al., our in vivo data suggest significant contribution of the NK cells in the anti-metastatic effect of Exo\textsuperscript{NM}; however, the in vitro data indicate NK-autonomous effects, which include tumor cell killing by fratricide and engulfment by macrophages. In addition, we observed that non-metastatic exosomes also promote differentiation and

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**Fig. 6** Exosomes from the sera of patients with non-recurrent melanoma suppress lung metastasis. 

(a) Representative TEM of exosomes isolated from the sera of melanoma patients. Scale bar: 100 nm.  

(b) Exosomes from the sera of healthy control donors and of patients with primary and recurrent melanoma were injected into athymic nude mice. Exosome-depleted serum from recurrent patients was used as control. Nude mice were injected i.v. three time, with 10 μg exosomes, 48 h apart followed by injection of CFSE-labeled A375 melanoma cells. The extravasated melanoma cells were counted in the lungs 24 h later.  

(c) Analysis of the data presented in b. \( * P < 0.05, ** P < 0.01, \text{ and } *** P < 0.001 \) calculated by Tukey’s multiple comparison test with Bonferroni post-test \( n = 9 \) for tumor-free, non-recurrent and recurrent groups, \( n = 5 \) for the group treated with exosome-depleted serum, five random images per animal were analyzed). Mean and s.d. values are shown.  

(d) Patients were separated into cohorts according to survival (death from melanoma over >5-year follow-up). Exosomal PEDF was measured by ELISA and normalized to the total protein content and correlated to survival. Statistical significance was determined by two-tailed t-test. \( P < 0.0003 \). \( n = 41 \) (survivors) and \( n = 79 \) (non-survivors). Median and s.e.m. values are shown.
polarization of macrophages, which then engage in tumor cell killing and phagocytosis. This sequence of events is summarized in Supplementary Figure 17.

In earlier studies, others and we have demonstrated that in melanoma, the switch to a highly aggressive and invasive metastatic state is associated with the loss of PEDF, a type 2 tumor suppressor41,42,47,58,59. Importantly, we were able to link the anti-metastatic, pro-immune properties of melanoma exosomes with PEDF, whereby tumor cells that lost PEDF expression no longer produce exosomes that maintain cancer surveillance by the innate immune system. This is the first study, which implicates cancer-derived exosomes in the induction of cancer immune surveillance and clearance.

Importantly, our results support the opposing roles of classical and non-classical (patrolling) monocytes in cancer. On the one hand, classical (inflammatory) CCR2+CX3CR1Ly6Chigh monocytes (M0) play a key role in the development of metastasis whereby they differentiate into inflammatory macrophages, which populate the pre-metastatic niche and support extravasation, survival, and proliferation of metastatic cancer cells60. Conversely, CCR2+CX3CR1Ly6Clow PMos are enriched in the microvasculature of tumor-challenged lung and reduce tumor metastasis37. In contrast to classical monocytes (M0), PMO rarely extravasate from the vasculature under normal circumstances; instead, they scrounge microvessels for the cell debris and particles36. In the presence of tumor cells, their patrolling behavior is disrupted and replaced by accumulation at the tumor site. Hanna et al. show that PMo recruitment to the tumor site is mediated by CX3CR1, which interacts with endothelial-derived CX3CII. However, the same study underscores the low abundance of PM0 in the lung. Our study provides a new mechanism underlying the increased PMo presence at the sites of metastasis, which is particular for pre-metastatic tumors. We discovered that early-stage, pre-metastatic melanoma expresses trigger(s) of innate clearance which are loaded onto exosome surface and delivered, to the monocyte progenitors in the BM of the host causing the expansion of CX3CR1Ly6Clow monocyte population.

In conclusion, our study provides a completely new mechanism responsible for the increased presence of PMo at the pre-metastatic niche and continuous elimination of circulating tumor cells in the tumor-bearing host. On the other hand, we for the first time demonstrate that prior to the acquisition of the metastatic state, tumors continuously alert host immune system by producing exosomes, which carry trigger(s) of innate immune responses and we identify one such trigger, PEDF. Furthermore, analysis of exosomes isolated from archival serum samples corroborates these findings. Taken together, our result point toward a potential new type of cancer immunotherapy based on the use of vesicular structures for delivery of such immune trigger. Interestingly, both previous studies and our current results demonstrate the anti-metastatic role and therapeutic potential of PEDF in melanoma37,61–64 and other cancers65–68. Although PEDF-derived peptide shows only mild anti-metastatic activity in our melanoma model, this could potentially be improved by its loading onto exosomes to increase targeting to the immune system and thus facilitate the development of PEDF-derived peptides into effective immunomodulatory, anti-metastatic therapy.

Methods

Cell culture. B16F10 melanoma cells (ATCC, Manassas, VA) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin. A375 melanoma cells (ATCC) were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin. sB22 cells were a gift from Dr. Maryn Herlyn (Wistar Institute) and C861L/C81-61 cells were a gift from Dr. Mary C. Hendrie (Stanley Manne Children’s Research Institute) and cultured in 50/50 DMEM/F12 containing 5% FBS, 1% penicillin/streptomycin, and RPMI in 10% FBS, 15 penicillin/streptomycin, respectively. PEDF was overexpressed in A375 and B16F10 melanoma cells using lentivirus encoding full-length human PEDF-CDNA inserted between XbaI and BamHI sites within the pUCAI GEGP lentiviral vector69. PEDF knockdown in C81-61 melanoma cells was performed using shRNA-mir in pGPx lentiviral vector VL2HS_221662 (Open Biosystems, Huntsville, AL). In brief, lentiviruses were generated by co-transfection into HEK 293 T cells using the Lent-i X HT packaging system. Culture supernatants were collected, concentrated, titrated, and used for cellular transduction. Cells stably expressing the construct were selected for using puromycin, then identified and sorted for GFP expression.

RAW macrophages (ATCC, Manassas, VA) were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin. Cells were passaged approximately every 3 days. Primary mouse macrophages were isolated from the BM as described below and cultured in 1/1 DMEM/F12 media supplemented with 10% FBS, 1% penicillin/streptomycin, and 20 ng ml−1 of M-CSF (Biolegend, San Diego, CA). All cell cultures were maintained in 5% CO2 at 37 °C. All cell lines were authenticated using MDACC Cell Bank Services and tested for mycoplasma contamination using MycoAlert™ Mycoplasma Detection Kit (Lonza, Walkersville, MD) on a monthly basis. For all cell lines, frozen aliquots of 106 cells were stored at a passage below 5 and a fresh aliquot used after 10 consecutive passages.

Isolation of BM macrophages. Mouse BM-derived macrophages were isolated and matured as described below69. Briefly, C57BL/6 mice were sacrificed and the hind limbs were removed using aseptic technique, leaving the femur and tibia intact. The muscle was removed from the bones with a razor blade. The bones were then cut at the joints and the BM flushed with sterile DPBS (calcium and magnesium free) using 1 ml syringe with a 27-gauge needle. BM cells were centrifuged at 500 × g, re-suspended in 50/50 DMEM/F12 media supplemented with 10% FBS, 1% penicillin/streptomycin and 20 ng ml−1 of M-CSF (Biolegend) and plated at 4 × 105 cells per 10 cm tissue culture dish. BM cells were incubated at 37 °C, 5% CO2 for 3 days at which point they were supplemented with additional 5 ml of cell culture medium containing 20 ng ml−1 of M-CSF. Cells were allowed to grow for an additional 4 days, harvested and used for further studies.

Experimental animals. Mice were housed and maintained at the Northwestern University Center for Comparative Medicine, according to the NIH guidelines and following protocols approved by the Northwestern University Animal Care and Use Committee. Female athymic nu/nu mice (4–6 weeks of age) were from Harlan Laboratories and female C57BL/6 mice (4–6 weeks of age) were from Jackson Laboratories. Age-matched female mice were used for all the studies and the group sizes calculated to achieve 80% power (see statistical methods below).

Exosomes collection and characterization. Melanoma exosomes were isolated from conditioned media (CM) by differential ultracentrifugation69. In brief, cells were cultured in DMEM containing 10% exosome-depleted FBS (Life Technologies) and 1% penicillin streptomycin for 72 h. The CM was collected and centrifuged at 2000 × g to remove dead cells and debris. Next, larger vesicles and finer debris were removed by centrifugation at 10,000 × g for 30 min. Exosomes were then centrifuged at 100,000 × g for 70 min on top of a 30% sucrose cushion, and washed in PBS by another centrifugation step (100,000 × g, 70 min). Exosomes were resuspended, pelleted and stored at −80 °C. Protein content was measured by BCA Protein assay (Thermo Scientific) and treatment doses were based on exosome protein concentration. Exosome size, morphology, and number were characterized using dynamic light scattering (Malvern), NanoSight imaging, and TEM (FEI Spirit G2 TEM). Exosome counts were determined in each nanotracking experiment and exosome number per μg protein calculated. For exosome tracing experiments, the lipophilic di-alkyl indocarbocyanine dyes, DiD or DiD (Life Technologies), were added to exosome preparations at a concentration of 2.5 μM after the first 100,000 × g ultracentrifugation step. The labeled exosomes were then washed twice in PBS by pelleting the exosomes and discarding the supernatant.

Exosome isolation by sucrose gradient ultracentrifugation. The exosome pellet isolated after the final 100,000 × g spin as outlined above was resuspended in 1 ml of PBS (1×, 20 mM HEPES, pH 7.4, 150 mM NaCl, 0.5 mM MgCl2), and loaded onto a sucrose gradient over the concentrations of 2.5 M sucrose, 20 mM NaOH, then centrifuged at 100,000 × g for 60 min at 4 °C. The exosome pellet was collected in 50 ml PBS and analyzed by Western blotting.

Exosome collection form human serum. Due to the low volumes of sera available from patients, we isolated exosomes using combinations of commercial reagents. Serum was first filtered via 0.45 μm syringe filters and exosomes were precipitated using ExoQuick reagent (System Biosciences), according to the manufacturer’s protocol. Membrane affinity spin columns (exoEasy Maxi, Qiagen) were then used to eliminate non-exosomal serum contaminants.

For PEDF analysis, ExoQuick-precipitated exosomes were purified using CD63+ (CD144, conjugated Dynabeads). The protein contents were measured using BCA assay and normalized to 100 μg ml−1. Exosomes were then characterized using TEM and western blotting as outlined above.
RT-PCR. RAW 264.7, THP-1, or BM-derived macrophages were treated with ExoM or ExoNM for 48 h. mRNA was isolated using an RNeasy kit (Qiagen), and 1 μg of RNA was converted to cDNA using iScript cDNA synthesis kit (Bio-Rad). RT-PCR was then used to amplify and measure the cDNA content using PerfeCTa SYBR Green mastermix (Quanta) or Eppendorf RealMasterMix Universal Detection System (Bio-Rad) (for primers, see Supplementary Table 3).

Flow cytometry. For analysis of the BM cell populations, C57BL/6 mice were treated with ExoM or ExoNM, hind limbs collected and BM cells collected from the femurs and tibiae as described above. Lymphocytes were then separated by centrifugation at 500 × g after layering on a Histopaque 1.077 g/mL density gradient. The cells at the interface were collected and residual red blood cells lysed in ACK lysis buffer for 10 min at 4 ºC. Lymphocytes were counted and resuspended in FACS buffer. Cells were incubating 1% bovine serum albumin (BSA), 0.1% sodium azide at 107 cells mL−1.

For analysis of lung monocytes/macrophages lungs were lavaged with DPBS, excised, and smashed in DPBS through a 70-μm cell strainer. Red blood cells were lysed in ACK buffer and the cells washed in FACS buffer, counted and resuspended in FACS buffer (107 cells mL−1).

For immunostaining, 100 μL of the cell suspension were blocked in Fc Block (BD Pharmingen) for 20 min at room temperature. The cells were incubated with fluorescein-conjugated antibodies (Supplementary Table 2) for 1 h at 4 ºC and washed in FACS buffer. Fluorescence was assessed on a BD LSR Fortessa Analyzer (Becton Dickinson, Franklin Lakes, NJ) or Nikon A1R fluorescent microscope (Northwestern Center for Advanced Microscopy) or Nikon Diaphot 2000 with 3× objective. Alternatively, the images were analyzed in a blinded manner prior to data analysis, using Nikon Elements software.

Tumor and immunogold labeling. Exosomes isolated as described above were stained with uranyl acetate 1. Briefly, exosomes resuspended in 2% paraformaldehyde were added to the formvar-carbon-coated EM grids (Electron Microscopy Sciences) for 20 min. The grids were then washed in 100 μL PBS, fixed in 1% glutaraldehyde for 5 min and washed with water 7 × 2 min. To contrast the samples, each grid was transferred to a 50-μL drop of uranyl-oxide (1:1 mix of 4% uranyl acetate and 0.15 M oxalic acid) pH 7 for 3 min. The grids were then embedded in 2% methylcellulose, 4% uranyl acetate (9:1 v/v ratio), for 10 min on ice. Grids were removed from embedding solution with micro-forceps and excess fluid blotted on Whatman no. 1 filter paper. Grids were allowed to dry for 10 min and stored in grid boxes. Exosomes were visualized using a FEI Tecnai Spirit G2 120 kV TEM.

For immunogold labeling, after paraformaldehyde fixation and adsorption onto EM grids, exosomes were washed 3 × 5 min in PBS 3 and incubated 3 min in 50 mM glycine in PBS to quench free aldehydes groups. Next, exosomes were blocked 10 min with 5% BSA in PBS, PDEF antibody (BioProducts MD) was diluted in 5% BSA at 10 μg mL−1 and layered on the EM grids for 30 min. Grids were then incubated in 2% BSA and 0.1% Protein A gold conjugates (Ted Pella, Inc.), diluted 1:200 in blocking buffer and incubated on EM grids for 20 min. The grids were washed with PBS 8 × 2 min, followed by 5 min fixation with 1% glutaraldehyde and 8 × 2 min washes in water. Grids were then contrasted, embedded, and images taken as described above.

Limited trypsin digestion. Exosomes were incubated with 0.05% trypsin for 5 min at room temperature to digest surface proteins only. After digestion, 4× Laemmli buffer was added to the samples, proteins resolved by gel electrophoresis and western blotting performed with antibodies for PEDF and β-actin (luminal marker).

Phagocytosis assay. RAW 264.7 macrophages and A375 melanoma cells expressing GFP tag were seeded in 24-well plates at 30:1 ratio. After 24 h, ExoM or ExoNM were added to the cells at 0.01, 0.5, and 2.0 μg mL−1. Cells were live imaged at 48 h. Before imaging, cover glass was removed and placed in Attofluor® Cell Chamber (Life Technologies) filled with 1 mL of cell superfusion buffer (0.35 mM Na2HPO4, 110 mM NaCl, 0.44 mM KH2PO4, 5.4 mM KCl, 1 mM MgSO4, 1.3 mM CaCl2, 25 mM HEPES, and the pH was adjusted to 7.4). The culture chamber was placed on the microscope platform for imaging. Nomarski/DIC images and confocal images were obtained using the Zeiss AxioVert 200 inverted fluorescence microscope, AxioCam camera, and AxioVision software. Cells were imaged using a 63x objective (N.A. 1.4; oil). The process length and the macrophage surface area were quantified using ImageJ. Images were taken randomly and analyzed in a blinded manner. In an independent experiment, cells were plated in glass bottom dishes treated with exosomes at indicated concentrations and cells were imaged every 15 min for 16 h.

Apoptosis assays. Terminal dUTP nick-end Labeling Assay (TUNEL): The cells were grown on glass coverslips coated with 0.1% gelatin (45 min at 37 ºC). RAW 264.7 macrophages were grown for 24 h in co-culture with A375 melanoma (3:1 ratio) or as monocytes. Exosomes were then added at a concentration of 3 μg mL−1. Cells were incubated for additional 24 h, then fixed in 4% paraformaldehyde and permeabilized with 0.1% TritonX-100 for 2 min at 4 ºC. Apoptotic cells were detected and apoptotic cells detected using in situ Cell Death Detection Kit (Roche) and visualized by fluorescence microscopy. Images were taken and evaluated in a blinded manner prior to data analysis using Nikon Elements software (Melville, NY, a minimum of eight images per condition).

Annexin V staining: Cells were grown as above, harvested and stained in suspension using Annexin V/propidium iodide apoptosis detection kit (Biolegend). For cell type-specific detection of cell death, the cells were co-stained with antibody for macrophage marker F4/80 (Brilliant Violet Fluorophore) and analyzed by flow cytometry.

Macrophage differentiation assay. RAW 264.7 macrophages (3 × 105) were plated in 35-mm glass-bottom dishes, allowed to adhere for 24 h and treated with ExoM or ExoNM at a concentration of 3 μg mL−1. Images were taken every 15 min for 16 h in a Nikon Biostation (5% CO2 at 37 ºC) focusing on the formation of dendriform-like projections. For quantification studies the cells were seeded in 6-well dishes. NIS-Elements AR 4.0.03 (Nikon) was used for analysis following imaging. The z-stacks were merged, ROI’s selected, and the surface area and the multi-point function were used to measure area and length, respectively.

Lung colonization assay. C57BL6 mice were randomly assigned into groups and injected intravenously with 10 μg (2.6 × 106 particles) ExoM or ExoNM at a concentration of 3 μg mL−1. Images were taken every 15 min for 16 h in a Nikon Biostation (5% CO2 at 37 ºC) focusing on the formation of dendriform-like projections. For quantification studies the cells were seeded in 6-well dishes. NIS-Elements AR 4.0.03 (Nikon) was used for analysis following imaging. The z-stacks were merged, ROI’s selected, and the surface area and the multi-point function were used to measure area and length, respectively.

Lung extravasation assay. Extravasation was assessed in a modified lung colonization assay 12. Female C57BL6 mice were randomly assigned into groups and perfluorocarbon (PFC) exosomes (1 μg) were added to the tumor cell inoculation. Two days after the second exosome treatment, the mice were inoculated with 106 B16F10 melanoma cells labeled with CFSE fluorescent dye. Mice were sacrificed at 3 and 24 h after tumor cell inoculation, the lungs excised and fluorescence-labeled cells were visualized with a Nikon AZ-100 fluorescent microscope (Northwestern Center for Advanced Microscopy). Images were evaluated in a blinded manner prior to data analysis, using Nikon Elements software.
aggressive C8161 (C8161 HA or C81-61 PA, respectively). Images were taken and evaluated in a blinded manner prior to data analysis using Nikon Elements software (>5% per condition).

**Clodronate depletion of macrophages and monocytes.** C57BL/6 mice were depleted of macrophages and monocytes using clodronate containing liposomes4. In brief, mice were given intravenous injections of clodronate or control PBS liposomes (200 µg/mouse), 96 and 48 h prior to exosome treatment and inoculation of CAF tags melanoma cells and assessment of extravasation (see above). Six mice in each group were sacrificed and the lungs stained for F4/80 and CD11b, to ascertain the depletion of monocyteic cells.

**Depletion of NK cells with anti-asialo GM1 antibody.** C57BL/6 mice were pretreated with anti-asialo GM1 antibodies (Wako Chemicals) diluted 5x in PBS and were injected intraperitoneal 24 h prior to exosome treatment and extravasation assay. Staining splenocytes with an anti-NK1.1 antibody and analyzing using flow cytometry was used to assess NK cell depletion.

**Functional analysis of patient exosomes.** Exosomes from melanoma patients and healthy controls were isolated from serum collected at the New York University School of Medicine following protocol number 10362 approved by the Institutional Review Board and carried out in accordance with guidance provided by the patients provided informed consent prior to serum collection. Melanoma patient serum samples were completely de-identified prior to transfer to Northwestern University. De-identified samples were separated into three groups, healthy control, patients with primary melanoma and no recurrence, and patients with recurrence after resection of the primary melanoma. Melanoma recurrence was determined after a minimum of 5-year follow-up. Patient data are outlined in Supplementary Table 1. For the depletion of exosomes, sera of recurrent melanoma patients were ultra centrifuged at 100,000 x g for 24 h. After ultracentrifugation, the supernatant was removed and used for analysis.

**Statistical analysis of the quantitative results.** Microscopy and flow cytometry results were analyzed in a double-blinded fashion, using ImageJ and FACS Express, respectively. All experiments were repeated at least three times and representative experiments of three are shown. For microscopy data we used at least three biological and five technical replicates. GraphPad Prism version 6 (GraphPad, San Diego, CA) was used for all statistical analysis. Data are presented as means, using standard deviation (s.d.) or standard error meaning (s.e.m.) of at least triplicate measurements, as a measure of sample variance. Mean values ± s.d., P-values, and sample sizes are reported in figures and figure legends. Differences between groups/populations were analyzed using Student’s t-tests for normal data sets and using non-parametric test (Mann–Whitney) for non-normal data sets. For more than two groups of data we were using Tukey’s multiple comparison test followed by Bonferroni post-test. Normality of the data sets was determined by Kolmogorov–Smirnov test. The differences were considered significant at P ≤ 0.05.

**Group sizes were determined using power analysis, based on pilot experiments and data variance (standard deviation). We conservatively assume that exosomes increase or decrease the mean colony number by at least 50% while compared to the untreated control with a common standard deviation of 25% (an effect size of 1.25). To achieve 80% power, a minimum of three mice per group is required.**

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Author contributions
M.P.P., N.L.A., S.F., S.D.M., and M.P.P. designed and carried out the experiments.
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interests.

Competing interests:
O.V.V. and J.H. declare financial interest in Pandemco LLC, a start-up biotechnology company, which develops PEDF peptide mimetics for cancer therapy. The remaining authors declare no competing financial interests.

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