

The Biodistribution and Immune Suppressive Effects of Breast Cancer-Derived Exosomes

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Abstract

Small membranous secretions from tumor cells, termed exosomes, contribute significantly to intercellular communication and subsequent reprogramming of the tumor microenvironment. Here, we use optical imaging to determine that exogenously administered fluorescently labeled exosomes derived from highly metastatic murine breast cancer cells distributed predominantly to the lung of syngeneic mice, a frequent site of breast cancer metastasis. At the sites of accumulation, exosomes were taken up by CD45⁺ bone marrow-derived cells. Subsequent long-term conditioning of naïve mice with exosomes from highly metastatic breast cancer cells revealed the accumulation of myeloid-derived suppressor cells

in the lung and liver. This favorable immune suppressive microenvironment was capable of promoting metastatic colonization in the lung and liver, an effect not observed from exosomes derived from nonmetastatic cells and liposome control vesicles. Furthermore, we determined that breast cancer exosomes directly suppressed T-cell proliferation and inhibited NK cell cytotoxicity, and hence likely suppressed the anticancer immune response in premetastatic organs. Together, our findings provide novel insight into the tissue-specific outcomes of breast cancer-derived exosome accumulation and their contribution to immune suppression and promotion of metastases. *Cancer Res*; 76(23); 6816–27. ©2016 AACR.

Introduction

Despite improvements in screening and therapy, breast cancer remains the most common type of cancer and the second-leading cause of cancer-related death in women (1). Currently, it is not possible to accurately predict the risk of developing metastatic disease or the response of patients to treatment, and this is reflected in up to 20% of patients who ultimately die of metastatic breast cancer (1, 2). The cross-talk between cancer cells and their surrounding stroma is essential in regulating tumor progression and systemic spread (3). While tumor cells are classically described to communicate via direct cell-to-cell contact and the secretion of soluble factors, such as cytokines and growth factors (4), alternative mechanisms have recently been described. One of these involves small membranous particles secreted from cancer

cells, termed exosomes, which contribute significantly to the intercellular communication and subsequent reprogramming of the tumor microenvironment (5, 6). Exosomes are extracellular vesicles of endocytic origin with a size of 30 to 120 nm that are released under both physiological and pathological conditions (5). The content of exosomes reflects the cell of origin and includes lipids, proteins, messenger RNA and microRNA, which are transferred from donor to target cells. In target cells, the content is thought to induce functional changes capable of promoting metastatic progression, including contribution to premetastatic niche formation (7–10). Therefore, cancer-secreted exosomes and their molecular contents have received much attention as potential biomarkers and therapeutic targets for cancer (5, 6, 11).

Reduced immune surveillance is a key mechanism through which primary tumors create permissive environments in secondary organs that favor the development of metastasis (premetastatic niche formation; refs. 12–14). Studies have reported that tumor-derived exosomes can have detrimental effects on the immune system by suppressing specific T-cell immunity and skew innate immune cells toward a protumor phenotype (15–17). Most recently, pancreatic cancer exosomes were shown to increase liver metastatic burden by transferring macrophage migration inhibitory factor (MIF) to liver macrophages and by recruiting immune cells to initiate premetastatic niche formation in the liver (12). However, it remains to be determined whether exosomes can induce premetastatic niches in other types of cancers, such as breast cancer. Moreover, it remains unclear which immune cell lineage in specific organs is largely responsible for the uptake of circulating tumor-secreted exosomes and its subsequent impact on anticancer immune responses during metastasis.

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Here, we report a detailed analysis of the tissue distribution of intravenously injected exosomes from breast cancer cells with differing metastatic potential, their uptake by various immune cell lineages, and the immunosuppressive outcomes of exosome accumulation in premetastatic organs.

Materials and Methods

Mice

Female C57BL/6 and BALB/c mice were purchased from the Walter and Eliza Hall Institute (Australia) and used at 8 to 12 weeks of age. All animal procedures were conducted in accordance with Australian National Health and Medical Research regulations on the use and care of experimental animals, and approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee (P1499).

Cell culture

The murine C57BL/6 EO771 and isogenic BALB/c 4T1 and 67NR cells were generated and maintained as previously described (18–22). No authentication protocol exists for these cell lines according to our knowledge. The cells tested negative for *mycoplasma* contamination and this testing was conducted every 3 months and after taking cells into culture.

Exosome isolation

Exosomes from EO771 cells were purified from cell culture supernatants by a combination of ultracentrifugation, ultrafiltration, and size exclusion purification as previously described (23). Briefly, cultured cells at 60% to 70% confluence were washed three times in PBS and grown for 24 hours in serum-free media. Conditioned media were collected, with dead cells and large debris removed by centrifugation ($500 \times g$; 10 minutes), followed by filtration (0.22 μm). The resulting cell-free medium was concentrated by ultrafiltration using the Centricon Plus-70 Centrifugal Filter (100 kDa; Merck Millipore) at $3,500 \times g$, 4°C.

Purification of exosomes from concentrated media was performed by overlaying on qEV size exclusion columns (Izon Science Ltd) followed by sample concentration in Amicon Ultra-4 10-kDa nominal molecular weight centrifugal filter units (Merck Millipore) to a final volume of 200 μL for further analysis (23).

Isolation of exosomes from 4T1 and 67NR cells was carried out as previously described (12, 15, 24). Briefly, cell lines were grown in FBS-supplemented culture media that was depleted of exosomes. Supernatant fractions were collected from 48-hour cell cultures, followed by centrifugation ($500 \times g$; 10 minutes) and filtration (0.22 μm) to remove dead cells and large debris. Exosomes were collected, washed in PBS, and pelleted by ultracentrifugation at $100,000 \times g$ for 90 minutes at 4°C.

Electron microscopy

Electron microscopy imaging was performed as described (25) with modifications. Briefly, purified exosomes were fixed with paraformaldehyde and transferred to Formvar-carbon-coated electron microscopy grids. Grids were transferred to 1% (v/v) glutaraldehyde for 5 minutes, followed by eight washes with water. For contrast, grids were negatively stained with 1% (w/v) uranyl-oxalate solution, pH 7 for 5 minutes before transferring to methyl-cellulose-UA for 10 minutes. Excess fluid was removed and exosomes were imaged in a JEOL 1011 transmission electron microscope at 60 kV.

Western blotting

Exosome preparations were solubilized with Laemmli sample buffer, protein quantified using a standard Bradford Assay, and analyzed by Western blotting as previously described (20). The membrane was probed with the following primary antibodies: mouse anti-flotillin-1 (610821; BD Biosciences), goat anti-tsg101 (M-19sc-6037; Santa Cruz Biotechnology), rabbit anti-CD9 (ab92726; Abcam), mouse anti-HSP70 (610608; BD Biosciences), and rabbit anti-GM130 (ab52649; Abcam). Samples were further incubated with the appropriate secondary antibodies: goat anti-rabbit HRP (1858415; Pierce), goat anti-mouse HRP (1858413; Pierce), or rabbit anti-goat HRP (A5420; Sigma-Aldrich).

Size distribution analysis by tunable resistive pulse sensor

The quantification and size distribution analysis of exosomes was performed using the Izon qNano system by tunable resistive pulse sensor (TRPS) technology (Izon Science Ltd) with the NP100 nanopore and 70-nm calibration beads (CPS70) as previously reported (26, 27).

In vivo imaging of fluorescently labeled exosomes and tracking

Purified exosomes were fluorescently labeled using Vybrant DiD (Life Technologies) according to the manufacturer's instructions with modifications. Briefly, exosomes and liposomes were incubated for 10 minutes with DiD (1:1000 dilution in PBS). Excess dye was removed by washing in 20 mL of PBS at $100,000 \times g$ (90 minutes) to receive the final DiD-stained exosome preparation. DiD-labeled exosomes derived from EO771, 4T1, or 67NR cells were injected intravenously either into syngeneic C57BL/6 or BALB/C mice (20 μg of exosomes/mouse). At 4, 24, and 48 hours after injection, various tissues were harvested (lung, spleen, kidney, liver, heart, and bone marrow) for *in vivo* and *ex vivo* imaging. The intensity of fluorescence was quantified using the IVIS Spectrum and Living Image Software (PerkinElmer) to assess tissue distribution of DiD-labeled exosomes. Additionally, immune populations in the lung, spleen, and bone marrow that had taken up DiD-labeled exosomes were assessed using flow cytometry.

Premetastatic niche formation and experimental metastasis studies

To initiate premetastatic niche formation, C57BL/6 or BALB/c mice were injected intravenously (tail vein) with either 10 μg (EO771) or 5 μg (4T1 and 67NR) exosomes, every 3 days for 30 days, or a once-off injection of 50 μg of EO771 exosomes. EO771 exosomes (1 μg) is equivalent to approximately 7.9×10^9 particles, and 1 μg of 4T1 and 67NR exosomes is equivalent to approximately 5.8×10^9 particles as determined by the TRPS and Bradford assays. After exosome injection, lungs, spleen, and bone marrow were harvested, and immune cell composition was assessed using flow cytometry. Alternatively, after exosome conditioning (exosomes injected every 3 days for 30 days), mice received 1×10^5 EO771 cells via the tail vein (experimental metastasis model) and metastatic burden in the lung was assessed 21 days later, or 2.5×10^5 4T1-luciferase cells via the tail vein, and metastatic burden in the lung and liver assessed 14 days later. Control mice received an equivalent particle number of synthetic unilamellar 100-nm liposomes (Encapsula Nanoscience) as determined using TRPS or PBS.

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Flow-cytometry analysis

Flow cytometry was carried out on single-cell suspensions of whole lung, spleen, bone marrow, and/or liver tissue. A standard protocol was used to prepare single-cell suspensions: (i) lungs were minced and then digested with 0.2 mg/mL collagenase type IV (Worthington Biochemical Corp.) for 20 minutes at 37 °C; (ii) spleen was minced to release splenocytes; (iii) liver was minced, digested with 0.2 mg/mL collagenase type IV for 30 minutes at 37°C, and hepatocytes removed by Percoll gradient; (iv) bone marrow cells were flushed from both the tibia and femur. All cell preparations were passed through a 40- μ m filter to obtain single-cell suspensions and treated with Ammonium chloride red cell lysis buffer. Samples were stained with the appropriate antibodies, together with Fc receptor blocking using anti-CD16/32 before resuspension in FACS buffer containing 2% FBS and viability dye. 7-Aminoactinomycin D (7-AAD) was used as the viability dye, or Zombie Yellow Fixable Viability Kit (BioLegend) for fixed samples. DiD-labeled exosome-positive cells were detected using red laser excitation and 640-nm emission. Flow-cytometric acquisition was completed using a LSR-Foressa (BD Biosciences), and analysis was performed using FlowJo (Tree Star).

T-cell proliferation assay

CD4⁺/CD11c⁻ and CD8⁺/CD11c⁻ cells were sorted using fluorescence-activated cell sorting (FACS) from whole spleen tissue from C57BL/6 mice. A standard T-cell proliferation assay using 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; 5 μ mol/L) was performed with modifications (28). Briefly, CFSE-labeled CD4⁺/CD11c⁻ and CD8⁺/CD11c⁻ T cells were cocultured with irradiated splenocytes, monoclonal anti-CD3 (0.5 μ g/mL) for T-cell stimulation and with varying protein amounts (μ g) of EO771-derived exosomes for 72 hours prior to flow-cytometry analysis. Control CD4⁺/CD11c⁻ and CD8⁺/CD11c⁻ T cells received the same volume of PBS or an equivalent particle number of synthetic unilamellar 100-nm liposomes (Encapsula Nanoscience) as determined using TRPS.

⁵¹Cr release cytotoxicity assay

NK1.1⁺/CD3⁻ cells were sorted using FACS from whole spleen tissue from C57BL/6 mice and cultured as previously described (29). NK cells were expanded in culture media containing IL2 (1,000 units/mL) for 5 days. ⁵¹Cr-labeled YAC-1 target cells were used in a standard 5-hour NK cell cytotoxicity assay at different target (YAC-1 cells) to effector cell (NK cells) ratios and performed as previously described (29, 30). NK1.1⁺/CD3⁻ cells were treated with varying protein amounts (μ g) of EO771-derived exosomes for 3 hours prior to incubation with target YAC-1 cells. Control NK1.1⁺/CD3⁻ cells received the same volume of PBS.

Statistical analysis

Quantitative data are presented as mean \pm SEM. Non-parametric data were analyzed by two-tailed Mann-Whitney *U* tests. Parametric data were analyzed using ANOVA with *post hoc* comparison (Tukey method). The Holm-Sidak multiple testing correction method was used. Adjusted *P* < 0.05 was considered statistically significant.

Results

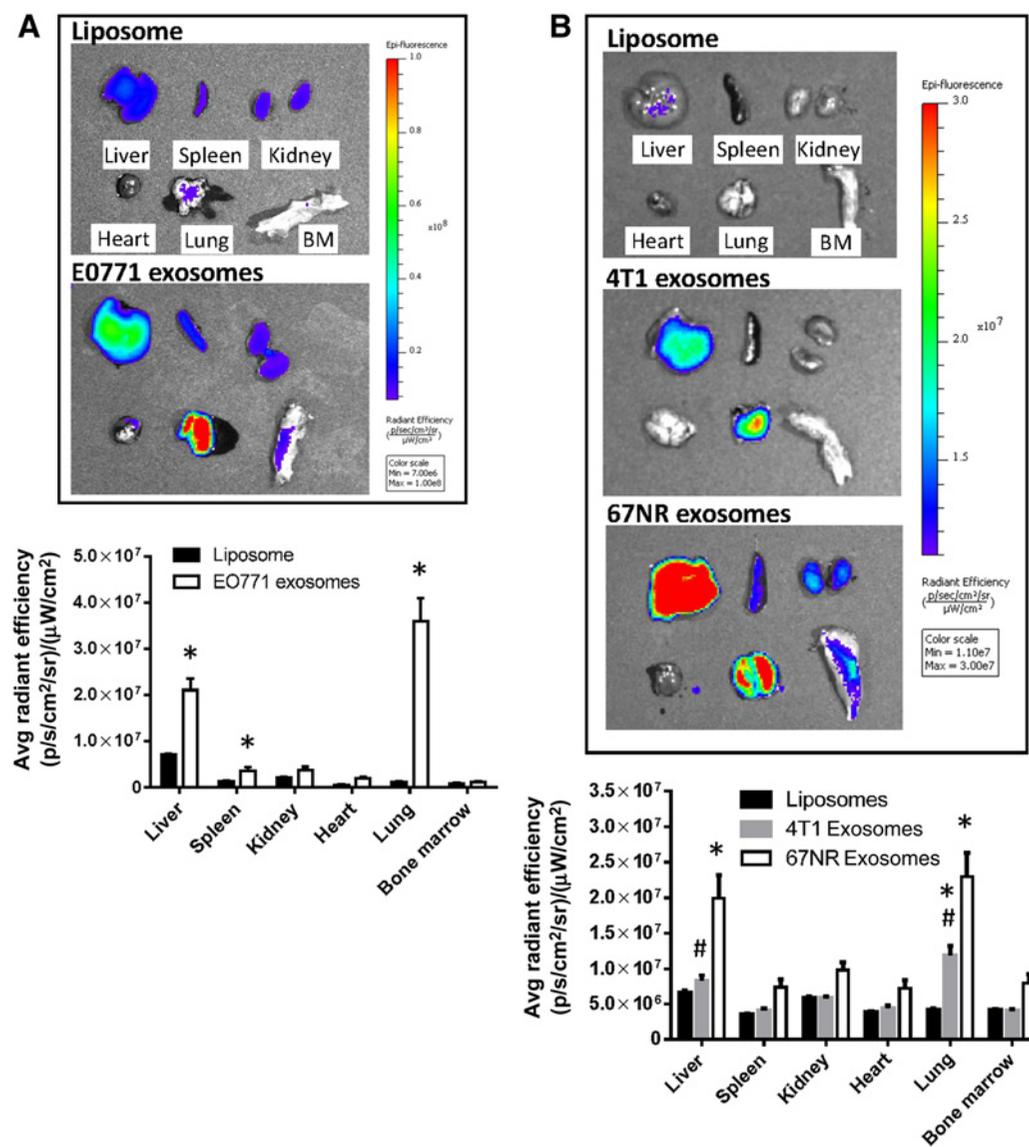
Spatial and temporal distribution of breast cancer-derived exosomes

We first characterized and confirmed exosome isolation from the conditioned medium of C57BL/6 EO771 and BALB/c 4T1-syngeneic murine breast cancer cell lines (Supplementary Fig. S1). The morphology of isolated exosomes, as assessed by transmission electron microscopy, showed the typically associated double-layered spherical structure (Supplementary Fig. S1A). Additionally, exosomes were of the correct size and exhibited various marker proteins (Supplementary Fig. S1A–S1C; ref. 6). Particles were positive for exosomal core protein markers, including CD9, HSP70, and TSG101 (Supplementary Fig. S1B, E lanes), and negative for the cis-Golgi marker GM130, which was only present in cell lysates (Supplementary Fig. S1B, C lanes). Using TRPS technology, we determined the mode size of exosomes to be 86 nm and 80 nm for EO771 and 4T1 cells, respectively (Supplementary Fig. S1C). Furthermore, EO771 (1.14×10^{12} particles/ml) and 4T1 cells (1.07×10^{12} particles/ml) displayed similar levels of exosome secretion (Supplementary Fig. S1D).

To elucidate the role of breast cancer exosomes in intercellular communication and its target effects, it is of utmost importance to first determine the *in vivo* fate of exosomes. Here, we studied the biodistribution of breast cancer exosomes in syngeneic mice after systemic delivery. As a majority of mortalities from breast cancer are due to metastatic disease (1, 2) and high exosome abundance in patient plasma correlates to tumor grade and poor patient outcomes (15, 31, 32), we examined the tissue distribution of exosomes derived from highly metastatic EO771 and 4T1 cells, and compared it to nonmetastatic 67NR cells to assess if differences in metastatic potential between isogenic breast cancer cells can influence the uptake and tissue biodistribution of tumor exosomes. 67NR cells produce approximately 13-fold fewer exosomes than 4T1 cells (Supplementary Fig. S1E). EO771-, 4T1-, and 67NR-derived exosomes were labeled with a lipid-associating fluorescent dye, DiD, and administered intravenously. Exosome biodistribution in various organs (lung, spleen, kidney, liver, heart, and bone marrow) was assessed 24 hours after injection using *in vivo* and *ex vivo* imaging and compared with liposome controls.

In vivo and *ex vivo* fluorescence quantification determined significant accumulation of EO771-derived exosomes in the lung (13-fold signal increase), liver, and spleen (2.5-fold signal increase in both tissues) within 24 hours after injection (Fig. 1A; and Supplementary Fig. S2). By 48 hours, this signal was significantly decreased (Supplementary Fig. S2). For this reason, we chose to use the 24-hour time point for further analysis. Accumulation of EO771 exosomes in the bone marrow was detected in some long bones of individual mice, but overall fluorescence quantification showed that this was not statistically significant when compared with liposome-treated controls (Fig. 1A).

Similarly, 4T1- and 67NR-derived exosomes accumulated primarily in the lung (2.8-fold and 5.5-fold signal increase, respectively) within 24 hours after injection compared with liposome-injected control animals (Fig. 1B), further suggesting the retention of tumor exosomes in specific tissues. However, in contrast to 4T1, exosomes from nonmetastatic 67NR cells also accumulated significantly in the liver (3-fold increase; Fig. 1B). Additionally, despite injecting the same number of DiD-labeled

**Figure 1.**

Visualization and *ex vivo* tracking of breast cancer exosomes. Animals received a single intravenous injection of DiD-labeled EO771-, 4T1-, or 67NR-derived exosomes (20 μg; 1.6×10^{11} EO771 exosomes and 1.2×10^{11} 4T1/67NR exosomes). DiD-labeled liposomes served as control. Tissues were harvested 24 hours after intravenous injection, and fluorescence was visualized and quantified using the IVIS Spectrum (PerkinElmer). Representative *ex vivo* images and quantification (average radiant efficiency) of DiD-labeled EO771 exosomes (A) and DiD-labeled 4T1 and 67NR exosomes (B). Results are presented as mean ± SEM ($n = 5-7$ /group) as analyzed by ANOVA; *, $P < 0.01$ compared with liposome control; #, $P < 0.01$ compared with 67NR DiD exosomes.

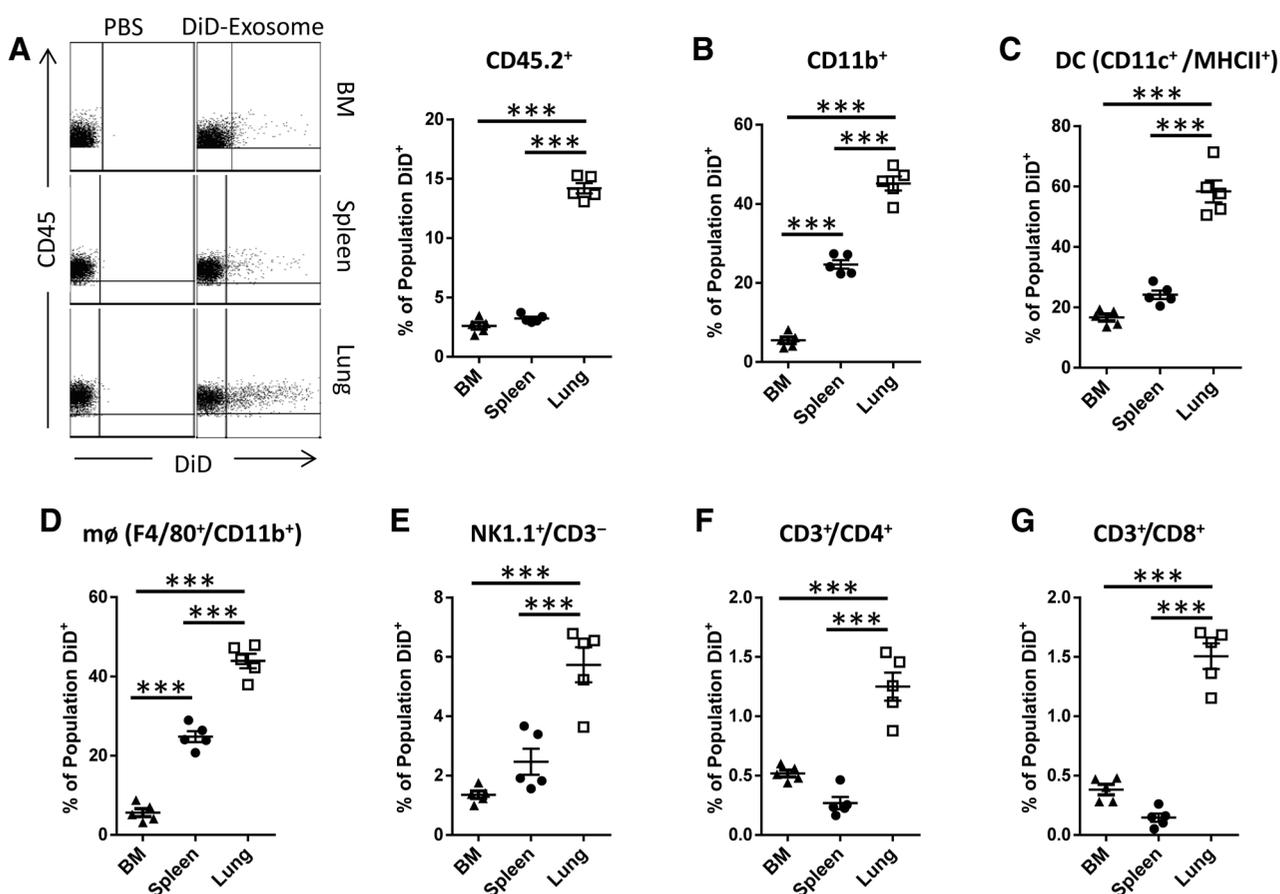
vesicles, both the liver and lung showed higher retention of 67NR exosomes after 24 hours compared with 4T1 exosomes (Fig. 1B). Overall, this suggests metastatic potential and/or cellular origin has an important influence on the biodistribution pattern of exosomes.

Breast cancer–derived exosomes are internalized and affect immune cell populations

Next, to determine the cell lineages that were taking up the fluorescent-labeled EO771 exosomes, CD45⁺ cells in the lung and spleen were assessed as these organs showed significant exosome accumulation (Fig. 2). CD45⁺/DiD⁺ cells represented

$14.2\% \pm 0.44$ and $3.2\% \pm 0.15\%$ of CD45⁺ cells in the lung and spleen, respectively (Fig. 2A). Despite no major EO771 exosome retention in the bone marrow (Fig. 1A, *ex vivo* fluorescence quantification), more in-depth flow cytometry analysis demonstrated $2.6\% \pm 0.28\%$ of bone marrow CD45⁺ cells to have taken up exosomes (Fig. 2A). Within each CD45⁺ cell subpopulation, CD11b⁺ myeloid cells (Fig. 2B), dendritic cells (DC; CD11C⁺/MHCII⁺; Fig. 2C), and macrophages (mφ; CD11b⁺/F4/80⁺; Fig. 2D) showed high uptake of EO771-derived exosomes, ranging approximately between 40%–60% uptake in the lung and 20%–30% uptake in the spleen. In contrast, EO771 exosome uptake was much lower by NK cells (NK1.1⁺/CD3⁻; Fig. 2E), CD4 T cells

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**Figure 2.**

Uptake of EO771 breast cancer exosomes by immune cell lineages. C57BL/6 mice received a single intravenous injection of DiD-labeled EO771-derived exosomes (20 μg ; 1.6×10^{11} particles) or PBS (control). Exosome uptake by immune populations in the bone marrow (BM), spleen, and lung was analyzed 24 hours later by flow cytometry. **A**, Representative flow cytometric plots (left) and quantification (right) of distinct DiD⁺ population within CD45⁺ cells. **B–G**, Frequency of the DiD⁺ subpopulation within myeloid cells (CD11b⁺; **B**), DCs (CD11c⁺/MHCII⁺; **C**), macrophages (m ϕ ; CD11b⁺/F4/80⁺; **D**), NK cells (NK1.1⁺/CD3⁻; **E**), CD4 T cells (CD3⁺/CD4⁺; **F**), and CD8 T cells (CD3⁺/CD8⁺; **G**). Results are presented as mean \pm SEM ($n = 5/\text{group}$) as analyzed by ANOVA; ***, $P < 0.001$.

(CD3⁺/CD4⁺; Fig. 2F), and CD8 T cells (CD3⁺/CD8⁺; Fig. 2G). In line with the degree of exosome uptake by CD45⁺ cells, EO771 exosome uptake by various target immune subpopulations was highest in the lung, followed by the spleen, with least uptake observed in the bone marrow.

A similar uptake pattern by immune populations was observed for fluorescent-labeled exosomes derived from metastatic 4T1 and nonmetastatic 67NR cells (Supplementary Fig. S3). CD45⁺ cells in the lung displayed highest uptake of exosomes from both tumor cell lines when compared with the spleen and bone marrow, along with minimal uptake of liposome control vesicles (Supplementary Fig. S3A). The uptake of 67NR exosomes by CD11b⁺ cells, CD11b⁺/Gr1⁺ cells, DCs, and macrophages was significantly higher compared with 4T1 exosomes in most organs assessed (Supplementary Fig. S3B–S3E). Similar to EO771 exosomes, there was minimal uptake of both 4T1- and 67NR-derived exosomes by CD8 and CD4 T cells (Supplementary Fig. S3F–S3G).

To assess the functional consequences of breast cancer exosome accumulation on CD45⁺ cell lineages, we first determined the composition of immune cells after an acute intravenous injection

of unlabeled EO771 exosomes. There were no significant changes in the frequency of myeloid cells, DCs, macrophages, or NK cells in the lung 24 hours after one injection of EO771 exosomes (50 $\mu\text{g}/\text{mouse}$; Supplementary Fig. S4A–S4D). However, a significant decrease in the frequency of CD4 and CD8 T cells (Supplementary Fig. S4E–F) compared with liposome-injected control animals was observed. This suggests the acute ability of breast cancer exosomes to alter immune microenvironments at distant organs. In organs such as the bone marrow, that showed minimal, nonsignificant accumulation of EO771 exosomes, no change in immune cell composition was observed (Supplementary Fig. S5).

To further examine the role of breast cancer–derived exosomes in conditioning the lung to potentially create a premetastatic niche, EO771-derived exosomes were injected intravenously into mice every 3 days for 30 days (Fig. 3A). Subsequent characterization of infiltrating immune cells in the lung confirmed significant changes in immune composition. EO771-derived exosomes increased overall CD45⁺ cell abundance in the lung, suggesting higher accumulation or infiltration of immune cells to the tissue environment (Supplementary Fig. S6A). While absolute numbers of CD8 T cells, macrophages, and NK cells were unchanged, the

frequency of these cells was altered (Fig. 3; Supplementary Fig. S6B–S6E). Specifically, EO771-derived exosomes reduced the frequency of CD8 T cells and NK cells, but increased macrophage frequency compared with liposome control animals (Fig. 3B and 3E–F). Despite no change in overall CD4 T-cell frequency (Fig. 3B), exosome conditioning did decrease the subpopulation of naïve CD4 T cells (CD44^{low}/CD62L^{high}) while increasing frequency of effector memory CD4 T cells (CD44^{high}/CD62L^{low}; Fig. 3C). Importantly, EO771 exosomes increased both the frequency and absolute numbers of CD11b⁺/Ly6C^{med} granulocytic myeloid-derived suppressor cells (gMDSC), but not CD11b⁺/Ly6C^{high} monocytic MDSCs (mMDSC; Fig. 3G, and Supplementary Fig. S6F). The MDSC population has been previously shown to create premetastatic niches permissive to metastatic colonization (30) and suppress T-cell activity (33, 34).

Indeed, we showed that increased gMDSCs accumulation in the lung correlated with a decreased ratio of CD8⁺ T-cell numbers to gMDSCs (Supplementary Fig. S6G). These data indicate that breast cancer exosomes may be capable of initiating premetastatic niche development by skewing the lung microenvironment to an immunosuppressive state. Assessment of the spleen did not indicate significant changes in immune composition after exosome conditioning, except for a slight decrease in NK cell frequency (Supplementary Fig. S7).

We next determined if the overall immunosuppressive environment created in the lung by EO771-derived exosomes can functionally increase metastasis. Following the 30-day conditioning of naïve mice with EO771-derived exosomes, animals received 1×10^5 EO771 cells via the tail vein. Mice injected with EO771-derived exosomes had a higher metastatic burden in the lung

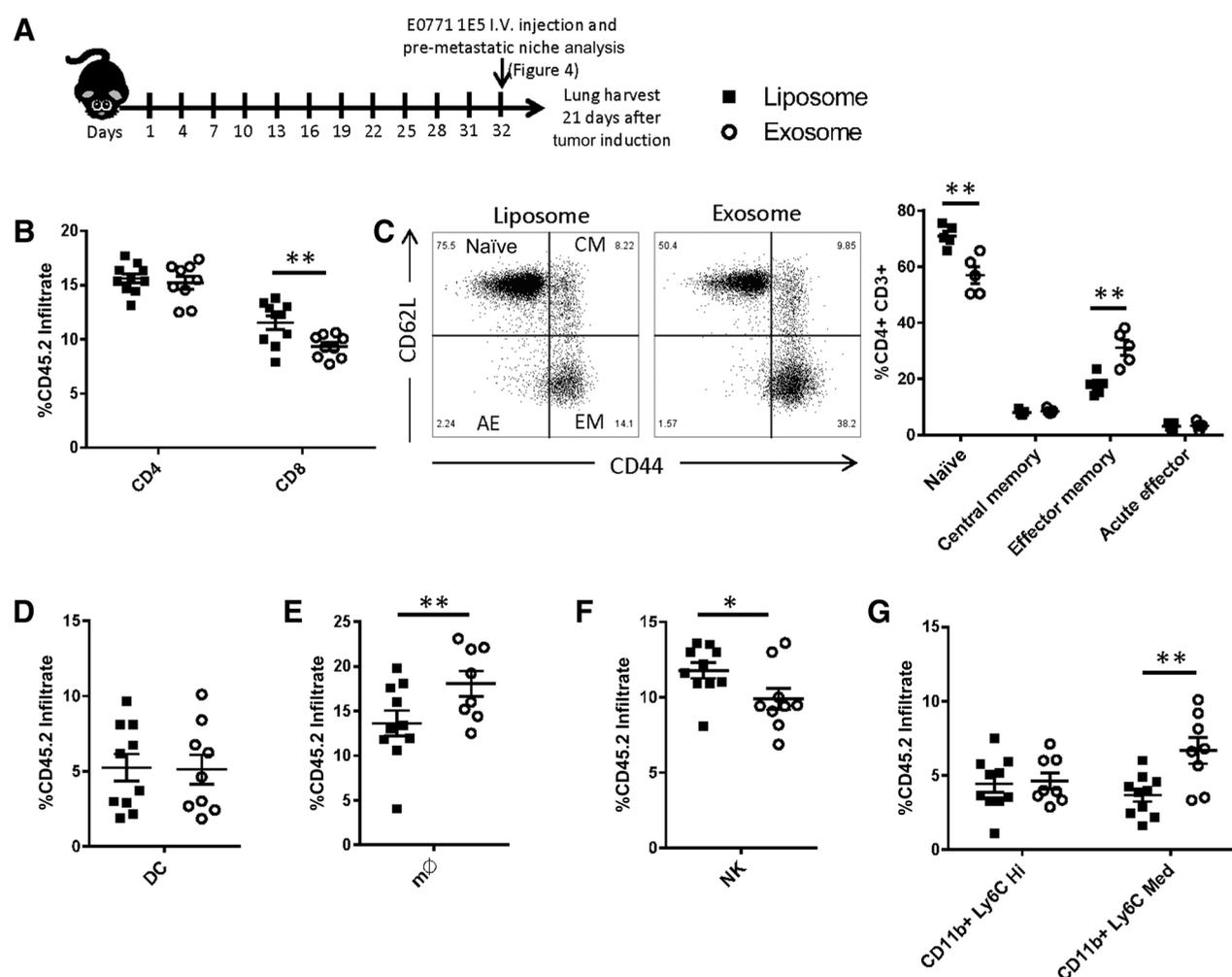
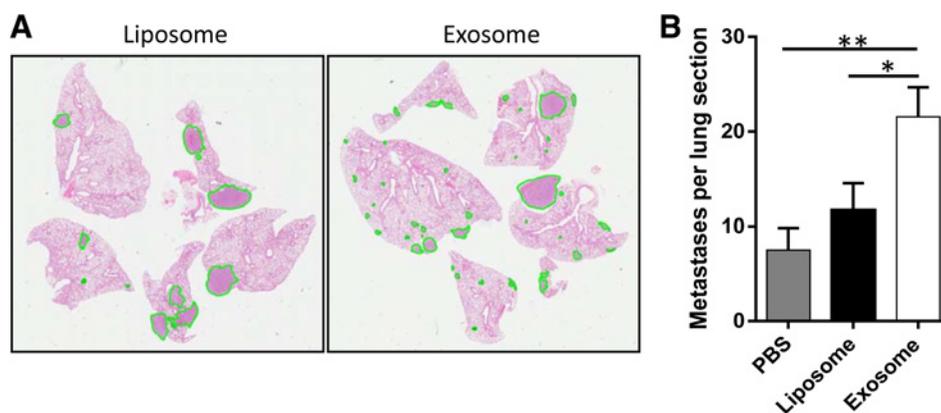


Figure 3.

Breast cancer exosomes create an immunosuppressive premetastatic niche in the lung. **A**, C57BL/6 mice received $10 \mu\text{g}$ (7.8×10^{10} particles) of EO771-derived exosomes or liposomes every 3 days for 30 days (intravenous). The frequency of various immune populations in the lung was quantified by flow cytometry at endpoint. **B**, Frequency of CD4 (CD3⁺/CD4⁺) and CD8 T cells (CD3⁺/CD8⁺) in the lung. **C**, Representative flow-cytometric plots of naïve (CD44^{low}/CD62L^{high}), central memory (CM; CD44^{high}/CD62L^{high}), effector memory (EM; CD44^{high}/CD62L^{low}), and acute effector CD4⁺ T cells (AE; CD44^{low}/CD62L^{low}; left). These subpopulations of CD4⁺ T cells were quantified in the lung (right). Frequency of DCs (CD11C⁺/MHCII⁺; **D**), macrophages (m ϕ ; CD11b⁺/F4/80⁺; **E**), NK cells (NK1.1⁺/CD3⁻; **F**), and CD11b⁺/Ly6C^{med} granulocytic MDSCs and CD11b⁺/Ly6C^{high} monocytic MDSCs (**G**). Results are presented as mean \pm SEM ($n = 5$ –10 animals/group) and analyzed by two-tailed Mann-Whitney U tests and the Holm-Sidak multiple testing correction method; *, $P < 0.05$; **, $P < 0.01$.

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**Figure 4.**

Breast cancer exosomes promote metastatic colonization in the lung. C57BL/6 mice received $10 \mu\text{g}$ (7.8×10^{10} particles) of EO771-derived exosomes, liposomes, or PBS every 3 days for 30 days (intravenous). After exosome conditioning, mice received 1×10^5 EO771 cells and metastatic burden in the lung assessed 21 days later. **A**, Representative lung sections (hematoxylin and eosin) identifying metastases (circled in green). **B**, Number of metastases per lung section (two sections per lung analyzed). Results are presented as mean \pm SEM ($n = 5$ animals/group) and analyzed by ANOVA. *, $P < 0.05$; **, $P < 0.01$.

(measured by histology) compared with liposome-treated and PBS control mice (Fig. 4).

Exosomes from highly metastatic breast cancer cells are required to promote metastasis

Given that breast cancer exosomes seem to create a premetastatic niche capable of promoting metastatic outgrowth, we next examined if this effect was restricted only to highly metastatic breast cancer cells. For this approach, we again conditioned naïve animals with exosomes derived this time from highly metastatic 4T1 and nonmetastatic 67NR isogenic breast cancer cells every 3 days for 30 days (Fig. 5A). Liposome-treated animals served as the control cohort. We first examined changes in T-cell, NK cell, and MDSC frequency in the lung and liver as these tissues displayed highest accumulation of systemically injected 4T1- and 67NR-derived exosomes (Fig. 5B and C). In the liver, 4T1 exosomes significantly increased the frequency of MDSCs ($\text{CD11b}^+/\text{Gr1}^+$) overall compared with both 67NR exosomes and liposome-treated groups (Fig. 5B). However, when we examined the particular MDSC subsets in the liver, we found 4T1 exosomes specifically increased the frequency of $\text{CD11b}^+/\text{Ly6C}^{\text{high}}/\text{Ly6G}^-$ cells (mMDSCs), accompanied by decreased NK cells compared with liposomes (Fig. 5B). In the lung, animals treated with 67NR-derived exosomes showed a reduced frequency of $\text{CD11b}^+/\text{Gr1}^+$ cells, but increased NK cells compared with both 4T1 exosomes- and liposome-treated animals (Fig. 5C). No change in CD4 and CD8 T-cell frequency was observed in both organs after exosome conditioning (Fig. 5B and C). Overall, the data suggest that exosomes from highly metastatic 4T1 cells are more capable than nonmetastatic 67NR exosomes at inducing MDSC recruitment.

We next examined if premetastatic niche development is exclusive to exosomes derived from cancer cells with high metastatic potential. Following the conditioning of naïve mice with 4T1- and 67NR-derived exosomes for 30 days, animals received 2.5×10^5 4T1-luciferase cells via the tail vein. Mice injected with 4T1-derived exosomes had a higher metastatic burden in both the lung (Fig. 6A) and liver (Fig. 6B) compared with 67NR exosomes, liposome-treated, and PBS control mice. This suggests that exosomes from highly metastatic breast cancer cells are required to initiate a premetastatic niche capable of promoting metastasis.

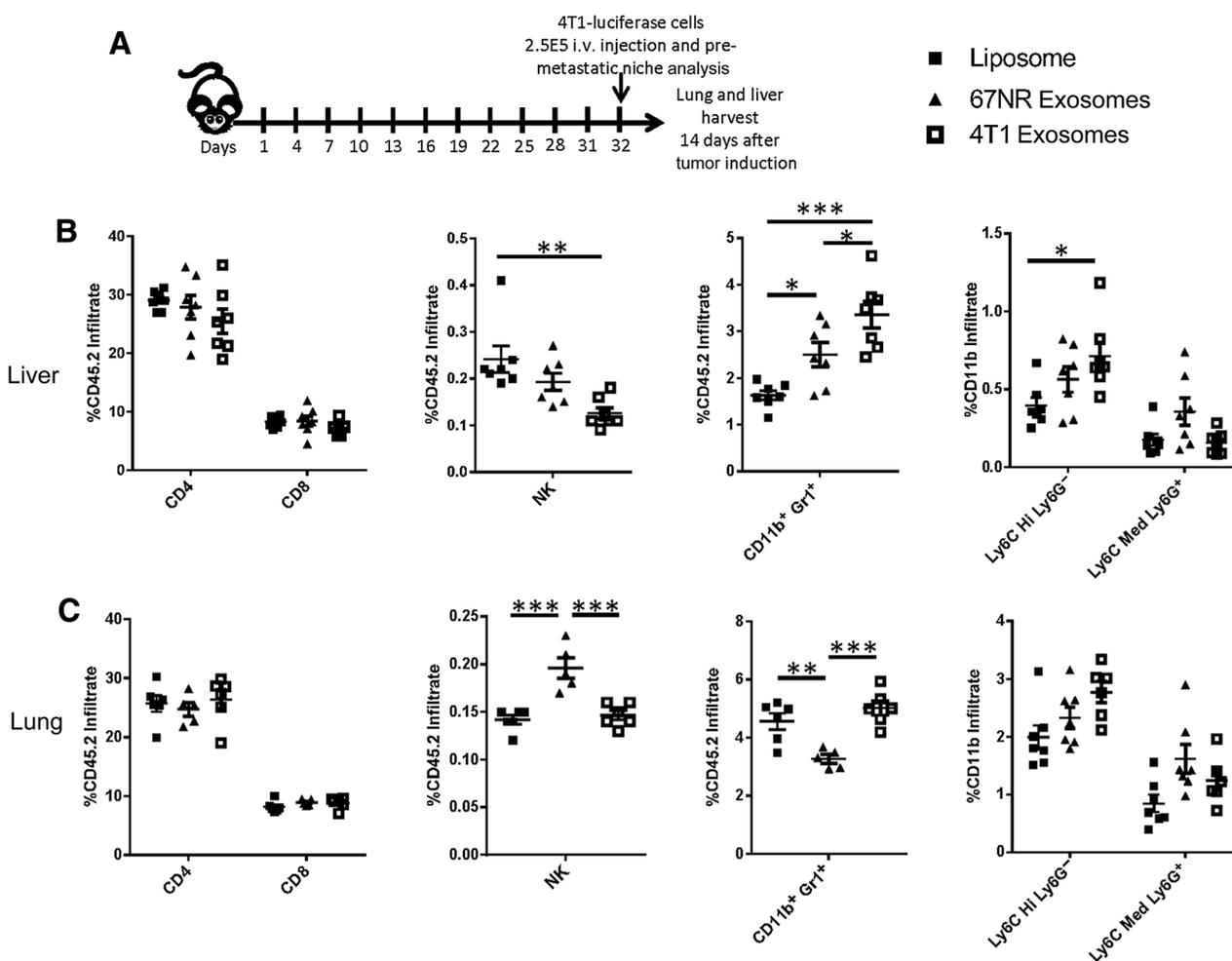
Impact of breast cancer–derived exosomes on T-cell and NK-cell functions

Given changes in the frequencies of NK cells and T cells in lungs conditioned with exosomes, we next wanted to determine if breast cancer–derived exosomes can affect these cells directly. We isolated T cells and NK cells from naïve mice and exposed them to EO771 exosomes *in vitro*. Breast cancer EO771 exosomes were capable of suppressing proliferation of both CD8 and CD4 T-cells in a dose-dependent manner (Fig. 7A and B). Flow-cytometric analysis indicated that T-cell apoptosis may be one reason for the observed reduction in CD8 and, to a lesser extent, CD4 T-cell proliferation (Supplementary Fig. S8A). This was a tumor-exosome–specific effect as liposome vesicles did not suppress T-cell proliferation (Supplementary Fig. S8B–S8C). Furthermore, the cytotoxic activity of NK cells against target tumor cells was reduced after exposure to breast cancer–derived exosomes (Fig. 7C). Together, these data demonstrate a direct effect of breast cancer–derived exosomes on the proliferative and anticancer capacities of T and NK cells.

Discussion

Metastatic spread of tumor cells to distant sites is the most common cause of cancer-related death (35). While primary tumor cells can directly release cytokines and growth factors to recruit bone marrow–derived cells to prime secondary sites for metastatic growth, we show that tumor-secreted exosomes also play a critical role. The present study uses a comprehensive *in vivo* imaging approach to describe the tissue-specific effects of exosomes from highly metastatic breast cancer cells on immune composition, and its ability to establish a premetastatic niche capable of increasing metastatic colonization.

In this study, fluorescently tagged exosomes allowed direct *in vivo* visualization to interrogate its systemic biodistribution and uptake by CD45^+ cells. Here, we report that exogenously administered (intravenous) murine breast cancer exosomes distribute predominantly to the lung regardless of metastatic potential, a frequent site of breast cancer metastasis. To a much lesser extent, breast cancer exosomes can also accumulate in the spleen. Interestingly, despite the propensity of 4T1 breast cancer cells to form

**Figure 5.**

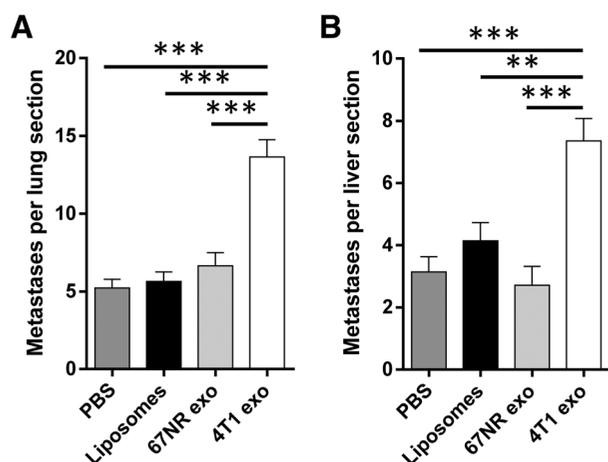
Exosomes from highly metastatic 4T1 cells are more capable than nonmetastatic 67NR exosomes at inducing MDSC recruitment. **A**, BALB/c mice received 5 μ g (2.9×10^{10} particles) of 4T1- or 67NR-derived exosomes every 3 days for 30 days (i.v., intravenous). Liposomes served as control. The frequency of T cells, NK cells, and MDSC subpopulations were quantified by flow cytometry at endpoint in the liver (**B**) and lung (**C**). Results are presented as mean \pm SEM ($n = 6-7$ animals/group) and analyzed by ANOVA; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

liver metastases, its secreted exosomes did not accumulate specifically in the liver compared with liposome particles, indicating that tumor exosomes may not necessarily mimic the metastatic distribution of host cells. Supporting this observation, exosomes from 67NR cells distribute significantly to both the liver and lung despite the nonmetastatic potential of host cells. Our results are in contrast to those of a recent report that showed the biodistribution of human breast cancer exosomes mimics the organotropic distribution of the cell line of origin (36). Importantly, this report showed that organotropic tumor exosomes can redirect the metastatic distribution of tumor cells (36). While the distribution of exosomes from tissue-specific breast cancer cells was shown to be consistent regardless of route of injection (i.e., retro-orbital venous sinus, the tail vein or intracardiac; ref. 36), the distribution pattern of exosomes derived from non-tumor cell lines (HEK293T epithelial cells) is highly dependent on the route of injection (37). Taken together, it is likely that the organ specificity of exosome biodistribution is driven by a multitude of factors, including cell source, injection route, tissue microenvironment,

and exosome surface markers, all of which warrants further investigation to elucidate relative impact. Given the limited number of studies reporting exosome tracking (12, 36, 38, 39) and the increasing interest of using exosomes as targeted vesicles of therapeutic delivery, our study provides further evaluation and confirmation of the tissue accumulation of breast cancer exosomes. Our studies suggest that the intravenous injection of exosomes is an ideal route for *in vivo* investigations because it results in the accumulation of systemic exosomes in the lung, a frequent site of metastasis in breast cancer as well as many other cancers (e.g., colorectal, bladder, prostate, and kidney cancer). Therefore, this ideal model allows us to adequately study the consequence of exosome accumulation in premetastatic organs and speculate if its accumulation can make the lung environment more prone to metastatic tumor cell colonization.

A key hallmark and prerequisite for tumor cells to metastasize and sustain neoplastic progression is to evade immune detection and destruction (4). Commonly, this involves the concurrent co-opting of multiple immunosuppressive cell populations (40).

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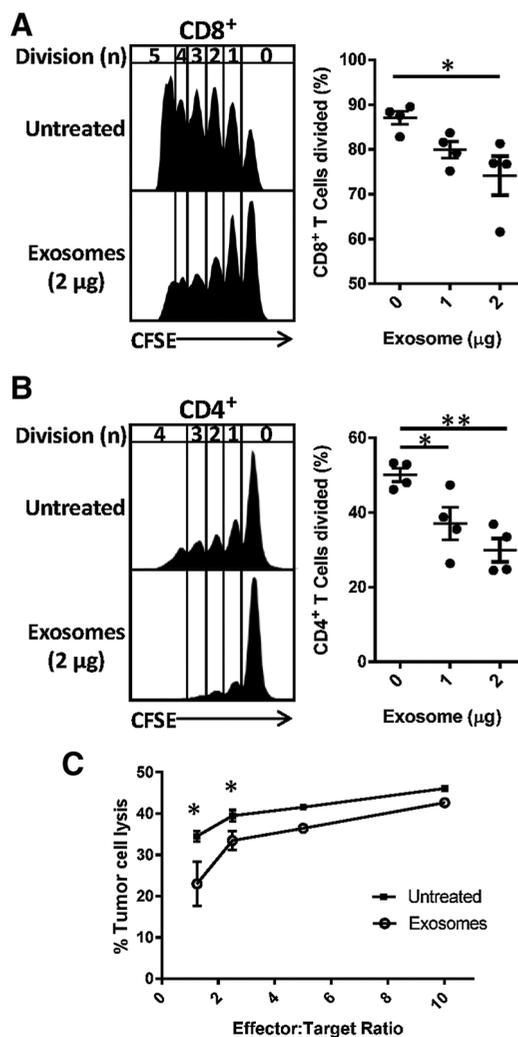
**Figure 6.**

Exosomes from metastatic breast cancer cells are required to initiate a premetastatic niche capable of promoting metastases. BALB/c mice received $5 \mu\text{g}$ (2.9×10^{10} particles) of 4T1- or 67NR-derived exosomes every 3 days for 30 days (intravenous). PBS and liposomes served as control treatment. After exosome conditioning, mice received 2.5×10^5 4T1-luciferase cells and metastatic burden in the lung and liver assessed 14 days later by histology. Number of metastases per lung section (A) and liver section (B). Two sections per organ were analyzed. Results are presented as mean \pm SEM ($n = 7$ animals/group) and analyzed by ANOVA. **, $P < 0.01$; ***, $P < 0.001$.

Here, we report the uptake of systemic breast cancer exosomes by various immune cell lineages in organs of high exosome accumulation and the subsequent establishment of a premetastatic niche. Up to 15% of CD45^+ cells in the lung were shown to take up EO771 breast cancer exosomes, a majority of which were macrophages, CD11b^+ myeloid cells, and DCs, and to a much lesser extent, lymphocytes. This uptake pattern was also comparable between highly metastatic 4T1 and nonmetastatic 67NR isogenic breast cancer cells. It is unclear from this study if exosome uptake is cell specific. Besides immune cells, lung-tropic exosomes are known to colocalize highly with S100A4-positive fibroblasts and surfactant protein C (SPC)-positive epithelial cells in the lung (36). Target cell specificity for binding of exosomes may be determined by adhesion molecules, such as tetraspanins, galectins, and integrins, as well as major histocompatibility complex class II present on exosomes (36, 41–43). Proteomic profiling combined with selective targeting of surface proteins will be required in future studies to determine exosome markers important for specific uptake by different immune cell populations. It is most certain that the overall organ-specific response induced by tumor-derived exosomes is a collective interplay between many target cell types, including fibroblasts, immune and epithelial populations.

It is becoming increasingly clear from both proteomic and genomic profiling studies that exosomes from metastatic and benign tumors are distinctly different and reflective of the disease state (12, 31, 44–46). Our findings provide key evidence that metastatic potential is also a key determinate of exosome function in the progression of metastasis. We demonstrate that exosomes from highly metastatic breast cancer cells (4T1 and EO771 cells) can condition a favorable microenvironment that promotes metastatic colonization in the lung and liver, an effect not observed from exosomes derived from

nonmetastatic cells (67NR cells) and liposome control vesicles. Specifically, the continuous accumulation and uptake of EO771 breast cancer exosomes in the lung was shown to recruit $\text{CD11b}^+/\text{Ly6C}^{\text{med}}$ gMDSCs (a subdivision of MDSCs), but decrease T-cell and NK-cell frequency, indicative of an immunosuppressive microenvironment. gMDSCs are a subpopulation of immature myeloid cells that are expanded in states of cancer and are associated with disease progression and poor prognosis, most often due to the suppression of T-cell immunity (33, 34, 47). Indeed, we showed that continuous conditioning of naïve mice with EO771-derived exosomes decreased the ratio of CD8 T-cell numbers to gMDSCs in the lung, suggesting that gMDSC recruitment by tumor exosomes may

**Figure 7.**

Breast cancer exosomes suppress T-cell proliferation and reduces NK-cell cytotoxicity. T cells and NK cells isolated from naïve mice were exposed to EO771-derived exosomes or PBS (untreated) *in vitro*. Representative flow-cytometric histograms (left) and percentage of divided $\text{CD8}^+/\text{CFSE}^+$ T cells (right; A) and $\text{CD4}^+/\text{CFSE}^+$ T-cells (B). C^{51}Cr release cytotoxicity assay for percent lysis of target tumor cells by effector NK cells at indicated effector-to-target ratios ($n = 3$ independent experiments in triplicate). Results are presented as mean \pm SEM and analyzed by ANOVA. *, $P < 0.05$; **, $P < 0.01$.

additionally regulate T cells. Furthermore, the frequency of MDSCs, as defined by the CD11b⁺/Gr1⁺ cells in a 4T1/BALB/c model (48), was also increased in the liver and lung following conditioning of naïve mice with exosomes from highly metastatic 4T1 cells. However, T-cell frequency in these organs remained unchanged. Several possible mechanisms for MDSC differentiation and recruitment by tumor-derived exosomes have been postulated by previous studies (17, 49–51). For example, exosomal PGE2 and TGF-beta secreted by breast cancer cells were reported to promote the differentiation of bone marrow myeloid cells to proinflammatory MDSCs (17). Others reported a pivotal role for Hsp72 and MyD88–Toll-like receptor (TLR) signaling in tumor exosome-mediated expansion of MDSCs and tumor progression (49, 50). Furthermore, exosomes derived from human colorectal and melanoma cells can impair the differentiation of peripheral blood monocytes to functional DCs, instead skewing them toward the phenotype of MDSCs (51). Interestingly, it was noted that 4T1 exosomes increased lung metastases despite inducing similar frequencies of MDSCs in the lung as liposome-treated controls. This suggests that exosomes may have equally important roles in MDSC accumulation, function regulation (e.g., gMDSC vs. mMDSC and cytokine secretion), and interplay with other immune cells (e.g., regulatory T cells) to promote metastatic outgrowth in premetastatic organs. Future studies to examine functional and gene expression consequences in MDSCs after exposure to tumor exosomes would be of interest to understand its overall contribution to premetastatic niche development.

Furthermore, continuous conditioning of the lung with EO771 breast cancer exosomes was shown to increase differentiation of naïve T cells (CD44^{low}CD62L^{hi}) to effector T cells (CD44^{hi}CD62L^{low}), a phenotype observed in other tumor microenvironments where T cells then proceed to terminal differentiation into "exhausted" T cells (52). Exhausted T cells are no longer functional and express high levels of immune inhibitory receptors, leading to cancer immune evasion (52). In line with our *in vivo* observation, the immunosuppressive nature of breast cancer exosomes was confirmed *in vitro* where it inhibited T-cell proliferation by initiating cell apoptosis and NK-cell cytotoxicity. It is possible that breast cancer exosomes are directly inducing apoptosis in activated T cells by the transfer of the death ligands FasL and TRAIL, a mechanism demonstrated in exosomes derived from other cancer types (16, 53, 54). Future studies to extensively characterize other immune cells types that demonstrated high exosome uptake, such as macrophages and DCs, would be important. Additionally, systemically injected breast cancer exosomes may have additional effects in organs other than the liver and lung (e.g., lymphoid organs) that contribute to tumor progression and warrant further investigation.

Overall, understanding the role and function of tumor-derived exosomes has important implications not just for the understanding of metastatic progression in breast cancer, but also in limiting the efficacy of current clinical and preclinical immunotherapeutics, which is currently not taken into consideration. Given that tumor-derived exosomes can target immune cells to alter its composition and induce an immunosuppressive tissue environment, they present potential targets of novel anticancer therapeutics in breast cancer. It may therefore be beneficial to selectively deplete tumor-specific exosomes in circulation by extracorporeal hemofiltration approaches (55), or develop inhibitors that interfere with tumor-exosome uptake. Future studies will focus on detailed proteomic and RNA profiling of breast cancer-derived exosomes to identify exosomal content responsible for driving immune regulation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.W. Wen, L.G. Lima, C.S.F. Wong, M. Becker, S. Krumeich, R.J. Lobb, K.N. Wong, S. Ellis

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.W. Wen, C.S.F. Wong, M. Becker, S. Krumeich

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Other (generated data by performing experiments crucial to the paper): V. Castillo

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