Extracellular vesicles from highly metastatic osteosarcoma cells induce pro-tumorigenic macrophage phenotypes

Katherine H. Griffin[1,2✉], Rachel Mizenko[3✉], Randy P. Carney[3#], and J. Kent Leach[1,3#]
[1]Department of Orthopaedic Surgery, UC Davis Health, Sacramento, CA; [2]School of Veterinary Medicine, University of California, Davis, CA; [3]Department of Biomedical Engineering, University of California, Davis, CA
khgriffin@ucdavis.edu

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INTRODUCTION: Osteosarcoma (OS) is the most common primary malignant bone cancer in children and adolescents, and metastasis is the principal factor in poor prognosis for individuals with the disease. Understanding the events that lead to metastasis is critical to develop better interventions for this disease. Alveolar macrophages are potentially involved in priming the lung microenvironment for OS metastasis, yet the mechanisms involved in this process remain unclear. Since extracellular vesicles (EVs) are a known actor in primary tumor development, we interrogated their potential role in OS metastagenesis by influencing macrophage polarization. To this end, we isolated EVs from two murine OS cell lines with varied metastatic potential to understand the immunomodulatory paracrine effects that EVs might play in distant metastatic sites. We hypothesize that EVs isolated from OS (OS-EVs) with increased metastatic potential will differentially modulate the immune microenvironment *via* macrophage migration, proliferation, and polarization state.

METHODS: EVs were isolated from highly metastatic (K7M2) and less metastatic (K12) OS cells with collection bioreactors (CeLLineAD1000) and differential ultracentrifugation. Here, we use the general term EVs to mean all material isolated during differential ultracentrifugation. This isolate likely contains multiple subtypes of EVs and EV-like particles (*e.g.*, exosomes, ectosomes, exomeres) since there is overlap of size and density of each, as well as non-vesicular co-isolates (*e.g.*, protein, cell debris) since no enrichment strategy can fully separate EVs from co-isolates. EVs were characterized with nanoparticle tracking analysis (NTA), resistive pulse sensing (RPS), single particle flow cytometry, and negative-stain transmission electron microscopy. We established an EV concentration that induced consistent IC-21 murine macrophage migration, and further characterized OS-EV interactions with macrophages with uptake, EdU proliferation, and flow cytometric polarization assays. During the uptake study, we labelled EVs with CFSE and interrogated the inherent differences in endocytic activity of macrophages with different polarization states. Thus, macrophages were polarized to the M0, M1, M2 phenotype prior to EV treatment. We also examined the role of CD47 in these interactions through inhibitory studies with a functionally blocking anti-CD47 antibody. Statistical analysis was performed using unpaired t-test assuming Gaussian distribution or two-way analysis of variance (ANOVA) with *post hoc* Tukey's test. Groups with different letters are statistically significant differences ($p<0.05$), while groups with the same letters are not significant. Data are presented as mean ± SD.

RESULTS: OS-EVs for both cell types exhibited the expected size characteristics, where NTA and RPS showed log-linear increases in particles to the detection limit of the instruments (100 nm and 65 nm, respectively) (**Fig. 1**). Single EV flow cytometry showed membranous CD44 and CD47, as expected for OS-derived EVs. We found that 10^4 EVs/cell induced increased migratory behavior in macrophages compared to both lower and higher EV doses (**Fig. 2**), and that when treated with this concentration of EVs, macrophage proliferative behavior remains unchanged, regardless of EV cell type. During the uptake study, by confocal microscopy, EVs were seen to interact with M1 macrophages both K12 and K7M2 EV treated macrophages compared to no treatment or CFSE control. There was also significant interaction for M2 macrophages treated with K7M2 EVs but not K12 EVs. However, there was no significant interaction of any EVs with M0 macrophages. Interestingly, when CD47 was inhibited, we did not observe differences in K7M2 and K12 EV interactions with M1 macrophages, but there was a significant decrease in K7M2 EV interactions with M2 macrophages. Lastly, when naïve macrophages were treated with EVs isolated from K7M2s, we observed a significant downregulation of M1 macrophages compared to those treated with K12 EVs, yet there was no change in M2 macrophage levels (**Fig 3**).

CONCLUSION: Together these data suggest that K7M2-EVs have unique interactions with macrophages that could contribute to the production of a higher proportion of pro-tumor type macrophages, thereby accelerating metastasis. Future studies are needed to identify the mechanism of these interactions.

SIGNIFICANCE/CLINICAL RELEVANCE: Overall, this work shows that OS-EVs may have different interactions with macrophages based on their metastatic potential. These differences could influence the profile of primary and metastatic niche macrophages. While most studies indicate that the presence of macrophages is a marker of poor prognosis, many studies suggest that they can also be protective, including in osteosarcoma. The heterogeneous effects found here may indicate a complex yet an unexplored mechanism for therapeutic targets to inhibit or treat OS metastasis.

IMAGES AND TABLES:

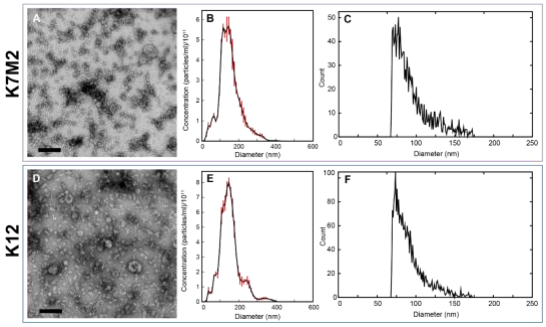

Fig. 1. **EVs isolated from highly metastatic and less metastatic OS have similar morphology and size profiles.** EVs from K7M2 (A-C) and K12 (D-F) cells were characterized by negative stain TEM (A, D), NTA (B, E) and RPS (C, F). Scale bar = 200 nm.

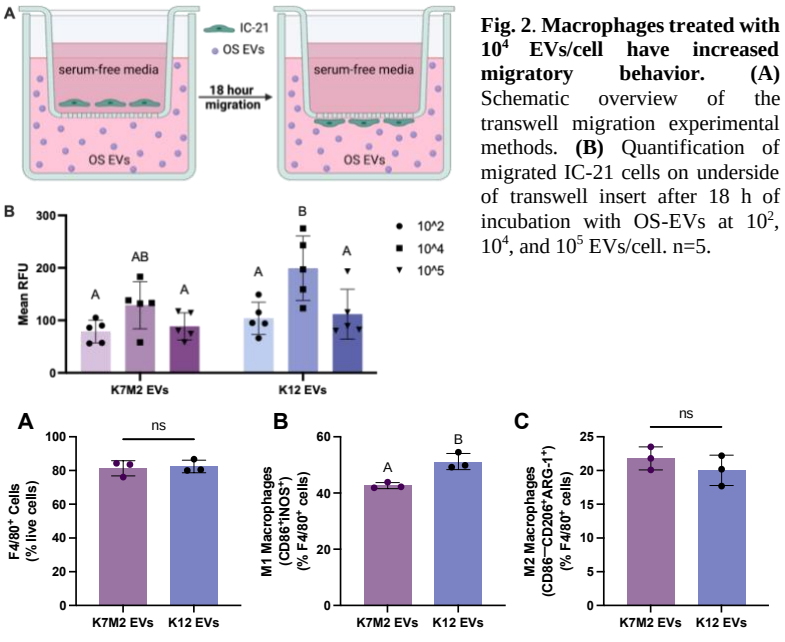

Fig. 2. **Macrophages treated with 10^4 EVs/cell have increased migratory behavior. (A)** Schematic overview of the transwell migration experimental methods. **(B)** Quantification of migrated IC-21 cells on underside of transwell insert after 18 h of incubation with OS-EVs at 10^2, 10^4, and 10^5 EVs/cell. n=5.

Figure 3 (right). **EVs isolated from highly metastatic OS downregulate M1 macrophages.** Flow cytometric analyses of IC-21 macrophages treated with OS-EVs for 24 h. Quantifications of **(A)** Live cells, **(B)** $F4/80^+$ cells, **(C)** $CD86^+iNOS^+$ (M1) macrophages, and **(D)** $CD86^-CD206^+Arg1^+$ (M2) macrophages. n=3.