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Stimulatory role of exosomes in the context of therapeutic anti-cancer vaccines

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Exosomes, the smallest of extracellular vesicles (EVs) produced by all cells and circulating freely in all body fluids, have been of interest as communication vehicles between cells (1). Much attention has been given to exosome origin and biogenesis as well as to the molecular/genetic cargo exosomes carry and deliver to recipient cells. Exosomes originate in the endosomal compartment of a parent cell. This biogenesis endows exosomes with special characteristics that differentiate them from other EVs, such as microvesicles (MVs) which "pinch off' the surface membrane of the parent cell (2). It also means that the exosome content is derived from the parent cell packaging machinery, so that a part of what that cell processes is incorporated in the exosome cargo (3). Thus, exosomes have the potential to serve as biomarkers of the parent cell activity (4). But perhaps the most intriguing aspect of exosomes concerns the delivery and the fate of the messages that they bring to recipient cells. While it remains unclear whether these messages are "edited" or "addressed" by the parent cell, numerous studies have shown that exosomes isolated from body fluids carry a large variety of stimulatory as well as inhibitory factors and deliver them "in bulk" to cells (5). The delivery of this complex cargo involves the engagement of receptors on the surface of recipient cells and the uptake of exosomes into the cell followed by reprogramming of its functions. The ability of exosomes to alter cellular functions of recipient cells and the mechanisms involved in this process are being intensely investigated. It appears that stimulatory and inhibitory signals exosomes carry are delivered simultaneously, but how this mode of delivery translates into specific functional alterations remains to be determined. Nevertheless, the potential impact of this type of exosome-driven reprogramming in health and disease is huge, and exosomes are emerging as future biomarkers based on the messages they carry but also as regulatory elements facilitating or directing functional attributes of recipient cells.

In the recent paper published in *Molecular Therapy*, Leaf Huang and his group (6) address the role of exosomes in potentiating efficacy of the nanoparticle lipid calcium phosphate (LCP)-based anti-tumor vaccine the group has been working on. The vaccine delivers a peptide derived from tyrosinase-related protein 2 (TRP2), a melanoma antigen, to B16F10 melanoma-bearing C57BL/6 mice. This vaccine, when administered subcutaneously (SC) to tumor-bearing mice together with CpG oligonucleotides (CpG ODNs) used as adjuvant, not only induces TRP2-specific T-cell responses but significantly inhibits the growth of

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established melanoma. The investigators decided to replace CPG ODNs in the vaccine with exosomes produced by the murine macrophage cell line (RAW246.7). The cell line was cultured in the presence of LPS, IFN-γ and GM-CSF to skew cell differentiation into M1-polarized macrophages. Exosomes isolated from supernatants of these M1 macrophages by ultracentrifugation were used in lieu of CPG ODNs as an adjuvant for the LPC vaccine. The objective was to evaluate the possibility of using exosomes derived from activated macrophages for enhancing immunogenicity and efficacy of tie anti-cancer vaccine that already inhibited tumor growth.

In a series of elegant *in vivo* experiments, the investigators demonstrated the localization of SC-injected exosomes labeled with a dye to the inguinal and axillary lymph nodes (LN) draining the injection site (6). These exosomes were readily taken up by macrophages and dendritic cells (DCs) populating the LNs. Importantly, these M1 macrophage-derived exosomes induced *in situ* production of proinflammatory cytokines, shifting the cytokine signature in the LNs to one rich in IL-6, IFN-γ and IL-12 but low in IL-10 and favoring the Th1 immune response. Monitoring of T-cell responses in the mice receiving the vaccine showed that the addition of M1-derived exosomes to the vaccine significantly boosted the frequency and anti-tumor cytolytic activity of antigen-specific CTLs. Further, these robust immune responses were accompanied by enhanced therapeutic efficacy of the vaccine as measured by the inhibition of tumor growth. The ability of exosomes produced by M1 macrophages to boost anti-tumor immunity and reduce growth of an already established melanoma deserves special attention. Adding this observation to the previously published data (7,8) permits us to entertain yet another important activity for the already bulging portfolio of exosome-mediated effects. Further, this is a vaccine-stimulatory activity, which is much in demand in the tumor microenvironment (TME), where immune suppression, including negative signaling by tumor-derived exosomes, creates unfavorable conditions for most therapeutic anti-cancer vaccines.

The environment of a growing tumor, such as B16F10 melanoma, is known to be strongly immunosuppressive (9). Macrophages accumulating in the TME actively participate in modulating anti-tumor immune responses. A majority of these macrophages are polarized to the M2 phenotype and contribute to creating the milieu favoring tumor growth (10). It should be remembered that tumor cells are avid producers of exosomes, and that tumor-derived exosomes carry numerous immunosuppressive ligands (4). The TME is thus saturated by these suppressive exosomes. The delivery to this TME of *ex vivo* generated M1-derived exosomes (but not M2-derived exosomes) introduces a major shift in exosomes populating the TME. The delivered exosomes, being derived from activated M1 macrophages, are presumably richly decorated by co-stimulatory ligands and are avidly internalized by native macrophages and DCs (as expected from phagocytic cells). This preferential uptake by antigen-presenting cells (APCs) of exosomes delivering co-stimulatory signals, and potentially also stimulatory cytokines, leads to rapid reprograming of recipient cells. These cells presumably upregulate expression levels of the antigen processing machinery (APM) components and increase production of pro-inflammatory cytokines. Mechanistically, this could involve miRNAs delivered by exosomes (11) and/or surface mediated co-stimulation translating into activation of cellular stimulatory pathways (12).

This scenario of exosomes competing for uptake by cellular inhabitants of the TME is not restricted to myeloid-derived cells. Immune cells, especially T cells, are also prominent infiltrating cells in the TME. They are also targeted by exosomes (13). However, T cells, unlike other immune cells, do not readily take up exosomes (12). Instead, exosome-mediated signals, handled via the receptor-ligand route and leading to downstream activation of cellular pathways, may be the major mechanism of their reprogramming. T cells in the TME are functionally dysregulated and overexpress inhibitory receptors, including PD −1 (14). Anti-tumor functions of these T cells are partly blocked via the checkpoint inhibitors or other tumor-orchestrated mechanisms (15). The inhibitory signaling pervading the TME is driven by tumor-derived exosomes carrying inhibitory ligands, such as PD-L1, and signaling via cognate receptors, i.e., PD-1, on T cells. Thus, restoration of T-cell anti-tumor activity rests upon overcoming this inhibitory signaling. That M1-derived, exogenous exosomes are able to do this, albeit only when delivered together with the LCP-based vaccine, is significant. It suggests that reprograming of APCs by costimulatory exosomes is critical for restoration of anti-tumor functions of T cells and for mounting of robust immune responses to the antigen present in the vaccine. Thus, not only timely delivery of co-stimulatory M1-derived exosomes but also the presence of the immunogen that is formatted for optimal presentation to APCs is critical for reprogramming the inhibitory TME into one permissive for the vaccine. In retrospect, it would have been informative if the authors defined co-stimulatory cargo of M1- *vs*. M2-derived exosomes or evaluated the upregulation of the APM components ± exosomes in addition to the cytokine profiles in DCs or macrophages in the LN of vaccinated animals.

One other point deserves a comment. The vaccine used in this study and M1-derived exosomes were administered SC to tumor-bearing mice. This mode of exosome and vaccine delivery might influence the efficacy of immune therapy. In the local milieu, direct infusion of co-stimulatory exosomes to the tumor site might favor stimulatory activity of exosomes for immune cells simply based on the numbers of delivered exosomes and the opportunity for their repeated delivery. For vaccines plus exosomes given IV, requirements for the delivery of an immunogen as well as exosomes to the tumor-draining LN, where tumor-specific T cell are generated and matured, may be different from those required for local vaccine administrations and perhaps more problematic.

Clearly, more *in vivo* studies are needed to study the role of exosomes as potentiators of the efficacy of anti-tumor vaccines. Huang *et al.* demonstrated the ability of exosomes to create local inflammatory milieu that favors a shift in the balance from immune suppression to immune stimulation in the presence of the growing tumor. This finding recommends exosomes as a potentially more effective adjuvant than those traditionally utilized in anti-cancer vaccines. However, the selection of immunopotentiating exosomes for use in cancer vaccines is of utmost importance. The membrane of these exosomes should be enriched in co-stimulatory receptor/ligands and phosphatidyl serine to, respectively, ensure strong costimulatory signaling and efficient uptake of exosomes by APC, especially immature DCs. The exosome lumen should be enriched in the miRNA species that upon internalization will re-direct the cytokine program of recipient cells to the production of IL-12, TNF-α, IFN-γ and other cytokines promoting activation of APCs. To accomplish this, it is necessary to rely either on the parent cell to provide exosomes with the necessary attributes, as did the authors

of the reviewed article, or to *ex vivo* modify exosomes to fit the requirements. The rational selection by Huang *et al.* (6) of M1 polarized macrophages as a source of stimulatory exosomes to be combined with vaccine undoubtedly contributed to the success of their experiments.

This study by Huang's group significantly contributes to our understanding of how correctly selected exosomes incorporated into an anticancer vaccine induce a desirable proinflammatory shift of balance in the TME. However, the report leaves us with many open questions about the possibility of moving this exosome-based stimulation platform to human therapeutic anti-tumor vaccines. The TME of human solid tumors appears to be more suppressive and harsher for immune cells than that of murine tumors. Therapeutic anti-tumor vaccines in humans have been challenging to implement, despite many different strategies developed for their production and delivery to patients (16). These vaccines have not been an overwhelming success so far, and it is certainly worthwhile to consider more effective adjuvant strategies for improving vaccine efficacy. As indicated above, the source of immunostimulatory exosomes for human anti-cancer vaccines is critical and will require substantial investigation. It is likely that in the near future, exosomes can be modified *ex vivo* to carry and deliver CpG ODNs or other adjuvant-like constructs to immune cells, thereby converting them into highly effective stimulators of adaptive immune responses. While we look forward to engineering exosomes to serve as adjuvants for therapeutic anti-cancer vaccines, it is crucial to bear in mind that human tumors evolve numerous strategies for blocking anti-tumor immune responses (5). This means that altering the immunosuppressive TME balance is likely to be challenging, the major challenge represented by tumor-derived immunosuppressive exosomes present in excess in the TME of all human tumors.

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