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Exosomes, microvesicles, and other extracellular vesicles—a Keystone Symposia report

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COMPETING INTERESTS

Niek Dekker is employed by AstraZeneca R&D. Michele de Palma is an inventor on a patent application filed by EPFL (WO2017134100A1) on engineered dendritic cell vaccines and serves on the scientific advisory board of EVIR Therapeutics, a start-up focused on the development of engineered dendritic cell vaccines for cancer therapy.

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Abstract

Extracellular vesicles (EVs) are small, lipid-bilayer-bound particles released by cells that can contain important bioactive molecules, including lipids, RNAs, and proteins. Once released in the extracellular environment, EVs can act as messengers locally as well as to distant tissues to coordinate tissue homeostasis and systemic responses. There is a growing interest in not only understanding the physiology of EVs as signaling particles but also leveraging them as minimally invasive diagnostic and prognostic biomarkers (e.g., they can be found in biofluids) and drug-delivery vehicles. On October 30–November 2, 2022, researchers in the EV field convened for the Keystone symposium "Exosomes, Microvesicles, and Other Extracellular Vesicles" to discuss developing standardized language and methodology, new data on the basic biology of EVs and potential clinical utility, as well as novel technologies to isolate and characterize EVs.

Keywords

exomere; exosome; extracellular RNA; extracellular vesicle; mitovesicle; supermere

INTRODUCTION

Extracellular vesicles (EVs) are naturally released by nearly all cell types in the body. They are generally delimited by a lipid bilayer and can contain protein, RNA, and lipid cargo indicative of the cell of origin. EVs comprise several types of particles, such as exosomes, ectosomes, microvesicles, and others, that are typically defined based on size and/or how they are produced. EVs are present in all bodily fluids. Once released from a cell, they can diffuse into circulation, carrying their cargo throughout the body and signal to distant tissues. For example, EVs released by tumor cells can have systemic effects on immune cells, enabling tumors to "remotely" modify the immune system to be more permissive.[1] Because the surface and cargo of EVs are derived from the cell from which they are produced, they can reveal important information about the cells and tissue of origin. There is growing interest in their potential use as minimally invasive disease and/or predictive biomarkers.[2] In addition, because EVs can travel to places where cells cannot easily go, they may represent attractive therapeutic agents themselves to deliver functional molecules to, or engage signaling pathways on, target cells.

On October 30–November 2, 2022, several investigators in the EV field met for the Keystone symposium "Exosomes, Microvesicles, and Other Extracellular Vesicles" to discuss new findings on the basic biology and clinical utility of EVs, including their roles in disease pathogenesis, novel therapeutic approaches, and biomarker developments in areas

including cancer and neurodegenerative, cardiovascular, and metabolic disorders. As the EV field grows and evolves, the need to standardize nomenclature and methods to purify and characterize EVs becomes paramount. Toward these goals, several speakers presented novel methodologies and tools for studying EVs.

EV ISOLATION, CHARACTERIZATION, AND NORMALIZATION

Establishing common ground: The 2018 MISEV guidelines

Kenneth W. Witwer from the Johns Hopkins University School of Medicine gave an overview of the minimal information for studies of extracellular vesicles (MISEV) guidelines. The guidelines, developed by the International Society for Extracellular Vesicles (ISEV), with input from experts and community members, were published in 2018 and are currently under review for an update.[2] The MISEV guidelines established a framework for EV studies for nomenclature and communicating key concepts to conducting translational studies. Because EVs are so diverse,[3] it is important to establish a common language that defines EV subtypes based on agreed upon criteria as the field evolves.[4] The guidelines also discuss the collection and preprocessing of EVs—defining which variables should be reported when, for example, drawing blood to collect EVs—and expand upon separation and concentration methods. EVs are just one of the many types of extracellular particles and are typically in low abundance. While the MISEV guidelines do not recommend a specific method for separation or concentration, they do advise that researchers be aware of the specificity of each method and whether it is appropriate for their research question.[5] Similarly, the guidelines do not recommend a universal negative or positive marker to characterize EVs but provide guidance on evaluating the purity of a sample and demonstrating the presence of EVs.[2] An adjunct to the guidelines is EV-TRACK, a central knowledgebase of EV separation and characterization data.[6] In addition to the general recommendations in MISEV, more specific recommendations are being created by task forces set up by the ISEV. For example, minimal information about a flow cytometry experiment, MIFlowCyt-EV, establishes a framework for standardized reporting of EV flow cytometry experiments.[7] Witwer stressed that these guidelines are meant to create common ground within the community and should not be seen as stifling innovation or a barrier to new EV investigators.

Exosomes as EGF receptor signaling moieties in cancer

Robert J. Coffey from Vanderbilt University Medical Center presented work on the role of EVs in trafficking EGF receptor (EGFR) ligands to epithelial cells, with a focus on colorectal cancer (CRC).[8] Coffey's group combined sequential ultracentrifugation and fluorescence-activated vesicle sorting to show that several EGFR ligands are present in exosomes from breast and CRC cell lines. They found that exosomes containing the EGFR ligand amphiregulin markedly enhanced the invasiveness of recipient cancer cells, inspiring the term *exosomal-targeted receptor activation* (ExTRAcrine) as a new mode of EGFR signaling.[9,10] Coffey's group has also developed new methods to isolate EVs and exomeres (non-membranous nanovesicles with a size ≤50 nm), including an optimized density gradient fractionation technique that revealed the presence of nonvesicular materials resembling the recently characterized exomeres.[11] Exomeres were discovered by David

Lyden's lab using asymmetric flow field-flow fractionation.[12] Coffey's group has developed a simpler, more cost-effective way to isolate exomeres based on ultracentrifugation.[13] They also discovered and characterized supermeres, small nanoparticles that contain both disease biomarkers and therapeutic targets. In particular, they found that supermeres are enriched in transforming growth factor beta induced (TGFBI) and contain secreted RNA, including small nuclear RNA and microRNA (miRNA). Studies are underway to elucidate the ontogeny of supermeres and to explore whether their cargo may contain potential biomarkers for CRC.[14]

Mapping extracellular RNA-binding proteins and their cargo

Aleksandar Milosavljevic from Baylor College of Medicine discussed work on mapping the extracellular RNA-binding proteins (exRBPs) and extracellular RNA cargo in human biofluids. The work leverages data from the exRNA Atlas, a project launched by the NIH Common Fund Extracellular RNA Communication Consortium that identified approximately 7000 small RNAseq profiles from different human biofluids.[15] In a second phase of the project, researchers are focusing on exRBPs and how they mediate the export and transport of exRNA. Correlation footprinting has identified exRBPs and their exRNA cargo in human biofluids, revealing six exRNA cargo types: two classes of EVs distinguished by their RNA cargo, three subtypes of RNA-protein particles, and a class of lipoproteins. Milosavljevic showed that different exRBPs tend to carry similar biotypes across biofluids, while lipoproteins associate with numerous exRBPs. Milosavljevic hopes that continued work on mapping the complete set of RNA-binding proteins (RBPs) will provide the foundation for understanding the role of exRBPs in extracellular communication.

Capturing EV heterogeneity with EV Fingerprinting

Ariana K. von Lersner from Alissa Weaver's group at Vanderbilt University presented work on EV Fingerprinting, a new methodology that uses multiparametric flow cytometry data to capture the heterogeneity of EV populations: EVs are labeled with a lipophilic dye, di-8-ANEPPS (di8), and detected via imaging flow cytometry. The unique spectral properties of di8 provide information on EV size and lipid composition through the dimensional reduction of multiparametric features (Figure 1). von Lersner explained how this method can be used to characterize and quantify EVs. As proof of principle, she showed how EV Fingerprinting can quantify the selective loss of small EV populations upon knockdown of Rab27a, which is involved in EV secretion.[16]

The impact of cell culture conditions on EVs

Lizandra Jimenez, also from Alissa Weaver's group at Vanderbilt University, presented work on the impact of cell culture conditions on small EVs. Jimenez proposed that using attempting to use "EV-depleted" fetal bovine serum (FBS) during EV purification can actually confound results. Multiple reports have shown that ultracentrifugation does not fully remove bovine EVs from FBS and that bovine RNA and lipoproteins remain in EV-depleted FBS.[17,18] She presented unpublished data on the effect of serum type on RBPs, including Ago2, and miRNA in EVs. In brief, Jimenez's work indicates that media conditions can

strongly affect the presence of both intravesicular and extravesicular RBPs and that serum can be a source of contaminating EV RBPs.

EV Paint—a new technique to study cell–cell communication

Ferdinando Pucci from Oregon Health & Science University presented a new technology to identify target cells of EVs *in vivo*—EV Paint—to study communication between native EVs and target cells. In EV Paint, parental cells are genetically engineered to express a transmembrane enzyme that localizes to the surface of EVs. Once an EV is released in the extracellular environment and engages with a target cell, the enzyme forms a covalent bond between a fluorescent substrate (secreted by the same parental cells) and cell surface proteins. EV Paint is 10 times more sensitive than first-degree labeling (such as CD63-GFP)[a] (Figure 2).[19] Pucci's group has verified the method *in vitro*[19] and shown how it can be used *in vivo*.[20] In mice, EV Paint was used to identify which immune cells in sentinel lymph nodes are targeted by EVs released by engineered tumor cells. On top of the already known interaction with subcapsular sinus macrophages, Pucci discovered that lymph node B cells are a major target of native tumor EVs. In a separate application of EV Paint, Pucci's group showed that restricting expression of the enzyme to cancer stem cells (CSCs) allowed specific study of tumor–host interactions within the CSC niche. This work suggested that MHC-II$^-$ macrophages and PD1$^+$ T cells may play important roles in CSC biology by closely interacting with CSCs and/or their EVs.[20] Overall, Pucci's work demonstrates the importance of studying native EVs without any *in vitro* manipulation in order to understand how they are involved in local and remote cell–cell communication.

BIOGENESIS AND TRANSFER OF EVs

EV biogenesis from cilia—a potential role in ADPKD pathology

Maureen M. Barr from Rutgers University presented work on cilia-derived vesicle biogenesis and signaling using *Caenorhabditis elegans* as a model organism. Cilia are present on most nondividing cells. Barr's group has shown that cilia can act as a specialized venue for regulated EV biogenesis by shedding EVs from their tip via budding. Cilia dysfunction is associated with many different disorders. Barr's group is using *C. elegans* to understand the biology of EV shedding and the function of PKD1 and PKD2. Mutations in these genes (*PKD1* and *PKD2*) lead to autosomal dominant polycystic kidney disease (ADPKD), a genetic condition that results in large, cystic kidneys.[21–23] Several lines of evidence suggest that EVs may play a role in the pathology of ADPKD. Urinary EVs in people with ADPKD differ from EVs of unaffected individuals, and EVs from ADPKD patients promote cystogenesis in cell culture.[24,25] Loss of cilia in the kidney suppresses cyst growth, suggesting a role for cilia-derived EVs in cyst formation.[26] Barr showed that in *C. elegans*, EVs are shed by cilia from both the tip, which they think may play a role in long-distance communication, and the base, which may play a role in interanimal communication.[27] PKD2 and lov-1, the *C. elegans* homolog of PKD-1, localize to male-specific cilia and EVs;[21,23] however, these two proteins localize to different EVs, indicating that cilia can shed different types of EVs.[28,29] They also showed that ciliary release of EVs can be regulated by mechanical stress.[30] Work on understanding the proteome of ciliary EVs identified approximately 3000 candidate EV cargos, including many nucleic acid–binding

proteins that do not colocalize with PKD2. [28,29] Barr's group has developed an app as a resource for researchers, MyEVomeapp, that includes single-cell transcriptomic data for proteins identified as EV cargo candidates.

Engineering EVs for drug delivery

Niek Dekker from AstraZeneca (AZ) described the company's efforts to engineer EVs for drug delivery and regenerative therapy. AZ has identified several proteins as promising EV-sorting candidates for protein cargo loading or ligand display. The group has further characterized EVs using single-particle analyses to quantify the percentage of vesicles that contain a desired cargo, as well as single-molecule analyses that enable them to quantify how many cargo molecules are present in a given vesicle.[31] The ability of these vesicles to deliver functional cargo was demonstrated *in vitro* using EVs loaded with Cas9 and a light-inducible dimerizing protein. Dekker showed that they were able to achieve efficient cargo loading, with approximately 25 Cas9 molecules per EV, and that the vesicles were able to conduct CRISPR gene editing *in vitro*, with a gene-editing rate of 25–51%, depending on cell type.[32] The critical bottleneck for functional delivery of EV cargo is endosomal escape—while EVs are efficiently taken up by cells, they are unable to escape from the endosome. Dekker showed that delivery efficiency can be improved with agents that induce endosomal rupture.[33,34] AZ is exploring a hybrid EV (HEV) containing a built-in endosomal escape mechanism. He showed unpublished data on the *in vivo* distribution of HEVs and delivery of CRISPR/Cas9 with *in vivo* gene editing. One of the key applications for which AZ is pursuing EVs is cardiac regeneration. In mice, exosomes secreted by cardiac progenitor cells (CPCs) are critical for regeneration and cardioprotection. Injecting these exosomes into injured mouse hearts recapitulates the regenerative and functional effects of CPC transplantation.[35] AZ is working to develop EVs for cardiac regeneration, initially by conducting *in vivo* efficacy studies with engineered HEVs that contain RNA cargo meant to further enhance cardiac regeneration.

Regulation of RNA cargo loading at ER–endosomal contact sites

Bahnisikha Barman from Alissa Weaver's lab at Vanderbilt University presented work on delineating the mechanisms by which RNAs are incorporated into EVs. There are several ways by which RNA can be incorporated into EVs.[36] Barman is specifically interested in the biogenesis of RNA-containing EVs at the endoplasmic reticulum (ER) membrane, given that several studies have demonstrated that ER–endosomembrane contact sites play a role in intraluminal vesicle (ILV) formation.[37–39] Barman confirmed that the ER and endosomes make contact with each other and that several miRNAs are present at these contact sites. She identified VAP-A as a key regulator of ER–endosomal contact formation that controls the biogenesis of a subpopulation of RNA-containing EVs. Further investigation on the mechanisms of VAP-A's effects showed that interactions between VAP-A and CERT at ER membrane contact sites promote the incorporation of ceramide into nascent ILVs.[40] Ceramide then induces negative curvature in the endosomal membrane, which is necessary for the budding of EVs.[40,41]

Designing enveloped protein nanocages for drug delivery

Daniel Humphrys from Neil King's lab at the Institute for Protein Design (IPD) at the University of Washington presented work on developing enveloped protein nanocages (EPNs) to deliver biocargo to cells. Researchers at the IPD had previously computationally designed a self-assembling protein nanocage[42] that was further engineered to induce its release from cells within a membrane envelope. These EPNs contain membrane binding and ESCRT recruitment modifications that result in the budding of EVs containing multiple protein nanocages. Pseudotyping EPNs with the viral membrane fusion protein VSV-G enables them to deliver proteins from cell to cell.[43] Humphrys presented unpublished work which has expanded this system to actively recruit other membrane proteins to the EPN surface. This affords new functionalities to EPNs, such as the ability to deliver biocargoes to specific cell types. Humphrys is also investigating the potential of EPN-based vaccines by incorporating the SARS-CoV-2 spike protein onto the EPN surface.

Regulation of exosome secretion

Justin Williams from Randy Schekman's group at the University of California Berkeley presented unpublished work on the role of annexin A6 in mediating calcium-dependent exosome secretion in response to plasma membrane damage.

EVs AND CANCER

Leveraging tumor-derived EVs in cancer vaccines

Michele de Palma from the Swiss Federal Institute of Technology in Lausanne (EPFL) presented work on leveraging tumor-derived extracellular vesicles (tEVs) to improve the activity of dendritic cell (DC) cancer vaccines. DC vaccines are typically produced by isolating monocytes from a patient, maturing them in the presence of tumor antigen, and infusing them back into the patient. The hope is to generate tumor-associated antigen (TAA)–presenting DCs that can activate tumor-specific cytotoxic T cells. This approach has several limitations, however. It requires *a priori* knowledge of relevant TAAs and uses monocyte-derived DCs, which are not as adept at antigen presentation as other types of DCs, such as type 1 conventional DCs (cDC1).[44] De Palma's group has developed a way to induce DCs to take up tEVs *in vivo*; because tEVs contain many TAAs, this approach obviates the need to identify TAAs in advance. In brief, DCs are engineered to express an extracellular vesicle–internalizing receptor (EVIR), in which the extracellular domain binds to a known tumor antigen present on tEVs. Using HER2 as a proof-of-principle, de Palma showed that DCs that express an anti-HER2 EVIR preferentially internalize HER2-expressing tEVs and promote antigen-specific T cell proliferation. Further work on the mechanism of antigen presentation revealed that DC cross-dressing, in which a preformed tumor antigen/MHCI complex is transferred from the cancer cell to the DC, is the preferred mechanism of T cell activation.[45,46] De Palma also presented unpublished data investigating the ability of DCs expressing an anti-GD2 EVIR to take up melanoma-derived EVs, as well as a protocol to use DC progenitors in their EVIR platform as opposed to monocytes in the hope of inducing cDC1 cells *in vivo*.

Immunosuppression via tumor-derived EVs

Wei Guo from the University of Pennsylvania presented work on understanding the impact of tEVs on immune suppression. One of the ways in which tumor cells evade T cell–mediated cytotoxicity is by expressing the immune checkpoint protein PD-L1: the interaction of PD-L1 with PD-1 expressed on T cells leads to the exhaustion of tumor-infiltrating T cells.[47] Guo's group has shown that tEVs also contain PD-L1 on their surface and can promote the exhaustion of $CD8^+$ T cells.[48] Guo showed that tEVs preferentially interact with activated $CD8^+$ T cells via ICAM-1 on tEVs and LFA-1 on $CD8^+$ T cells. This interaction enables PD-$L1^+$ tEVs to home in on activated $CD8^+$ T cells and protect the tumor from the immune system at a distance.[49] Guo's group has also elucidated the players involved in loading PD-L1 onto tEVs. He showed that HRS, a pivotal component of the ESCRT complex, directly interacts with PD-L1 and recruits it onto exosomes. HRS is a substrate of ERK, which is often aberrantly activated in melanoma. Using phosphomimics, Guo showed that phosphorylated HRS promotes selective loading of PD-L1 to tEVs. This in turn inhibits $CD8^+$ T cell proliferation and activity *in vitro* and promotes tumor growth and blocks T cell infiltration *in vivo*.[50] Guo hopes that phosphorylated HRS and exosomal PD-L1 may provide important diagnostic information to identify patients likely to benefit from anti–PD-1 treatment.

Tumor-derived EVs in pancreatic cancer

Despite the profound impact of immunotherapy (IO) on cancer survival, it has had minimal impact in pancreatic cancer.[51] **Nuno Bastos** from Sonia Melo's group at the Institute for Research and Innovation in Health is interested in understanding whether targeting tEVs can improve the efficacy of IO in pancreatic ductal adenocarcinoma and/or be used as a biomarker to identify patients more likely to benefit from IO. He presented unpublished data on the impact of Rab27a, which regulates EV release, on the tumor microenvironment and disease progression.

EVs IN THE NERVOUS SYSTEM

EVs as pathologic entities and diagnostic biomarkers in neurodegenerative diseases

Andrew F. Hill from Victoria University presented work on the role of EVs in neurodegenerative diseases. EVs can be efficient carriers of the misfolded proteins that are characteristic of several neurodegenerative diseases.[52,53] Hill showed data from various cell culture models that EVs can efficiently transfer infectious prion particles from cell to cell.[54–60] His lab is now working on understanding the specific components in EVs that mediate prion transfer. Hill also showed that EVs can promote misfolding of α-synuclein, which is implicated in Parkinson's disease. While other groups have shown that lipids can influence α-synuclein misfolding, these studies typically use vesicles that are not biologically relevant.[61] Hill's group has shown that small EVs can accelerate the misfolding of α-synuclein and promote the production of higher-order, neurotoxic species. They confirmed that an intact EV membrane is important to support and enhance α-synuclein misfolding.[62,63] Hill also discussed the potential of EVs as diagnostic biomarkers for neurodegenerative diseases. His lab has identified a panel of nine miRNAs that are dysregulated in exosomes from prion-infected neuronal cells in mice.[56] Tracking the

expression of these miRNAs over time in prion-infected mice revealed different signatures associated with preclinical and clinical stages of the disease. This raises the potential of identifying prion disease before symptom onset and intervening before the advent of irreversible neuropathologic changes.[64] Hill's group is also investigating the potential of EV miRNAs to aid in the diagnosis of Alzheimer's disease. They have narrowed down a 12-miRNA panel that may be able to accurately detect AD from blood samples.[65,66]

Mitovesicle secretion in aging and neurodegenerative disorders

Efrat Levy from New York University Langone Medical Center presented work to understand the role of *mitovesicles* in neuronal integrity. Dysfunction in the neuronal endosomal–lysosomal pathways is one of the earliest pathologies observed in Alzheimer's disease and has been observed in individuals with Down syndrome and during aging. Since EVs reflect changes in the endosomal pathway, Levy's group isolated and characterized EVs from brain extracellular space.[67] In doing so, they identified a novel type of mitochondrial-derived EV, mitovesicles (Figure 3).[68,69] Levy showed that mitovesicles are double-membrane vesicles with high electron density which contain a subset of mitochondrial proteins that enable them to achieve some of the functions of mitochondria, such as producing ATP.[68,69] *In vitro*, mitovesicle secretion is induced by mitochondrial stress.[68] Levy's group has also identified several conditions that promote mitovesicle secretion *in vivo*, including aging, Down syndrome, and chronic cocaine exposure.[68,70–72] Levy proposed that mitovesicles may play a role in mitochondria quality control and responses to oxidative stress. Her lab is working to understand the functional roles of mitovesicles, including possibly in neurotransmission, and to delineate the mechanisms involved in mitovesicle formation and secretion.

EVs in neuronal communication

Michael P. Hantak from Jason Shepherd's group at the University of Utah presented work on understanding the impact of Arc-containing EVs in neuronal communication. Arc is a neuronal protein involved in long-term memory; it induces the endocytosis of the AMPA receptor at the neuronal synapse to tune its strength. Shepherd's group has shown that Arc is a so-called *repurposed* virus-like gene that can form virus-like capsids.[73] Given the functional similarities between EVs and viruses—both can package proteins from the cytoplasm and transfer them between cells—Hantak is interested in whether Arc is similarly released from EVs. He showed unpublished data on how Arc EVs represent a novel form of intercellular signaling in the brain.

EVs AND CARDIO-METABOLIC PHYSIO/PATHOLOGY

EVs as adipocyte–cardiac messengers

Clair Crewe from Washington University School of Medicine in St. Louis showed how EVs can act as interorgan messengers between adipocytes and the heart. Adipose tissue is a large, metabolic organ that becomes dysfunctional in the setting of obesity. Work in Philipp Scherer's lab at the University of Texas Southwestern Medical Center has shown that mitochondrial dysfunction contributes to adipocyte dysfunction over the course of obesity. Using a mouse model that mimics obesity-related mitochondrial dysfunction in adipocytes,

Crewe and colleagues observed that these mice also had high levels of oxidative stress in the cardiac tissue.[74] Crewe showed that EVs act as the messenger between these two organs —cardiac and adipose tissue. *In vitro*, stress-stimulated adipocytes increase EV secretion, which can subsequently induce oxidative stress in cardiomyocytes. This mechanism was confirmed *in vivo* by exposing adipocytes to palmitate to mimic the stress experienced during obesity and injecting the secreted EVs into mice. This resulted in transient cardiac oxidative stress followed by a long-term antioxidant response. Interestingly, the EVs seemed to have a protective effect against cardiac injury.[75] These results illustrate an example of interorgan mitohormesis, a phenomenon in which cells are subjected to mitochondrial stress and, consequently, upregulate pathways that increase resiliency: When a second, more severe stress occurs, the cells are better protected from damage.[76] While the short-term impact of adipocyte-derived EVs appears to be protective toward cardiac tissue, the long-term impacts—and whether they play a role in the increased risk of cardiovascular disease associated with obesity—remain to be seen.

EVs in cardiovascular calcification

Elena Aikawa from Harvard Medical School presented work on the role of EVs in cardiovascular calcification. Microcalcification is an independent risk factor for cardiac plaque rupture, which can lead to myocardial infarction.[77] Aikawa showed that within atherosclerotic lesions both macrophages and smooth muscle cells can release EVs that contribute to microcalcification. In macrophages, EV release is driven by S100A9, a calcium-binding protein.[78,79] In smooth muscle cells, the osteogenic conditions of atherosclerotic plaques alter mitochondrial calcium signaling and promote the release of EVs that contain proteins such as annexin A1 that promote EV aggregation and the formation of microcalcifications. Blocking annexin A1, either via a neutralizing antibody or siRNA, inhibits EV aggregation and calcification of smooth muscle cells.[80] EV aggregates can become trapped between collagen fibers and nucleate hydroxyapatite, forming microcalcifications.[81] Aikawa's group also identified sortilin as a key player in the generation of EVs from smooth muscle cells. Both phosphorylation and dimerization of sortilin are important for regulating trafficking of tissue-nonspecific alkaline phosphatase (TNAP, a key enzyme responsible for tissue mineralization) into EVs, thereby inducing calcification.[82,83] Serum sortilin levels also correlate with aortic calcification and cardiovascular risk in humans, indicating that sortilin may be a useful plasma biomarker.[84]

Impact of EVs on inflammation

Alan M. Adamczyk from Matias Ostrowski's lab at the University of Buenos Aires presented work on the impact of EVs on macrophage-driven inflammation. Macrophages are one of the main cell types that drive inflammatory responses to infection, but they also play a key role in resolving inflammation once the infection is cleared. Adamczyk showed unpublished data that support a role for plasma EVs in dampening the inflammatory effect of stimulated macrophages.

EVs as interorgan drug delivery particles

Tamires M. Zanotto from C. Ronald Kahn and Mario Saad's lab at the State University of Campinas presented unpublished work on using exosomal miRNA to target PCSK9, an enzyme released by hepatocytes that promotes LDL receptor (LDL-R) internalization and degradation. PCSK9 has become a promising therapeutic target for conditions like hypercholesterolemia, since inhibiting its activity increases LDL-R levels and reduces cholesterol.[85] Work in the Kahn lab has shown that adipose tissue is a key source of circulating miRNAs that can impact distant organs, including the liver.[86] One of the ways miRNAs can enter circulation is via exosomes. Zanotto is investigating the potential to decrease PCSK9 levels in the liver by inducing exosomal loading of miRNAs that target PCSK9 in the adipose tissue.

NONMAMMALIAN EVs AND VIRUSES

The role of EVs in inflammation and reservoir dynamics during chronic HIV infection

Matias Ostrowski from the University of Buenos Aires presented work on the impact of plasma EVs on inflammation and the viral reservoir in HIV-infected individuals. While effective antiretroviral therapy has turned HIV infection from a near-certain AIDS diagnosis and subsequent death to a manageable chronic condition, two obstacles stand in the way of a cure: inflammation and the viral reservoir. Ostrowski stressed that these two obstacles are linked. Residual inflammation can contribute to HIV persistence both by activating and promoting the homeostatic proliferation of latently infected cells.[87] Ostrowski's group is investigating the role of EVs from infected cells on inflammation and the viral reservoir, which has historically been controversial. Some studies have demonstrated that EVs activate latent HIV, while others have shown they promote viral latency.[88–91] Ostrowski showed that plasma EVs isolated from HIV-infected individuals stimulate macrophages, thus promoting inflammation.[92] Subsequent work has revealed that while plasma EVs themselves cannot activate viral reservoirs, EV-treated macrophages do. Ostrowski's group has narrowed down the moiety responsible for this effect to galectin-1, a soluble, secreted protein produced by myeloid cells that binds to glycans on cell membranes and can impact various aspects of T cell physiology. Galectin-1 levels are higher in patients infected with HIV-1, and levels correlate with viral reservoir size. Ostrowski proposed that HIV infection promotes changes in the composition of plasma EVs that induce macrophages to secrete inflammatory cytokines and galectin-1. The increase in inflammatory cytokines contributes to the inflammatory diseases that are prevalent in HIV-infected individuals, while galectin-1 interacts with $CD4^+$ T cells and promotes latency reactivation. This work demonstrates a role for EVs in fueling two of the major obstacles toward a cure for HIV.[93]

EV packing of RNA during SIV infection

Tanina Arab from Kenneth Witwer's lab at Johns Hopkins University, and representing first author Yiyao Huang, presented unpublished data on EV packaging of the small nuclear RNA U6 during SIV infection.[b] There are many similarities between the biogenesis of EVs and enveloped viruses—both processes use similar host machinery, such as the ESCRT complex and tetraspanins. EVs generated by infected cells can also contain viral protein and RNA fragments, and infection can impact the loading of host proteins and other components

into EVs.[94] Arab hopes that understanding the impact of viral infection on EVs may lead to novel biomarkers and/or therapeutic targets that can help to eradicate HIV from viral reservoirs.

Detecting virus-related EVs for diagnostics and prognosis

Daniel C. Rabe from Shannon Stott's lab at Massachusetts General Hospital presented unpublished data on the development of a microfluidic device that detects SARS-CoV-2 infection and virus-related EVs. The Stott lab has developed microfluidic-based techniques to isolate EVs from patient plasma.[95] Rabe showed how they are modifying the device to make it more amenable to commercialization and clinical uses. He demonstrated the utility of this technique as a diagnostic tool to detect SARS-CoV-2 from patient plasma, as well as to capture infection-related EVs, which may be useful in predicting severe disease.

Bacteria-derived vesicles in cancer

Aadil Sheikh from Leigh Greathouse and Joe Taube's groups at Baylor University presented work on characterizing bacteria-derived outer membrane vesicles (OMVs) from *Bacteroides fragilis*. Pathogenic strains of *B. fragilis*, e.g., enterotoxigenic *B. fragilis* (ETBF), have been implicated in inflammatory bowel disease and CRC.[96] Sheikh presented unpublished data on how OMVs from both ETBF and commensal strains of *B. fragilis* differ in composition and their impact on host epithelial cells.

Impact of bacteria-derived EVs on pancreatic cells

Danilo Rodrigues da Silva from Graciela Lorca's group at the University of Florida presented work on the impact of *Lactobacillus johnsonii* N6.2–derived nanovesicles on cellular responses. *L. johnsonii*, a Gram-positive bacterium originally isolated from the stool of diabetes-resistant rats,[97] has been studied as a probiotic in a phase 1 clinical trial, where it was deemed to be safe and associated with beneficial effects, including a decrease in stomachache, bloating, cramping, and abdominal pain.[98] Lorca's group is currently recruiting for a phase 2 trial to investigate the impact of *L. johnsonii* in adults with diabetes. *L. johnsonii* produces EVs from the cytosolic membrane; Da Silva showed that these nanovesicles have a distinct lipid and protein profile compared to the cell membrane, specifically, they are enriched in cytoplasmic proteins, including those involved in glycolysis and protein synthesis.[99] Da Silva showed that *L. johnsonii*–derived nanovesicles may play a role in the protective effects against diabetes. Bacteria-derived nanovesicles increased insulin secretion in glucose-stimulated human islets and reduced cytokine-induced apoptosis in pancreatic beta cells. Investigations into the mechanism of these effects showed that upon internalization, the nanovesicles release RNA cargo that induces the expression of RNA-sensing and aryl hydrocarbon receptor genes.[100]

NOVEL TECHNOLOGICAL DEVELOPMENTS

New tools for single-particle and EV repertoire analyses

Jennifer C. Jones from the National Cancer Institute (NCI/NIH) presented work on developing new tools to isolate and characterize EVs, both at a single-particle and repertoire levels (the tools and protocols are available to researchers at https://nano.ccr.cancer.gov)

Jones's group is interested in distinguishing between different types of EVs, that is, those that originate from immune, tumor, or vascular cells, to better understand their biology. This requires techniques to identify biomarkers that define each subset as well as optimizing RNA-seq methods to characterize each subset. Jones's group has developed single-EV methods for labeling and sorting EVs via flow cytometry.[101,102] In addition, new software tools provide more accurate methods to characterize single EVs, via FCM_{pass}, or EV repertoires via MPA_{pass}.[103,104] A third software, RPS_{pass}, provides more accurate single-particle size and concentration measurements and a live-acquisition interface that enables researchers to view data in real-time. These tools provide the foundation for the NIH-sponsored exRNA Atlas, which Jones's group is developing with Aleksandar Milosavljevic's group at Baylor College of Medicine. They have also created customized RNA-seq methods for EV cargo that tackle the problem of low RNA content of EVs, which usually falls below the 1-ng threshold needed for most methodologies used in transcriptomics studies.

A novel EV isolation strategy

Chioma Okeoma from New York Medical College presented a novel technology for isolating native EVs. There is no one-size-fits-all approach when it comes to isolating EVs; current isolation methods, such as ultracentrifugation and precipitation, have several limitations, e.g., they can alter EV morphology and it can be difficult to remove contaminants. And not all methods are amenable to retrieving EV subpopulations for subsequent functional studies or preparative purposes for clinical uses. Okeoma's method, particle purification liquid chromatography (PPLC), addresses many of these limitations. PPLC is a one-step gradient size exclusion chromatographic separation technique that can be tuned to achieve the desired resolution. Analytes are monitored via UV/Vis spectra to determine particle size, concentration, and purity. PPLC can separate EVs from other analytes, such as lipoproteins, albumin, and other extracellular condensates that may masquerade as EVs, including viral particles. Okeoma showed how they used this technique to isolate blood EVs from patients with breast cancer, with the aim of identifying markers that predict response to neoadjuvant chemotherapy. They found that nonresponders had higher levels of EVs in their blood than responders and that the EVs had distinct proteomes that may be able to distinguish who is likely to respond to neoadjuvant therapy. In particular, EVs from nonresponders were enriched in several proteins, an example being HSPB1, which is known to cause resistance to doxorubicin in breast cancer cells and is a prognostic marker of poor outcome.[105]

A nanomembrane-based approach for EV purification

Thomas Gaborski from the Rochester Institute of Technology discussed efforts to develop new ways to purify and separate EVs. Gaborski's group focuses on developing and using nanomembranes for various purposes, such as filtration and coculture models. They have developed a tangential flow–based method, tangential flow for analyte capture (TFAC), to isolate and purify EVs from complex biofluids that uses an ultrathin silicone nanomembrane. In this approach, as the sample flows across the membrane, a slight transmembrane pressure captures and holds vesicles while smaller proteins pass through nanopores. A cleaning step washes away the remaining contaminants, and vesicles

are then released by reversing the membrane pressure.[106] Gaborski noted that there are several limitations with TFAC; notably, it does not differentiate between EVs and similarly sized lipoproteins, and the fluid shear stress can deform and damage EVs. Gaborski's group has adapted TFAC by incorporating nanopocket-containing membranes. The system incorporates concepts from chromatography and filtration to capture and protect nanovesicles. The membrane contains nanopockets of different sizes that can capture nanovesicles and protect them from fluid shear forces.[107] Pockets are arranged to capture and release small, medium, and large vesicles sequentially, thus combining purification and separation. Gaborski's group is now working to functionalize the nanopockets to enable them to distinguish between small EVs and similarly sized species like lipoproteins.

Supermeres—a novel amembranous nanoparticle

Qin Zhang from Robert Coffey's lab at Vanderbilt University Medical Center presented work characterizing supermeres. Zhang originally discovered supermeres while developing an ultracentrifugation method to isolate exomeres.[13,14] Zhang found that small, 25–30 nm amembranous extracellular nanoparticles, which she later dubbed supermeres, were present in the supernatant of her exomere preparations. Supermeres can be taken up by cells, likely through macropinocytosis. They exhibit distinct RNA and proteomic profiles that differ from those of exomeres, sEVs, and NVs. In particular, supermeres are enriched in TGFBI, several clinically relevant shed membrane proteins, such as amyloid precursor protein, as well as several miRNAs, including miR-1246, a potential cancer biomarker. Zhang's work supports the role of supermeres as distinct, functional extracellular nanoparticles and represents an important step toward properly assigning protein/RNA cargo to their correct carriers.[14]

A novel field-flow fractionation method to separate EVs

Olesia Gololobova from Kenneth Witwer's group at Johns Hopkins University presented work on separating the subclasses of extracellular RNA carriers in human plasma. She showed unpublished data on a plasma EV separation workflow that uses asymmetric flow field-flow fractionation (AF4) to separate EVs, lipoproteins, and plasma proteins in a single run.

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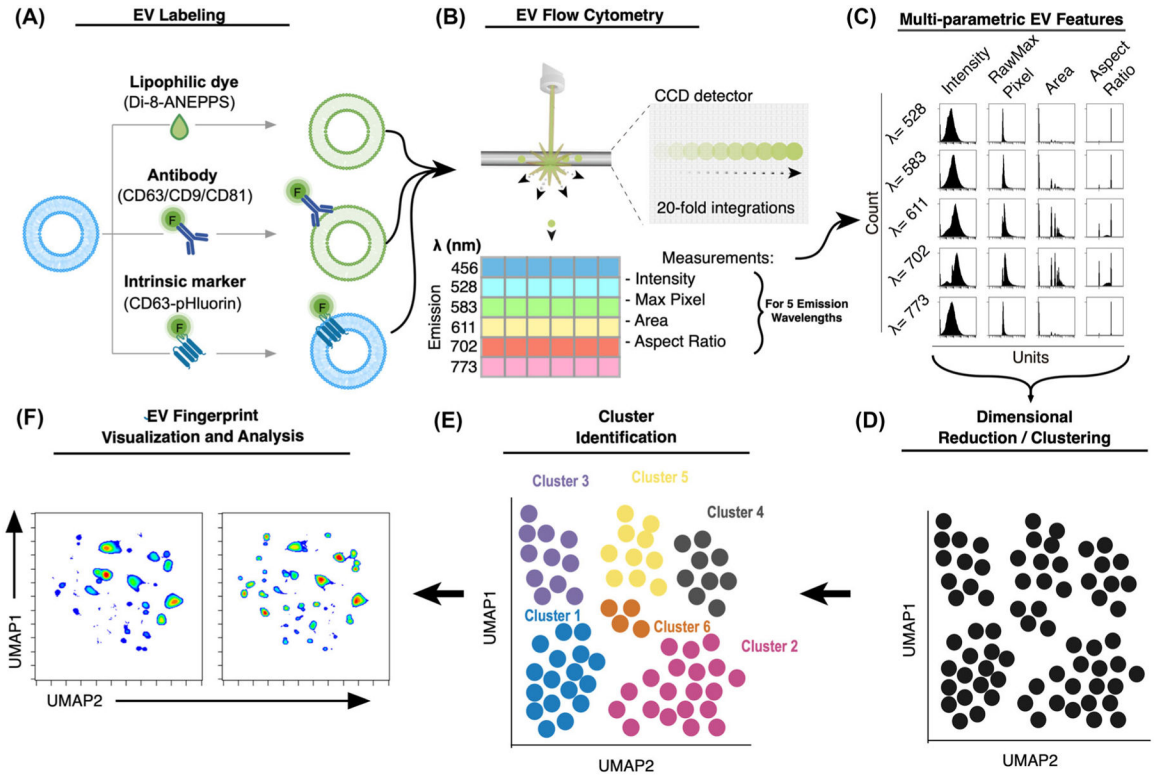

FIGURE 1.

EV Fingerprinting, a new methodology that uses multiparametric flow cytometry data to capture the heterogeneity of EV populations.

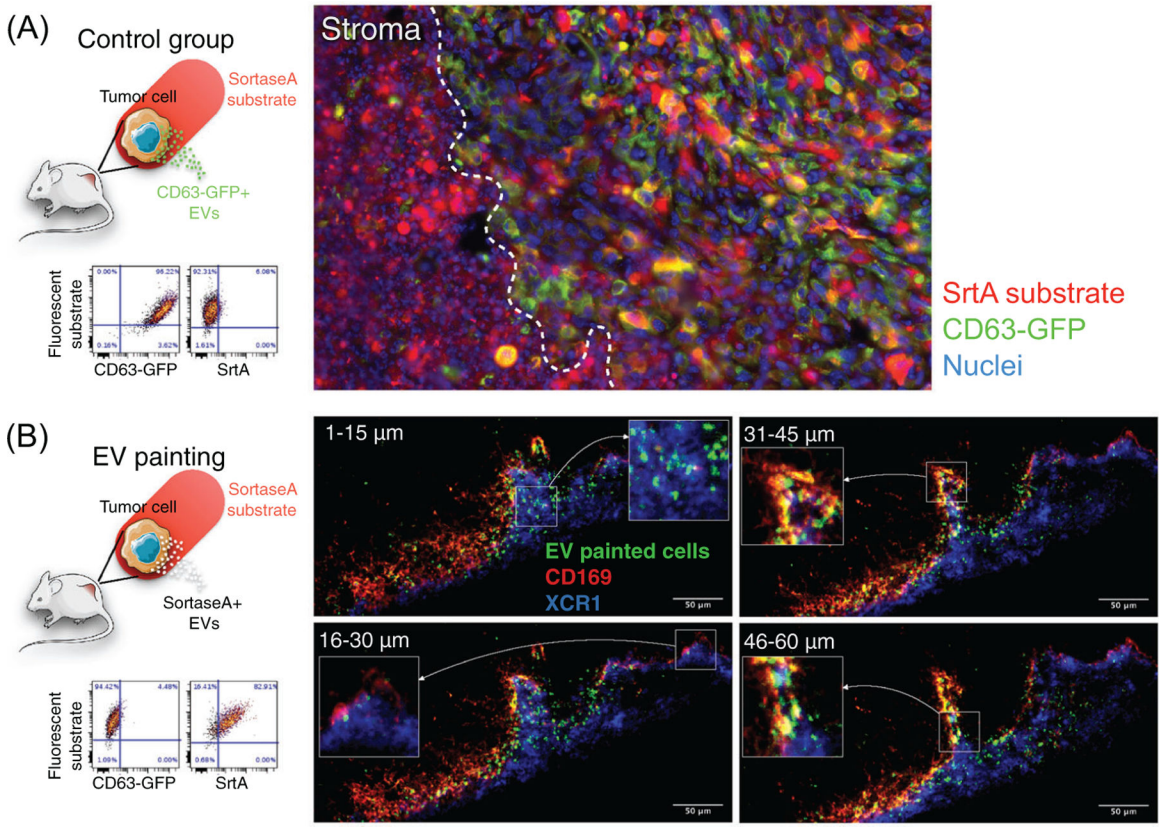

FIGURE 2.

EV Painting: cell surface labeling via EVs. Cells of choice (in the depicted example, tumor cells) are engineered to express the CD63-GFP fusion protein (A) or a membrane-bound form of Sortase-A (B) and its fluorescent substrate (as secreted protein). *In vitro* flow cytometric analyses of the cells are shown. The secreted fluorescent substrate can be observed in the tumor stroma *in vivo* (A, right), suggesting that the Sortase-A substrate is released from tumor cells and it drains into sentinel lymph nodes (not shown). In tumor-draining lymph nodes (B, right), 3D imaging reconstructions show $CD169^{+}$ sub-capsular sinus macrophages are extensively labeled by EVs, as expected. Migratory and resident $XCR1^{+}$ dendritic cells are mostly lacking EV binding, possibly as a result of internalization and degradation of EV-bound antigens for cross-presentation. Of note, several small, round, lymphoid-like cells have been EV painted. Scale bar: 50 um. Each image is a projection of 15um-deep volumes taken at the indicated depths. [Correction added on 23 March 2023, after first online publication: Legend for Figure 2 was updated.]

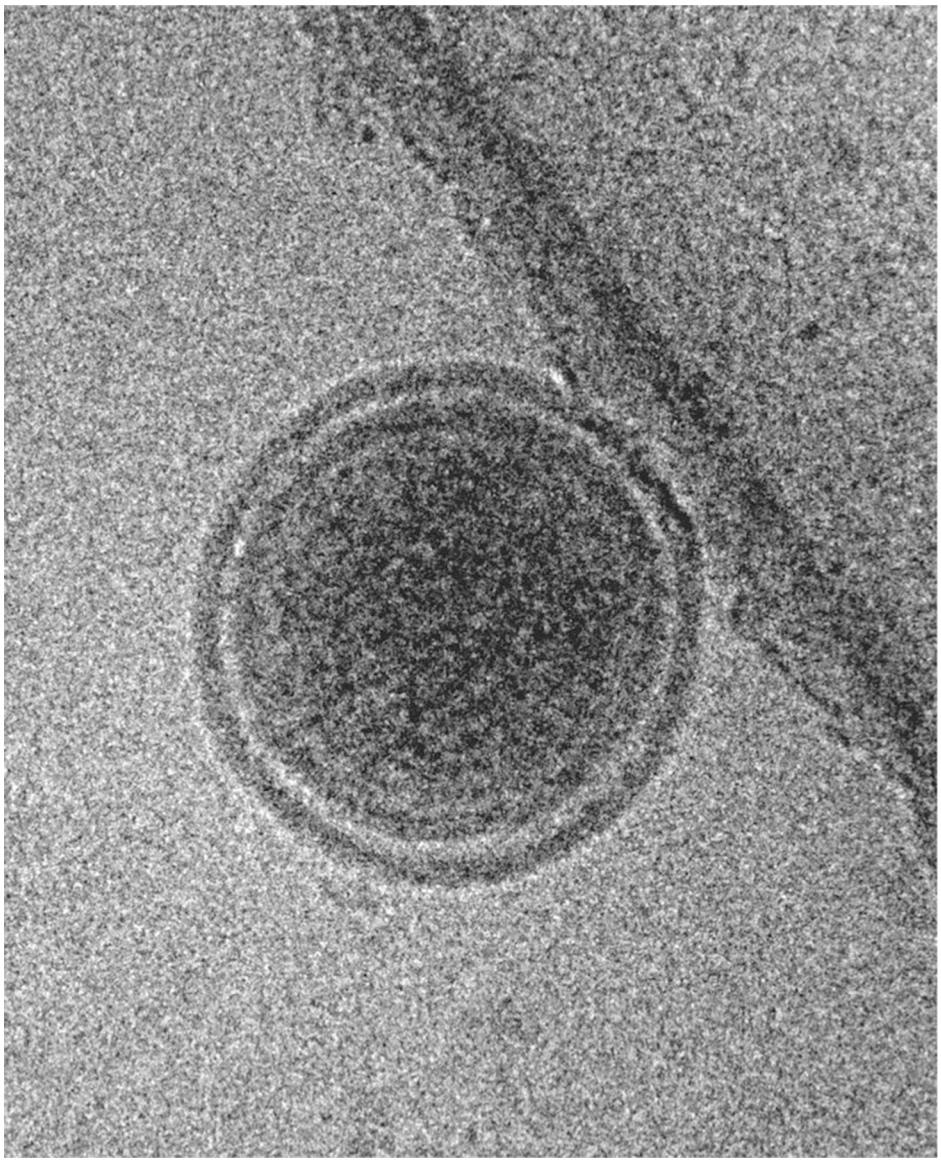

FIGURE 3.
CryoEM of a mitovesicle.