

EXOSOMES: MESSAGE IN A VESICLE

EDITED BY: Suman Dutta, Gal Bitan, Kendall Van Keuren-Jensen and
Satish Balasaheb Nimse

PUBLISHED IN: Frontiers in Pharmacology, Frontiers in Immunology,
Frontiers in Cell and Developmental Biology and
Frontiers in Microbiology





frontiers

Frontiers eBook Copyright Statement

The copyright in the text of individual articles in this eBook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this eBook is the property of Frontiers.

Each article within this eBook, and the eBook itself, are published under the most recent version of the Creative Commons CC-BY licence.

The version current at the date of publication of this eBook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or eBook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714

ISBN 978-2-83250-288-4

DOI 10.3389/978-2-83250-288-4

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.

Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

EXOSOMES: MESSAGE IN A VESICLE

Topic Editors:

Suman Dutta, University of California, Los Angeles, United States

Gal Bitan, University of California, Los Angeles, United States

Kendall Van Keuren-Jensen, Translational Genomics Research Institute (TGen),
United States

Satish Balasaheb Nimse, Hallym University, South Korea

Citation: Dutta, S., Bitan, G., Van Keuren-Jensen, K., Nimse, S. B., eds. (2022).

Exosomes: Message in a Vesicle. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-83250-288-4

Table of Contents

- 05 Editorial: Exosomes: Message in a Vesicle**
Suman Dutta, Satish Balasaheb Nimse, Kendall Van Keuren-Jensen and Gal Bitan
- 09 Generation and Application of a Reporter Cell Line for the Quantitative Screen of Extracellular Vesicle Release**
Jonathan Shpigelman, Fitzgerald S. Lao, Shiyin Yao, Chenyang Li, Tetsuya Saito, Fumi Sato-Kaneko, John P. Nolan, Nikunj M. Shukla, Minya Pu, Karen Messer, Howard B. Cottam, Dennis A. Carson, Maripat Corr and Tomoko Hayashi
- 24 The Depolarization-Evoked, Ca^{2+} -Dependent Release of Exosomes From Mouse Cortical Nerve Endings: New Insights Into Synaptic Transmission**
Guendalina Olivero, Francesca Cisani, Danilo Marimpietri, Daniela Di Paolo, Maria Cristina Gagliani, Marina Podestà, Katia Cortese and Anna Pittaluga
- 37 Blood Nanoparticles – Influence on Extracellular Vesicle Isolation and Characterization**
Marija Holcar, Maša Kandušer and Metka Lenassi
- 57 Multi-Omics Analysis of Microglial Extracellular Vesicles From Human Alzheimer's Disease Brain Tissue Reveals Disease-Associated Signatures**
Whitaker Cohn, Mikhail Melnik, Calvin Huang, Bruce Teter, Sujyoti Chandra, Chunni Zhu, Laura Beth McIntire, Varghese John, Karen H. Gylys and Tina Bilousova
- 72 The Aquatic Invertebrate *Hydra vulgaris* Releases Molecular Messages Through Extracellular Vesicles**
Maria Moros, Eugenio Fergola, Valentina Marchesano, Margherita Mutarelli, Giuseppina Tommasini, Beata Miedziak, Giuliana Palumbo, Alfredo Ambrosone, Angela Tino and Claudia Tortiglione
- 86 Exosomes Recovered From the Plasma of COVID-19 Patients Expose SARS-CoV-2 Spike-Derived Fragments and Contribute to the Adaptive Immune Response**
Elisa Pesce, Nicola Manfrini, Chiara Cordiglieri, Spartaco Santi, Alessandra Bandera, Andrea Gobbini, Paola Gruarin, Andrea Favalli, Mauro Bombaci, Alessandro Cuomo, Federica Collino, Giulia Cricri, Riccardo Ungaro, Andrea Lombardi, Davide Mangioni, Antonio Muscatello, Stefano Aliberti, Francesco Blasi, Andrea Gori, Sergio Abrignani, Raffaele De Francesco, Stefano Biffo and Renata Grifantini
- 103 Biogenesis and Biological Functions of Extracellular Vesicles in Cellular and Organismal Communication With Microbes**
Yuan Fang, Zhiwen Wang, Xili Liu and Brett M. Tyler
- 118 The Role of Extracellular Vesicles in Systemic Lupus Erythematosus**
Chenghui Zheng, Lin Xie, Haihong Qin, Xiao Liu, Xi Chen, Fan Lv, Li Wang, Xiaohua Zhu and Jinhua Xu

- 130 ***A Novel Tissue Atlas and Online Tool for the Interrogation of Small RNA Expression in Human Tissues and Biofluids***
Eric Alsop, Bessie Meechoovet, Robert Kitchen, Thadryan Sweeney, Thomas G. Beach, Geidy E. Serrano, Elizabeth Hutchins, Ionita Ghiran, Rebecca Reiman, Michael Syring, Michael Hsieh, Amanda Courtright-Lim, Nedyalka Valkov, Timothy G. Whitsett, Jorge Rakela, Paul Pockros, Joel Rozowsky, Juan Gallego, Matthew J. Huentelman, Ravi Shah, Peter Nakaji, M. Yashar S. Kalani, Louise Laurent, Saumya Das and Kendall Van Keuren-Jensen
- 151 ***Extracellular Vesicle-Mediated Delivery of Ultrasmall Superparamagnetic Iron Oxide Nanoparticles to Mice Brain***
Naseer A. Kutchy, Rong Ma, Yutong Liu, Shilpa Buch and Guoku Hu
- 160 ***A Triple High Throughput Screening for Extracellular Vesicle Inducing Agents With Immunostimulatory Activity***
Nikunj M. Shukla, Fumi Sato-Kaneko, Shiyin Yao, Minya Pu, Michael Chan, Fitzgerald S. Lao, Yukiya Sako, Tetsuya Saito, Karen Messer, Tomoko Hayashi, Howard B. Cottam, Maripat Corr and Dennis A. Carson
- 176 ***Differential Effects of APOE Genotype on MicroRNA Cargo of Cerebrospinal Fluid Extracellular Vesicles in Females With Alzheimer's Disease Compared to Males***
Ursula S. Sandau, Trevor J. McFarland, Sierra J. Smith, Douglas R. Galasko, Joseph F. Quinn and Julie A. Saugstad
- 197 ***Extracellular Vesicles From TNF α Preconditioned MSCs: Effects on Immunomodulation and Bone Regeneration***
Miya Kang, Chun-Chieh Huang, Praveen Gajendrareddy, Yu Lu, Sajjad Shirazi, Sriram Ravindran and Lyndon F. Cooper
- 211 ***Exosome: The "Off-the-Shelf" Cellular Nanocomponent as a Potential Pathogenic Agent, a Disease Biomarker, and Neurotherapeutics***
Satyajit Ghosh and Surajit Ghosh
- 228 ***Potentiality of Exosomal Proteins as Novel Cancer Biomarkers for Liquid Biopsy***
Chunmiao Hu, Wei Jiang, Mingjin Lv, Shuhao Fan, Yujia Lu, Qingjun Wu and Jiang Pi
- 247 ***Antiviral Activity of Extracellular Vesicles Derived From Respiratory Syncytial Virus-Infected Airway Epithelial Cells***
Tiziana Corsello, Yue Qu, Teodora Ivanciuc, Roberto P. Garofalo and Antonella Casola



OPEN ACCESS

EDITED AND REVIEWED BY

Heike Wulff,
University of California, Davis,
United States

*CORRESPONDENCE

Suman Dutta,
sduttacla@gmail.com

†PRESENT ADDRESS

Suman Dutta,
International Institute of Innovation and
Technology, Kolkata, India

SPECIALTY SECTION

This article was submitted to
Experimental Pharmacology and Drug
Discovery,
a section of the journal
Frontiers in Pharmacology

RECEIVED 14 August 2022

ACCEPTED 16 August 2022

PUBLISHED 08 September 2022

CITATION

Dutta S, Nimse SB,
Van Keuren-Jensen K and Bitan G
(2022), Editorial: Exosomes: Message in
a vesicle.
Front. Pharmacol. 13:1018928.
doi: 10.3389/fphar.2022.1018928

COPYRIGHT

© 2022 Dutta, Nimse, Van Keuren-Jensen and Bitan. This is an open-access article distributed under the terms of the [Creative Commons Attribution License \(CC BY\)](#). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Editorial: Exosomes: Message in a vesicle

Suman Dutta^{1*†}, Satish Balasaheb Nimse²,
Kendall Van Keuren-Jensen³ and Gal Bitan^{1,4,5}

¹Department of Neurology, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA, United States, ²Institute of Applied Chemistry and Department of Chemistry, Hallym University, Chuncheon, South Korea, ³Neurogenomics Division, The Translational Genomics Research Institute, Arizona, AZ, United States, ⁴Brain Research Institute, University of California, Los Angeles, Los Angeles, CA, United States, ⁵Molecular Biology Institute, University of California, Los Angeles, Los Angeles, CA, United States

KEYWORDS

extracellular vesicle (EV), intercellular communication, biomarker, drug delivery vehicle, therapeutics

Editorial on the Research Topic Exosomes: Message in a vesicle

Exosomes are a class of extracellular vesicles (EVs) comprising a heterogeneous population of biological nano-vesicles enclosed by a lipid bilayer (Yan et al., 2019). All eukaryotic cells produce and release EVs into the surrounding extracellular space, including exosomes. Because the term ‘exosome’ refers to a specific biogenesis pathway that produces some EVs (Lötvall et al., 2014), and once they leave the cell, it is difficult to separate exosomes from other similar types of extracellular vesicles, we refer to all extracellular vesicles as EVs in this editorial. EVs carry various cargoes including nucleic acids, such as miRNA and mRNA, proteins, and lipids. Although EVs originally was thought to be a disposal mechanism of unwanted biomaterials, it became evident in recent years that they play a crucial role in intricate signaling pathways and intercellular communication (Figure 1). Collaborative efforts across the globe have helped elucidate the pathway of EV biogenesis and their involvement in several human diseases. This Research Topic is a collection of original articles, review articles, and one brief research report that highlight current knowledge on different isolation and characterization methods for EVs, their roles in intercellular communications, therapeutic applications, potential as a source of biomarkers, and their use as a vehicle for drug delivery. In total, the Research Topic contains sixteen papers by experts in the corresponding fields describing and discussing enthralling breakthroughs over the past several years in the EV research field.

Shpigelman et al., at the University of California, San Diego, CA, United States, are reporting a pioneering study developing a reporter cell line for quantitative measurement of EV release from antigen-presenting cells (APCs) in a phenotypic high-throughput screening format. They show that the new, dual-reporter cell line exhibits robust responses and has the potential to become an excellent research tool for the screening

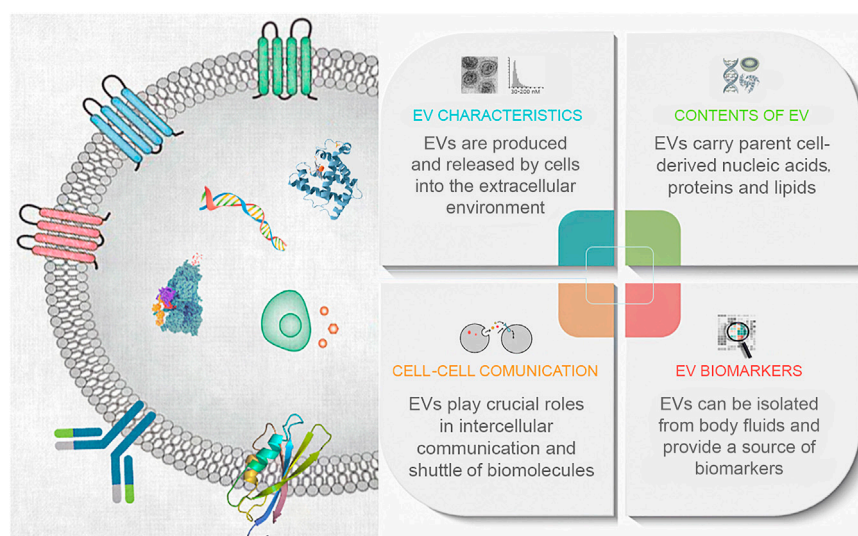


FIGURE 1
An infographic overview of extracellular vesicles.

of compounds that induce or inhibit EV release by APCs. An article by [Shukla et al.](#), at the University of California, San Diego, CA, United States, aimed at identifying small molecules that could induce EV release in a human monocytic leukemia THP-1 reporter cell line. They described a process of screening the EV release that could add a new dimension to structure-activity relationship studies of vaccine adjuvant activity. A study by [Olivero et al.](#), at the University of Genoa, Italy, addresses the important question of whether exosomes are actively released from presynaptic nerve terminals. Their findings provide strong evidence supporting the release of EVs from presynaptic structures and indicate an increased EV release upon exposure of synaptosomes to a depolarizing stimulus.

[Alsop et al.](#), from the group of one of our Research Topic Editors, Dr. Kendall Van Keuren-Jensen at the Translational Genomics Research Institute, Phoenix, AZ, United States, together with multiple collaborators, have generated an atlas of small RNAs from 30 different tissues and three different blood cell types. They analyzed various tissues for the enrichment of small RNA sequences, assessed their expression in different human biofluids (saliva, urine, CSF, and plasma) in cell-free circulation as well as from EVs ([Kalani et al., 2020](#)), and developed an online tool providing information about extracellular-RNA sequences found in different biofluids and tissues. The authors made the tool, which shows tissue-specific elevation of extracellular RNA sequences and their abundance in biofluids, freely available online. [Sandau et al.](#), at Oregon Health and Science University, Portland, OR, United States, with several collaborators, sought to understand the effect of Alzheimer's Disease (AD) on the miRNA cargo in cerebrospinal fluid-derived

EVs and to determine if AD risk factors have an impact on EV-associated miRNA expression patterns. Their study showed that APOE- ϵ 4 and female sex, two known risk factors for the disease, influence EV-associated different miRNA levels in the CSF of patients with AD. [Bilousova et al.](#), at UCLA, CA, United States, led a multi-omics study analyzing putatively microglia-originating small EVs from cryopreserved human brain tissue in search of novel biomarkers for Alzheimer's Disease. The analysis included lipids, proteins, and miRNAs in microglia-originating small EVs from human brain tissue and indicated the release of neuronal and myelin materials via EVs. Another important finding was a significant decrease in docosahexaenoic acid levels in AD, which potentially could be used as a biomarker. A review article by [Ghosh and Ghosh](#) from the Indian Institute of Technology Jodhpur, India, described in detail the role of small EVs as pathogenic agents transmitting toxic protein forms that propagate neurodegenerative disorders throughout the CNS. It is becoming increasingly evident how small EVs can be harnessed in identifying neurodegenerative disease biomarkers ([Hornung et al., 2020](#); [Dutta et al., 2021](#)).

A review article by [Hu et al.](#), from Guangdong Medical University, Dongguan, China, discussed the potential of small EV-associated cargo as a source of novel biomarkers for cancer. The authors gathered useful information regarding the clinical applicability of EV-associated proteins based on which they suggest that the advancement of EV isolation techniques and characterization strategy will play a significant role in diagnosis as well as immunotherapy of cancer. Another detailed review by [Holcar et al.](#), from the University of Ljubljana, Slovenia has summarized major contaminants of blood-derived EVs and

thoroughly discussed how different EV isolation methods might affect the EV yield and data interpretation of downstream experiments. The authors reiterated the importance of developing methods for isolation of pure subpopulations of EVs to which the origin of the parent cell type can be attributed and argued that such development will help understand the functions of EVs in a definitive way.

A timely study by [Pesce et al.](#), from the National Institute of Molecular Genetics (INGM), Milan, Italy, demonstrated that EVs isolated from the plasma of patients with COVID-19 harbor SARS-CoV-2-related protein materials. They observed that EVs recovered from patients with mild symptoms effectively modulated antigen-specific CD4-positive T-cell responses. The study also suggested that the patient-derived EV-associated proteins not only induced an immune response, but also the EV cargo could help identify the pathological state of patients in the acute phase of the disease.

[Moros et al.](#), at the Universidad de Zaragoza, Zaragoza, Spain, and Istituto di Scienze Applicate e Sistemi Intelligenti, Pozzuoli, Italy, isolated EVs from the freshwater cnidarian polyp *Hydra Vulgaris* and investigated their biochemical, morphological, and functional characteristics. The analysis showed that EVs released by this species transfer protein and major components of axial patterning, modulating foot and head regeneration. A review article by [Fang et al.](#), China Agricultural University, Beijing, China, and Dr. Brett M. Tyler Oregon State University, Corvallis, OR, United States compiles information regarding recent research on the biogenesis of EVs and their pathobiological functions in organismal communication of microbes. The authors highlighted key questions related to the transfer of genetic materials between species which requires further exploration in this direction.

The delivery of therapeutics across the blood-brain barrier for brain disorders has remained challenging despite recent progress ([Terstappen et al.](#), 2021). A study [Kutchy et al.](#), at the University of Nebraska Medical Center, Omaha, NE, United States, and collaborators in Grenada and China showed that EVs could be used as a vehicle for the effective delivery of drugs to the brain. The authors investigated the biodistribution of ultra-small, iron oxide-labeled, mouse astrocyte-originating EVs after intranasal perfusion in mice and suggested that engineered EVs could be utilized for targeted delivery of therapeutics into several organs including the brain. In another review article, [Zheng et al.](#), from Fudan University, Shanghai, China, summarized the current research advances on the involvement of EVs in the pathogenesis, diagnosis, and treatment of the autoimmune disease, systemic lupus erythematosus (SLE) and lupus nephritis (LN). In this article, the authors have discussed the role of several immune cell-originating EVs in the regulation of the innate immune responses and highlighted the necessity of further research to fully understand the involvement of EVs in the pathophysiological mechanisms of SLE and LN.

While EVs have been extensively studied in the field of cancer ([Xu et al.](#), 2018) and some selective neurodegenerative disorders ([Hill](#), 2019; [Hornung et al.](#), 2020), their involvement in bone regeneration is limited. [Kang et al.](#), at the University of Illinois, Chicago, IL, United States, studied the effects of the inflammatory cytokines on mesenchymal stem cell-derived EV-mediated immunomodulation. They concluded that TNF α preconditioning of human mesenchymal stem cells results in the generation and release of EVs that can alter the phenotype of macrophages, both *in vitro* and *in vivo*. [Corsello et al.](#), at the University of Texas Medical Branch at Galveston, TX, United States, investigated the presence of innate immune mediators, including interferons in EVs, released from airway epithelial cells infected with the respiratory syncytial virus. The study found significant levels of soluble and EV-associated interferon λ in nasopharyngeal secretions (NPS) and NPS-derived EVs from children with respiratory syncytial virus infections.

Overall, this Research Topic covers broad aspects of extracellular vesicle biogenesis, their isolation and characterization strategies, involvement in intercellular communication, and the roles EVs play in different human diseases. The topic also includes articles that investigated and discussed the possibilities of using small EVs as potential disease biomarkers and opportunities for therapeutic applications. These basic, translational, and clinical studies demonstrate the importance and high potential of EVs in human health and diseases including neurodegenerative disorders and cancer, as well as in understanding intercellular communication in low organisms, including microbes and polyps. Future scientific efforts to fully understand the mechanisms of EV-mediated cell-cell communication in health and different disease states are expected to provide critical insights into basic biological processes, development of biomarkers, identification of therapeutic targets, and harnessing EVs as a vehicle for drug delivery.

Author contributions

SD wrote the article. GB, KVK-J, and SN reviewed the article and provided critical feedback. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated

organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

- Dutta, S., Hornung, S., Kruayatidee, A., Maina, K. N., Del Rosario, I., Paul, K. C., et al. (2021). α -Synuclein in blood exosomes immunoprecipitated using neuronal and oligodendroglial markers distinguishes Parkinson's disease from multiple system atrophy. *Acta Neuropathol.* 142 (3), 495–511. Epub 2021 May 15. Erratum in: *Acta Neuropathol.* 2021 May 24; PMID: 33991233; PMCID: PMC8357708. doi:10.1007/s00401-021-02324-0
- Hill, A. F. (2019). Extracellular vesicles and neurodegenerative diseases. *J. Neurosci.* 39 (47), 9269–9273. PMID: 31748282; PMCID: PMC6867808. doi:10.1523/JNEUROSCI.0147-18.2019
- Hornung, S., Dutta, S., and Bitan, G. (2020). CNS-derived blood exosomes as a promising source of biomarkers: Opportunities and challenges. *Front. Mol. Neurosci.* 13, 38. PMID: 32265650; PMCID: PMC7096580. doi:10.3389/fnmol.2020.00038
- Kalani, M. Y. S., Alsop, E., Meechoovet, B., Beecroft, T., Agrawal, K., Whitsett, T. G., et al. (2020). Extracellular microRNAs in blood differentiate between ischaemic and haemorrhagic stroke subtypes. *J. Extracell. Vesicles* 9 (1), 1713540. PMID: 32128071; PMCID: PMC7034450. doi:10.1080/20013078.2020.1713540
- Lötvall, J., Hill, A. F., Hochberg, F., Buzás, E. I., Di Vizio, D., Gardiner, C., et al. (2014). Minimal experimental requirements for definition of extracellular vesicles and their functions: A position statement from the international society for extracellular vesicles. *J. Extracell. Vesicles* 3, 26913. PMID: 25536934; PMCID: PMC4275645. doi:10.3402/jev.v3.26913
- Terstappen, G. C., Meyer, A. H., Bell, R. D., and Zhang, W. (2021). Strategies for delivering therapeutics across the blood-brain barrier. *Nat. Rev. Drug Discov.* 20 (5), 362–383. Epub 2021 Mar 1. PMID: 33649582. doi:10.1038/s41573-021-00139-y
- Xu, R., Rai, A., Chen, M., Suwakulsiri, W., Greening, D. W., and Simpson, R. J. (2018). Extracellular vesicles in cancer - implications for future improvements in cancer care. *Nat. Rev. Clin. Oncol.* 15 (10), 617–638. PMID: 29795272. doi:10.1038/s41571-018-0036-9
- Yan, Z., Dutta, S., Liu, Z., Yu, X., Mesgarzadeh, N., Ji, F., et al. (2019). A label-free platform for identification of exosomes from different sources. *ACS Sens.* 4 (2), 488–497. Epub 2019 Jan 31. PMID: 30644736. doi:10.1021/acssensors.8b01564



Generation and Application of a Reporter Cell Line for the Quantitative Screen of Extracellular Vesicle Release

Jonathan Shpigelman^{1†}, Fitzgerald S. Lao^{1†}, Shiyin Yao¹, Chenyang Li², Tetsuya Saito^{1,3}, Fumi Sato-Kaneko¹, John P. Nolan⁴, Nikunj M. Shukla¹, Minya Pu^{1,5}, Karen Messer^{1,5}, Howard B. Cottam¹, Dennis A. Carson¹, Maripat Corr⁶ and Tomoko Hayashi^{1*}

¹Moores Cancer Center, University of California, San Diego, La Jolla, CA, United States, ²School of Pharmaceutical Sciences, Health Science Center, Shenzhen University, Shenzhen, China, ³Department of Rheumatology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan, ⁴Scintillon Institute, San Diego, CA, United States, ⁵Division of Biostatistics and Bioinformatics, Department of Family Medicine and Public Health, University of California, San Diego, La Jolla, CA, United States, ⁶Department of Medicine, University of California, San Diego, La Jolla, CA, United States

OPEN ACCESS

Edited by:

Maria Grazia Morgese,
University of Foggia, Italy

Reviewed by:

Simonetta Falzoni,
University of Ferrara, Italy
Ivan Poon,
La Trobe University, Australia

*Correspondence:

Tomoko Hayashi
thayashi@ucsd.edu

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Experimental Pharmacology
and Drug Discovery,
a section of the journal
Frontiers in Pharmacology

Received: 16 February 2021

Accepted: 30 March 2021

Published: 16 April 2021

Citation:

Shpigelman J, Lao FS, Yao S, Li C, Saito T, Sato-Kaneko F, Nolan JP, Shukla NM, Pu M, Messer K, Cottam HB, Carson DA, Corr M and Hayashi T (2021) Generation and Application of a Reporter Cell Line for the Quantitative Screen of Extracellular Vesicle Release. *Front. Pharmacol.* 12:668609. doi: 10.3389/fphar.2021.668609

Extracellular vesicles (EVs) are identified as mediators of intercellular communication and cellular regulation. In the immune system, EVs play a role in antigen presentation as a part of cellular communication. To enable drug discovery and characterization of compounds that affect EV biogenesis, function, and release in immune cells, we developed and characterized a reporter cell line that allows the quantitation of EVs shed into culture media in phenotypic high-throughput screen (HTS) format. Tetraspanins CD63 and CD9 were previously reported to be enriched in EVs; hence, a construct with dual reporters consisting of CD63-Turbo-luciferase (Tluc) and CD9-Emerald green fluorescent protein (EmGFP) was engineered. This construct was transduced into the human monocytic leukemia cell line, THP-1. Cells expressing the highest EmGFP were sorted by flow cytometry as single cell, and clonal pools were expanded under antibiotic selection pressure. After four passages, the green fluorescence dimmed, and EV biogenesis was then tracked by luciferase activity in culture supernatants. The Tluc activities of EVs shed from CD63Tluc-CD9EmGFP reporter cells in the culture supernatant positively correlated with the concentrations of released EVs measured by nanoparticle tracking analysis. To examine the potential for use in HTS, we first miniaturized the assay into a robotic 384-well plate format. A 2210 commercial compound library (Maybridge) was then screened twice on separate days, for the induction of extracellular luciferase activity. The screening data showed high reproducibility on days 1 and 2 (78.6%), a wide signal window, and an excellent Z' factor (average of 2-day screen, 0.54). One hundred eighty-seven compounds showed a response ratio that was 3SD above the negative controls in both day 1 and 2 screens and were considered as hit candidates (approximately 10%). Twenty-two out of 40 re-tested compounds were validated. These results indicate that the performance of CD63Tluc-CD9EmGFP reporter cells is reliable, reproducible, robust, and feasible for HTS of compounds that regulate EV release by the immune cells.

Keywords: extracellular vesicle, exosome, THP-1, CD63, CD9, luciferase, antigen-presenting cells

INTRODUCTION

Cells generate small vesicles by inward or outward budding of multivesicular bodies or late endosomes and release them into extracellular spaces (Woodman and Futter, 2008; Gireud-Goss et al., 2018). These extracellular vesicles (EVs) are produced by multiple cell types and include exosomes, microvesicles, and oncosomes (Stahl and Raposo, 2018). They are found abundantly in body fluids, including saliva, blood, urine, and breast milk, presumably as means of disposal or dissemination from their source to distal targets (Zwicker et al., 2009; Yanez-Mo et al., 2015; Foster et al., 2016). EVs are thought to play an essential role in cell-to-cell communication as they carry cell-type-specific molecules, including those involved in innate immune responses, such as cytokines, chemokines, intercellular adhesion molecules (ICAMs), coding and non-coding RNAs (including microRNAs), lipids, proteins, peptides and DNA fragments (Valadi et al., 2007; Skog et al., 2008; Cossetti et al., 2014; Yanez-Mo et al., 2015). Certain molecules integrated into the EV outer surface membrane direct adhesion to potential target cells, and their cargos can confer specific intercellular communications. These properties enable EVs to play modulating roles in mediating immune responses to pathogens and tumors (Campos et al., 2015; Wang et al., 2017). Chemically controlled EV biogenesis could lead to novel immunomodulation modalities, but the mechanisms that control cargo loading and EV formation are yet to be fully defined. Quantifying EVs' cellular biogenesis in small samples would enable chemical screening to define pathways involved in EV production and release.

In immune responses, EVs have been reported to play immune-stimulatory and suppressive roles. We previously reported that tumor cells deficient in large tumor suppressor kinase (LATS)1/2 (part of the Hippo pathway) released immunostimulatory EVs carrying tumor-specific antigens and RNA (Moroishi et al., 2016). Such EVs from the LATS1/2 deficient tumor cell lines induced tumor-specific adaptive immune responses (Moroishi et al., 2016). EVs from antigen-presenting cells (APCs) have essential biological functions, as EVs released from dendritic cells (DCs) could stimulate pro-inflammatory responses and induce antigen-specific T cell responses (Zitvogel et al., 1998; Escudier et al., 2005; Morse et al., 2005; Besse et al., 2016). Pooled EVs shed from mature DCs expressing CD54, CD86, and MHC-class-I and II molecules effectively stimulated T cells (Wen et al., 2017). In contrast to immune-stimulating EVs, EVs can also act as immune suppressors. EVs from tumor cells suppressed tumor-specific immune responses by inhibiting APC maturation, inducing monocyte-derived suppressor cells and regulatory T cells (Treg), and promoting apoptosis of activated cytotoxic T lymphocytes (Taylor and Gercel-Taylor, 2011; Lindenberg and Stoorvogel, 2018).

High throughput screening (HTS) is indispensable for the current drug discovery process (Macarron and Hertzberg, 2011). Compounds that regulate EV release with various immunostimulatory or antigen-presenting properties would be potential candidates for cancer immune therapy or vaccine adjuvants for infectious diseases. Thus, phenotypic HTS technology quantifying EV release by immune reporter cells could be an essential initial step for drug development for immune related conditions. Reporter cells amenable for phenotypic HTS need to not only be relevant for the

biological activities in question, but also robust and reproducible in miniaturized 384- or 1536-well formats (Macarron and Hertzberg, 2011). Although a few reports have utilized tetraspanins (TSs) to monitor or label EVs in the culture supernatant, these reporter cells used non-immune cells and were not optimized for the HTS platform (Hikita et al., 2018; Cashikar and Hanson, 2019; Gupta et al., 2020). Thus, this study aimed to develop a stable human monocytic reporter cell line (THP-1 cells) that could quantitatively measure EV release and screen for compounds that alter EV release levels from innate immune cells. To monitor EV release into culture supernatants, we employed TSs (CD9 and CD63) for the reporter construct (Andreu and Yanez-Mo, 2014; Kowal et al., 2016). The reporter cells were characterized and then used in a 384-well format robotic screen of over 2000 compounds. The data demonstrated the robustness and reproducibility of the CD63Tluc-CD9EmGFP THP-1 reporter cells for screening compounds that modulate EV release by the immune cells.

MATERIALS AND METHODS

Generation of CD63Tluc-CD9EmGFP Lentivirus

A synthetic gene sequence encoding CD63 fused to the Turbo luciferase gene and then linked to an internal ribosomal entry site (IRES) and CD9 encoding sequences fused to that for emerald green fluorescent protein (EmGFP, derived from *Aequorea Victoria* GFP) was assembled from synthetic oligonucleotides and/or PCR products (**Supplementary Figure S1A**). The fragment was inserted into the lentiviral vector pLenti6.3_V5-DEST_A244 (Thermo Fisher Scientific, Waltham, MA, United States) according to the manufacturer's instructions. Plasmid DNA was purified from transformed *Escherichia coli*, and the concentration was determined by UV spectroscopy. The sequence in the insertion sites in the final construct was verified using next-generation sequencing technology and was verified to be accurate. A map of the final construct (pLenti CD63Tluc-CD9EmGFP) is shown in **Supplementary Figure S1B**. Lentivirus was generated using a proprietary high titer yield protocol at Thermo Fisher Scientific. Briefly, HEK293T cells were transfected with the pLenti CD63-TlucCD9-EmGFP vector using ViraPower™ Lentiviral Packaging Mix (K497500, Thermo Fisher Scientific) and Lipofectamine™ 3000 (L3000075, Thermo Fisher Scientific). After 48 h, the released lentivirus was collected from the conditioned medium. The lentiviral preparation was concentrated ten-fold and the viral titer was determined using Lenti-X™ GoStix™ (#631280, Takara Bio United States Inc., Mountain View, CA, United States).

Transduction, Cloning and Selection of THP-1 Cells Stably Expressing CD63 Turbo-Luciferase and CD9-EmGFP

THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% dialyzed FBS (S12850H, Atlanta Biologicals), 100 U/ml

penicillin, 100 µg/ml streptomycin (15140122, Gibco, Thermo Fisher Scientific), 1 mM sodium pyruvate (11360-070, Gibco, Thermo Fisher Scientific) and 1 × MEM non-essential amino acids (11140-050, Gibco, Thermo Fisher Scientific) at 37°C in 5% CO₂. THP-1 cells were transduced with lentivirus encoding CD63Tluc-CD9EmGFP at varying dilutions (1:2, 1:5, 1:10, 1:50, 1:250 and 1:500) in the presence of polybrene (8 µg/ml). After transduction, the culture was centrifuged at 2,500 rpm for 30 min to improve the transduction efficiency. The antibiotic-resistant cells were collected and analyzed for EmGFP expression by flow cytometry. Tluc activity was measured using the TurboLuc™ Luciferase One-Step Glow Assay Kit (88264, Thermo Fisher Scientific). A stable pool of cells expressing CD63Tluc and CD9EmGFP was expanded in RPMI containing 5 µg/ml blasticidin (Gibco Thermo Fisher Scientific). Single EmGFP^{hi} cells were sorted by flow cytometry into individual wells of six 96-well plates to establish clones. EmGFP expression was validated by flow cytometry and Tluc expression was verified by the TurboLuc™ Luciferase One-Step Glow Assay Kit as described above.

Culture and Treatment of CD63 Turbo-Luciferase-CD9EmGFP Reporter Cells and Parental THP-1 Cells

The selected clone (designated as CD63Tluc-CD9EmGFP onward) was cultured in HEPES buffered RPMI 1640 medium (72400, Thermo Fisher Scientific) supplemented with 10% dialyzed FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 1 × MEM non-essential amino acids, and 5 µg/ml blasticidin. Parental THP-1 cells were cultured in RPMI 1640 medium (11875, Thermo Fisher Scientific) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-mercaptoethanol. Both cell types were maintained in humidified conditions with 5% CO₂ at 37°C.

Prior to treatment, CD63Tluc-CD9EmGFP reporter cells and parental THP-1 cells were washed with their base medium and re-suspended in their complete culture medium substituted with exosome-depleted FBS (EXO-FBS-250A-1, System Biosciences, Mountain View, CA, United States, or Gibco™ Exosome-Depleted FBS, Thermo Fisher Scientific). In the experiments performed for **Figures 2A–G** (Thermo Fisher), CD63Tluc-CD9EmGFP reporter cells were treated with graded concentrations of phorbol myristate acetate (PMA) (tlrl-pma, InvivoGen, San Diego, CA, United States) or immunostimulatory compounds (5 and 81) (Sato-Kaneko et al., 2018; Chan et al., 2019). Cell concentrations were 5×10^4 cells/200 µl/well for 96-well format and 2.5×10^4 cells/80 µl/well for 384-well format unless otherwise indicated in the figure legends. In the experiments performed for **Figures 2H, 3, and 4, and Tables 1, 2**, CD63Tluc-CD9EmGFP reporter cells and parental THP-1 cells were treated at 2.5 or 5×10^5 cells/ml in 6-, 96 well tissue culture plates or flasks with 10 ng/ml lipopolysaccharide (LPS, Ultrapure LPS, tlrl-3pelps, InvivoGen), 50 ng/ml or graded concentrations of PMA, 5 µM compound 5, 5 µM compound 81, 5 µM GW4869 (GW, 13127, Cayman Chemical Company, Ann Arbor, MI, United States) or 1 µM manumycin A (MA,

TABLE 1 | Tluc activity in CD63TlucCD9EmGFP reporter cells and culture supernatants.

Treatment	Cells ($\times 10^3$) ^a	Supernatant ($\times 10^3$) ^{a,b}	% Release ^{a,b}
Vehicle	37.3 ± 19.4	1.03 ± 0.06	3.6 ± 2.2
LPS	138.9 ± 97.6	2.59 ± 0.9 ^d	2.4 ± 1
PMA	201.2 ± 75.1 ^c	3.66 ± 0.62 ^d	2.0 ± 0.5
#5	86.2 ± 23.8	3.12 ± 0.33 ^d	3.8 ± 1.1
#81	85.5 ± 18.3	3.34 ± 0.41 ^d	4.1 ± 1.1

^amean ± SD of four independent experiments. The raw data were presented in the **Supplementary Table S1**.

^b% release = luciferase activity of supernatant/(Tluc activities of cells + supernatant).

^cp < 0.005 vs. vehicle treated condition.

^dp ≤ 0.0001 vs. vehicle treated condition.

10010497, Cayman Chemical Company). All treatments were performed for 48 h in humidified conditions with 5% CO₂ at 37°C.

Extracellular Vesicle Isolation

Four EV isolation methods, Total exosome isolation reagent™, ExoQuick-TC™ exosome isolation reagent, polyethylene glycol (PEG) precipitation and differential ultracentrifugation, were used.

Total Exosome Isolation Reagent™

The culture plates were centrifuged at 2,000 g for 10 min. The supernatants were first mixed with an aliquot of Total Exosome Isolation Reagent (Thermo Fisher Scientific) equal to 1/2 the total volume of culture supernatant and then incubated at 4°C overnight. Following a 10,000 g spin for 1 h at 4°C, all traces of supernatant were discarded, and pellets were re-suspended in particle-free PBS.

ExoQuick-TC Exosome Isolation Reagent

After treatment, culture supernatants were spun at 250 g to remove cells. Supernatants were then filtered using a 0.45 µm syringe filter (MilliporeSigma, Sigma-Aldrich, St. Louis, MO, United States). Filtrates were then mixed with an aliquot of ExoQuick-TC Exosome Isolation Reagent (System Biosciences) equal to 1/5 the total volume of culture supernatant and incubated at 4°C overnight. Mixtures were then spun at 3,000 g for 15 min at 4°C, and an aliquot of this supernatant (EV-depleted fraction) was saved. Following another 3,000 g spin for 15 min at 4°C, all remaining traces of fluid were removed. Pellets were then resuspended in particle-free PBS.

Eight % PEG Precipitation

The culture plates were centrifuged at 300 g for 5 min. The supernatants were centrifuged at 2,000 g for 10 min at RT, and 160 µl supernatant were transferred to the new plates. An equal volume of cold 16% PEG/1 M NaCl (final 8% PEG) was mixed by pipetting, and the mixtures were incubated at 4°C overnight. The mixtures were centrifuged at 4,300 g at 4°C for 60 min. The supernatants were discarded, and pellets washed with 200 µl cold 5% PEG/1M NaCl/PBS (dilute 16% PEG/1 M NaCl with filtered PBS to final 5% PEG) at 4,300 g at 4°C for 15 min. The pellets were resuspended in particle-free PBS.

TABLE 2 | Profiles of EVs released from CD63TlucCD9EmGFP reporter cells and parental THP-1 cells.

Samples	CD63TlucCD9GFP reporter cells			Parental THP-1 cells		
	Total protein (μg)/ 10^6 cells ^a	Particles ($\times 10^9/\text{ml}$)/ 10^6 cells ^a	Mean size (nm) ^a	Total protein (μg)/ 10^6 cells ^a	Particles ($\times 10^9/\text{ml}$)/ 10^6 cells ^a	Mean size (nm) ^a
Vehicle	18.1 $\pm$ 0.88	1.5 $\pm$ 0.07	161.0 $\pm$ 0.7	11.6 $\pm$ 0.35	1.4 $\pm$ 0.25	160.8 $\pm$ 2.7
PMA (50 ng/ml)	18.5 $\pm$ 1.5	2.0 $\pm$ 0.09 ^b	148.7 $\pm$ 5.9	18.5 $\pm$ 1.8	2.0 $\pm$ 0.12 ^b	156.1 $\pm$ 3.1

^aMean $\pm$ SD of two independent experiments.^bp < 0.05 by Student's t test vs. vehicle treated condition.^cp < 0.0001 by Student's t test vs. vehicle treated condition.

Differential Ultracentrifugation

The reporter cells or parental THP-1 cells were treated at a density of $5 \times 10^5/\text{ml}$ with vehicle or PMA (50 ng/ml) for 48 h in tissue culture flasks. Conditioned culture media (12 ml or 32 ml) was spun at 300 g for 10 min. Supernatants were subsequently spun at 2,000 g for another 10 min. Next, depending on volume, supernatants were transferred to 10 ml or 31.5 ml open-top polypropylene UC tubes (358120 and 358126, respectively, Beckman Coulter Life Sciences, CA, United States) and spun at 10,000_g for 30 min in an SW40Ti rotor (K-Factor: 2,771) or SW28 rotor (K-Factor: 2,554), respectively. 28.5 ml of supernatants were then transferred to new UC tubes and spun at 130,000_g (SW40Ti; K-Factor: 213) for 3 h or 100,000_g (SW28; K-Factor: 246) for 2 h by Beckman Optima XL-90 Ultracentrifuge (Beckman Coulter Life Sciences), respectively. The supernatants were then gently aspirated (leaving $\sim 50 \mu\text{l}$), and pellets resuspended to their previous step volumes in cold PBS. Re-suspended pellets were then spun under the same conditions as their previous spin, followed by another round of gentle aspiration and resuspension to a final volume of either 100 μl (SW40Ti) or 150 μl (SW28) in cold PBS. All centrifugation steps were performed at 4°C, and resultant samples were stored at -80°C until use.

Additional details for each procedure are described in the figure legends.

Measurement of Turbo-Luciferase Activity

Culture supernatant or isolated EV samples were plated in 96-well black wall, clear bottom assay plates (3603, Corning) or 384-well (164564, Thermo Fisher Scientific) and were allowed to equilibrate to RT. An equal volume of working Tluc assay reagent was added. The plate was incubated with agitation for 10 min at RT in the dark. Luminescence was then measured on the BMG CLARIOstar (BMGLabtech, Cary, NC) or TECAN Infinite M200 plate reader (Tecan, Männedorf, Switzerland).

Measurement of Extracellular Vesicle Concentrations and Size Distributions

Particle concentrations and size distributions were determined using nanoparticle tracking analysis (NTA) with a Nanosight NS300 instrument (Malvern Panalytical, Malvern, United Kingdom) or microfluidic resistive pulse sensing with an nCS1 instrument (Spectradyne LLC, United States).

NanoSight NS300

Isolated EVs were diluted in particle-free PBS to the optimal concentration (10–150 particles per camera frame). A minimum of 700 μl of diluted sample was then manually injected using a 1-ml syringe before being installed on a syringe pump and infused at a rate of “100” for the duration of the video acquisitions. Three 60-s acquisitions of each sample were obtained and averaged for subsequent analysis. The camera level and detection threshold parameters in the NTA 3.1 software were maintained at “13” and “5”, respectively, for accurate and consistent measurement of particle concentration, mean diameter, and size distribution.

Spectradyne nCS1

Samples were analyzed using TS-400 cartridges for particles between 65 and 400 nm in diameter. Isolated EVs were diluted in particle-free PBS to the optimal cartridge particle concentration (10^7 – 10^{11} particles/ml). 10-s measurements were performed for each sample at least 20 times and averaged. Unconditioned media without cells were measured for subtraction of background particles, including protein aggregates from exosome-depleted FBS. Mean size and particle concentrations were analyzed using the nCS1 Data Analyzer (Spectradyne). Only particles from 75 to 400 nm were included in the analysis to minimize the inclusion of false particle events.

Electron Microscopy

EVs were isolated by ExoQuick-TC, resuspended to 1/500 original volume of culture supernatant, and stored at -80°C . For the physical characterization of EVs, negative stain transmission electron microscopy was performed as follows. Formvar-carbon-coated copper grids (100 mesh, Electron Microscopy Sciences, Hatfield, PA) were placed on 5 μl drops of each sample solution displayed on a parafilm sheet. After allowing the material to adhere to the grids for 10 min, grids were washed times times by rinsing through 200 μl drops of milli-Q water before being left for 1 min on 2% (wt/vol) uranyl acetate (Ladd Research Industries, Williston, VT) in water. Excess solution was removed with Whatman 3MM blotting paper, and grids were left to dry for a few minutes before viewing. Grids were examined using a Tecnai G2 Spirit BioTWIN transmission electron microscope equipped with an Eagle 4k HS digital camera (FEI, Hillsboro, OR, United States).

Immunoblotting

For immunoblotting, EVs (30 µg protein) were mixed with 4 × NuPAGE sample buffer (NP0007, Thermo Fisher Scientific), with or without dithiothreitol (DTT) (D9779-5G, Sigma-Aldrich), and a protein inhibitor cocktail (Roche, Basel, Switzerland). The total protein in the samples was quantitated by Pierce BCA Protein Assay Kit. Samples were then mixed by pipetting and incubated on ice for 15 min. When DTT, a reducing agent, was used, samples were also denatured at 70°C for 10 min prior to loading. After fractionation on NuPAGE 4–12% Bis-Tris Gels (Thermo Fisher Scientific), samples were blotted onto Immobilon-P PVDF membranes (IPVH00010, Sigma) and blocked for 1 h in 5% BSA-TBS-T at RT. The blots were then incubated with primary antibodies (Ab): anti-CD63 Ab (1:1000 dilution) (sc-5275, Santa Cruz Biotechnology, Dallas, TX, United States), anti-CD81 Ab (1:500 dilution) (sc-23962; Santa Cruz Biotechnology), anti-Tsg101 Ab (1:500 dilution) (MA1-23296; Thermo Fisher Scientific), and anti-calnexin Ab (1:1000 dilution) (2679S; Cell Signaling, Danvers, MA, United States), overnight at 4°C with gentle agitation. After washing, the blots were incubated with anti-mouse secondary antibody (7076S; Cell Signaling) for blots probed against CD63, CD81, and Tsg101 or anti-rabbit secondary antibody (7074S; Cell Signaling) for blots probed against Calnexin for 30 min at RT with gentle agitation. Blots were developed with ProSignal Dura ECL Reagent (Prometheus Protein Biology Products, Genesee Scientific, San Diego, CA, United States) and visualized using a ChemiDoc Imaging System (Bio-Rad Laboratories, Hercules, CA, United States). Two molecular weight markers, AccuRuler Prestained Protein Ladder (G02101, Lamda Biotech, St. Louis, MO, United States) and Precision Plus Protein™ Dual Color Standards (1610374, Bio-Rad Laboratories) were used.

Vesicle Flow Cytometry Assessing Extracellular Vesicle Number, Size and Composition

EV number, size and composition were determined by single vesicle flow cytometry (Stoner et al., 2016) using a commercial assay kit (vFC™ Assay kit, Cellarcus Biosciences, San Diego, CA, United States) and flow cytometer (CytoFlexS, Beckman Coulter, Indianapolis, IN, United States). Briefly, samples were stained with the fluorogenic membrane stain vFRed™ and, where indicated, carboxyfluorescein diacetate succinimidyl ester (CFSE, 40 µM, Cellarcus Biosciences) and antibodies against individual tetraspanins (**Supplementary Table S2**) or a mixture of antibodies against CD9, CD63, and CD81-labeled with phycoerythrin (PE, vTag™ anti-human TS PE mix, Cellarcus Biosciences) for 1 h at RT, diluted 1,000-fold in the buffer, and analyzed using membrane fluorescence to trigger detection. Data were analyzed using FCS Express, and included calibration using a vesicle size and fluorescence intensity standards. The analysis included a pre-stain dilution series in determining the optimal initial sample dilution and multiple positive and negative controls, as recommended by guidelines from the International Society for Extracellular Vesicles (ISEV) (Thery et al., 2018). A detailed description of

vFC™ methods and controls are presented in **Supplementary Information**.

Cell Viability Assay by 3-[4,5-Dimethylthiazol-2-yl]-2,5-dipheyl Tetrazolium Bromide (MTT)

0.5 mg/ml MTT (Thermo Fisher Scientific) solution was added to cell pellets in each well and incubated 6 h and formazan crystals were then lysed with lysis buffer (15% SDS and 0.12% 12 N HCl). The absorbance was measured at 570 nm using 650 nm as a reference with a TECAN plate reader. Percent viability was normalized to the vehicle control (100%).

Robotic Screen Using CD63 Turbo-Luciferase-CD9EmGFP Reporter Cells

A total of 2210 compounds were purchased from the Maybridge Library series, including the Maybridge HitFinder library (1035 compounds) and the Maybridge HitCreator library (1175 compounds). A medium-throughput screen was performed by the SelectScreen™ service, Thermo Fisher Scientific (Madison, WI Biosciences). Briefly, the CD63Tluc-CD9EmGFP reporter cells were cultured in growth media containing 20% FBS. The cells were harvested and resuspended in assay media containing 10% Exosome-Depleted FBS (Thermo Fisher Scientific) at 2×10^5 cells/ml, 50 µl/well of 384-well plates (3674, Corning). PMA (10 ng/ml at the final concentration) was used for a full stimulation (100%). Both media and vehicle (0.1% DMSO) conditions were included as negative controls. PMA and compounds (10 µM at the final concentration) were added to the cells using Echo Acoustic Liquid Handlers (Labcyte Inc. Indianapolis IN, United States), and the cells were incubated for 48 h at 37°C. Subsequently, the plate was centrifuged at 2,000 g for 10 min, and 25 µl supernatant was transferred to the plate (3570, Corning). TurboLuc reagent (25 µl) was added and incubated for 10 min at RT. Chemiluminescent activities were measured by the BMG CLARIOstar. The viability of cells was assessed using Presto Blue reagent (Thermo Fisher Scientific). Briefly, Presto Blue reagent (2.5 µl) was added to the cells left in the wells, and the plates were incubated for 30 min at RT. Fluorescent signals were read using the BMG CLARIOstar. The response ratio was calculated using the following formula; % response = $100 \times (\text{compound RLU} - \text{vehicle RLU}) / (\text{PMA RLU} - \text{vehicle RLU})$. Z' factor was calculated as previously reported (Zhang et al., 1999). Hit compounds were selected by a “Top X” approach in which the means of the negative control + 3SD of the unstimulated wells were calculated within each plate, and any compounds that gave signals above the cut-off were called hit compounds. Forty hit compounds were selected from the original library and used for validation studies at the UCSD laboratory.

Statistical Analysis

Data are presented as means with a standard error of the mean (SEM) or standard deviation (SD). A two-tailed Student's *t*-test was used to compare two groups, and one-way ANOVA with

Dunnett's post hoc was used to compare multiple groups against a control group. Spearman's rank correlation test was performed to assess linear relationships. Analyses were performed using Prism 8 software (GraphPad Software, San Diego, CA, United States). $P < 0.05$ was considered statistically significant.

RESULTS

Generation of Stable THP-1 Cells Expressing CD63-Turbo-Luciferase and CD9-EmGFP

To quantitate EV release, a human monocytic cell line with APC-like features, THP-1, was employed to generate a reporter cell line. The schematic of the process is shown in **Supplementary Figure S2**. A gene containing CD63 fused to Tluc, and CD9 fused to EmGFP was synthesized and cloned under the cytomegalovirus (CMV) promoter of the lentiviral vector pLenti6.3_V5-DEST_A244 (**Supplementary Figures S1A,B**). The transducing titer for the lenti-CD63Tluc-CD9EmGFP was $\sim 5 \times 10^6$ transducing units/ml as determined by Lenti-X™ GoStix™. THP-1 cells were transduced with the lenti-CD63Tluc-CD9EmGFP, followed by centrifugation to improve the transduction efficiency. The transduced cells were cultured for 21 days with 5 $\mu\text{g}/\text{ml}$ blasticidin. The EmGFP and Tluc expressions in the cell pool were then assessed by flow cytometer and the TurboLuc™ Luciferase One-Step Glow assay, respectively, and compared to the parental THP-1 cells. Ten percent of the antibiotic-resistant pooled cells expressed EmGFP (**Supplementary Figures S1C,D**), and significantly higher Tluc activity in the pooled cells was also observed (**Supplementary Figure S1E**). These results indicated that the antibiotic-resistant pooled cells were enriched with CD63Tluc and CD9EmGFP expressing cells.

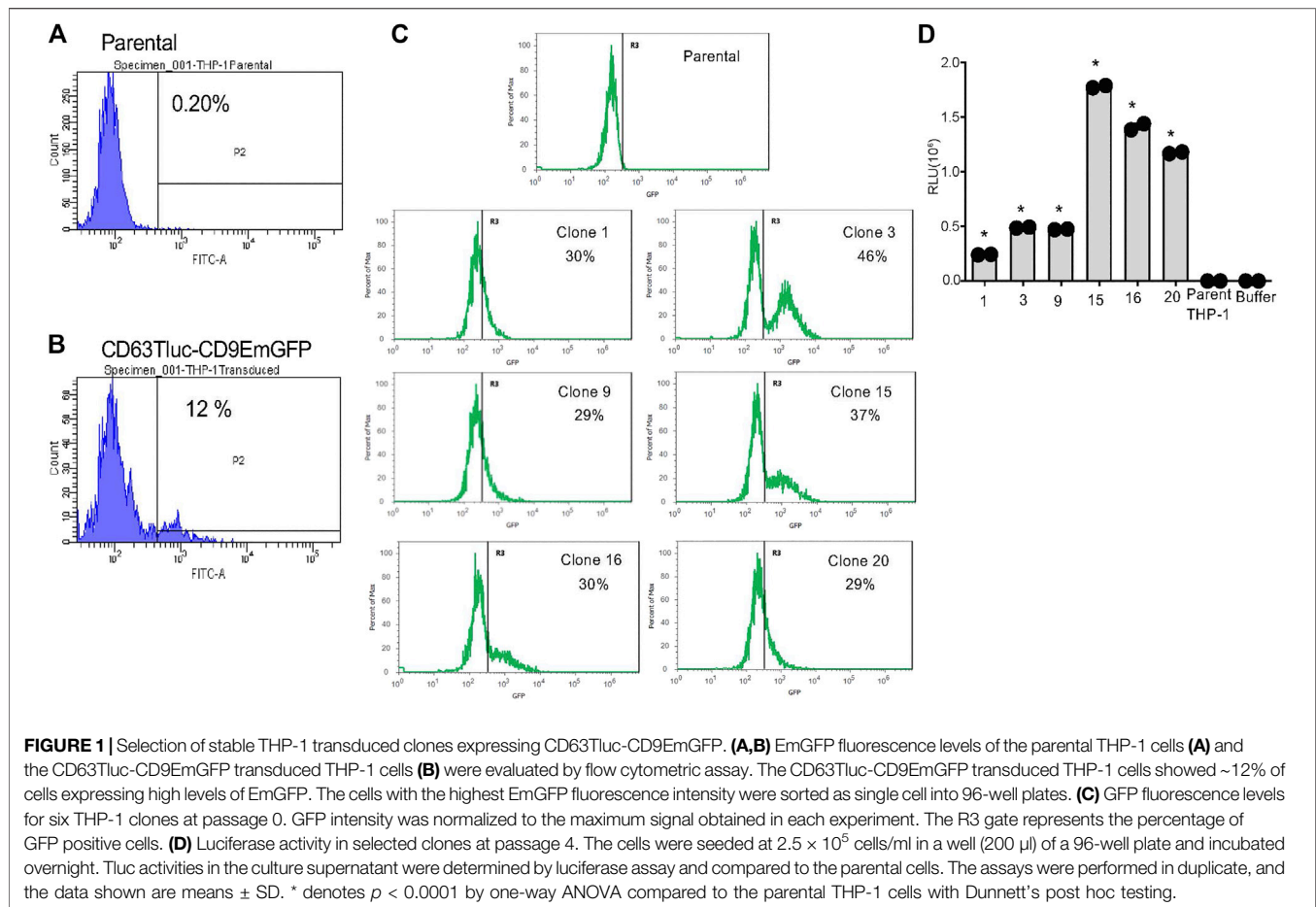
Cloning of Stable THP-1 Cells Expressing CD63Tluc and CD9EmGFP

The antibiotic-resistant pooled cells were further expanded in a medium containing 5 $\mu\text{g}/\text{ml}$ blasticidin for 21 days and sorted by flow cytometry for THP-1 cell clones expressing CD9EmGFP. Approximately 12% of the pool expressed high levels of CD9EmGFP (**Figures 1A,B**); this subpopulation was sorted into six 96-well plates of clones, twenty-two of which were chosen for assessment of Tluc activity. Six of these clones (1, 3, 9, 15, 16 and 20) showed high expression of EmGFP (**Figure 1C**) and were selected for further expansion. In some clones, e.g., clone 3 and 15, the distinct EmGFP positive and negative populations were detected (**Figure 1C**), suggesting the presence of multiple clones. At passage 4, the EmGFP expression of the selected 6 clones dimmed markedly compared to passage 0 (**Figure 1C** and **Supplementary Figure S3**); however, the clones maintained high Tluc activity (**Figure 1D**). On the basis of Tluc and EmGFP activity at passage 10, clone 15 was selected for the

subsequent feasibility studies of a reporter-based screening method for EV release (**Supplementary Figure S4**).

Feasibility and Robustness of the Selected CD63Tluc-CD9EmGFP Transduced THP-1 Cell Clone for Compound Screening

To evaluate whether the CD63Tluc-CD9EmGFP transduced THP-1 cell line (clone 15, designated as the CD63Tluc-CD9EmGFP reporter cells) was sufficiently robust for a quantitative compound screening of EV release, we tested 1) the optimal cell density, 2) optimal plate format feasible for HTS and 3) DMSO tolerance. To determine an optimal cell density, we seeded CD63Tluc-CD9EmGFP reporter cells in a culture medium containing exosome depleted FBS in 96-well plates at 1.25, 2.5, or 5×10^5 cells/ml and treated them with graded concentrations of PMA for 48 h. Plates were then spun at 2,000 g for 30 min to remove cells and debris. These supernatants were either used directly in the luciferase assay, or EVs were first isolated by the Total Exosome Isolation kit and resuspended in particle-free PBS. Samples derived from wells seeded with $5 \times 10^5/\text{ml}$ exhibited the best resolution and dynamic range (**Figures 2A,B**). Tluc activity increased in a dose-dependent manner in both culture supernatants (**Figure 2A**) and enriched EVs (**Figure 2B**). The Tluc activities in the culture supernatant and enriched EVs were positively correlated ($R^2 = 0.9971$; **Figure 2C**). To evaluate whether the CD63Tluc-CD9EmGFP reporter cells would be amenable for HTS, the assay was carried out in both 96-well and 384-well formats. The reporter cells were plated at densities of 5×10^5 cells/ml in a well (200 μl) of 96-well plate or 3.125×10^5 cells/ml in a well (80 μl) of a 384-well plate and were treated with serially diluted PMA and two immunostimulatory compounds (**Figures 2D–G**). Compounds 5 and 81 are hit compounds from a prior HTS, in which a library comprised of approximately 170,000 compounds (Small Molecule Discovery Center, University of California, San Francisco, CA, United States) was screened using THP-1 NF- κB -*bla* and THP-1 interferon-sensitive response element (ISRE)-*bla* reporter cell lines (Sato-Kaneko et al., 2018; Shukla et al., 2018; Chan et al., 2019). Compounds 5, and 81 were identified as prolonging NF- κB - and ISRE activation induced by LPS and type I IFN, respectively, (Sato-Kaneko et al., 2018; Shukla et al., 2018; Chan et al., 2019). PMA stimulated a dose-dependent release of Tluc activity into culture supernatants (**Figures 2D,F**). Compounds 5 and 81 induced EV release at concentrations above 0.55 μM (**Figures 2E,G**) in a dose-responsive manner. During these assays, the cells tolerated up to 0.2% dimethyl sulfoxide (DMSO). The average Z' value for Tluc in the supernatants of PMA and vehicle treated cells in the 384-well format was 0.63. The CD63Tluc-CD9EmGFP reporter cells were also tested for known EV release inhibitors. The reporter cells were incubated with 5 μM GW4869 (GW) or 1 μM manumycin A (MA) for 48 h and Tlu activities in the culture supernatant were examined (Essandoh et al., 2015; Datta et al., 2017; Cai et al., 2018; Datta et al., 2018). Both inhibitors significantly suppressed Tluc levels in the culture supernatant (**Figure 2H**). These results support the conclusion that the selected CD63Tluc-



CD9EmGFP reporter cell line is amenable for screening assays using both 96-well and 384-well formats.

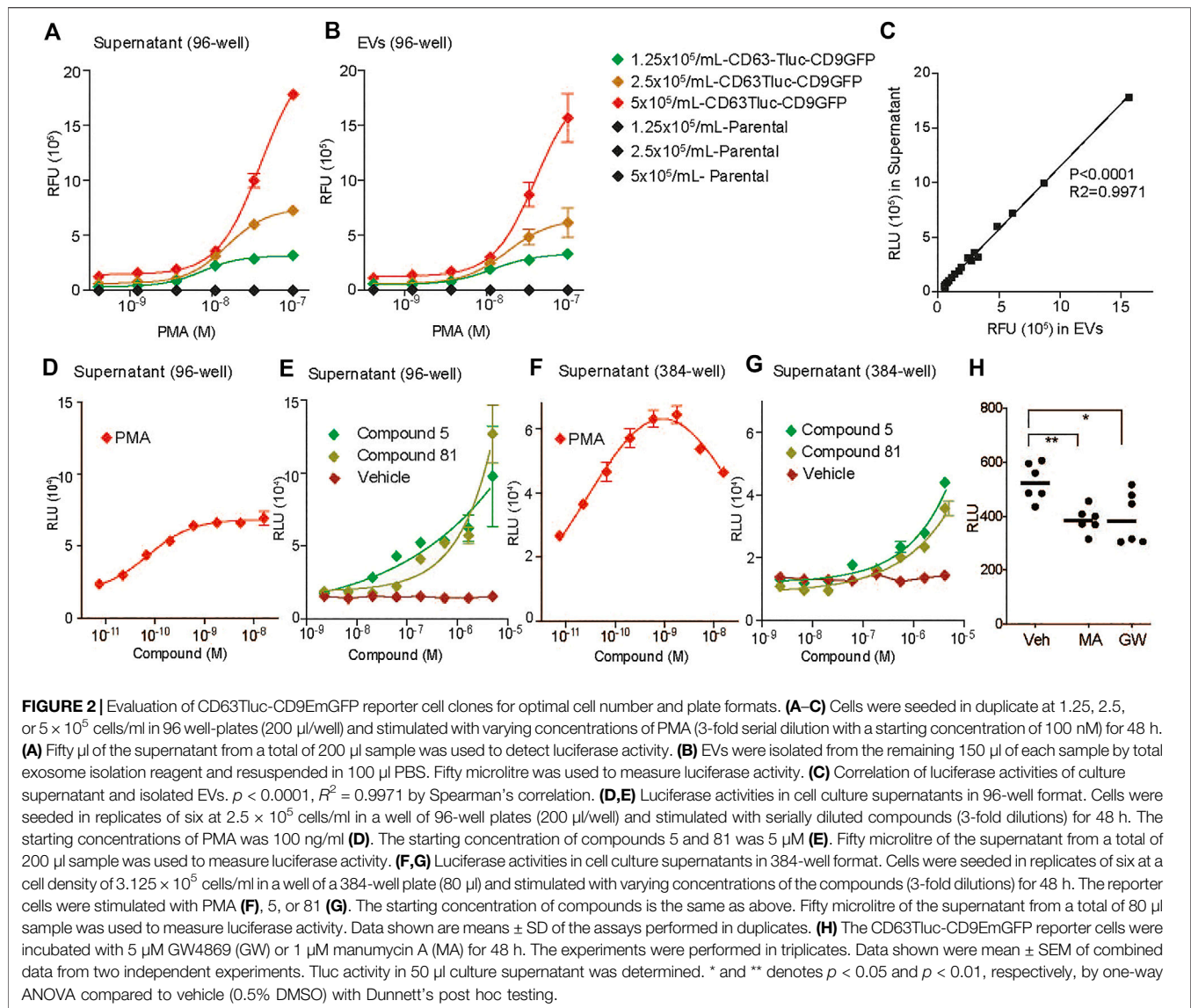
Correlation of Extracellular Vesicles Release and Tluc Activities in the Culture Supernatant of CD63Tluc-CD9EmGFP Reporter Cells

The above data indicated that CD63Tluc-CD9EmGFP reporter cells released EVs carrying CD63Tluc. However, because a portion of CD63Tluc may not be associated with EVs, we examined Tluc activities in the cultured cells (**Table 1** and **Supplementary Table S1**), the EV enriched fractions (EV; **Figure 3A**), and the remaining supernatants (EV-depleted; **Figure 3A**). The CD63Tluc-CD9EmGFP reporter cells were incubated with PMA [50 ng/ml (8.17 nM)] for 48 h, and Tluc activities of the cell pellets and culture supernatants were measured (**Table 1**). The Tluc activities were primarily retained in the cell pellets, and only 2–4% was released into the culture supernatants (**Table 1**). To further test whether Tluc activities were associated with the EV fraction, the Tluc activities of isolated EVs and the residual culture supernatants were determined using two different isolation methods; ExoQuick and differential ultracentrifugation (UC) (**Figures 3A–C**). The

percent of total Tluc activity in the EV-associated fractions was comparable across both methods (vehicle vs. PMA; 61 vs. 63% by ExoQuick; 88 vs. 88% by UC) (**Figures 3B,C**). Furthermore, a dose-response study using graded concentrations of PMA showed that the luciferase activities and particle numbers in the culture supernatants were positively correlated (Spearman's correlation $r = 0.9339$, $p < 0.0001$, **Figures 3D,E**).

Characterization of Extracellular Vesicles Released by CD63 Tluc-CD9EmGFP Reporter Cells

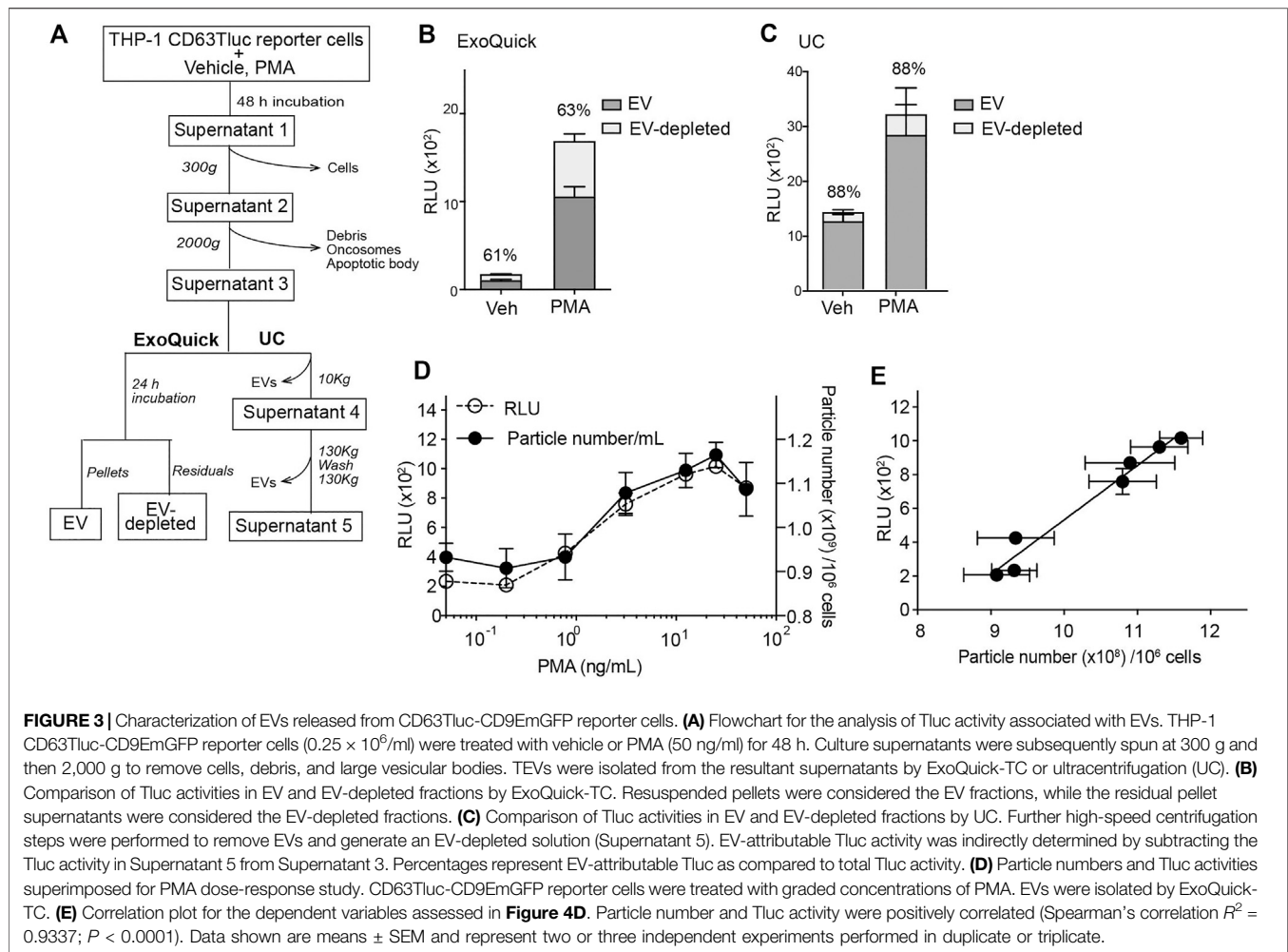
As the engineered CD63Tluc-CD9EmGFP reporter cells constitutively express CD63 driven by a CMV promoter, we examined whether overexpression of CD63 attenuated EV biogenesis and release. We compared the quantity and phenotype of EVs released from the CD63Tluc-CD9EmGFP reporter cells and parental THP-1 cells using four approaches; immunoblotting, NTA, TEM, and vesicle flow cytometry. The expression of TSs (CD63, CD81, and Tsg101) was evaluated by immunoblotting (**Figure 4A** and **Supplementary Figure S5** for the original blots). The EV expression of CD63, CD81, and Tsg101 were similar in the CD63Tluc-CD9EmGFP reporter cells and parental THP-1 cells (**Figure 4A**). Calnexin, an



endoplasmic reticulum-associated protein, was not detected in any EV samples (**Figure 4A**). Particle concentrations and mean diameters of isolated EVs were determined by NanoSight (**Table 2**). The mean sizes of isolated EVs from the vehicle- or PMA-treated CD63Tluc-CD9EmGFP reporter cells were comparable to EVs released by the parental THP-1 cells (**Table 2**). The size distribution of EVs was similar between the reporter cells and parental THP-1 cells (**Figure 4B**). Isolated EVs from parental THP-1 cells and CD63Tluc-CD9EmGFP reporter cells were also morphologically similar as evaluated by TEM and predominantly had diameters within the size distributions detected by NTA (**Figure 4C** and **Supplementary Figure S6**).

EVs released by CD63Tluc-CD9EmGFP reporter cells and parental THP-1 cells were further characterized by vesicle flow cytometry. The EVs were detected by their membrane staining vFRedTM fluorescence, as well as their intra-vesicular esterase

activity using CFSE and their expression of common TS using a mixture of PE-conjugated anti-TS (CD9, CD63, and CD81) antibodies. Essential controls performed to demonstrate single EV specificity include buffer and reagent-only controls, dilution series to determine dynamic assay range and lack of coincidence, and detergent lability testing (**Supplementary Figures S7–S10**). EVs ranged in size from ~75 nm, a typical limit of detection for vFC on the CytoFlex, to about 250 nm (median ~105 nm). EVs from both reporter and parental THP-1 cells contained esterase activity, as detected by CFSE (**Figures 4D,E**). CFSE staining resulted in single EV fluorescence intensities that ranged from less than ~200 FITC molecule of an equivalent soluble fluorophore (MESF), the limit of detection (LOD) defined by background autofluorescence, to several thousand FITC MESF per EV (mean ~1500 MESF, mean ~3000 MESF), with ~40–50% positive above an arbitrarily set threshold of ~200 FITC MESF. As measured by immunofluorescence, EVs also bore surface TS



molecules with a mix of anti-CD9, CD63, and CD81 PE-labeled antibodies. EVs from both cells stained for TS, with single EV intensities that ranged from less than 20 PE MESF, the LOD defined by background autofluorescence of unstained EVs, to ~ 300 MESF per EV (median 8 MESF, mean ~ 30 MESF) (**Figures 4D,E**). Approximately 20–25% of anti-TS PE-stained EVs were positive above an arbitrary threshold of ~ 20 PE MESF. Equivalent number of EVs were released from the reporter cells and the parental THP-1 cells (**Figure 4F**). Over 90% of TS positive particles were detergent sensitive in both cell lines as expected for EVs (**Figure 4G**; **Supplementary Figure S9**). Taken together, EVs released from CD63Tluc-CD9EmGFP reporter cells and parental THP-1 cells were similar in size, numbers, and EV components.

Compound Screen Using CD63Tluc-CD9EmGFP Reporter Cells

To evaluate whether the CD63Tluc-CD9EmGFP reporter cells were acceptable for HTS, we transferred the reporter cells to the Thermo Fisher SelectScreen Facility (Madison, WI), where the protocols were further optimized for HTS using their robotic instruments

(**Figure 5A**). A total of 2210 compounds on seven plates were screened twice on separate days. The scatter plots showed that the signal window was adequate between the positive control (PMA) and negative control (0.1% DMSO), and the average Z' factor was 0.54 ± 0.07 (mean $\pm$ SEM) (**Figure 5B** and **Supplementary Figure S11A,B**). The confirmation rate of the two assay dates (days 1 and 2) was 78.6%, and % response of day 1 and 2 data points were positively correlated (Spearman's correlation $r = 0.4912$, $p < 0.0001$ two-tailed) (**Figure 5C**). The hit rates of the day 1 and 2 screens were 10 and 11%, respectively (**Table 3**). One hundred eighty-seven compounds that showed % response above the cut-off value (mean of negative control values + 3SD) in days 1 and 2 were selected as hit candidates.

Forty out of the 187 compounds were selected from the original library plates and re-tested to induce Tluc activity in the same reporter cell line in the UCSD laboratory. The percent response ratios measured at the SelectScreen facility and our manual rescreen positively correlated (Spearman's correlation $r = 0.4445$, $p = 0.004$ two-tailed) (**Figure 5D**). A negative correlation between cell viability and % response was observed in the validation study (Spearman's correlation $r = -0.5728$, $p = 0.0001$, **Figure 5E**). Twenty-two of the 40 re-

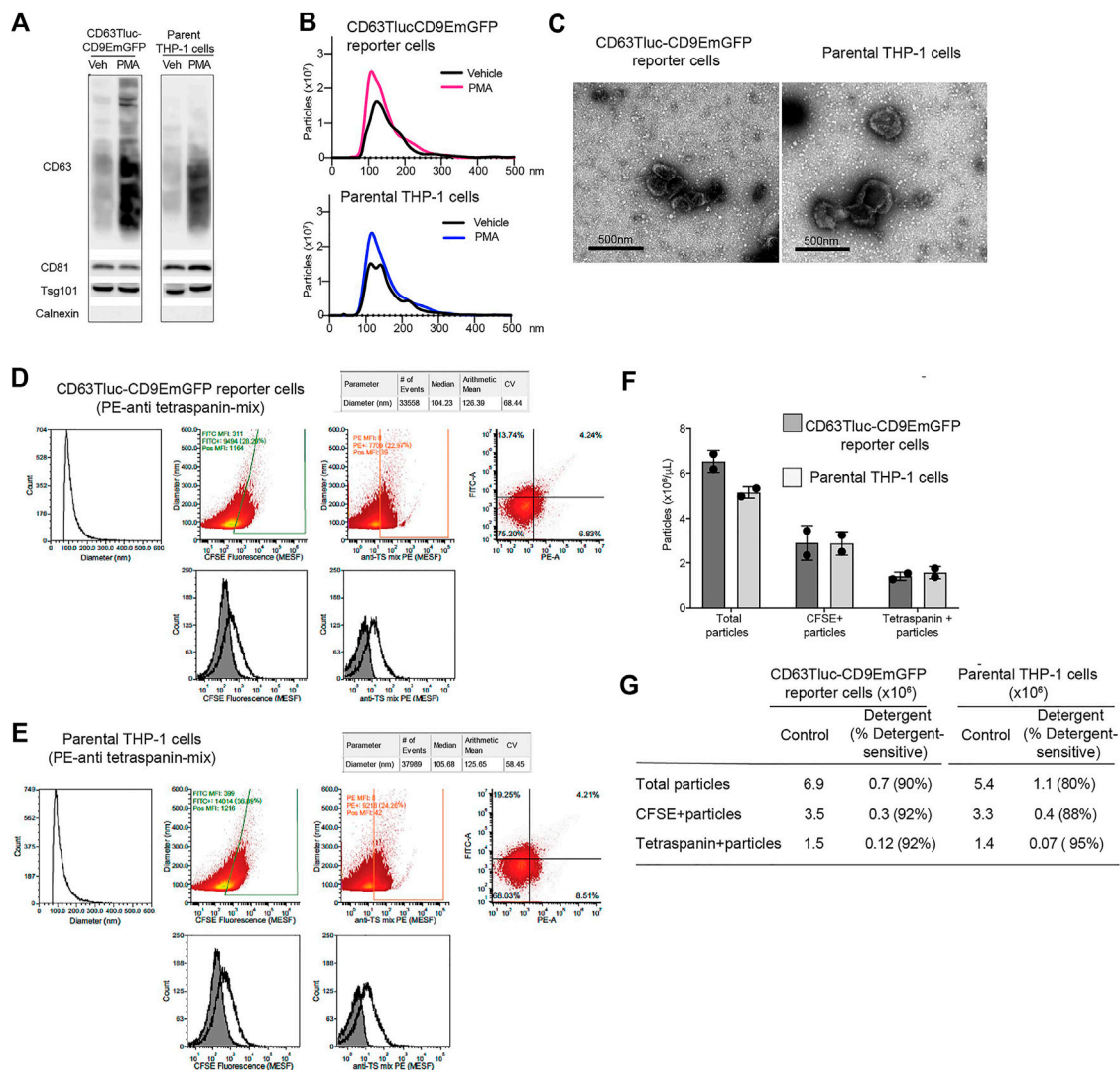
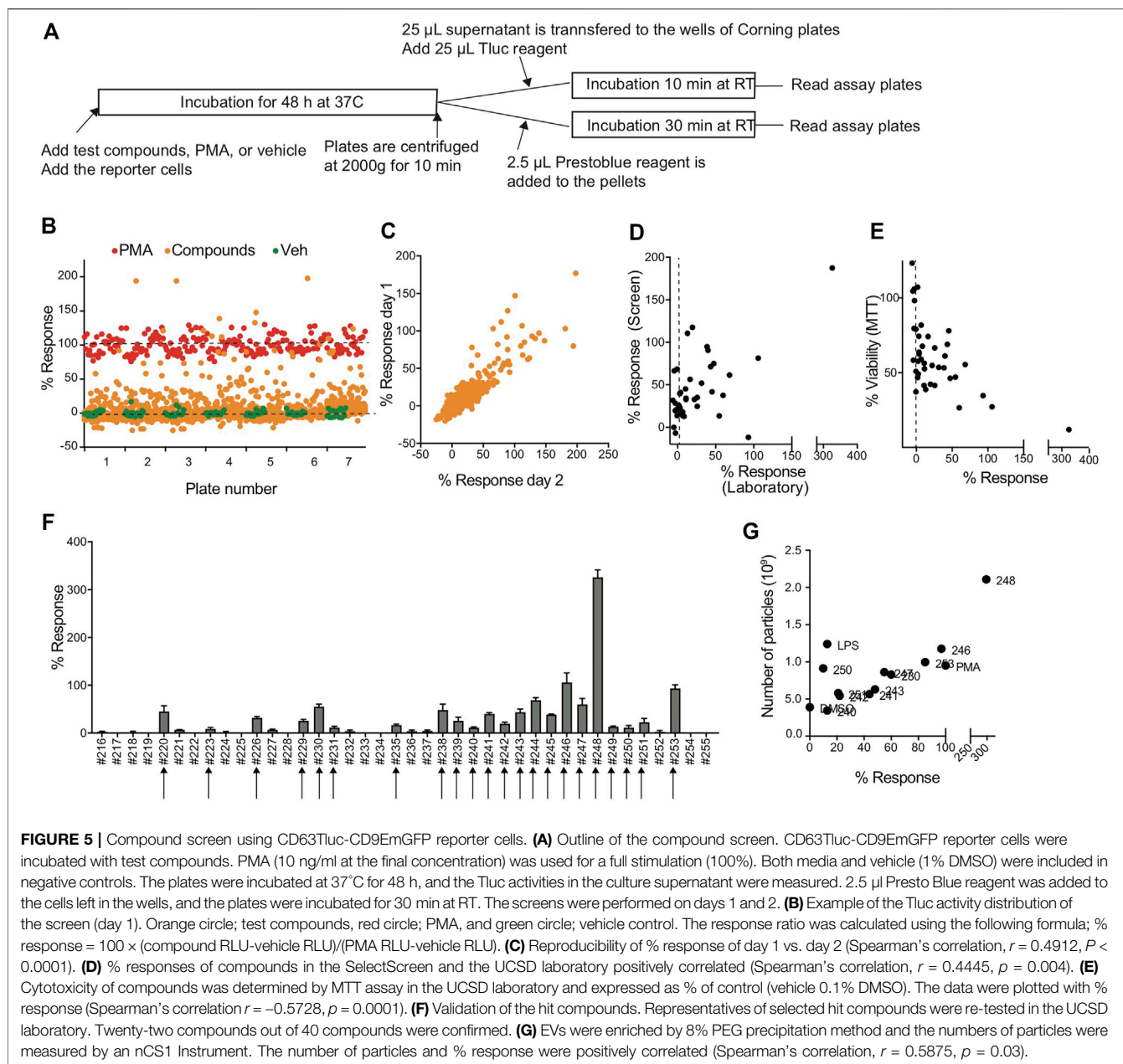


FIGURE 4 | Comparison of EVs released from CD63Tluc-CD9EmGFP reporter cells and parental THP-1 cells. **(A–C)** 0.5×10^6 cells/ml CD63Tluc-CD9EmGFP reporter cells or parental THP-1 cells were treated with 50 ng/ml PMA, 5 μ M compounds 81, or 5 for 48 h. EVs were isolated using ExoQuick and were resuspended in particle-free PBS to 1/500 of the original volume of culture supernatant. **(A)** Immunoblot of EVs isolated from CD63Tluc-CD9EmGFP reporter cells and parental THP-1 cells. EVs (30 μ g protein/well) were separated in a 4–12% NuPAGE gel. Blots were probed with anti-CD63, anti-CD81, anti-Tsg101, or anti-Calnexin Abs. The images shown are representative of three independent experiments. The original blots are presented in **Supplementary Figure S4**. **(B)** Size distributions of isolated EVs released from CD63Tluc-CD9EmGFP reporter cells (**top**) and parental THP-1 cells following treatment with PMA (50 ng/ml) or vehicle as measured by NanoSight. **(C)** Morphological examination of EVs from vehicle-treated CD63Tluc-CD9EmGFP reporter cells and parental THP-1 cells by transmission electron microscopy. Scale bars represent 500 nm. **(D–G)** Vesicle flow cytometric (vFC) analysis of EVs isolated from CD63Tluc-CD9EmGFP reporter cells (**D,F,G**) or parental THP-1 cells (**E–G**) using differential ultracentrifugation. EVs were stained with vFRedTM, CFSE, or a mixture of anti-TS antibodies. The gating strategy, positive and negative control samples and isotype staining are presented in **Supplementary Figure S8**. **(F)** The number of total EVs are reported (vFRedTM-positive), as are the number of CFSE-positive and TS-positive EVs that stained above an arbitrarily placed threshold determined from the background of the relevant unstained samples. **(G)** The vesicular nature of the particles was validated by detergent treatment (0.1% Empigen, **Supplementary Figure S9**). **(A–E,G)** The data shown are representative of two independent experiments. **(F)** Means $\pm$ SD of two independent experiments are presented.

tested compounds had confirmed activity using the same cut-off values (means of negative controls + 3SD (arrows in **Figure 5F**). We further confirmed for representative compounds that the % response positively correlated to particle numbers (Spearman's correlation $r = 0.5875$, $p = 0.03$, two-tailed, **Figure 5G**) as determined by nCS1 Instruments (Spectradyn). When we compared % cell

viability of top five compounds, compound #244, which presented with more than 50% cell viability after 48 h incubation, was chosen as a possible hit compound (**Supplementary Figure S11C**). Collectively, the CD63Tluc-CD9EmGFP reporter cells were amenable to a robotic HTS operation and provided wide signal windows between positive and negative controls, excellent Z' factors, and a positive



correlation of EV release and % response, indicating that this reporter cell line is suitable for large screens.

DISCUSSION

EVs are lipid-bilayer-bound vesicles that have multiple biological functions in mediating cell to cell communications (Margolis and Sadovsky, 2019). Recently the role of EVs in initiating and regulating immune responses has generated interest in immunotherapy. EVs from APCs or diseased cells can present antigens to T and B cells or enhance the functions of immune cells as a by-stander effect (Segura et al., 2005; Luketic et al., 2007; Wen

et al., 2017). Hence, pharmacological agents that reliably influence the biogenesis, biophysical properties, and physiological functions of EVs could add to immune-based therapeutic strategies. In this study, we aimed to develop a cell-based screening system for EV release from immune cells.

TABLE 3 | Number of hit, and non-hit compounds.

		Day 1	
		Non-hit	Hit
Day 2	Non-hit	1909	63
	Hit	51	187

We generated a luciferase reporter linked to CD63 (CD63Tluc) in a human monocytic cell line, THP-1. The cloned CD63Tluc-CD9EmGFP reporter cells, when treated with a known stimulus, shed EVs at a level comparable to that of the parent cell line and with similar phenotypic and morphological properties. The number of EVs measured by NTA correlated with the Tluc activity in culture supernatants. Also, Tluc activity in the culture supernatant of reporter cells increased in a dose-dependent manner following stimulation with either PMA or two other immunostimulatory compounds.

In the design of the reporter construct, we co-opted the well-described essential roles of TSs in EV biogenesis (Petersen et al., 2011; van Niel et al., 2011; Andreu and Yanez-Mo, 2014; Datta et al., 2018). CD63 is a member of the TS family of proteins transferred from the plasma membrane to endosomal compartments and also delivers selected cargo to intraluminal vesicles for exocytosis (Stoorvogel et al., 2002; Pols and Klumperman, 2009). The reporter construct contains the CD63 fusion gene downstream from the CMV promoter, resulting in a slight increase in CD63 protein expression (**Figure 4A**). Other groups have used CD63 for reporter constructs for EV research (Datta et al., 2018; Cashikar and Hanson, 2019). Cashikar et al. developed a plasmid-based construct with luciferase that delivered an EV-associated signal in several cell types indicating that the choice of CD63 is sufficiently robust for cell-based reporter constructs (Cashikar and Hanson, 2019). Datta et al. optimized the CD63 reporter cells in a prostate cancer cell line C4-2B that measure cellular CD63 biogenesis in a HTS format (Datta et al., 2018). Our data, however, showed that CD63Tluc-CD9EmGFP THP-1 cells were sensitive enough to quantitatively measure CD63-carrying EVs directly in culture supernatant.

We initially attempted to prepare dual reporter cells using the CD63Tluc-CD9EmGFP construct. However, GFP fluorescence by the transduced THP-1 cells was dimmed after 4th passage and did not recover thereafter. The CD9EmGFP fusion may have been driven off of the IRES element, and there may have been incomplete transcription after repeated passages and cell division. Alternatively, overexpression of GFP, particularly with an SV40 promoter, might have impaired cell survival (Liu et al., 1999). Although overexpression of EmGFP or CD9 was not reported as toxic in THP-1 cells, it is fully surmisable that the overexpression of the fused protein attributed to the cytotoxicity of THP-1 cells. To test EV induction we used PMA, a protein kinase C activator, which increases intracellular Ca^{2+} levels in THP-1 and other cell types (Reisine and Guild, 1987; Gonczi et al., 2002; Yu et al., 2003). PMA stimulates increased expression levels of CD63 compared to vehicle-treated THP-1 cells (Chakraborty et al., 2019) and the increase in intracellular Ca^{2+} generates exosome secretion (Savina et al., 2003; Messenger et al., 2018). These reports are consistent with our finding that PMA enhanced CD63 expression in both parent THP-1 and the reporter cells and led to the choice of PMA as a positive control for the HTS.

To characterize EVs from the reporter line, we used four enrichment methods: Total Exosome Isolation Reagent™, ExoQuick, 8% PEG precipitation, and differential ultracentrifugation. The differential ultracentrifugation has been recognized as the “gold standard” purification method consisting of low-speed centrifugation to remove live and dead cells and cell debris and high-speed ultracentrifugation to pellet

EVs. Total Exosome Isolation Reagent, ExoQuick, and 8% PEG precipitation method use a principle of PEG precipitation that co-precipitate proteins and nucleic acids in the environment (Garcia-Romero et al., 2019). Tluc activities were more highly retained in the EV fractions of differential ultracentrifugation samples, compared to ExoQuick samples. This finding suggests that the isolation methods could significantly contribute to the apparent phenotypes of isolated EVs (Bozic et al., 2019; Garcia-Romero et al., 2019; Brennan et al., 2020).

Further characterization by immunoblot and single vesicle flow cytometry demonstrated that TSs were expressed on the surface of individual EVs. The number, size distribution, and TS staining were similar for EVs released from the reporter cells and parental THP-1 cells, as was staining with CFSE, a marker for cytoplasmic esterases inside the EVs. EV anti-TS PE fluorescence ranged from less than 20 MESF, the LOD defined by background autofluorescence of unstained EVs, to ~300 MESF per EV, which estimated the number of antigen epitopes to within a factor of two, given the bivalent nature of the IgGs used. Approximately 20–25% percent of EVs were positive above an arbitrary threshold of ~20 MESF, but the characterization of EVs as “positive” or “negative” in this context is inappropriate, owing to the vagaries of making measurements near the detection limit of the instrument. CFSE staining, which is often considered a “generic” marker of EVs, ranged from less than ~200 FITC MESF, the LOD defined by background autofluorescence, to several thousand FITC MESF per EV, with ~40–50% positive above an arbitrarily set threshold of ~200 FITC MESF. As for immunofluorescence measurements of EVs, it is inappropriate to refer to these as CFSE “positive” and “negative” EVs, as these values depend upon the performance features of the instrument as much as the properties of the EVs and we use them here simply as a marker of EVs with entrapped cytoplasm.

Our goal for developing robust reporter cells measuring EV release from immune cells is to screen for compounds that enhance or suppress EV release into tissue culture supernatant. We assessed the robustness of the responses after the prolonged culture. The luciferase activities remained stable in the CD63Tluc-CD9EmGFP reporter cells for over 30 passages. The cell numbers and conditions were miniaturized to a 384-well format suitable for a robotic HTS operation (Datta et al., 2018; Im et al., 2019). One of the limitations of a HTS study is the small number of replicates; however further confirmation in subsequent studies with additional assays were used here to support the key findings. The screening data of 2210 compounds demonstrated that the assays using CD63Tluc-CD9EmGFP reporter cells were reproducible and showed a wide assay window and excellent Z' factors. Compound #244, 3-(3-(methylthio)-1-phenyl-1H-pyrazol-4-yl)-5-(thiophen-2-yl)-1,2,4-oxadiazole, was chosen as a possible hit candidate and carried out for the validation study. This compound had no prior reported biologic activity; however, 1,2,4-oxadiazole derivatives are reported to show a broad range of biologic activities, e.g., anti-tumor, anti-microbial, and anti-inflammatory, anti-allodynic, anti-convulsant, anti-Alzheimer agents, anti-insomnia, and others (Biernacki et al., 2020).

The validation study using 184 confirmed hit candidates showed that the cytotoxicity data and % response ratio were negatively

correlated, suggesting cytotoxic compounds enhance EVs release (**Figure 5E**). The exosome function from dying or apoptotic cells was recently discussed (Kakarla et al., 2020). Dying cells due to infection or tissue injury, release EVs that transfer the proinflammatory information to the recipient cells (Baxter, 2020). THP-1 cells that are undergoing lytic cell death release more EVs compared with viable or apoptotic cells *in vitro*, and it is essential to distinguish between Tluc associated with EV and free Tluc (Baxter et al., 2019). Therefore, it is likely that the cytotoxicity of the compounds causes higher EV release. It is also essential to distinguish Tluc associated with EVs and free Tluc. Our study indicated that only 60–88% Tluc was associated with EV fractions in the reporter cell culture supernatants following vehicle or PMA stimulation. After the treatment with cytotoxic compounds, the proportion of free Tluc in the total Tluc in the culture supernatants might be attenuated by incubation time and compound concentration. Hence, in the HTS, further validation screening of confirmed hit candidates using NTA or nanoparticle flow cytometry is indispensable for properly identifying the hit compounds.

In summary, we developed a human reporter cell line to quantitatively measure EV release from APCs. A CD63Tluc-CD9EmGFP reporter construct was transduced into the human monocytic cell line, THP-1. The Tluc activities in the culture supernatant positively correlated with the number of EVs released by the reporter cells as measured by NTA. Tluc assay using the CD63Tluc-CD9EmGFP reporter cells was sensitive and amenable to a 384-well robotic screening format with a Z' factor over 0.5. These results indicated that the CD63Tluc-CD9EmGFP reporter cell line exhibited reliable, reproducible, and robust responses that makes it an excellent tool for the screening of compounds that enhance EV release by APCs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

REFERENCES

- Andreu, Z., and Yáñez-Mó, M. (2014). Tetraspanins in extracellular vesicle formation and function. *Front. Immunol.* 5, 442. doi:10.3389/fimmu.2014.00442
- Baxter, A. A., Phan, T. K., Hanssen, E., Liem, M., Hulett, M. D., Mathivanan, S., et al. (2019). Analysis of extracellular vesicles generated from monocytes under conditions of lytic cell death. *Sci. Rep.* 9 (1), 7538. doi:10.1038/s41598-019-44021-9
- Baxter, A. A. (2020). Stoking the fire: how dying cells propagate inflammatory signalling through extracellular vesicle trafficking. *Ijms* 21 (19), 7256. doi:10.3390/ijms21197256
- Besse, B., Charrier, M., Lapierre, V., Dansin, E., Lantz, O., Planchard, D., et al. (2016). Dendritic cell-derived exosomes as maintenance immunotherapy after first line chemotherapy in NSCLC. *Oncoimmunology* 5 (4), e1071008. doi:10.1080/2162402X.2015.1071008
- Biernacki, K., Daško, M., Ciupak, O., Kubiński, K., Rachon, J., and Demkowicz, S. (2020). Novel 1,2,4-oxadiazole derivatives in drug discovery. *Pharmaceuticals* 13 (6), 111. doi:10.3390/ph13060111

AUTHOR CONTRIBUTIONS

Study conception and design: CL, DC, FK, JS, MC, TH, and TS. Acquisition of data: CL, FL, FS-K, JN, JS, SY, and TS. Analysis and interpretation of data: DC, FS-K, JN, JS, FL, MC, SY, TH, TS, and NS. Drafting of the manuscript: JS, MC, and TH. Critical revision: DC, FL, FS-K, HC, JN, JS, MC, SY, TH, and TS.

FUNDING

This work was supported by the National Institute of Health/National Institute of Allergy and Infectious Diseases under contracts HHSN272201400051C and 75N93019C00042. CL was supported by the Medical Scientific Research Foundation of Guangdong Province, China (2018037), and the National Natural Science Foundation of China (81503215).

ACKNOWLEDGMENTS

We appreciate Jan Olof Lötvald for his critical comments to improve the manuscript. We thank Chetana Revankar, David Piper, and SelectScreen™ service team, Thermo Fisher Scientific, for their technical expertise in the generation of the CD63Tluc-CD9EmGFP reporter cell line. We also appreciate Timothy Meerloo for his technical assistance with TEM. We are also grateful to Antonio De Maio and David Cauvi for sharing their expertise and allowing us to use the NanoSight instrument.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2021.668609/full#supplementary-material>.

- Bozic, D., Sitar, S., Junkar, I., Stukelj, R., Pajnic, M., Zagar, E., et al. (2019). Viscosity of plasma as a key factor in assessment of extracellular vesicles by light scattering. *Cells* 8 (9). doi:10.3390/cells8091046
- Brennan, K., Martin, K., FitzGerald, S. P., O'Sullivan, J., Wu, Y., Blanco, A., et al. (2020). A comparison of methods for the isolation and separation of extracellular vesicles from protein and lipid particles in human serum. *Sci. Rep.* 10 (1), 1039. doi:10.1038/s41598-020-57497-7
- Cai, C., Koch, B., Morikawa, K., Suda, G., Sakamoto, N., Rueschenbaum, S., et al. (2018). Macrophage-derived extracellular vesicles induce long-lasting immunity against hepatitis C virus which is blunted by polyunsaturated fatty acids. *Front. Immunol.* 9, 723. doi:10.3389/fimmu.2018.00723
- Campos, J. H., Soares, R. P., Ribeiro, K., Cronemberger Andrade, A., Batista, W. L., and Torrecilhas, A. C. (2015). Extracellular vesicles: role in inflammatory responses and potential uses in vaccination in cancer and infectious diseases. *J. Immunol. Res.* 2015, 1. doi:10.1155/2015/832057
- Cashikar, A. G., and Hanson, P. I. (2019). A cell-based assay for CD63-containing extracellular vesicles. *PLoS One* 14 (7), e0220007. doi:10.1371/journal.pone.0220007
- Chakraborty, J., Roy, S., Murab, S., Ravani, R., Kaur, K., Devi, S., et al. (2019). Modulation of macrophage phenotype, maturation, and graft integration

- through chondroitin sulfate cross-linking to decellularized cornea. *ACS Biomater. Sci. Eng.* 5 (1), 165–179. doi:10.1021/acsbiomaterials.8b00251
- Chan, M., Lao, F. S., Chu, P. J., Shpigelman, J., Yao, S., Nan, J., et al. (2019). Structure-activity relationship studies to identify affinity probes in bis-aryl sulfonamides that prolong immune stimuli. *J. Med. Chem.* 62, 9521–9540. doi:10.1021/acs.jmedchem.9b00870
- Cossetti, C., Iraci, N., Mercer, T. R., Leonardi, T., Alpi, E., Drago, D., et al. (2014). Extracellular vesicles from neural stem cells transfer IFN- γ via Ifng1 to activate Stat1 signaling in target cells. *Mol. Cell* 56 (2), 193–204. doi:10.1016/j.molcel.2014.08.020
- Datta, A., Kim, H., Lal, M., McGee, L., Johnson, A., Moustafa, A. A., et al. (2017). Manumycin A suppresses exosome biogenesis and secretion via targeted inhibition of Ras/Raf/ERK1/2 signaling and hnRNP H1 in castration-resistant prostate cancer cells. *Cancer Lett.* 408, 73–81. doi:10.1016/j.canlet.2017.08.020
- Datta, A., Kim, H., McGee, L., Johnson, A. E., Talwar, S., Marugan, J., et al. (2018). High-throughput screening identified selective inhibitors of exosome biogenesis and secretion: a drug repurposing strategy for advanced cancer. *Sci. Rep.* 8 (1), 8161. doi:10.1038/s41598-018-26411-7
- Escudier, B., Dorval, T., Chaput, N., André, F., Caby, M.-P., Novault, S., et al. (2005). Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of the first phase I clinical trial. *J. Transl Med.* 3 (1), 10. doi:10.1186/1479-5876-3-10
- Essandoh, K., Yang, L., Wang, X., Huang, W., Qin, D., Hao, J., et al. (2015). Blockade of exosome generation with GW4869 dampens the sepsis-induced inflammation and cardiac dysfunction. *Biochim. Biophys. Acta (Bba) - Mol. Basis Dis.* 1852 (11), 2362–2371. doi:10.1016/j.bbdis.2015.08.010
- Foster, B. P., Balassa, T., Benen, T. D., Dominovic, M., Elmadjian, G. K., Florova, V., et al. (2016). Extracellular vesicles in blood, milk and body fluids of the female and male urogenital tract and with special regard to reproduction. *Crit. Rev. Clin. Lab. Sci.* 53 (6), 379–395. doi:10.1080/10408363.2016.1190682
- García-Romero, N., Madurga, R., Rackov, G., Palacín-Aliana, I., Núñez-Torres, R., Asensi-Puig, A., et al. (2019). Polyethylene glycol improves current methods for circulating extracellular vesicle-derived DNA isolation. *J. Transl Med.* 17 (1), 75. doi:10.1186/s12967-019-1825-3
- Gireud-Goss, M., Reyes, S., Wilson, M., Farley, M., Memarzadeh, K., Srinivasan, S., et al. (2018). Distinct mechanisms enable inward or outward budding from late endosomes/multivesicular bodies. *Exp. Cell Res.* 372 (1), 1–15. doi:10.1016/j.yexcr.2018.08.027
- Gonczi, M., Papp, H., Biro, T., Kovacs, L., and Csernoch, L. (2002). Effect of protein kinase C on transmembrane calcium fluxes in HaCaT keratinocytes. *Exp. Dermatol.* 11 (1), 25–33. doi:10.1034/j.1600-0625.2002.110103.x
- Gupta, D., Liang, X., Pavlova, S., Wiklander, O. P. B., Corso, G., Zhao, Y., et al. (2020). Quantification of extracellular vesicles *in vitro* and *in vivo* using sensitive bioluminescence imaging. *J. Extracellular Vesicles* 9 (1), 1800222. doi:10.1080/20013078.2020.1800222
- Hikita, T., Miyata, M., Watanabe, R., and Oneyama, C. (2018). Sensitive and rapid quantification of exosomes by fusing luciferase to exosome marker proteins. *Sci. Rep.* 8 (1), 14035. doi:10.1038/s41598-018-32535-7
- Im, E.-J., Lee, C.-H., Moon, P.-G., Rangaswamy, G. G., Lee, B., Lee, J. M., et al. (2019). Sulfisoxazole inhibits the secretion of small extracellular vesicles by targeting the endothelin receptor A. *Nat. Commun.* 10 (1), 1387. doi:10.1038/s41467-019-09387-4
- Kakarla, R., Hur, J., Kim, Y. J., Kim, J., and Chwae, Y.-J. (2020). Apoptotic cell-derived exosomes: messages from dying cells. *Exp. Mol. Med.* 52 (1), 1–6. doi:10.1038/s12276-019-0362-8
- Kowal, J., Arras, G., Colombo, M., Jouve, M., Morath, J. P., Primdal-Bengtson, B., et al. (2016). Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc. Natl. Acad. Sci. USA* 113 (8), E968–E977. doi:10.1073/pnas.1521230113
- Lindenbergh, M. F. S., and Stoorvogel, W. (2018). Antigen presentation by extracellular vesicles from professional antigen-presenting cells. *Annu. Rev. Immunol.* 36, 435–459. doi:10.1146/annurev-immunol-041015-055700
- Liu, H.-S., Jan, M.-S., Chou, C.-K., Chen, P.-H., and Ke, N.-J. (1999). Is green fluorescent protein toxic to the living cells? *Biochem. Biophysical Res. Commun.* 260 (3), 712–717. doi:10.1006/bbrc.1999.0954
- Luketic, L., Delanghe, J., Sobol, P. T., Yang, P., Frotten, E., Mossman, K. L., et al. (2007). Antigen presentation by exosomes released from peptide-pulsed dendritic cells is not suppressed by the presence of active CTL. *J. Immunol.* 179 (8), 5024–5032. doi:10.4049/jimmunol.179.8.5024
- Macarrón, R., and Hertzberg, R. P. (2011). Design and implementation of high throughput screening assays. *Mol. Biotechnol.* 47 (3), 270–285. doi:10.1007/s12033-010-9335-9
- Margolis, L., and Sadovsky, Y. (2019). The biology of extracellular vesicles: the known unknowns. *Plos Biol.* 17 (7), e3000363. doi:10.1371/journal.pbio.3000363
- Messenger, S. W., Woo, S. S., Sun, Z., and Martin, T. F. J. (2018). A Ca²⁺-stimulated exosome release pathway in cancer cells is regulated by Munc13-4. *J. Cell Biol.* 217 (8), 2877–2890. doi:10.1083/jcb.201710132
- Moroishi, T., Hayashi, T., Pan, W.-W., Fujita, Y., Holt, M. V., Qin, J., et al. (2016). The Hippo pathway kinases LATS1/2 suppress cancer immunity. *Cell* 167 (6), 1525–1539. doi:10.1016/j.cell.2016.11.005
- Morse, M. A., Garst, J., Osada, T., Khan, S., Hobeika, A., Clay, T. M., et al. (2005). A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. *J. Transl Med.* 3 (1), 9. doi:10.1186/1479-5876-3-9
- Petersen, S. H., Odintsova, E., Haigh, T. A., Rickinson, A. B., Taylor, G. S., and Berditchevski, F. (2011). The role of tetraspanin CD63 in antigen presentation via MHC class II. *Eur. J. Immunol.* 41 (9), 2556–2561. doi:10.1002/eji.201141438
- Pols, M. S., and Klumperman, J. (2009). Trafficking and function of the tetraspanin CD63. *Exp. Cell Res.* 315 (9), 1584–1592. doi:10.1016/j.yexcr.2008.09.020
- Reisine, T., and Guild, S. (1987). Activators of protein kinase C and cyclic AMP-dependent protein kinase regulate intracellular calcium levels through distinct mechanisms in mouse anterior pituitary tumor cells. *Mol. Pharmacol.* 32 (4), 488–496.
- Sato-Kaneko, F., Wang, X., Yao, S., Hosoya, T., Lao, F. S., Messer, K., et al. (2018). Discovery of a novel microtubule targeting agent as an adjuvant for cancer immunotherapy. *Biomed. Res. Int.* 2018, 1. doi:10.1155/2018/8091283
- Savina, A., Furlán, M., Vidal, M., and Colombo, M. I. (2003). Exosome release is regulated by a calcium-dependent mechanism in K562 cells. *J. Biol. Chem.* 278 (22), 20083–20090. doi:10.1074/jbc.M301642200
- Segura, E., Amigorena, S., and Théry, C. (2005). Mature dendritic cells secrete exosomes with strong ability to induce antigen-specific effector immune responses. *Blood Cell Mol. Dis.* 35 (2), 89–93. doi:10.1016/j.bcmd.2005.05.003
- Shukla, N. M., Arimoto, K.-I., Yao, S., Fan, J.-B., Zhang, Y., Sato-Kaneko, F., et al. (2018). Identification of compounds that prolong type I interferon signaling as potential vaccine adjuvants. *SLAS DISCOVERY: Advancing Sci. Drug Discov.* 23 (9), 960–973. doi:10.1177/2472555218774308
- Skog, J., Würdinger, T., van Rijn, S., Meijer, D. H., Gainche, L., Curry, W. T., et al. (2008). Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* 10 (12), 1470–1476. doi:10.1038/ncb1800
- Stahl, P. D., and Raposo, G. (2018). Exosomes and extracellular vesicles: the path forward. *Essays Biochem.* 62 (2), 119–124. doi:10.1042/EBC20170088
- Stoner, S. A., Duggan, E., Condello, D., Guerrero, A., Turk, J. R., Narayanan, P. K., et al. (2016). High sensitivity flow cytometry of membrane vesicles. *Cytometry* 89 (2), 196–206. doi:10.1002/cyto.a.22787
- Stoorvogel, W., Kleijmeer, M. J., Geuze, H. J., and Raposo, G. (2002). The biogenesis and functions of exosomes. *Traffic* 3 (5), 321–330. doi:10.1034/j.1600-0854.2002.30502.x
- Taylor, D. D., and Gercel-Taylor, C. (2011). Exosomes/microvesicles: mediators of cancer-associated immunosuppressive microenvironments. *Semin. Immunopathol.* 33 (5), 441–454. doi:10.1007/s00281-010-0234-8
- Thery, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., et al. (2018). Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell Vesicles* 7 (1), 1535750. doi:10.1080/20013078.2018.1535750
- Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J., and Lötvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9 (6), 654–659. doi:10.1038/ncb1596
- van Niel, G., Charrin, S., Simoes, S., Romao, M., Rochin, L., Saftig, P., et al. (2011). The tetraspanin CD63 regulates ESCRT-independent and -dependent endosomal sorting during melanogenesis. *Dev. Cell* 21 (4), 708–721. doi:10.1016/j.devcel.2011.08.019

- Wang, J., Sun, X., Zhao, J., Yang, Y., Cai, X., Xu, J., et al. (2017). Exosomes: a novel strategy for treatment and prevention of diseases. *Front. Pharmacol.* 8, 300. doi:10.3389/fphar.2017.00300
- Wen, C., Seeger, R. C., Fabbri, M., Wang, L., Wayne, A. S., and Jong, A. Y. (2017). Biological roles and potential applications of immune cell-derived extracellular vesicles. *J. Extracellular Vesicles* 6 (1), 1400370. doi:10.1080/20013078.2017.1400370
- Woodman, P. G., and Futter, C. E. (2008). Multivesicular bodies: co-ordinated progression to maturity. *Curr. Opin. Cell Biol.* 20 (4), 408–414. doi:10.1016/j.ccb.2008.04.001
- Yáñez-Mó, M., Siljander, P. R.-M., Andreu, Z., Bedina Zavec, A., Borràs, F. E., Buzas, E. I., et al. (2015). Biological properties of extracellular vesicles and their physiological functions. *J. Extracellular Vesicles* 4, 27066. doi:10.3402/jev.v4.27066
- Yu, T., Morita, I., Shimokado, K., Iwai, T., and Yoshida, M. (2003). Amlodipine modulates THP-1 cell adhesion to vascular endothelium via inhibition of protein kinase C signal transduction. *Hypertension* 42 (3), 329–334. doi:10.1161/01.HYP.0000087199.34071.4F
- Zhang, J.-H., Chung, T. D. Y., and Oldenburg, K. R. (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* 4 (2), 67–73. doi:10.1177/108705719900400206
- Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., Flament, C., Tenza, D., et al. (1998). Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell derived exosomes. *Nat. Med.* 4 (5), 594–600. doi:10.1038/nm0598-594
- Zwicker, J. I., Liebman, H. A., Neuberg, D., Lacroix, R., Bauer, K. A., Furie, B. C., et al. (2009). Tumor-derived tissue factor-bearing microparticles are associated with venous thromboembolic events in malignancy. *Clin. Cancer Res.* 15 (22), 6830–6840. doi:10.1158/1078-0432.CCR-09-0371

Conflict of Interest: JN is the chief executive officer of Cellarcus Biosciences.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Shpigelman, Lao, Yao, Li, Saito, Sato-Kaneko, Nolan, Shukla, Pu, Messer, Cottam, Carson, Corr and Hayashi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



The Depolarization-Evoked, Ca^{2+} -Dependent Release of Exosomes From Mouse Cortical Nerve Endings: New Insights Into Synaptic Transmission

OPEN ACCESS

Edited by:

Jacob Raber,
Oregon Health and Science University,
United States

Reviewed by:

Michele Guescini,
University of Urbino Carlo Bo, Italy
Maria Angela Sortino,
University of Catania, Italy
Sergei V. Fedorovich,
Belarusian State University, Belarus

*Correspondence:

Anna Pittaluga
pittaluga@difar.unige.it

†Present Address:

Daniela Di Paolo,
Nuclear Medicine Unit, Santa Corona
Hospital, via XXV Aprile, 17027, Pietra
Ligure Savona, Genoa, Italy

Specialty section:

This article was submitted to
Neuropharmacology,
a section of the journal
Frontiers in Pharmacology

Received: 20 February 2021

Accepted: 22 June 2021

Published: 22 July 2021

Citation:

Olivero G, Cisani F, Marimpietri D,
Di Paolo D, Gagliani MC, Podestà M,
Cortese K and Pittaluga A (2021) The
Depolarization-Evoked, Ca^{2+} -
Dependent Release of Exosomes From
Mouse Cortical Nerve Endings: New
Insights Into Synaptic Transmission.
Front. Pharmacol. 12:670158.
doi: 10.3389/fphar.2021.670158

Guendalina Olivero¹, Francesca Cisani¹, Danilo Marimpietri², Daniela Di Paolo^{3†},
Maria Cristina Gagliani⁴, Marina Podestà², Katia Cortese⁴ and Anna Pittaluga^{5,6*}

¹Department of Pharmacy, DIFAR, Pharmacology and Toxicology Section, University of Genoa, Genoa, Italy, ²Stem Cell Laboratory and Cell Therapy Center, IRCCS Istituto Giannina Gaslini, Genoa, Italy, ³Laboratory of Experimental Therapies in Oncology, IRCCS Istituto Giannina Gaslini, Genoa, Italy, ⁴Department of Experimental Medicine, DIMES, Human Anatomy Section, University of Genoa, Genoa, Italy, ⁵Department of Pharmacy, DIFAR, Pharmacology and Toxicology Section, Centre of Excellence for Biomedical Research, 3Rs Center, University of Genoa, Genoa, Italy, ⁶IRCCS Ospedale Policlinico San Martino, Genoa, Italy

Whether exosomes can be actively released from presynaptic nerve terminals is a matter of debate. To address the point, mouse cortical synaptosomes were incubated under basal and depolarizing (25 mM KCl-enriched medium) conditions, and extracellular vesicles were isolated from the synaptosomal supernatants to be characterized by dynamic light scattering, transmission electron microscopy, Western blot, and flow cytometry analyses. The structural and biochemical analysis unveiled that supernatants contain vesicles that have the size and the shape of exosomes, which were immunopositive for the exosomal markers TSG101, flotillin-1, CD63, and CD9. The marker content increased upon the exposure of nerve terminals to the high-KCl stimulus, consistent with an active release of the exosomes from the depolarized synaptosomes. High KCl-induced depolarization elicits the Ca^{2+} -dependent exocytosis of glutamate. Interestingly, the depolarization-evoked release of exosomes from cortical synaptosomes also occurred in a Ca^{2+} -dependent fashion, since the TSG101, CD63, and CD9 contents in the exosomal fraction isolated from supernatants of depolarized synaptosomes were significantly reduced when omitting external Ca^{2+} ions. Differently, ($\pm$)-baclofen (10 μM), which significantly reduced the glutamate exocytosis, did not affect the amount of exosomal markers, suggesting that the GABA_B-mediated mechanism does not control the exosome release. Our findings suggest that the exposure of synaptosomes to a depolarizing stimulus elicits a presynaptic release of exosomes that occurs in a Ca^{2+} -dependent fashion. The insensitivity to the presynaptic GABA_B receptors, however, leaves open the

Abbreviations: ESCRT-I, endosomal sorting complex required for transport I; EVs, extracellular vesicles; LDH, lactate dehydrogenase; MVB, multivesicular body; PSD95, postsynaptic density protein 95; t-TBS, tris-buffered saline-tween; TEM, transmission electron microscopy; TSG101, tumor susceptibility gene 101 protein; WG, wingless.

question on whether the release of exosomes could be a druggable target for new therapeutic intervention for the cure of synaptopathies.

Keywords: synaptosomes, exosomes, calcium dependency, glutamate release, GABAB receptor

INTRODUCTION

Extracellular vesicles (EVs) have emerged in the last 3 decades as a novel way of cell-to-cell communication (Raposo and Stoorvogel, 2013). Exosomes are nanosized EVs (30–100 nm in diameter) of endosomal origin, secreted by most cells in the body, known to vehiculate complex cargoes, including proteins, lipids, and nucleic acids, whose composition is supposed to depend on the cell they originate from. Once released in the extracellular fluids, they are taken up by the selected target cells, influencing their functions (Mathieu et al., 2019).

Exosomes also mediate intercellular communication in the CNS, where they are actively released by all the CNS cells, including astrocytes (Verkhatsky et al., 2016), microglia (Paolicelli et al., 2018), oligodendrocytes (Frühbeis et al., 2013), and neurons (Chivet et al., 2012). Data in the literature demonstrated that the exosomal trafficking has important implications in CNS physiology and pathology, from supporting neurogenesis, neuroprotection, and brain homeostasis (Smalheiser, 2007; Saeedi et al., 2019; Schiera et al., 2020) to favoring the cell-to-cell spreading of “pathogenic proteins” (β amyloid peptides, tau, and prions; Holm et al., 2018; Caruso Bavisotto et al., 2019).

Emerging evidence suggests that exosomes are also involved in the modulation of synaptic transmission and plasticity. Cultured cortical neurons release exosomes, mainly from the somata and the dendrites, and this secretion is increased by depolarization and strictly dependent on synaptic glutamatergic activity. Furthermore, neuron-derived exosomes carry selected proteins involved in synaptic transmission, such as GluA2/3 AMPA receptor subunits, suggesting a new mechanism for regulating synaptic strength after neuronal activation (Faurè et al., 2006; Lachenal et al., 2011). Whether exosomes can also be actively released from presynaptic neuronal terminals of the CNS mammalian synapses, however, has not been clarified so far, although some evidence supports the hypothesis (Xu et al., 2013; Janas et al., 2016; Wang et al., 2017). As a matter of fact, at the *Drosophila* neuromuscular junction, which represents a model of presynaptic structure, a trans-synaptic transferring of the Wnt-family signaling protein Wingless (Wg) was reported to occur through exosomes released from the synaptic boutons and containing the Wg-binding protein Evenness Interrupted (Korkut et al., 2009; Koles et al., 2012). In the same model, synaptotagmin-4 was also found to be delivered through exosomes from the presynaptic motor neuron to the muscle fiber to mediate the activity-dependent synaptic growth (Korkut et al., 2013).

Synaptosomes are isolated nerve endings which carry the structural features and the properties of the *in vivo* neuronal terminals they originate from. They are widely recognized as an *in vitro* model for selectively studying the molecular events

occurring presynaptically, particularly the release of neurotransmitters and its modulation by presynaptic receptors (Raiteri, 2001; Raiteri, 2008; Langer, 2008).

Our study aimed at investigating whether synaptosomes can release EVs having the morphological and proteomic features of exosomes. The results support the conclusion, suggesting new unexpected aspects of the synaptic transmission that could represent the targets of new therapeutic interventions to control and restore the efficiency of the central synaptic connections.

METHODS

Animals

Mice (male, strain C57BL/6J) were obtained from Charles River (Calco, Italy) and housed in the animal facility of the Department of Pharmacy (DIFAR), Pharmacology and Toxicology Section (Genoa, Italy), under controlled environmental conditions (ambient temperature = 22°C, humidity = 40%) on a 12-h light/dark cycle with food and water *ad libitum*. The animal care and experimental procedures complied with the European Communities Parliament and Council Directive of September 22, 2010 (2010/63/EU) and with the Italian D.L. n. 26/2014 and were approved by the Local Committee for Animal Care and Welfare of the University of Genova and the Italian Ministry of Health (DDL 26/2014 and previous legislation; protocol number n° 75F11.N.IMY). All efforts were made to minimize the number of animals used and their suffering, and no *in vivo* technique was used.

Preparation of Synaptosomes

Synaptosomes can be easily obtained by homogenization of brain tissues, their membranes having “pinched off” at the point of connection with the neuronal axon, and then purified from other tissue components using density-gradient centrifugation techniques (Gray and Wittaker, 1962; Dunkley et al., 2008). Purified synaptosomes were prepared as previously described (Olivero et al., 2018) from the cortex of one animal/experiment, except for the experiments described in **Figure 4**, where the cortices of two mice/experiments were pooled together. The tissue was homogenized in 10 volumes of 0.32 M sucrose and buffered to a pH value of 7.4 with Tris (final concentration 0.01 M) using a glass/Teflon tissue grinder (clearance 0.25 mm). The homogenate was first centrifuged at 1,000 g for 5 min to remove nuclei and debris; the supernatant was gently layered on a discontinuous Percoll gradient (6, 10, and 20% v/v in Tris-buffered 0.32 M sucrose) and then centrifuged at 33,500 g for 6 min. The layer between 10 and 20% Percoll (synaptosomal fraction) was collected and washed by centrifugation at 19,000 g for 15 min (**Figure 1A**). Synaptosomes were then resuspended in

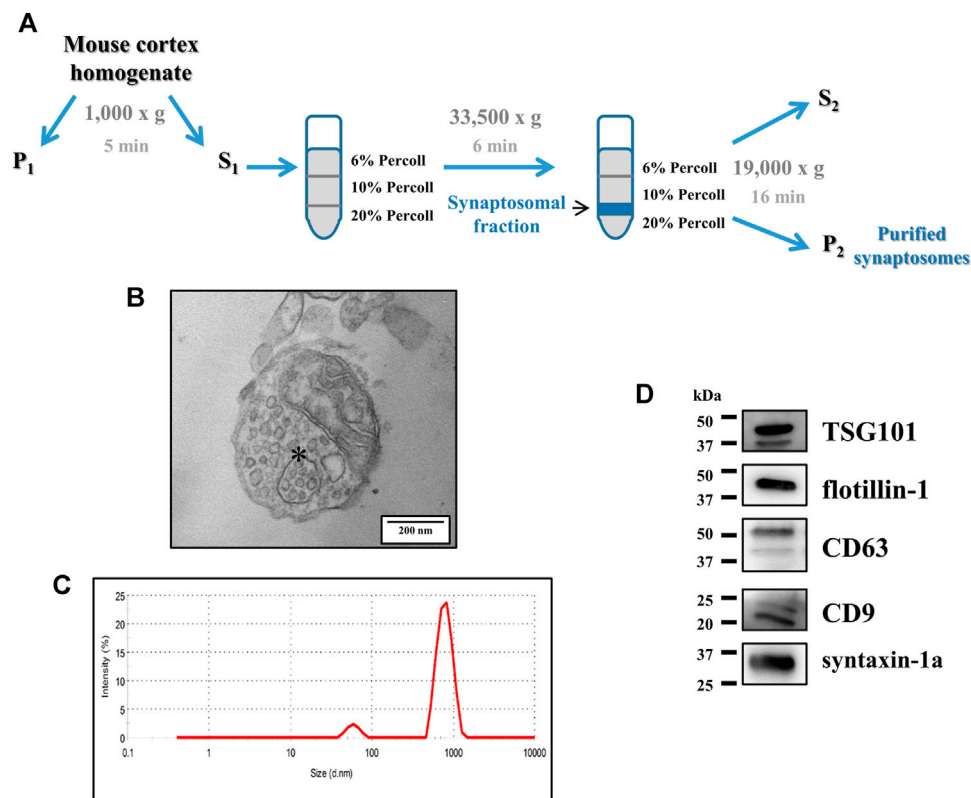


FIGURE 1 | Structural analysis of purified synaptosomes isolated from the mouse cortex. **(A)** Schematic representation of the discontinuous Percoll gradient isolation procedure of the purified synaptosomes from the mouse cortical homogenate. For further details, see the Methods section. **(B)** Ultrastructural analysis of synaptosomes by transmission electron microscopy (TEM). The figure shows an isolated synaptosome, containing multiple vesicles, a mitochondrion, and a multivesicular body-like organelle (asterisk). Scale bar: 200 nm. The image is representative of the TEM analysis of $n = 5$ different synaptosomal preparations. **(C)** Size distribution of synaptosomes assessed using a Zetasizer Nano ZS90 particle sizer. The curve is representative of the analysis of $n = 5$ different synaptosomal preparations. **(D)** Western blot analysis of the exosomal markers TSG101, flotillin-1, CD63, and CD9 in cortical synaptosomal lysates (5 μ g/lane). Syntaxin-1a was used as a synaptosomal marker. The image is representative of the analysis of $n = 6$ synaptosomal lysates.

a physiological medium having the following composition (mM): NaCl, 140; KCl, 3; MgSO₄, 1.2; CaCl₂, 1.2; NaH₂PO₄, 1.2; NaHCO₃, 5; HEPES, 10; glucose, 10; pH 7.4.

Depolarization and Pharmacological Treatments

The synaptosomal suspension was then divided into identical aliquots and preincubated for 5 min in the physiological medium in a water bath at 37°C under mild shaking to equilibrate the system. Synaptosomal aliquots were then incubated for 2 min with the physiological medium containing 3 mM KCl (3 mM KCl medium, basal condition, control synaptosomes) or with a KCl-enriched solution (25 mM KCl-containing medium, NaCl substituted for an equimolar amount of KCl, depolarized synaptosomes). At $t = 2$ min, cold 3 mM KCl medium was added to the reaction tubes to stop the incubation, and the synaptosomal suspensions were centrifuged at 19,000 g for 10 min to separate the synaptosomal pellets from the supernatants.

When indicated, synaptosomes were incubated for 2 min with a 25 mM KCl medium lacking Ca²⁺ ions (25 mM KCl/Ca²⁺-free

medium) or with a 25 mM KCl medium containing the GABA_B receptor agonist ($\pm$)-baclofen (10 μ M, Tocris Bioscience, Bristol, United Kingdom).

The synaptosomal pellets were quantified for the protein content using Pierce BCA assay (Thermo Fisher Scientific, Waltham, MA, United States) and then lysed in modified RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, protease inhibitors) for Western blot analysis or resuspended in PBS for transmission electron microscopy and dynamic light scattering analyses. The synaptosomal supernatants were centrifuged at 19,000 g for 30 min to remove any synaptosomal debris and collected for the isolation of exosomes.

Isolation of the Exosomes From the Synaptosomal Supernatants

Identical volumes of the synaptosomal supernatants from the different experimental conditions were incubated with the Total Exosome Isolation Reagent (from cell culture media, Invitrogen, Thermo Fisher Scientific) overnight at 4°C and then centrifuged

at 10,000 g for 1 h at 4°C. Pellets corresponding to the exosomal fractions were collected and resuspended in modified RIPA lysis buffer for the Western blot analysis or in PBS for flow cytometry, transmission electron microscopy, and dynamic light scattering analyses.

Western Blot Analysis

The exosomal lysates from the different experimental conditions were dissolved in SDS-PAGE sample buffer, boiled for 5 min at 95°C, subjected to 10% SDS-PAGE, and then blotted onto PVDF membranes (Merck, Darmstadt, Germany). Membranes were blocked for 1 h at room temperature with Tris-buffered saline-Tween (t-TBS: 20 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% Tween 20), containing 5% (w/v) nonfat dried milk, and then incubated overnight at 4°C with the following primary antibodies: rabbit anti-TSG101 (1:500, T5701, Sigma-Aldrich, St. Louis, MO, United States), rabbit anti-flotillin-1 (1:1,000, 18634, Cell Signaling Technology, Danvers, MA, United States), rabbit anti-CD63 (1:1,000, sc-5275, Santa Cruz Biotechnology, Dallas, TX, United States), and rabbit anti-CD9 (1:300, ab92726, Abcam, Cambridge, United Kingdom). After extensive washes in t-TBS, membranes were incubated for 1 h at room temperature with appropriate horseradish peroxidase-linked secondary antibodies (1:10,000, A9044 and A9169, Sigma-Aldrich).

Also the cortical synaptosomal lysates (5 µg/lane) were analyzed with immunoblot analysis, and membranes were probed with the primary antibodies mentioned above (concentrations as previously indicated) and with mouse anti-syntaxin-1a antibody (1:500, GTX18010, GeneTex, Irvine, CA, United States).

In a set of Western blot experiments, an equal amount of the synaptosomal and exosomal lysates (2 µg/lane) were compared for the content of selected proteins by using the primary antibodies mentioned already, at the concentrations indicated, and the following ones: mouse anti-synaptotagmin-1 (1:500, 105 011, Synaptic System, Goettingen, Germany) and rabbit anti-PSD95 (1:1,000, NBP1-40474, Novus Biologicals, Centennial, CO, United States).

Immunoblots were visualized using the enhanced chemiluminescence Western blotting detection system Immobilon Forte Western HRP substrate (Merck). Images were acquired by using an Alliance LD6 image capture system (Uvitec, Cambridge, United Kingdom) and analyzed using UVI-1D software (Uvitec).

Transmission Electron Microscopy

Freshly prepared synaptosomes were washed out in 0.1 M cacodylate buffer and immediately fixed in 0.1 M cacodylate buffer containing 2.5% glutaraldehyde (Electron Microscopy Science, Hatfield, PA, United States) for 1 h at room temperature. The synaptosomes were postfixed in osmium tetroxide for 2 h and 1% uranyl acetate for 1 h. Subsequently, samples were dehydrated through a graded ethanol series and embedded in resin (Poly-Bed; Polysciences, Inc., Warrington, PA, United States) for 24 h at 60°C. Ultrathin sections (50 nm) were cut and stained with 5% uranyl acetate in 50% ethanol.

Electron microscopic analysis on isolated vesicle preparations was performed as follows. The extracellular vesicle preparations were resuspended in 20 µL PBS (pH 7.4) and fixed by adding an equal volume of 2% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). Extracellular vesicles were then adsorbed for 10 min onto formvar-carbon-coated copper grids by floating the grids on 5 µL drops on parafilm. Subsequently, grids with adhered vesicles were rinsed in PBS and negatively stained with 2% uranyl acetate for 5 min at room temperature. Stained grids were embedded in 2.5% methylcellulose for improved preservation and air-dried before examination (Cortese et al., 2020). Electron micrographs were taken using a Hitachi TEM microscope (HT7800 series, Tokyo, Japan) equipped with a Megaview 3 digital camera and Radius software (EMSIS, Germany).

Dynamic Light Scattering and Zeta-Potential

Particle size, polydispersity, and zeta-potential were analyzed using a Zetasizer Nano ZS90 particle sizer at a fixed angle of 90° (Malvern Instruments, Worcestershire, United Kingdom). Vesicle size was evaluated by dynamic light scattering, allowing the analysis of particles within the range of 0.1–10 µm, as previously described (Marimpietri et al., 2013). Briefly, exosomes and synaptosomes were suspended in PBS, and the measure was performed at a constant temperature of 25°C in UV-transparent cuvettes. The translational diffusion coefficient of the solutions was calculated from the time autocorrelation of the scattered light intensity, and the translational diffusion coefficient was extracted from the correlogram using the method of cumulants as applied in proprietary Malvern software. The diameter of the exosomes was obtained from the application of the Stokes-Einstein equation as follows: $d(H) = kT/3\pi\eta D$, where $d(H)$ is the hydrodynamic diameter, k the Boltzmann constant, T the temperature, η the shear viscosity of the solvent, and D the diffusion coefficient of the particles.

Flow Cytometric Analysis

Exosomes were analyzed for the presence of CD9 and TSG101 by flow cytometry after vesicles adsorption onto latex beads as previously reported (Marimpietri et al., 2013). In brief, 1 ml of purified exosomes was incubated with 4 µL of aldehyde/sulfate latex beads with a diameter of 4 µm (Invitrogen, Thermo Fisher Scientific) for 2 h at room temperature; PBS supplemented with 4% fetal bovine serum was then added to each sample, and the incubation was prolonged for 30 min. After washing, exosome-coated beads were incubated for 30 min at 4°C with primary rat anti-mouse CD9 PE-conjugated monoclonal antibody (12-0091-81, Invitrogen, Thermo Fisher Scientific) or with unlabeled mouse anti-TSG101 monoclonal antibody (ab43, Abcam), plus further incubation for 20 min at 4°C with a goat anti-mouse PE-conjugated secondary antibody (PA5-33249, Thermo Fisher Scientific). As negative control, an isotype-matched primary monoclonal antibody was used. All antibodies were used in accordance with the manufacturer's instructions. Samples were then analyzed using a Gallios flow cytometer and Kaluza software (BeckmanCoulter, Brea, CA, United States).

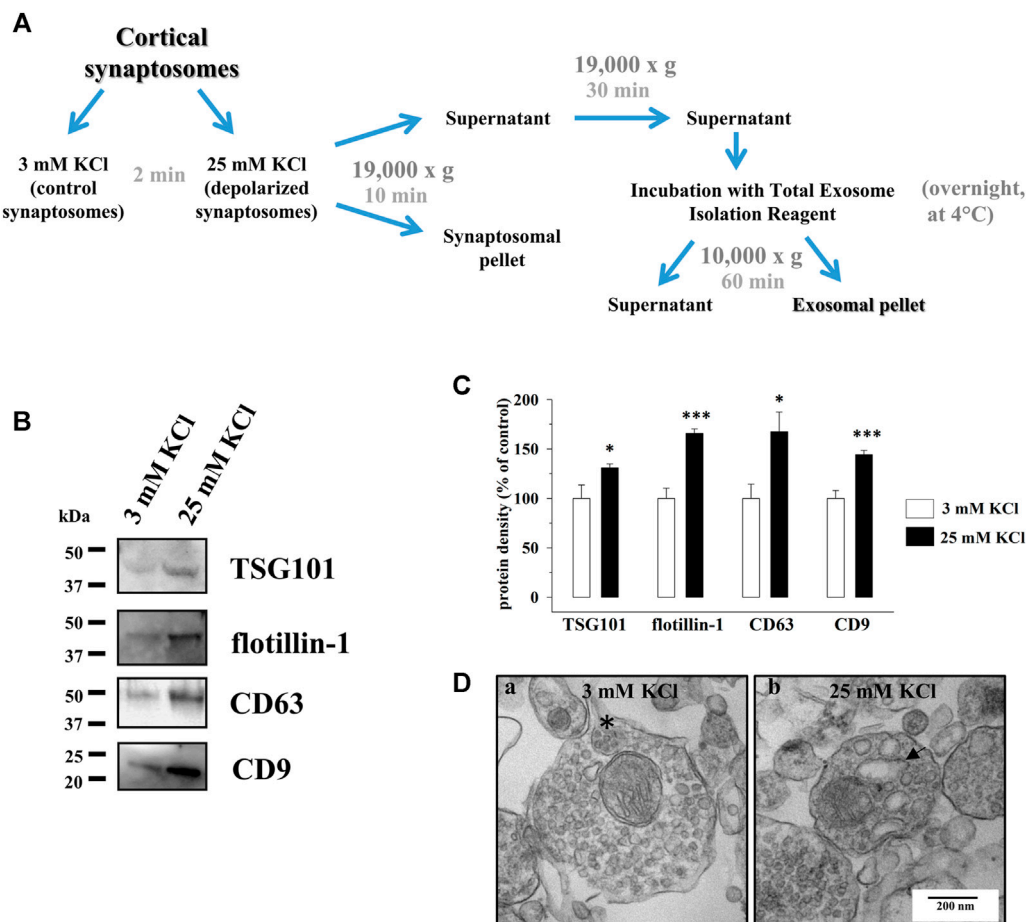


FIGURE 2 | Cortical synaptosomes release extracellular vesicles immunopositive for the exosomal markers TSG101, flotillin-1, CD63, and CD9, and their secretion is increased by depolarization. **(A)** Schematic representation of the isolation procedure of the exosomes from the supernatants of the synaptosomes incubated for 2 min in 3 mM KCl medium (control synaptosomes) or in 25 mM KCl medium (depolarized synaptosomes). For further details, see the Methods section. **(B)** Western blot analysis of TSG101, flotillin-1, CD63, and CD9 proteins in the exosomal fractions isolated from the supernatants of the control (lane 3 mM KCl) and depolarized synaptosomes (lane 25 mM KCl). The image is representative of the results of *n* = 5 (TSG101), *n* = 6 (CD63), and *n* = 11 (flotillin-1 and CD9) Western blot analyses. **(C)** Quantification of the change in TSG101, flotillin-1, CD63, and CD9 proteins in the exosomal fraction from the supernatants of depolarized synaptosomes (25 mM KCl, black bars) when compared to the control synaptosomes (3 mM KCl, empty bars). Results are expressed as percent of control (3 mM KCl). Data represent the mean ± SEM. **p* < 0.05 vs. respective control; ****p* < 0.001 vs. respective control. **(D)** Ultrastructural analysis of the control and depolarized cortical synaptosomes by TEM. The figure shows a representative image of synaptosomes incubated for 2 min in 3 mM KCl medium (a) and in 25 mM KCl medium (b). Asterisk, MVB-like organelle in control preparations; arrow, endosomal-like organelle in depolarized synaptosomes. Scale bar: 200 nm. The image is representative of *n* = 5 different synaptosomal preparations.

Quantification of the Endogenous Glutamate Content

The endogenous glutamate content in the synaptosomal supernatants was analyzed by high-performance liquid chromatography analysis after precolumn derivatization with ophthalaldehyde and separation on a C18 reverse-phase chromatographic column (10 mm × 4.6 mm, 3 μm; at 30°C; Chrompack, Middleburg, Netherlands) coupled with fluorimetric detection (excitation wavelength, 350 nm; emission wavelength, 450 nm). Buffers and the gradient program were previously described (Salamone et al., 2014). Homoserine was used as the internal standard. The amount of glutamate was expressed as pmol/mg of synaptosomal proteins.

LDH Assay

The LDH activity in the synaptosomal supernatants was evaluated by using Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific) according to the manufacturer's manual. The LDH activity in the supernatants was expressed as percent of the total synaptosomal LDH activity (LDH activity in synaptosomal pellets and in the supernatants).

Experimental Design and Statistical Analysis

Data represent the mean ± SEM of *n* independent replications (*n* indicated in the figure legends). Sigma Plot 10 data analysis and the graphing software package was used for data

handling/statistics and for graph drawing. Analysis of variance was performed using ANOVA, followed by Tukey's multiple-comparisons test; direct comparisons were performed using Student's *t*-test. Data were considered significant if $p < 0.05$, at least.

RESULTS

Cortical Synaptosomes Possess Proteins That Are Markers of Exosomes

Purified cortical synaptosomes were analyzed at the ultrastructural level by transmission electron microscopy (TEM). Consistent with previous data in the literature (Feligioni et al., 2006; Nisticò et al., 2015), synaptosomes appeared as round-shaped membrane-bound structures having a diameter in the range of 500–1,200 nm, containing intraterminal mitochondria, small clathrin-coated vesicles, and abundant synaptic vesicles. Some of them were endowed with endosome-like structures, including multivesicular body (MVB)-like organelles, with intraluminal vesicles (Figure 1B but also Figure 2Da).

Cortical synaptosomes were also analyzed by dynamic light scattering, which confirmed the size range observed in the TEM analysis, with a bell-shaped size distribution profile of the synaptosomal particles peaking at 790 ± 67 nm (Figure 1C).

The presence of MVB-like organelles in the purified synaptosomes prompted us to investigate the presence of proteins widely recognized as exosomal markers: the endosomal sorting complex required for transport I tumor susceptibility gene 101 protein (TSG101), the lipid raft-associated protein flotillin-1, and the tetraspanins CD63 and CD9 (Andreu and Yàñez-Mò, 2014; Zhang et al., 2019). The Western blot analysis unveiled a clear immunoreactivity having appropriate mass for all the proteins mentioned above (expected molecular weights: TSG101, 46 kDa; flotillin-1, 49 kDa; CD63, 30–60 kDa depending on the glycosylated forms of the protein; CD9, 25 kDa; Figure 1D).

Cortical Synaptosomes Exposed to a Depolarizing Stimulus Release Extracellular Vesicles Having the Biochemical and Morphological Features of Exosomes

Based on the presence of MVB-like organelles in cortical synaptosomes and on the immunoreactivity for the exosomal markers TSG101, flotillin-1, CD9, and CD63 in the synaptosomal lysates, we asked whether exosomes can also be actively released from axonal terminals.

To test the hypothesis, identical aliquots of cortical synaptosomes were incubated under basal, non-depolarizing condition (3 mM KCl medium, control synaptosomes) or in the presence of a mild depolarizing stimulus (25 mM KCl medium, depolarized synaptosomes). Extracellular vesicles (EVs) that had the dimension and the features of exosomes were then isolated by means of the Total Exosome Isolation Reagent from identical volumes of the

synaptosomal supernatants (Figure 2A, see also the Methods section) and analyzed for the presence of the exosomal markers listed above: TSG101, flotillin-1, CD63, and CD9. The Western blot analysis unveiled that the EV fractions isolated from the supernatants of both the control and depolarized synaptosomes were immunopositive for all the proteins (Figure 2B). A significant increase in the TSG101, flotillin-1, CD63, and CD9 immunoreactivities in the fraction from the depolarized synaptosomes emerged when compared to the control (Figure 2C). Concomitantly, the comparative ultrastructural analysis by TEM of the control and depolarized cortical synaptosomes unveiled that depolarized synaptosomes were almost devoid of MVB-like organelles when compared to the control ones but contained empty structures that Leenders et al. (2002) defined as endosomal-like organelles (Leenders et al., 2002; Figure 2Db).

The EVs isolated from the synaptosomal supernatants were then analyzed by dynamic light scattering for their size and zeta-potential. The size distribution profile of the EVs isolated from the depolarized synaptosomes did not differ significantly from that of the EVs isolated from the control synaptosomes. Both curves showed a bell-shaped profile that reached the peak at 50.78 ± 2.10 nm for the EVs isolated from the supernatants of control synaptosomes and at 52.54 ± 1.85 nm for the EVs isolated from the supernatants of depolarized synaptosomes (Figures 3A,B). The size range for both profiles was consistent with the expected size values for exosomes. Furthermore, the EVs from both control and depolarized synaptosomes showed a comparable zeta-potential (Figure 3A), suggesting a good and similar nanoparticle stability in terms of dispersion, aggregation, or flocculation in both preparations. However, the mean count rate of dynamic light scattering (the parameter that represents the average scattering intensity during the measurement) was almost double (125.41 ± 6.22 kcps) when analyzing the EVs isolated from the supernatants of depolarized synaptosomes when compared to that recorded during the analysis of the EVs from the supernatants of the control synaptosomes (66.79 ± 3.12 kcps, Figure 3A). Since the mean count rate is directly proportional to the concentration of the EVs in the analyzed sample, the data indirectly imply that the EVs isolated from the supernatants of the control and depolarized synaptosomes differ quantitatively but not qualitatively.

The latex bead cytofluorimetric assay of the EVs isolated from the supernatants of the 25 mM KCl-depolarized synaptosomes confirmed good levels of signals for CD9 and TSG101, two of the most typical exosomal surface markers (Figure 3C). Furthermore, TEM analysis demonstrated that the EVs released from the synaptosomes after depolarization have a round shape and a smooth surface, with a diameter ranging from 40 to 70 nm, which is consistent with the exosomal ultrastructure (Figure 3D). Based on these observations, the term “exosomes” will be used from here on out to indicate the released EVs.

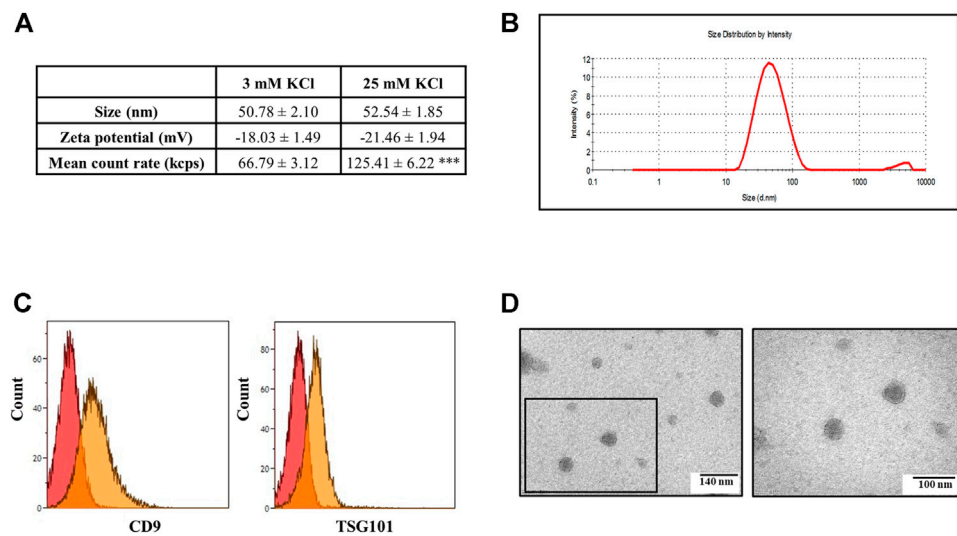


FIGURE 3 | Characterization of the exosomes released from cortical synaptosomes by light dynamic scattering, flow cytometry, and transmission electron microscopy (TEM). **(A)** Size (nm), zeta-potential (mV), and mean count rate (kcps) values detected using a Zetasizer Nano ZS90 particle sizer during the analysis of the exosomes isolated from the supernatants of the control (3 mM KCl) and depolarized synaptosomes (25 mM KCl). Data represent the mean ± SEM of $n = 5$ (control synaptosomes) and $n = 7$ (depolarized synaptosomes) analyses. *** $p < 0.001$ vs. the mean count rate value of 3 mM KCl. **(B)** Size distribution of the exosomes assessed using a Zetasizer Nano ZS90 particle sizer. The curve is representative of the analysis of $n = 8$ exosomal preparations. **(C)** Flow cytometric analysis of TSG101 and CD9 expression in the exosomes purified from the supernatants of depolarized synaptosomes and coated on latex beads. Images are representative of the analysis of $n = 5$ exosomal preparations. **(D)** Negative-stained exosomes isolated from the supernatants of depolarized synaptosomes observed by TEM. The left image shows the round and smooth morphology of isolated exosomes, and the right picture represents an enlargement (squared box) of the original image. The electron micrographs are representative of $n = 5$ exosomal preparations. Scale bar right: 140 nm; scale bar left: 100 nm.

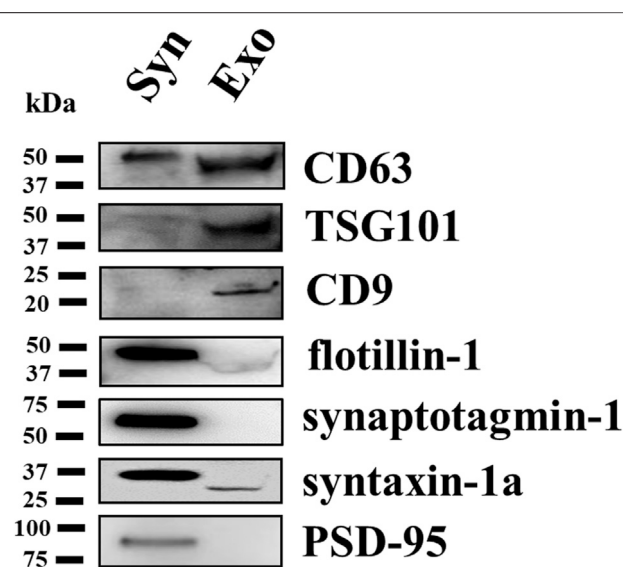


FIGURE 4 | Comparative Western blot analysis of selected proteins in the cortical synaptosomal and exosomal lysates. Identical amounts (2 µg proteins/lane) of the cortical synaptosomal lysate (lane Syn) and of the exosomal lysate (lane Exo) were loaded on 10% SDS-PAGE and analyzed for the contents of the exosomal markers TSG101, CD63, CD9, and flotillin-1, and of the synaptosomal markers synaptotagmin-1, syntaxin-1a, and PSD-95. The image is representative of the Western blot analysis on $n = 5$ synaptosomal and exosomal preparations.

Comparative Analysis of Exosomal and Synaptosomal Markers in Synaptosomal and Exosomal Lysates

Western blot analysis was carried out to compare the content of selected synaptosomal and exosomal markers in the lysates of the exosomes and of the depolarized synaptosomes they are released from. Identical amounts of proteins (2 µg/lane) of both lysates were analyzed for the exosomal markers TSG101, flotillin-1, CD63, and CD9. The exosomal lysates were particularly enriched in the TSG101, CD63, and CD9 proteins when compared to the synaptosomal ones, while flotillin-1 was expressed in synaptosomes and to a lesser extent in the exosomal fraction. We then focused on the following synaptic markers: synaptotagmin-1, a synaptic vesicle membrane protein; syntaxin-1a, which is located in presynaptic plasma membranes; and the postsynaptic density protein 95 (PSD-95), a membrane protein that has a postsynaptic localization (Bonanno et al., 2005; Bonfiglio et al., 2019; Franchini et al., 2019). The analysis unveiled that these proteins are present (although to a different level, PSD-95 showing a scarce immunoreactivity) in the synaptosomal lysates (expected protein weight: synaptotagmin-1, 60 kDa; syntaxin-1a, 36 kDa; PSD-95, 95 kDa) but not in the exosomal lysates where only a slight immunopositivity for syntaxin-1a was observed (**Figure 4**). These observations unveiled a qualitative difference in the protein composition of the synaptosomal and the exosomal preparations.

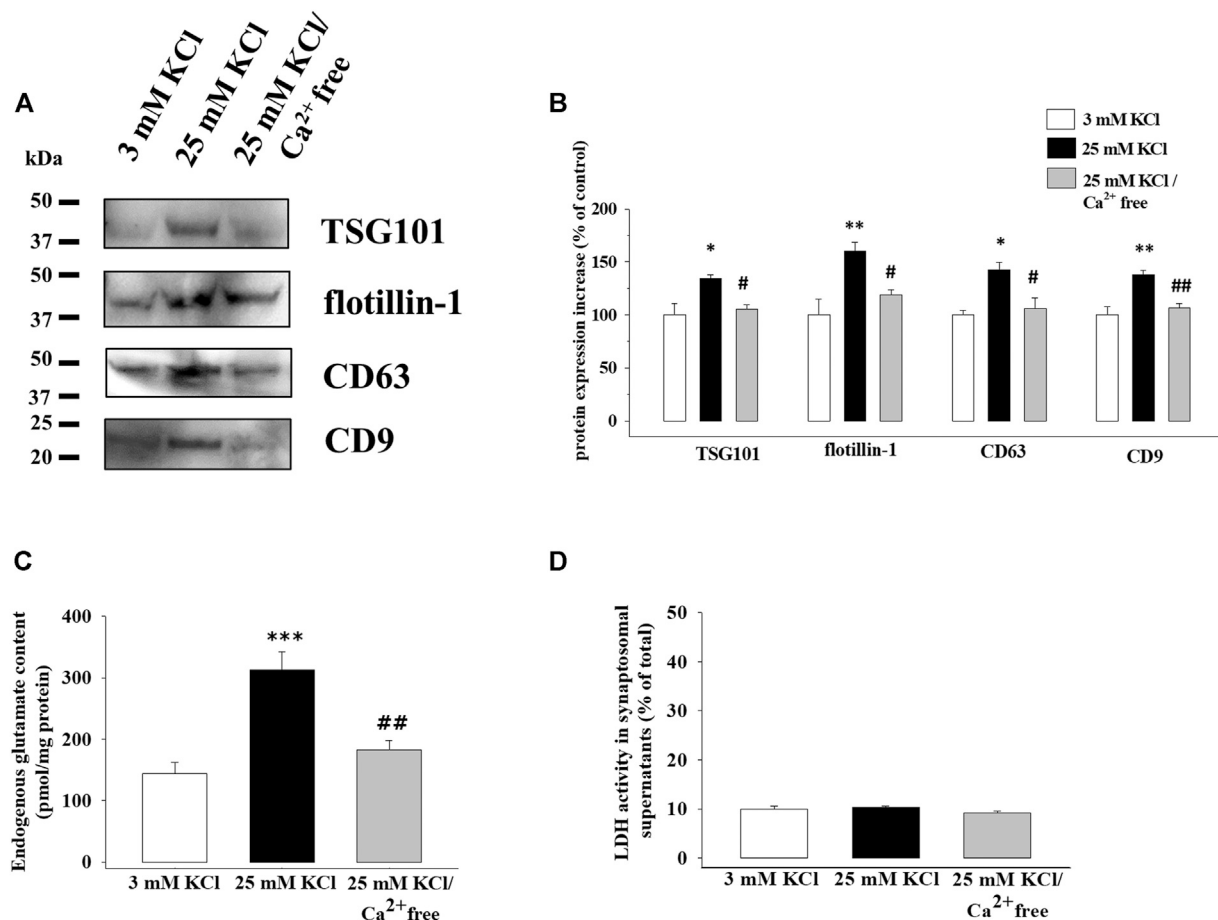


FIGURE 5 | Release of exosomes from cortical synaptosomes is a Ca^{2+} -dependent event. Synaptosomes were incubated for 2 min in basal condition, in a 25 mM KCl medium containing 1.2 mM Ca^{2+} ions or lacking Ca^{2+} ions (Ca^{2+} free medium). **(A)** Western blot experiments were carried out to analyze TSG101, flotillin-1, CD63, and CD9 protein content in the exosomal fractions isolated from the supernatants of the synaptosomes under the three experimental conditions (respectively, lane 3 mM KCl, lane 25 mM KCl, and lane 25 mM KCl/ Ca^{2+} free). The image is representative of the results of $n = 4$ (TSG101), $n = 5$ (CD63), $n = 6$ (CD9), and $n = 8$ (flotillin-1) Western blot analyses. **(B)** Quantification of the change in TSG101, flotillin-1, CD63, and CD9 proteins in the exosomal fraction from the supernatants of the synaptosomes exposed to 25 mM KCl/ Ca^{2+} -free medium (gray bars) when compared to 25 mM KCl/1.2 mM Ca^{2+} medium (25 mM KCl, black bars). Results are expressed as percent of control (3 mM KCl, empty bars). Data represent the mean $\pm$ SEM. * $p < 0.05$ vs. respective control; ** $p < 0.01$ vs. respective control; # $p < 0.05$ vs. 25 mM KCl; ## $p < 0.01$ vs. 25 mM KCl. **(C)** Endogenous glutamate content in the supernatants of the synaptosomes incubated in 3 mM KCl medium (empty bar), 25 mM KCl/1.2 mM Ca^{2+} -containing medium (25 mM KCl, black bar), and 25 mM KCl/ Ca^{2+} -free medium (gray bar). The endogenous glutamate content is expressed as pmol/mg synaptosomal proteins. Data represent the mean $\pm$ SEM of the analysis of $n = 11$ samples. *** $p < 0.001$ vs. 3 mM KCl; ## $p < 0.01$ vs. 25 mM KCl. **(D)** Lactate dehydrogenase (LDH) activity in the supernatants of the synaptosomes incubated in 3 mM KCl medium (empty bar), 25 mM KCl/1.2 mM Ca^{2+} -containing medium (25 mM KCl, black bar), and 25 mM KCl/ Ca^{2+} -free medium (gray bar). The LDH activity in the supernatants is expressed as percent of total LDH activity (LDH activity in the synaptosomal pellets and supernatants). Data represent the mean $\pm$ SEM of the analysis of $n = 5$ samples.

The Release of Exosomes From Cortical Synaptosomes is a Ca^{2+} -Dependent Event

Identical aliquots of cortical synaptosomes were exposed to the 25 mM KCl medium containing a physiological amount of Ca^{2+} ions (1.2 mM Ca^{2+} -containing medium) or to the 25 mM KCl medium lacking the divalent cation (Ca^{2+} -free medium). As shown in the representative Western blot in **Figure 5A**, a significant reduction in TSG101, flotillin-1, CD63, and CD9 protein content was observed in the exosomal fraction isolated from the supernatants of synaptosomes exposed to the 25 mM KCl/ Ca^{2+} -free medium when compared to synaptosomes depolarized with the 25 mM KCl/1.2 mM Ca^{2+} -containing medium (**Figure 5B**).

As expected, the endogenous glutamate content in the supernatants from synaptosomes exposed to the 25 mM KCl/1.2 mM Ca^{2+} -containing medium was significantly increased when compared to that from synaptosomes under basal condition (3 mM KCl), but the omission of calcium ions in the external medium almost totally prevented the releasing activity (**Figure 5C**).

To exclude the possibility that a nonspecific synaptosomal leakage might account for the results described so far, we verified the viability of the synaptosomes under the different experimental conditions by measuring the endogenous lactate dehydrogenase (LDH) activity in the supernatants. The LDH activity was

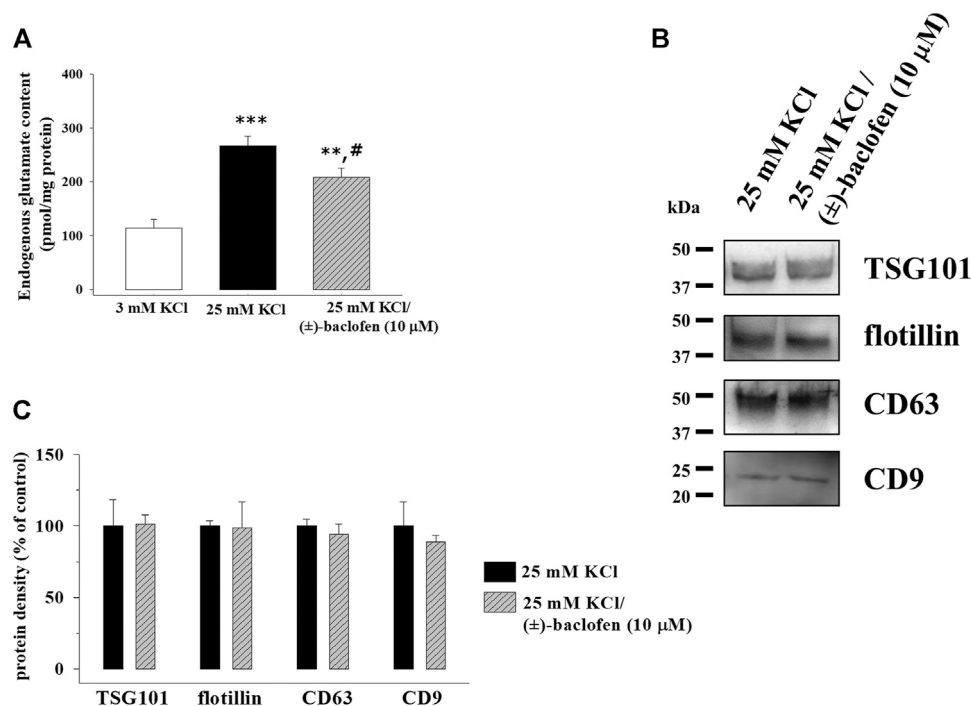


FIGURE 6 | Release of exosomes from cortical synaptosomes is not modified by presynaptic GABA_B receptor activation. Synaptosomes were incubated for 2 min in a 25 mM KCl medium in the absence or in the presence of the GABA_B receptor agonist (±)-baclofen (10 μM). **(A)** Endogenous glutamate content in the supernatants of the synaptosomes incubated in 3 mM KCl medium (empty bar), 25 mM KCl medium (black bar), and 25 mM KCl/(±)-baclofen (10 μM) medium (rising right hatched gray bar). The endogenous glutamate content is expressed as pmol/mg synaptosomal proteins. Data represent the mean ± SEM of the analysis of $n = 12$ samples. ** $p < 0.01$ vs. control; *** $p < 0.001$ vs. control; # $p < 0.05$ vs. 25 mM KCl. **(B)** Western blot experiments were carried out to analyze TSG101, flotillin-1, CD63, and CD9 protein content in the exosomal fractions isolated from the supernatants of synaptosomes stimulated with 25 mM KCl alone (lane 25 mM KCl) or in the presence of (±)-baclofen (10 μM) (lane 25 mM KCl/(±)-baclofen (10 μM)). The image is representative of the results of $n = 4$ (TSG101), $n = 6$ (CD63), $n = 9$ (flotillin-1), and $n = 10$ (CD9) Western blot analyses. **(C)** Quantification of the change in TSG101, flotillin-1, CD63, and CD9 proteins in the exosomal fraction from the supernatants of the synaptosomes exposed to 25 mM/(±)-baclofen (10 μM) medium (rising right hatched gray bars) when compared to 25 mM KCl medium (black bars). Results are expressed as percent of control (25 mM KCl).

unmodified in the supernatants of the control and depolarized synaptosomes, and also the removal of external Ca^{2+} ions from the 25 mM KCl medium did not affect this parameter (Figure 5D).

The Release of Exosomes From Cortical Synaptosomes is not Modulated by Presynaptic GABA_B Heteroreceptors

Glutamate exocytosis from cortical synaptosomes is an active process tuned by auto- and heteroreceptors presynaptically located in nerve terminals. In particular, presynaptic inhibitory GABA_B heteroreceptors exist in cortical glutamatergic nerve endings, whose activation reduces the adenylyl cyclase activity and the Ca^{2+} conductance, inhibiting glutamate exocytosis from the nerve endings (Bonanno and Raiteri, 1993; Grilli et al., 2004; Vergassola et al., 2018; Pittaluga 2019). Accordingly, the glutamate content in the supernatants of synaptosomes exposed to 25 mM KCl medium in the presence of the GABA_B receptor agonist (±)-baclofen (10 μM) was significantly lower than that in the supernatants of synaptosomes exposed to the depolarizing stimulus alone

(Figure 6A). Differently, the amount of TSG101, flotillin-1, CD63, and CD9 in the exosomal fraction isolated from the synaptosomes exposed to the depolarizing stimulus in the presence of (±)-baclofen (10 μM) did not significantly differ from that in the exosomal fraction isolated from synaptosomes exposed to the depolarizing stimulus alone (Figures 6B,C).

DISCUSSION

In recent years, exosomes have emerged as nonconventional messengers in intercellular communication in the CNS, and their role in the modulation of synaptic activity in physiological and pathological conditions has gained interest (Smalheiser, 2007; Saeedi et al., 2019; Schiera et al., 2020).

Although MVBs, the late endosomal organelles from which exosomes originate and are released from, are less present in central axonal terminals than in other neuronal compartments (i.e., somata and dendrites; Von Bartheld and Altick, 2011; Men et al., 2019), some studies supported their presynaptic location and the possibility that they can fuse with nerve terminal plasma membranes (Koles et al., 2012; Korkut et al., 2013; Janas et al.,

2016 and references therein; Zappulli et al., 2016; Zhang and Yang, 2018). Although these findings would support the presence and the release of exosomes from nerve endings, clear evidence demonstrating this conclusion is lacking. With the aim of answering this question, we focused on synaptosomes (Pittaluga, 2019), convinced that this preparation represents an appropriate model to study the presynaptic events. Synaptosomes are pinched-off nerve terminals (see **Figure 1B**). Which retain the main features of the synaptic boutons they originate from and contain structures with a presynaptic origin that account for the main presynaptic functional events (e.g., the uptake, the synthesis, the metabolism, the storage, and the release of transmitters). Synaptosomes are also endowed with naïve proteins and receptors, controlling these presynaptic functions, including the release of neurotransmitters. In a few cases, synaptosomes retain fragments of the postsynaptic membranes that remain attached to the presynaptic side by means of proteins (e.g., PSD95) but that do not reseal and cannot influence the functional synaptosomal responses (Raiteri and Raiteri, 2000).

The first finding of this study is that mouse cortical synaptosomal lysates are immunopositive for endosome-associated proteins, commonly considered as exosomal markers (i.e., TSG101, flotillin-1, CD63, and CD9), consistent with the presence of exosomes in isolated nerve endings. In line with the conclusion, the results from TEM ultrastructural analysis unveiled the presence of MVB-like organelles containing intraluminal vesicles in cortical synaptosomes.

Second, we demonstrated that mouse cortical synaptosomes release EVs with the structural and biochemical features of exosomes in response to a depolarizing stimulus. The conclusion relies on the following main findings:

- 1) the supernatants of cortical synaptosomes exposed to a mild depolarizing stimulus (25 mM KCl medium) were enriched with EVs that, in both dynamic light scattering and TEM analyses, displayed a size and shape consistent with those of exosomes.
- 2) the size and the zeta-potential of the EVs isolated from the supernatants of synaptosomes under both basal and depolarized conditions were largely conserved, but a significant increase in the mean count rate of dynamic light scattering was observed when analyzing EVs from depolarized terminals. We considered that the latter observation might be predictive of an accumulation of EVs at the outer side of synaptosomes upon the application of the depolarizing stimulus.
- 3) the EV fraction isolated from the supernatants of depolarized synaptosomes were more reactive for TSG101, flotillin-1, CD63, and CD9 than the EV fraction collected under the basal condition.
- 4) MVB-like organelles were rarely, if ever, detected in depolarized synaptosomes when compared to control synaptosomes, which might suggest the depletion of these organelles in depolarized nerve terminals because of the increased release of exosomes.

The comparative analysis of synaptosomal and exosomal proteins in identical amounts of synaptosomal and exosomal lysates unveiled a different proteomic profile of the two preparations, stressing that synaptosomes and exosomes are different in nature. Specifically, as far as the exosomal markers are concerned, the exosomal lysates were particularly enriched in the TSG101, CD9, and CD63 proteins. Differently, flotillin-1 was particularly expressed in the synaptosomal lysates and to a lower extent in the exosomal preparation. This is not surprising, since this protein concentrates within the cholesterol-enriched microdomains of the lipid rafts, which are also components of the neuronal plasma membranes, to control the synapsis formation (Swanwick et al., 2010).

Turning to the synaptosomal markers, synaptotagmin-1, a v-SNARE protein located in vesicular membranes, was largely expressed in synaptosomal lysates but absent in the exosomal fraction, which would exclude a contamination of synaptic vesicles in the exosomal preparation. Similarly, the lack of PSD95 immunopositivity, a specific marker for the postsynaptic compartment, in the exosomal preparation excludes the presence of postsynaptic impurities and the possibility that exosomes could originate from the postsynaptic membrane fragments, if present. Differently, syntaxin-1a, a t-SNARE membrane protein mediating vesicular exocytosis, was slightly detected in the exosomal lysates. This would reflect a contamination since, at least in the *Drosophila* neuromuscular junction (Koles et al., 2012), the protein assures the anchoring of MVBs to plasma membranes, participating in the membrane–membrane interaction that drives the docking and the fusion of MVBs and the consequent release of exosomes.

Another interesting observation of the study is that the amount of exosomal markers in the exosomal fraction isolated from the supernatants of depolarized synaptosomes was drastically reduced when exposing the nerve endings to the 25 mM KCl/Ca²⁺-free medium. The finding is in line with previous results already present in the literature (Savina et al., 2003; Krämer-Albers et al., 2007; Lachenal et al., 2011) and supports the conclusion that the release of exosomes is an active event which depends on triggering events linked to the influx of the divalent cation in the nerve terminals.

The influx of calcium ions is pivotal to several processes in nerve terminals, including the exocytosis of transmitters, which largely depends on the opening of the voltage-dependent Ca²⁺ channels (Raiteri and Raiteri, 2000; Nicholls, 2003). We asked whether a possible correlation exists linking the sorting of the exosomes triggered by the high-KCl stimulus to the vesicular exocytosis of transmitter(s) in depolarized synaptosomes. To answer the question, we focused on glutamate, which represents the most diffuse transmitter in the brain. Despite cortical synaptosomes being a heterogeneous population, the glutamatergic ones largely prevail, so the quantification of glutamate exocytosis permits us to monitor the release efficiency in the largest subpopulation of cortical synaptosomes (Grilli et al., 2004). As expected, we detected a significant increase in the glutamate overflow in the supernatants of the depolarized cortical synaptosomes from which the

exosomes were isolated. Interestingly, both the glutamate availability and the exosomal marker content was largely reduced when the external Ca^{2+} ions were omitted to levels comparable to those detected under basal, non-depolarizing, condition.

The huge correlation in terms of Ca^{2+} dependency between glutamate exocytosis and exosome release seems best interpreted by assuming that both events might involve common triggering pathway(s). To address this more directly, we focused on presynaptic receptors, which represent one of the main mechanisms of control of vesicular exocytosis (Raiteri, 2001; Langer, 2008; Pittaluga, 2019) and asked whether release-regulating receptors could also control the sorting of exosomes elicited by high KCl. We focused on presynaptic GABA_B receptors because of their wide distribution in cortical nerve endings (they exist on cortical GABA_{A} ergic, glutamatergic, peptidergic, and dopaminergic terminals; Bonanno and Raiteri, 1993) and their ability to modulate transmitter exocytosis through the inhibition of the voltage-dependent Ca^{2+} channels. The activation with ($\pm$)-baclofen of presynaptic GABA_B heteroreceptors efficiently inhibited the glutamate overflow in cortical synaptosomes, as already reported in the literature (Perkinton and Sihra, 1998). The agonist, however, failed to affect the secretion of exosomes, as suggested by the finding that the immunoreactivities of the exosomal markers isolated from the supernatant of depolarized synaptosomes exposed to ($\pm$)-baclofen were superimposable to those detected in the absence of the agonist. The more likely conclusion is that the depolarization-evoked, Ca^{2+} -dependent sorting of exosomes from isolated nerve endings escapes the presynaptic control of the GABA_B presynaptic receptors. This would imply that Ca^{2+} enters synaptosomes through accesses that are differently sensitive to the GABA_B receptors. Although further investigation is needed to correctly address the question, the hypothesis is supported by data in the literature showing that the N and the P/Q-type Ca^{2+} channels, which assure the influx of the divalent cation in nerve endings, are differently sensitive to the presynaptic inhibitory actions of the GABA_B receptors (Langer, 2008). These data unveil a multiplicity of calcium-dependent events that can occur in synaptosomes and could be differently modulated but could also lead to different functional outcomes.

To conclude, the results described in this study provide evidence supporting the release of exosomes from presynaptic structures. The structural and biochemical analysis confirmed the exosomal nature of the EVs in the synaptosomal supernatants, also unveiling their increased availability upon exposure of synaptosomes to a depolarizing stimulus. The exosomal particles show a proteomic profile distinct from that of the synaptosomes, suggesting that these nanosized vesicles differ in nature from the synaptosomes they are released from. Finally, the release of the exosomes from the synaptosomes is a Ca^{2+} -dependent event, which possibly depends on the firing of the nerve terminals. Interestingly, the exosomal sorting was not controlled by presynaptic GABA_B receptors, suggesting that the vesicular transmitter release and the exosome outflow are independent mechanisms which develop concomitantly at nerve endings. The finding, however, would not exclude the

existence of presynaptic mechanism(s) of control of the exosome sorting that would represent rather attractive target(s) for therapeutic purposes.

Exosomes have emerged in recent years as a cargo of proteins/lipids/genetic material rapidly transferred to other cells with an efficiency that would strictly depend on the kinetics of release and diffusion in the biophase. They were proposed to carry components that could be either deleterious to the course of neurological diseases (e.g., Alzheimer's disease, Goetzl et al., 2018; Parkinson's disease, McCormack et al., 2019; and multiple sclerosis, Sáenz-Cuesta et al., 2014) or beneficial because of their anti-inflammatory/immunomodulatory properties (Bahrini et al., 2015; Cui et al., 2018). In both cases, the possibility to pharmacologically tune the efficiency of their release and diffusion (and as a consequence, of the materials they vehiculate) by means of ligands acting at release-regulating receptors would give new therapeutic opportunities to manage the course of central disorders. Therefore, despite the disappointing results obtained with ($\pm$)-baclofen, we believe that the study of the mechanism of sorting of exosomes from nerve endings improves our knowledge on the mechanism of synaptic transmission, paving the road to new therapeutic treatments.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Local Committee for Animal Care and Welfare of the University of Genova and the Italian Ministry of Health (DDL 26/2014 and previous legislation; protocol number nÂ° 75F11.N.IMY).

AUTHOR CONTRIBUTIONS

GO designed the experiments, performed the animal experiments, the isolation of the exosomes, Western blot, HPLC and LDH analyses, and statistical analysis, and wrote the manuscript. AP designed the experiments, supervised the execution of research activity, and wrote the manuscript. FC helped with Western blot analysis. DM and MP performed dynamic light scattering and flow cytometry analysis. DD performed dynamic light scattering analysis. KC and MG performed transmission electron microscopy analysis. All authors revised the manuscript and approved its final version.

ACKNOWLEDGMENTS

This work was previously published as a preprint on Authorea, Olivero et al. (2020).

REFERENCES

- Andreu, Z., and Yáñez-Mó, M. (2014). Tetraspanins in Extracellular Vesicle Formation and Function. *Front. Immunol.* 5, 1–12. doi:10.3389/fimmu.2014.00442
- Bahrini, I., Song, J.-h., Diez, D., and Hanayama, R. (2015). Neuronal Exosomes Facilitate Synaptic Pruning by Up-Regulating Complement Factors in Microglia. *Sci. Rep.* 5 (5), 7989. doi:10.1038/srep07989
- Bonanno, G., Giambelli, R., Raiteri, L., Tiraboschi, E., Zappettini, S., Musazzi, L., et al. (2005). Chronic Antidepressants Reduce Depolarization-Evoked Glutamate Release and Protein Interactions Favoring Formation of SNARE Complex in hippocampus. *J. Neurosci.* 25, 3270–3279. doi:10.1523/JNEUROSCI.5033-04.2005
- Bonanno, G., and Raiteri, M. (1993). Multiple GABAB Receptors. *Trends Pharmacol. Sci.* 14 (7), 259–261. doi:10.1016/0165-6147(93)90124-3
- Bonfiglio, T., Olivero, G., Vergassola, M., Di Cesare Mannelli, L., Pacini, A., Iannuzzi, F., et al. (2019). Environmental Training Is Beneficial to Clinical Symptoms and Cortical Presynaptic Defects in Mice Suffering from Experimental Autoimmune Encephalomyelitis. *Neuropharmacology* 145, 75–86. doi:10.1016/j.neuropharm.2018.01.026
- Caruso Bavisotto, C., Scalia, F., Marino Gammazza, A., Carlisi, D., Bucchieri, F., Conway de Macario, E., et al. (2019). Extracellular Vesicle-Mediated Cell-Cell Communication in the Nervous System: Focus on Neurological Diseases. *Ijms* 20, 434–456. doi:10.3390/ijms20020434
- Chivet, M., Hemming, F., Pernet-Gallay, K., Fraboulet, S., and Sadoul, R. (2012). Emerging Role of Neuronal Exosomes in the central Nervous System. *Front. Physiol.* 3, 1–6. doi:10.3389/fphys.2012.00145MAY
- Cortese, K., Marconi, S., Aiello, C., Gagliani, M. C., Pilato, S., Zappacosta, R., et al. (2020). Liposomes Loaded with the Proteasome Inhibitor Z-Leucinyl-Leucinylnorleucinal Are Effective in Inducing Apoptosis in Colorectal Cancer Cell Lines. *Membranes* 10, 91–11. doi:10.3390/membranes10050091
- Cui, G. H., Wu, J., Mou, F. F., Xie, W. H., Wang, F. B., Wang, Q. L., et al. (2018). Exosomes Derived from Hypoxia-Preconditioned Mesenchymal Stromal Cells Ameliorate Cognitive Decline by Rescuing Synaptic Dysfunction and Regulating Inflammatory Responses in APP/PS1 Mice. *FASEB J.* 32 (2), 654–668. doi:10.1096/fj.201700600R
- Dunkley, P. R., Jarvie, P. E., and Robinson, P. J. (2008). A Rapid Percoll Gradient Procedure for Preparation of Synaptosomes. *Nat. Protoc.* 3 (11), 1718–1728. doi:10.1038/nprot.2008.171
- Fauré, J., Lachenal, G., Court, M., Hirrlinger, J., Chatellard-Causse, C., Blot, B., et al. (2006). Exosomes Are Released by Cultured Cortical Neurons. *Mol. Cell. Neurosci.* 31, 642–648. doi:10.1016/j.mcn.2005.12.003
- Felicioni, M., Holman, D., Haglerod, C., Davanger, S., and Henley, J. M. (2006). Ultrastructural Localisation and Differential Agonist-Induced Regulation of AMPA and Kainate Receptors Present at the Presynaptic Active Zone and Postsynaptic Density. *J. Neurochem.* 99, 549–560. doi:10.1111/j.1471-4159.2006.04087.x
- Franchini, L., Stanic, J., Ponzoni, L., Mellone, M., Carrano, N., Musardo, S., et al. (2019). Linking NMDA Receptor Synaptic Retention to Synaptic Plasticity and Cognition. *iScience* 19, 927–939. doi:10.1016/j.isci.2019.08.036
- Frühbeis, C., Fröhlich, D., Kuo, W. P., Amphornrat, J., Thilemann, S., Saab, A. S., et al. (2013). Neurotransmitter-Triggered Transfer of Exosomes Mediates Oligodendrocyte-Neuron Communication. *Plos Biol.* 11, e1001604. doi:10.1371/journal.pbio.1001604
- Goetzl, E. J., Abner, E. L., Jicha, G. A., Kapogiannis, D., and Schwartz, J. B. (2018). Declining Levels of Functionally Specialized Synaptic Proteins in Plasma Neuronal Exosomes with Progression of Alzheimer's Disease. *FASEB J.* 32 (2), 888–893. doi:10.1096/fj.201700731R
- Gray, E. G., and Whittaker, V. P. (1962). The Isolation of Nerve Endings from Brain: an Electron-Microscopic Study of Cell Fragments Derived by Homogenization and Centrifugation. *J. Anat.* 96, 79–88.
- Grilli, M., Raiteri, L., and Pittaluga, A. (2004). Somatostatin Inhibits Glutamate Release from Mouse Cerebrocortical Nerve Endings through Presynaptic Sst2 Receptors Linked to the Adenyl Cyclase-Protein Kinase A Pathway. *Neuropharmacology* 46, 388–396. doi:10.1016/j.neuropharm.2003.09.012
- Holm, M. M., Kaiser, J., and Schwab, M. E. (2018). Extracellular Vesicles: Multimodal Envoys in Neural Maintenance and Repair. *Trends Neurosci.* 41, 360–372. doi:10.1016/j.tins.2018.03.006
- Janas, A. M., Sapoń, K., Janas, T., Stowell, M. H. B., and Janas, T. (2016). Exosomes and Other Extracellular Vesicles in Neural Cells and Neurodegenerative Diseases. *Biochim. Biophys. Acta (Bba) - Biomembranes* 1858, 1139–1151. doi:10.1016/j.bbmem.2016.02.011
- Koles, K., Nunnari, J., Korkut, C., Barria, R., Brewer, C., Li, Y., et al. (2012). Mechanism of Evenness Interrupted (Evi)-Exosome Release at Synaptic Boutons. *J. Biol. Chem.* 287, 16820–16834. doi:10.1074/jbc.M112.342667
- Korkut, C., Ataman, B., Ramachandran, P., Ashley, J., Barria, R., Gherbesi, N., et al. (2009). Trans-Synaptic Transmission of Vesicular Wnt Signals through Evi/Wntless. *Cell* 139, 393–404. doi:10.1016/j.cell.2009.07.051
- Korkut, C., Li, Y., Koles, K., Brewer, C., Ashley, J., Yoshihara, M., Budnik, V., et al. (2013). Regulation of Postsynaptic Retrograde Signaling by Presynaptic Exosome Release. *Neuron* 77 (6), 1039–1046. doi:10.1016/j.neuron.2013.01.013
- Krämer-Albers, E. M., Bretz, N., Tenzer, S., Winterstein, C., Möbius, W., Berger, H., et al. (2007). Oligodendrocytes Secrete Exosomes Containing Major Myelin and Stress-Protective Proteins: Trophic Support for Axons? *Proteomics Clin. Appl.* 1, 1446–1461. doi:10.1002/prca.200700522
- Lachenal, G., Pernet-Gallay, K., Chivet, M., Hemming, F. J., Belly, A., Bodon, G., et al. (2011). Release of Exosomes from Differentiated Neurons and its Regulation by Synaptic Glutamatergic Activity. *Mol. Cell. Neurosci.* 46, 409–418. doi:10.1016/j.mcn.2010.11.004
- Langer, S. Z. (2008). Presynaptic Autoreceptors Regulating Transmitter Release. *Neurochem. Int.* 52, 26–30. doi:10.1016/j.neuint.2007.04.031
- Leenders, A. G., Scholten, G., de Lange, R. P., Lopes da Silva, F. H., and Ghijsen, W. E. (2002). Sequential Changes in Synaptic Vesicle Pools and Endosome-like Organelles during Depolarization Near the Active Zone of central Nerve Terminals. *Neuroscience* 109 (1), 195–206. doi:10.1016/s0306-4522(01)00450-xPMID:11784710
- Marimpietri, D., Petretto, A., Raffaghello, L., Pezzolo, A., Gagliani, C., Tacchetti, C., et al. (2013). Proteome Profiling of Neuroblastoma-Derived Exosomes Reveal the Expression of Proteins Potentially Involved in Tumor Progression. *PLoS One* 8, e75054. doi:10.1371/journal.pone.0075054
- Mathieu, M., Martin-Jaulat, L., Lavieu, G., and Théry, C. (2019). Specificities of Secretion and Uptake of Exosomes and Other Extracellular Vesicles for Cell-To-Cell Communication. *Nat. Cell Biol.* 21, 9–17. doi:10.1038/s41556-018-0250-9
- McCormack, A., Keating, D. J., Chegeni, N., Colella, A., Wang, J. J., and Chataway, T. (2019). Abundance of Synaptic Vesicle-Related Proteins in Alpha-Synuclein-Containing Protein Inclusions Suggests a Targeted Formation Mechanism. *Neurotox. Res.* 35 (4), 883–897. doi:10.1007/s12640-019-00014-0
- Men, Y., Yelick, J., Jin, S., Tian, Y., Chiang, M. S. R., Higashimori, H., et al. (2019). Exosome Reporter Mice Reveal the Involvement of Exosomes in Mediating Neuron to Astroglia Communication in the CNS. *Nat. Commun.* 10, 4136. doi:10.1038/s41467-019-11534-w
- Nicholls, D. G. (2003). Bioenergetics and Transmitter Release in the Isolated Nerve Terminal. *Neurochem. Res.* 28 (10), 1433–1441. doi:10.1023/a:1025653805029
- Nisticò, R., Florenzano, F., Mango, D., Ferraina, C., Grilli, M., Di Prisco, S., et al. (2015). Presynaptic c-Jun N-Terminal Kinase 2 Regulates NMDA Receptor-Dependent Glutamate Release. *Sci. Rep.* 5, 1–14. doi:10.1038/srep09035
- Olivero, G., Grilli, M., Vergassola, M., Bonfiglio, T., Padolecchia, C., Garrone, B., et al. (2018). 5-HT_{2A}-mGlu_{2/3} Receptor Complex in Rat Spinal Cord Glutamatergic Nerve Endings: A 5-HT_{2A} to mGlu_{2/3} Signalling to Amplify Presynaptic Mechanism of Auto-Control of Glutamate Exocytosis. *Neuropharmacology* 133, 429–439. doi:10.1016/j.neuropharm.2018.02.030
- Olivero, G., Cisani, F., Marimpietri, D., Di Paolo, D., Gagliani, M. C., Podestà, M., et al. (2020). The Depolarization-Evoked, Ca²⁺-dependent Release of Exosomes from Mouse Cortical Nerve Endings: New Insights into Synaptic Transmission. *Authorea*. doi:10.22541/au.159164649.94507195
- Paolicelli, R. C., Bergamini, G., and Rajendran, L. (2019). Cell-to-cell Communication by Extracellular Vesicles: Focus on Microglia. *Neuroscience* 405, 148–157. doi:10.1016/j.neuroscience.2018.04.003
- Perkinton, M. S., and Sihra, T. S. (1998). Presynaptic GABAB Receptor Modulation of Glutamate Exocytosis from Rat Cerebrocortical Nerve Terminals: Receptor Decoupling by Protein Kinase C. *J. Neurochem.* 70 (4), 1513–1522. doi:10.1046/j.1471-4159.1998.70041513.x
- Pittaluga, A. (2019). Acute Functional Adaptations in Isolated Presynaptic Terminals Unveil Synaptosomal Learning and Memory. *Ijms* 20, 3641–3656. doi:10.3390/ijms20153641

- Raiteri, L., and Raiteri, M. (2000). Synaptosomes Still Viable after 25 Years of Superfusion. *Neurochem. Res.* 25, 1265–1274. doi:10.1023/A:1007648229795
- Raiteri, M. (2001). Presynaptic Autoreceptors. *J. Neurochem.* 78, 673–675. doi:10.1046/j.1471-4159.2001.00485.x
- Raiteri, M. (2008). “Presynaptic Metabotropic Glutamate and GABAB Receptors,” in *Handb Exp Pharmacol.* Editors T. C. Südhof and K. Starke, 184, 373–407. doi:10.1007/978-3-540-74805-2_12
- Raposo, G., and Stoorvogel, W. (2013). Extracellular Vesicles: Exosomes, Microvesicles, and Friends. *J. Cell Biol.* 200, 373–383. doi:10.1083/jcb.201211138
- Saeedi, S., Israel, S., Nagy, C., and Turecki, G. (2019). The Emerging Role of Exosomes in Mental Disorders. *Transl Psychiatry* 9, 122. doi:10.1038/s41398-019-0459-9
- Salamone, A., Mura, E., Zappettini, S., Grilli, M., Olivero, G., Preda, S., et al. (2014). Inhibitory Effects of Beta-Amyloid on the Nicotinic Receptors Which Stimulate Glutamate Release in Rat hippocampus: The Glial Contribution. *Eur. J. Pharmacol.* 723, 314–321. doi:10.1016/j.ejphar.2013.11.011
- Savina, A., Furlán, M., Vidal, M., and Colombo, M. I. (2003). Exosome Release Is Regulated by a Calcium-dependent Mechanism in K562 Cells. *J. Biol. Chem.* 278, 20083–20090. doi:10.1074/jbc.M301642200
- Sáenz-Cuesta, M. a., Osorio-Querejeta, I. a., and Otaegui, D. (2014). Extracellular Vesicles in Multiple Sclerosis: What Are They Telling Us? *Front. Cel. Neurosci.* 8 (8), 100. doi:10.3389/fncel.2014.00100
- Schiera, G., Di Liegro, C. M., and Di Liegro, I. (2020). Cell-to-Cell Communication in Learning and Memory: From Neuro- and Glia-Transmission to Information Exchange Mediated by Extracellular Vesicles. *Ijms* 21, 266. doi:10.3390/ijms21010266
- Smalheiser, N. R. (2007). Exosomal Transfer of Proteins and RNAs at Synapses in the Nervous System. *Biol. Direct* 2, 35–15. doi:10.1186/1745-6150-2-35
- Swanwick, C. C., Shapiro, M. E., Vicini, S., and Wenthold, R. J. (2010). Flotillin-1 Promotes Formation of Glutamatergic Synapses in Hippocampal Neurons. *Dev. Neurobiol.* 70, 875–883. doi:10.1002/dneu.20828
- Vergassola, M., Olivero, G., Cisani, F., Usai, C., Bossi, S., Puliti, A., et al. (2018). Presynaptic mGlu1 Receptors Control GABAB Receptors in an Antagonist-like Manner in Mouse Cortical GABAergic and Glutamatergic Nerve Endings. *Front. Mol. Neurosci.* 11, 324–335. doi:10.3389/fnmol.2018.00324
- Verkhratsky, A., Matteoli, M., Parpura, V., Mothet, J. P., and Zorec, R. (2016). Astrocytes as Secretory Cells of the central Nervous System: Idiosyncrasies of Vesicular Secretion. *EMBO J.* 35, 239–257. doi:10.15252/embj.201592705
- Von Bartheld, C. S., and Altick, A. L. (2011). Multivesicular Bodies in Neurons: Distribution, Protein Content, and Trafficking Functions. *Prog. Neurobiol.* 93, 313–340. doi:10.1016/j.pneurobio.2011.01.003
- Wang, Y., Balaji, V., Kaniyappan, S., Krüger, L., Irsen, S., Tepper, K., et al. (2017). The Release and Trans-synaptic Transmission of Tau via Exosomes. *Mol. Neurodegeneration* 12, 1–25. doi:10.1186/s13024-016-0143-y
- Xu, J., Chen, Q., Zen, K., Zhang, C., and Zhang, Q. (2013). Synaptosomes Secrete and Uptake Functionally Active microRNAs via Exocytosis and Endocytosis Pathways. *J. Neurochem.* 124, 15–25. doi:10.1111/jnc.12057
- Zappulli, V., Friis, K. P., Fitzpatrick, Z., Maguire, C. A., and Breakefield, X. O. (2016). Extracellular Vesicles and Intercellular Communication within the Nervous System. *J. Clin. Invest.* 126, 1198–1207. doi:10.1172/JCI81134
- Zhang, G., and Yang, P. (2018). A Novel Cell-Cell Communication Mechanism in the Nervous System: Exosomes. *J. Neuro Res.* 96, 45–52. doi:10.1002/jnr.24113
- Zhang, Y., Liu, Y., Liu, H., and Tang, W. H. (2019). Exosomes: Biogenesis, Biologic Function and Clinical Potential. *Cell Biosci* 9, 1–18. doi:10.1186/s13578-019-0282-2

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Olivero, Cisani, Marimpietri, Di Paolo, Gagliani, Podestà, Cortese and Pittaluga. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Blood Nanoparticles – Influence on Extracellular Vesicle Isolation and Characterization

Marija Holcar*, Maša Kandušer and Metka Lenassi

Institute of Biochemistry and Molecular Genetics, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

OPEN ACCESS

Edited by:

Suman Dutta,
University of California, Los Angeles,
United States

Reviewed by:

Anand Kumar Rai,
University of California, Los Angeles,
United States
Simon Hornung,
Technical University of Munich,
Germany

Ghulam Hassan Dar,
Government of Jammu & Kashmir,
India

*Correspondence:

Marija Holcar
marija.holcar@mf.uni-lj.si

Specialty section:

This article was submitted to
Experimental Pharmacology and Drug
Discovery,
a section of the journal
Frontiers in Pharmacology

Received: 10 September 2021

Accepted: 25 October 2021

Published: 10 November 2021

Citation:

Holcar M, Kandušer M and Lenassi M
(2021) Blood Nanoparticles – Influence
on Extracellular Vesicle Isolation
and Characterization.
Front. Pharmacol. 12:773844.
doi: 10.3389/fphar.2021.773844

Blood is a rich source of disease biomarkers, which include extracellular vesicles (EVs). EVs are nanometer-to micrometer-sized spherical particles that are enclosed by a phospholipid bilayer and are secreted by most cell types. EVs reflect the physiological cell of origin in terms of their molecular composition and biophysical characteristics, and they accumulate in blood even when released from remote organs or tissues, while protecting their cargo from degradation. The molecular components (e.g., proteins, miRNAs) and biophysical characteristics (e.g., size, concentration) of blood EVs have been studied as biomarkers of cancers and neurodegenerative, autoimmune, and cardiovascular diseases. However, most biomarker studies do not address the problem of contaminants in EV isolates from blood plasma, and how these might affect downstream EV analysis. Indeed, nonphysiological EVs, protein aggregates, lipoproteins and viruses share many molecular and/or biophysical characteristics with EVs, and can therefore co-isolate with EVs from blood plasma. Consequently, isolation and downstream analysis of EVs from blood plasma remain a unique challenge, with important impacts on the outcomes of biomarker studies. To help improve rigor, reproducibility, and reliability of EV biomarker studies, we describe here the major contaminants of EV isolates from blood plasma, and we report on how different EV isolation methods affect their levels, and how contaminants that remain can affect the interpretation of downstream EV analysis.

Keywords: extracellular vesicles, blood nanoparticles, contaminants, isolation methods, characterization methods

INTRODUCTION

Biological fluids are an ideal source for early disease discovery and for monitoring of disease progression or the success of a treatment (Alix-Panabières and Pantel, 2017; Bracht et al., 2018; Morrison and Goldkorn, 2018). The most widely used and studied source of disease biomarkers is peripheral circulating venous blood, as its collection is minimally invasive and can be performed repeatedly (González and Falcón-Pérez, 2015). Blood has an important role in the transmission of information between cells and tissues, and it can accumulate diverse signals as a result of disease or distress, in the form of nucleic acids, proteins, metabolites, and extracellular vesicles (EVs). Blood will thus reflect the health of the organs and tissues.

Extracellular vesicles are nanometer-to micrometer-sized spherical particles (diameter, 40–1,000 nm; density, 1.110–1.190 g/cm³) that are enclosed by a phospholipid bilayer; they can be secreted by most cell types, and they cannot replicate (Mathieu et al., 2019). EVs are very heterogeneous in size, biophysical characteristics, molecular content, function, biogenesis, and release pathways (Zaborowski et al., 2015; Zhang Q. et al., 2019; Dong et al., 2020). Based on their

sizes and cellular origins, EVs released by living cells can be split into two major groups: exosomes (diameter, 50–150 nm), which originate from multivesicular bodies, and microvesicles (diameter, $\leq 1 \mu\text{m}$), which bud from the plasma membrane. However, the cellular origins of EVs are difficult to ascertain using simple methods, such as measurements of size, and even more so *in vivo*; e.g., for EVs in body fluids after they have already been released into the systemic circulation (Karasu et al., 2018). Therefore, the Minimal Information for the Study of EVs (MISEV 2018) guidelines have proposed the use of descriptive terms for EV subtypes that refer to their physical characteristics, such as size (diameters: <100 nm, small EVs; 100–200 nm, medium/large EVs; >200 nm, large EVs) or density (low, medium, high densities, with each range defined) (Théry et al., 2019). Importantly, the composition and the physiological state of the cell of origin is reflected by the molecular cargo of EVs, as these can contain functional nucleic acids (e.g., mRNAs, miRNAs, DNA), proteins (e.g., cytoskeletal proteins, tetraspanins, integrins), and specific enrichment of molecules typical of lipid rafts (e.g., ceramide, cholesterol, phosphatidylserine) (Ghosh et al., 2014; Doyle and Wang, 2019; Jeppesen et al., 2019).

Extracellular vesicles used to be regarded as merely a disposal mechanism used by cells, or even as “cellular dust”. However, they can also cross extracellular space and even biological barriers, and they can activate specific pathways or directly transfer biological contents upon binding to their target cells, which indicates that they can influence the physiology of recipient cells and tissues. EVs are important mediators of adaptations to micro-environmental changes, and they participate in intercellular communication and immune responses (Clotilde et al., 2009; Karasu et al., 2018; Shao et al., 2018). Therefore, EVs represent a promising source of novel diagnostic and prognostic biomarkers, particularly in terms of their traceability to their cell type or tissue origin and its physiological state, their stability, their protection of their internal cargo from degradation in the extracellular environment, and their presence in circulating blood at estimated concentrations of 10^{10} EV/ml (Dickhout and Koenen, 2018; Johnsen et al., 2019; Vasconcelos et al., 2019; Holcar et al., 2020; Hoshino et al., 2020; Kamal and Shahidan, 2020; Laurenzana et al., 2021). Even more importantly, in the future, should they be modified to encapsulate different drugs, they can potentially be used as new therapeutic options for a nonimmunogenic delivery system with high target specificity upon systemic administration (Dang et al., 2020).

Molecular components of blood EVs have generally focused on proteins and miRNAs, and these have already been studied in the context of many diseases, as they might represent easily detectable and disease-specific biomarkers. The presence and concentrations of specific EV-associated proteins have been analyzed in many different diseases. Particular examples can be seen for cancers: the correlation of baseline EV-related PD-L1 with tumor response to treatment for melanoma; dysregulation of tumor-associated antigens (including BAGE, PD-L1, MAGE-3, AKAP4) in EVs of patients with nonsmall-cell lung cancer; higher expression of LZH8, HER2

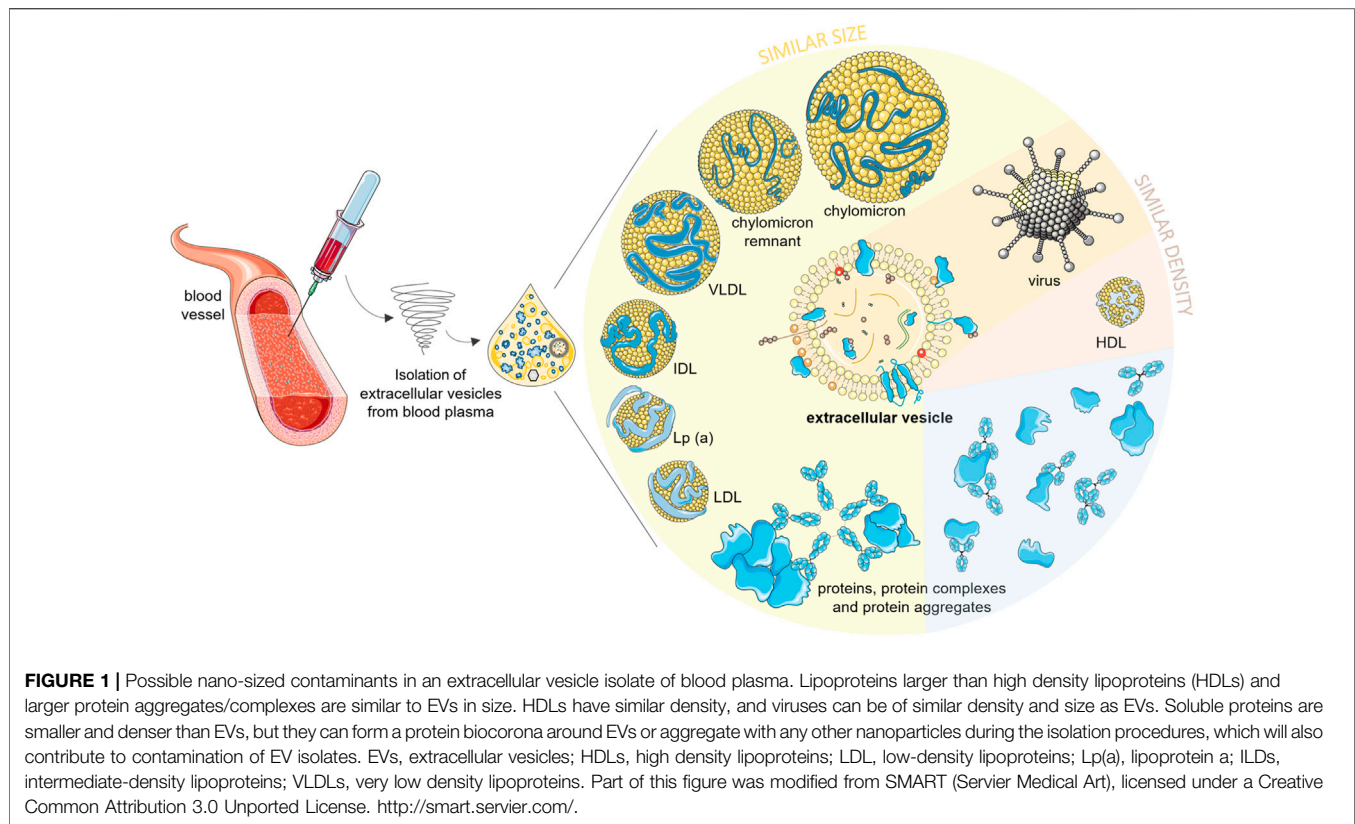
and PSA in EVs from lymphoma patients; and even separation of tumor-derived EVs from those derived from healthy tissue according to their contents of VCAN, TNC, and THBS2 (Liu et al., 2019; Wu et al., 2019; Cordonnier et al., 2020; Hoshino et al., 2020). In neurodegenerative diseases, the amyloid- β and tau proteins have been detected in brain-secreted EVs from blood from patients with Alzheimer’s disease, and similarly for the α -synuclein protein in patients with Parkinson’s disease (Badhwar and Haqqani, 2020; Yu et al., 2020). In the autoimmune disease rheumatoid arthritis, patients with more IgM rheumatoid-factor-positive EVs have higher disease activity (Arntz et al., 2018). Higher levels of CD31 and annexin A5 have been reported for EVs of patients who later develop major adverse cardiovascular and cerebral events, which also indicates the involvement of EVs in cardiovascular diseases (Johnsen et al., 2019).

As well as such changes in their molecular composition in blood, the EV concentrations and size profile might be linked to physiological factors like hypoxia, autophagy or stress, and hence be typical for a diverse set of diseases (Vasconcelos et al., 2019). Indeed, increases in plasma EV levels have been reported in connection with several cancers (Navarro et al., 2019; Badovinac et al., 2021), and also for cardiovascular and autoimmune diseases (Dickhout and Koenen, 2018; Maione et al., 2020).

Despite great interest in EVs as biomarkers of pathologies, it remains challenging to entirely separate EVs from other blood nanoparticles, such as proteins and lipoproteins, and potentially viruses, due to their overlapping characteristics (**Figure 1; Table 1**). Consequently, isolation and downstream analysis of EVs isolated from blood plasma remain a unique challenge (Bracht et al., 2018; Freitas et al., 2019; Geeurickx and Hendrix, 2020; Nieuwland et al., 2020), with important impact on biomarker study outcomes. To help to improve rigor, reproducibility, and reliability of EV biomarker studies, we describe here the major contaminants of EV isolates from blood plasma, and we report on how different EV isolation methods can affect their levels, and how remaining contaminants can affect the interpretation of downstream EV analysis.

CONTAMINANTS OF EXTRACELLULAR VESICLE ISOLATES FROM BLOOD PLASMA AND THEIR RELEVANCE FOR EXTRACELLULAR VESICLE ISOLATION AND CHARACTERIZATION

Blood is the most commonly used body fluid for liquid biopsies – a term that refers to the assessment of biomarkers in biological fluids, as a minimally invasive alternative to tissue biopsies. EVs are emerging as promising new biomarkers in liquid biopsies; however, blood plasma is a very complex biofluid (Lötvall et al., 2014; Lu et al., 2019; Notarangelo et al., 2019; Ruhen and Meehan, 2019; Vasconcelos et al., 2019; Pang et al., 2020). The abundance of different types of plasma nanoparticles thus leads to challenges for EV isolation and characterization.



In the context of diseases, the main plasma nanoparticles of interest are EVs, which can accumulate in the blood after their release from specific pathological tissues, while the blood itself also contains a variety of cells that release EVs. It has been shown that up to 30% of blood EVs are erythrocyte-derived EVs, and up to 20% are leucocyte-derived EVs (Berckmans et al., 2019). Platelets are an anuclear component of blood, and these require special attention as **platelet-derived EVs** are the most abundant EVs in human blood, as they represent 50–90% of all circulating large EVs in healthy subjects (Table 1) (Berckmans et al., 2019; Taus et al., 2019). Their elevated concentrations in blood are linked to vascular diseases and even to some types of cancers, and thus these can carry important clinical information (González and Falcón-Pérez, 2015). On the other hand, due to the important role of platelets in the process of thrombosis, these are particularly susceptible to activation during blood collection and handling. This can lead to abundant *ex-vivo* platelet vesiculation, especially if there is a delay between sample collection and processing (Brahmer et al., 2019; Taus et al., 2019; Tripisciano et al., 2020; Antich-Rosselló et al., 2021; Puhm et al., 2021). This uncontrolled release of nonphysiological EVs can adversely affect downstream EV analysis; e.g., it has been shown that contaminating platelet-derived EVs can skew the isolated miRNAs populations in patient and control samples (Palviainen et al., 2020). Further important pre-analytical factors that can affect isolation and characterization of EVs include size of the needle used to draw the blood, correct handling of blood samples, and prompt and complete

separation of plasma from uncoagulated blood cells and platelets (i.e., to limit their activation). The purity of EV preparations can be evaluated by flow cytometric quantification of CD41⁺, CD42⁺ or CD62P + nanoparticles, which are characteristic of platelet-derived EVs (Pugholm et al., 2016; Berckmans et al., 2019; Brahmer et al., 2019).

Proteins and protein aggregates are the most common nonEV contaminants of EV preparations from blood, and these can considerably impact the downstream analyses (Table 1) (Yuana et al., 2014; Sódar et al., 2016; Takov et al., 2019; Théry et al., 2019). Blood plasma contains approximately 60 mg/ml to 80 mg/ml protein, with wide ranges of concentrations of different proteins (i.e., pg/mL to mg/mL) and a vast heterogeneity of their glycosylation profiles, as up to 50% of plasma proteins are glycosylated (Anderson and Anderson, 2002; Shamsi et al., 2012). About 50–60% of all plasma proteins are albumins, and 40% are globulins, of which 10–20% are immunoglobulins G (Leeman et al., 2018). Coagulation factors are the next most abundant proteins in the blood (e.g., fibrinogen, 4%), followed by lipoproteins (1%) and iron-binding/transferring proteins (1%), with <1% of the total circulating proteins represented by different hormones, lysosomal proteins, proteins released from dead or damaged cells, proteins related to diseases or infection (e.g., cytokines, components of the complement), and also biotherapeutic proteins used as drugs (Karimi et al., 2018; Geyer et al., 2019; Pietrowska et al., 2019). These proteins can all contribute to the formation of protein aggregates, which can have similar biophysical properties as EVs, such as size, charge,

TABLE 1 | Characteristics of blood plasma nanoparticles.

Blood-plasma nanoparticle	Subgroup	Diameter (nm)	Density (g/cm ³)	Concentration in blood plasma (particles/mL)	Detection in extracellular-vesicle isolates	References
Extracellular vesicles (EVs)	All plasma EVs	40–1,000	1.08–1.21 (1.110–1.190)	10 ⁸ –10 ¹³ (on average, 10 ¹⁰)	Presence/concentration of EV-derived proteins (e.g., tetraspanins CD9, CD63, CD81, TSG101, Flotillin1, others)	Simonsen, (2017); Berckmans et al. (2019); Johnsen et al. (2019); Mathieu et al. (2019); Tian et al. (2020)
	Platelet-derived EVs			10 ⁸ –10 ¹⁰	Presence/concentration of platelets in plasma prior to EV isolation; Presence/concentration of CD41, CD42a, CD61, CD62p EVs in EV isolate	
Lipoproteins	High density	5–12	1.063–1.210	10 ^{16a}	Presence/concentration of ApoA1	Nakajima et al. (2001); Wojcynski et al. (2011); Sabaka et al. (2013); Tsimikas et al. (2018); Tian et al. (2020)
	Low density	18–25	1.019–1.063	10 ^{15a}	Presence/concentration of ApoB100	
	Intermediate density	25–35	1.006–1.019	10 ^{12a,b}	Presence/concentration of ApoB100	
	Lipoprotein (a)	12–500	1.048–1.086	10 ^{12b}	Presence/concentration of ApoB100 and ApoA1	
	Very low density	30–80	0.930–1.006	10 ^{12a,b}	Presence/concentration of ApoB100	
	Chylomicrons	75–1,200	<0.930	10 ^{13a,c}	Presence/concentration of ApoB48	
	Chylomicron remnants	30–80	0.950–1.006	10 ¹² –10 ^{13a,c,d}	Presence/concentration of ApoB48	
Protein aggregates	-	<1–15,000	~1.4 (dense packing)	10 ^{17b} of albumin 10 ^{16b} of globulins	Protein concentration (absorbance at 280 nm, bicinchoninic acid/Bradford assay)	Buis et al. (1996); Stanyon and Viles, (2012); Simonsen, (2017)
Viruses	-	30–300	1.16–1.18 (most retroviruses)	Depends on the infection status	Presence of viral genome (DNA/RNA extraction and quantification)	Nolte-'t Hoen et al. (2016), Raab-Traub and Dittmer, (2017)

^aNumbers can change significantly with prandial status and diet composition.

^bNumbers of particles calculated from reported mass concentrations in plasma.

^cMostly present post-prandially.

^dNumbers of particles calculated from reported mass concentrations of their specific protein (ApoB48) in plasma.

and buoyant density, and which can thus co-isolate and contaminate EV preparations (Sódar et al., 2016; Simonsen, 2017; Johnsen et al., 2019). The concentration of cell-free nucleic acids can also increase in correlation to different diseases such as cancers, autoimmune diseases, and inflammatory reactions (Endzelinš et al., 2017; Zhang L. et al., 2019; Duvvuri and Lood, 2019). These cell-free nucleic acids and the proteins bound to them can also form insoluble aggregates in the blood (Duvvuri and Lood, 2019).

Recent studies on EVs and previous studies on nanoparticles of nonbiological origin have suggested that the same sets of proteins can bind physiologically to the surface of EVs in body fluids to form the protein corona, which thus coats the EVs (Palviainen et al., 2019; Priyanka et al., 2020). The composition of the protein corona depends on the composition of the biofluid and its protein concentration, on the fluid conditions (i.e., static vs. flowing), and on the temperature and nanoparticle properties (Nguyen and Lee, 2017). In blood, the EV protein corona typically consists of immunoglobulins, complement proteins, coagulation factors,

cytokines, enzymes, DNA, and RNAs (Cvjetkovic et al., 2016; Buzás et al., 2018). When blood proteins are part of the protein corona, they can have notable effects on EVs, including their mobility, interactions with their surroundings or target surfaces, and recognition by the immune system, which will affect the physiological role of the EVs (Strojan et al., 2017; Buzás et al., 2018; Charoenviriyakul et al., 2018; Skliar et al., 2018; Ezzat et al., 2019). It is therefore important to efficiently remove unbound blood proteins from EV isolates, while still appreciating the physiological roles of blood proteins that are bound to EVs as the protein corona. The purity of EV preparations can also be evaluated by determination of the nanoparticle-to-protein ratio or the nanoparticle-to-lipid ratio (Théry et al., 2019).

Another common contaminant of blood EV isolates are the **lipoproteins** (Table 1). These are spherical particles that transport the major lipids in the bloodstream of humans throughout the body. They consist of an amphipathic surface of protein(s), free cholesterol, and phospholipids, which surround a hydrophobic core that contains cholesterol esters and triacylglycerols (FERENCE et al., 2020). Depending on the lipid

and protein contents, these circulating lipoproteins are separated into high density lipoproteins (HDLs), low density lipoproteins (LDLs), intermediate density lipoproteins (IDLs), very low density lipoproteins (VLDLs), lipoprotein a (Lp(a)), chylomicrons, and chylomicron remnants (**Table 1**). Lipoproteins can be identified based on their sizes, chemical compositions, physicochemical and flotation characteristics, and electrophoretic mobilities (Nakajima et al., 2001; Feingold and Grunfeld, 2010; Pasquetto et al., 2016; Karimi et al., 2018). Importantly, lipoproteins share either similar sizes or densities with EVs (**Figure 1**; **Table 1**) (Karimi et al., 2018; Johnsen et al., 2019). In blood, these lipoproteins are at concentrations far higher than those of EVs (Johnsen et al., 2019). They also fluctuate intra- and inter-individually, and are significantly affected by prandial status (Varga et al., 2014; Simonsen, 2017). Additionally, HDLs and LDLs have both been shown to transport miRNAs, which can co-isolate with EV-associated RNAs (Vickers et al., 2011; Mateescu et al., 2017). Also, LDLs added to pure EV preparations can associate with the EV surface *in vitro*, and lipoprotein–EV interactions appear to even have roles in the pathogenesis of atherothrombosis, which highlights the likelihood of lipoprotein co-isolation with EVs (Sódar et al., 2016; Chiva-Blanch and Badimon, 2019). It is thus important to take special care to remove lipoproteins from EVs in samples before downstream analysis, and to sufficiently test for lipoprotein contamination in EV isolates. The purity of EV preparations can be evaluated by determining the apolipoprotein concentrations (ApoA1 for HDLs; ApoB, ApoE for other lipoproteins) in samples by enzyme-linked immunosorbent assays (ELISA) or Western blotting (Dong et al., 2020; Matsumoto et al., 2020; Zhang et al., 2020; Ji et al., 2021).

In the context of infectious diseases, infectious agents like **viruses** can also co-isolate with blood EVs (**Table 1**). Viral particles share similar biophysical properties with EVs, such as size, molecular composition, and physical features (Martin-Jaular et al., 2021). For example, enveloped viruses can share several biogenesis pathways with EVs, such as seen for Human immunodeficiency virus and Hepatitis C virus. This results in secretion of diverse types of nanoparticles from infected cells, such as naked virions, EVs containing infective viral genomes and quasi-enveloped viruses, classical complete viral particles, and also EVs modified by the infection and EVs that are not altered by the infection (Nolte-‘t Hoen et al., 2016; Sódar et al., 2016; Ramirez et al., 2018; Martins and Alves, 2020; Martin-Jaular et al., 2021). Even in cases of nonenveloped viruses (e.g., Hepatitis A virus), EVs can provide an ‘envelope’ for the viruses and thus propagate infection to other cells (Nolte-‘t Hoen et al., 2016). Consequently, the separation of host EVs from any virions is extremely challenging, and at present this is limited to affinity-based purification strategies (Jung et al., 2020; Martin-Jaular et al., 2021). It is important to note that isolation of EVs from biological samples of patients with viral infections can also present safety risks for laboratory operators, which was indeed highlighted by the recent COVID-19 pandemic that was caused by Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-

CoV-2 infection primarily affects the upper respiratory tract and lungs, but the virus can also be detected in blood (Nunez Lopez et al., 2021). Even though additional treatments of clinical samples prior to EV isolation from blood can have a profound impact on their isolation and related contaminants, inactivation of SARS-CoV-2 by heat or solvent–detergent treatments is recommended (Jureka et al., 2020; Frigerio et al., 2021).

Finally here, bacterial EVs have also been found and quantified in human plasma samples (Tulkens et al., 2020). Collected, non-sterile blood samples can also be contaminated with fungi, which release fungal EVs that can co-purify with blood EVs (Sódar et al., 2017). It is, therefore, important to evaluate the possible biological contaminants of all body fluid samples also from the safety perspective, to eliminate or minimize the potential risk to laboratory personnel during the handling of samples. The presence of microbial contaminants in blood and EV isolates can be evaluated by checking for their genetic material using PCR.

To conclude, blood contains diverse nanoparticles besides EVs, which include proteins, lipoproteins and viruses. As these can be similar to EVs in terms of certain characteristics, they can be co-isolated with EVs from blood. It is thus important to select method(s) for isolation of EVs from blood that are compatible with the planned downstream analysis, and to determine the levels of contaminants in EV preparations. This is also highly encouraged by the recent guidelines for minimal information for studies on EVs, as supported by the International Society for Extracellular Vesicles (Théry et al., 2019). However, to improve the reliability and reproducibility of EV biomarker studies there remain important challenges to be overcome in terms of how preanalytical variables can affect the levels of nonEV nanoparticles in blood samples, and how these nonEV nanoparticles might affect EV isolation and downstream analysis.

THE SELECTION OF ISOLATION METHOD AFFECTS THE PURITY OF EXTRACELLULAR VESICLE ISOLATION FROM BLOOD

Extracellular vesicles can be isolated from biological fluids using diverse methods according to their sizes, densities, charges, or specific markers, such as size-exclusion chromatography (SEC), ultrafiltration, asymmetric flow field-flow fractionation (AF4), ultracentrifugation (over density gradients), precipitation, and immunoaffinity approaches (Witwer et al., 2013; Ramirez et al., 2018; Gandham et al., 2020). For in-depth descriptions of various isolation methods, please see previous publications (Monguió-Tortajada et al., 2019; Coccozza et al., 2020; Sidhom et al., 2020). The selection of the EV isolation method also influences the co-isolation of other blood nanoparticles with similar properties. Therefore, the choice of the method used is crucial, and should be made with the downstream analyses in mind. **Table 2** provides a summary of the characteristics of the commonly used methods for isolation of EVs from human plasma.

TABLE 2 | Characteristics of the most commonly used methods for isolation of extracellular vesicles (EVs) from human plasma.

Characteristic	Size exclusion chromatography	Ultrafiltration	Differential ultracentrifugation	Density gradient ultracentrifugation	Precipitation	Immunoaffinity purification	Asymmetric flow field-flow fractionations	Microfluidics	References
Plasma volume (ml)	≤0.5	≥3.0	≥3.0	0.5–3.0	0.5–3.0	≤0.5 ml	≤0.5 ml	≤0.5 ml	Dong et al. (2020); Veerman et al. (2021)
Time (h)	1.5–2.0	0.5	3.0–4.0	16.0–90.0	2.0–16.0	4.0–overnight (without coupling of beads)	<1.0	<1.0	Coumans et al. (2017); Doyle and Wang, (2019); Monguió-Tortajada et al. (2019); Patel et al. (2019); Zhang and Lyden, (2019)
Cost	High	Medium	Low	Low	Low	High	Medium	Very high	Doyle and Wang, (2019); Sidhom et al. (2020)
Principle of isolation	Difference in hydrodynamic size and shape between particles; particles flow through or around the stationary phase; larger particles elute first	Difference in hydrodynamic size between particles; particles larger than cut-off size of membrane retained in concentrate	Difference in sedimentation coefficient (hydrodynamic size and density) of particles; particles with different sedimentation coefficients pellet at different centrifugation speeds	Difference in sedimentation and flotation coefficient of particles; during centrifugation particles distribute through density gradient matrix according to sedimentation and flotation index	Difference in charge/solubility between particles, leading to precipitation	Interaction between specific protein and antibody; antibodies often coupled to magnetic beads and bound particles separated using magnetic separation	Difference in hydrodynamic size of particles, leading to differential transport velocity of particles in laminar flow profile according to particle position above the semipermeable membrane	Separation on microchip; combination of different approaches	Zhang and Lyden, (2019); Gandham et al. (2020)
Nanoparticles efficiently removed	Soluble proteins, high density lipoproteins	Soluble proteins	None	Larger lipoproteins, protein aggregates (depends on gradient)	None	All particles except protein of interest	Soluble proteins; also lipoproteins (depends on protocol)	Depends on isolation method(s) used	Bo et al. (2014); Vergauwen et al. (2017); Wu et al. (2020)
Major contaminants in EV isolates ^a	Lipoproteins of similar size to EVs	Lipoproteins of similar size or larger than EVs, larger protein aggregates	Protein aggregates, aggregates of proteins and cell-free nucleic acids, lipoproteins	High density lipoproteins (depends on gradient and protocol)	Lipoproteins, soluble proteins, complexes of proteins and cell-free nucleic acids	Nonspecific binding of abundant plasma proteins	Lipoproteins of similar size to EVs (efficiency depends on programmable cross-flow intensity)	Depends on isolation method(s) used	Gandham et al. (2020); Holcar et al. (2020); Tian et al. (2020)
Optimization of critical steps	Use fasting plasma; optimize eluted fractions	Membrane type (regenerated cellulose, pore size	Number of ultracentrifugation steps, acceleration,	Top versus bottom loading, gradient	N.A.	Elution of sample from antibodies	Optimize cross-flow velocity and channel thickness	N.A.	Sitar et al. (2015); Mørk et al. (2016);

(Continued on following page)

TABLE 2 | (Continued) Characteristics of the most commonly used methods for isolation of extracellular vesicles (EVs) from human plasma.

Characteristic	Size exclusion chromatography	Ultrafiltration	Differential ultracentrifugation	Density gradient ultracentrifugation	Precipitation	Immunoaffinity purification	Asymmetric flow field-flow fractionations	Microfluidics	References
	concentrated into final sample	10 kDa best for centrifugal ultracentrifugation)	rotor, solution viscosity, duration	medium, gradient used					Vergauwen et al. (2017); Sidhom et al. (2020)
Frequency of use as primary EV isolation method^{b,c}	Used exclusively, it is the third most often used method; frequency of use is increasing; can be used as a first step in combined protocols, followed by other methods	Not used on its own	Used exclusively, it is the most often used method; frequently used in combination with ultrafiltration and precipitation	Usually used in combination with other methods	Used exclusively, it is the second most often used method; frequently used with differential ultracentrifugation	Used commonly, if starting plasma volume <1.0 ml	Low, use is increasing in last years	Low, use is increasing in last years	Gardiner et al. (2016); Royo et al. (2020)
Frequency of use as additional clean-up/purification method^b	Third most often used method	Frequently used to concentrate samples (e.g., after size-exclusion chromatography, before density gradient ultracentrifugation)	The most commonly used method (ultracentrifuge wash)	Second most often used method	Often follows differential ultracentrifugation	D.N.R.	Can be used for further separation of pre-isolated EV samples, usually coupled to different detectors	D.N.R.	Sitar et al. (2015), Sitar et al. (2017); Gardiner et al. (2016); Holcar et al. (2020); Royo et al. (2020); Tian et al. (2020)
Concentration of isolated particles (particles/mL plasma)	1.4×10^{11d} – 6.5×10^{11e}	1.5×10^{12e} – 3.2×10^{12d}	4.9×10^{8d} – 1.5×10^{12e}	1×10^{10e}	1.4×10^{11} – $5.5 \times 10^{12d,e}$	1×10^{8e}	1.1×10^{1f}	N.A.	Sitar et al. (2015); Askeland et al. (2020); Dong et al. (2020); Tian et al. (2020); Wei et al. (2020); Veerman et al. (2021)
Recovery efficiency (%)	100 ^e ; 65 ^d	80–84 ^e ; 37 ^d	5.0–22 ^e ; 40 (for single step); ~16 (for twice at 1,000,00× g) ^d	30 (using iodixanol gradient) ^e	56 ^e	>90 ^e	5-fold that of differential ultracentrifugation ^{a,h} ; 90 ^j	82 ^j	Kol et al. (2010); Lobb et al. (2015); Busatto et al. (2018); Chen et al. (2019); Geurickx et al. (2019); Dong et al. (2020) Marioli and Kok, (2020); Pang et al. (2020)

(Continued on following page)

TABLE 2 | (Continued) Characteristics of the most commonly used methods for isolation of extracellular vesicles (EVs) from human plasma.

Characteristic	Size exclusion chromatography	Ultrafiltration	Differential ultracentrifugation	Density gradient ultracentrifugation	Precipitation	Immunoaffinity purification	Asymmetric flow field-flow fractionations	Microfluidics	References
Purity of EV isolate (%)	28	11	78	Similar to differential ultracentrifugation	5–19	Greater than differential ultracentrifugation	D.N.R.	N.A.	Lobb et al. (2015); Tian et al. (2020)
Functionality of isolated EVs	Good	Medium	Medium	Good	Medium	Poor	D.N.R.	Depends on isolation method(s) used	Sidhom et al. (2020)
Typical markers analyzed in samples	EV protein and miRNA cargoes	Depends on combined isolation method	Used for every marker	Morphology of EVs and EV protein cargoes	EV RNA cargoes	EV protein and miRNA cargoes	Depends on coupled detector(s)	Depends on isolation method(s) used	Coumans et al. (2017); Chen et al. (2020); Gaspar et al. (2020); Pang et al. (2020); Sidhom et al. (2020)
Prevalent end-point method/ application	Flow cytometry; functional studies	<i>In-vivo</i> and <i>in-vitro</i> functional studies	<i>In-vivo</i> and <i>in-vitro</i> functional studies	Flow cytometry; proteomics; basic science of EV heterogeneity and biology; translational studies	RNA analysis	Flow cytometry and proteomics	D.N.R.	Depends on isolation method(s) used	Nordin et al. (2015) Gardiner et al. (2016) Pang et al. (2020)
Analysis methods affected by major contaminants^k	Methods for label-free quantification of EVs; RNA analysis	Depends on combined isolation method	Methods for label-free quantification of EVs; proteomics	N.A.	Methods for label-free quantification of EVs; electron microscopy; DNA/RNA analysis; mass spectrometry	Methods for label-free quantification of EVs; atomic force microscopy; functional studies ^l	Methods for label-free quantification of EVs	Depends on isolation method(s) used	Nordin et al. (2015); Guerreiro et al. (2018); Doyle and Wang, (2019)

^aExcept for immunoaffinity precipitation, if viral particles are present, they will remain as a contaminant after all of the isolation protocols (Jung et al., 2020; Martins and Alves, 2020).

^bFrequency of use for each method when starting material is plasma is estimated based on two worldwide studies conducted in 2016 and 2020 by ISEV; for exact frequency of use combining all types of starting materials, see Gardiner et al. (2016) and Royo et al. (2020).

^cBased on EV-TRACK data (search parameters: biofluid: blood plasma, species: Homo sapiens) at <http://evtrack.org>, collected to 14 October 2021.

^dMeasured by nano-flow cytometry.

^eMeasured by nanoparticle tracking analysis.

^fMeasured by multi-angle light scattering detection.

^gEVs, isolated from cultured media, not blood plasma.

^hMeasured by tunable resistive pulse sensing.

ⁱMeasured and calculated from analyzing polystyrene latex nanoparticles in phosphate-buffered saline.

^jEVs, isolated directly from full blood.

^kSpecial care should be taken when analyzing samples without further EV, sample processing.

^lFunctional EV, studies require EV, samples to be devoid of antibody contaminants.

N.A., not applicable; D.N.R., data not reported.

Extracellular Vesicle Isolation Based on Particle Size

Size-Exclusion Chromatography

As for ultrafiltration (see below), SEC is used to isolate EVs based on their sizes. In SEC, the particles move through the fixed stationary phase (beads with pores with specific diameters) with the fluid flow, either under gravity or under a small differential pressure (Taylor and Shah, 2015). Any particle that is small enough to enter the bead pores is delayed due to its increased path length. Instead, particles that are larger than the pore diameter cannot enter the beads, and thus travel along with the void volume of the fluid (Bo et al., 2014). Isolation of EVs from different body fluids by SEC usually leads to EV preparations that are free from significant protein contamination, where the EVs retain their structures and physiological functions, accompanied by very high vesicle yields (Baranyai et al., 2015; Gámez-Valero et al., 2016; Geeurickx et al., 2019). However, low levels of albumin contamination of such EV isolates have been reported (Baranyai et al., 2015; An et al., 2018). While SEC is mostly successful in removing HDLs, other lipoproteins of similar sizes to EVs (e.g., LDLs, IDLs, VLDLs, Lp(a), chylomicrons) can be co-isolated, which is especially problematic for blood with high lipoprotein concentrations (Sódar et al., 2016; Karimi et al., 2018; Takov et al., 2019; Holcar et al., 2020). In our hands, in EV-enriched samples compared to plasma, SEC leads to a 100-fold reduction in ApoA1 concentrations (HDL marker) and a 50-fold reduction in ApoB100 concentrations (LDL, IDL, VLDL, Lp(a) marker) (Holcar et al., 2020). Such EV preparations are especially problematic when used for size and concentration determinations, as most techniques do not differentiate between the different nanoparticle types, such as EVs and lipoproteins. Care should also be taken when analyzing EV-bound miRNAs, as lipoproteins are known to transport RNA molecules, and also when performing functional assays, as lipoproteins can have biological effects (Vickers et al., 2011; Allen et al., 2018; Chiva-Blanch and Badimon, 2019; Freitas et al., 2019).

Ultrafiltration

In ultrafiltration, the use of membrane filters provides enrichment of EVs depending on their size in relation to the membrane pore size. In addition to centrifugal ultrafiltration, tangential flow filtration can also be used, where a sample does not flow through the membrane, but moves in a stream across the ultrafiltration membrane, in 'tangential flow' (Liangsupree et al., 2021). The isolation process of ultrafiltration can be influenced by the selection of filters with different pore sizes (e.g., 0.8, 0.45, 0.22, 0.1 μm) or of different materials (e.g., regenerated cellulose, stabilized cellulose, polyethersulfone, cellulose triacetate, anodic aluminum oxide, track-etched polycarbonate) (Vergauwen et al., 2017; Liangsupree et al., 2021). As several blood nanoparticles are similar in size to EVs, protein aggregates and lipoproteins are typically abundantly present in EV samples isolated from blood plasma by ultrafiltration (Ramirez et al., 2018). When isolating EVs from such complex biofluids, ultrafiltration is mainly used to concentrate down large volumes of sample in conjunction with other EV isolation methods, such as SEC or AF4 (Muller et al., 2014; Vergauwen

et al., 2017; Dong et al., 2020). The chosen combination of methods affects the yields and characteristics of the EV isolates.

Unspecific binding of EVs to membranes used for ultrafiltration can also dramatically affect the yield of EV isolates. In a comparison of five different commonly used centrifugal filter types for their efficiency in concentrating recombinant GFP-labeled EVs spiked into phosphate-buffered saline, Vergauwen et al. (2017) investigated the membrane material (regenerated cellulose, Hydrosart, polyethersulfone, cellulose triacetate) and the pore size (10, 100 kDa). They concluded that regenerated cellulose membranes with a pore size of 10 kDa provided more than 100% recovery efficiency. Further, less than 40% recovery was achieved with the other filters, possibly due to binding of EVs to the membranes (Vergauwen et al., 2017).

As pressure or centrifugal force is commonly used to speed up ultrafiltration methods, EVs can also be deformed, or their size profiles can be shifted to smaller sizes. Finally, removal of all cells and cell remnants from blood plasma prior to ultrafiltration is necessary, as these can be similarly deformed into smaller particles when pushed through filter membranes, which can then be indistinguishable from physiological EVs.

Asymmetric Flow Field-Flow Fractionation

Particles can also be separated based on their diffusion coefficient using AF4, which has recently gained popularity for EV isolation (Sitar et al., 2015, 2017; Contado, 2017; Leeman et al., 2018; Oeyen et al., 2018; Zhang et al., 2018; Zhang and Lyden, 2019; Holcar et al., 2020; Wu et al., 2020). High-resolution separation (from a few nanometers up to micrometers) is achieved in a channel within a parabolic flow profile, against which a perpendicular cross-flow is applied. The particles are driven by the cross-flow toward the accumulation wall at the bottom of the channel, while also diffusing back into the channel because of the counteracting Brownian motion. Small particles with high diffusion coefficients float closer to the channel center and are displaced by the faster flow of the parabolic stream, whereas larger particles with smaller diffusion coefficients remain closer to the accumulation wall and are displaced by the slower flow. Thus, the particles are fractioned from smaller to larger sizes (Sitar et al., 2015; Contado, 2017). Importantly, due to the absence of a stationary phase, the separation in the channel is gentle and the surface area available for unwanted interactions is limited, which helps to preserve particle structure and avoid particle aggregation and loss (Wagner et al., 2014). However, blood nanoparticles of similar sizes to EVs can co-isolate, as seen for other methods of separation based on size. If AF4 is connected to a UV detector, protein aggregates and lipoprotein contaminants can be detected at 280 nm (Scheffer et al., 1997).

Extracellular Vesicle Isolation Based on Particle Sedimentation Rate

Ultracentrifugation

Ultracentrifugation is the most commonly used method for isolating EVs (Furi et al., 2017). It involves centrifugation at high centrifugal forces ($\geq 1,000,00\times g$) to separate particles

depending on their sizes, shapes, and flotation densities. EV yields are dependent on the centrifugal force, rotor type (i.e., fixed angle vs. swinging bucket), pelleting efficiency (i.e., rotor and tube k-factors), and sample viscosity (Taylor and Shah, 2015). For example, different rotor pelleting efficiencies can result in different EV yields when isolating EVs from the same fluid, even when centrifuged for the same length of time (Witwer et al., 2013). Before ultracentrifugation, it is necessary to remove the cells, cellular debris, larger protein aggregates, and lipoproteins that differ in density to EVs, using differential centrifugation. This complex process leads to lower EV recovery, which can be additionally affected by the trapping of EVs by protein aggregates (Baranyai et al., 2015; Helwa et al., 2017). Repeated washing steps can reduce protein contaminants to some extent, but Western blotting indicates that albumin can remain in such EV preparations (Baranyai et al., 2015).

Another common contaminant in EV preparations after ultracentrifugation are HDLs, which are smaller but have similar flotation densities to EVs (Yuana et al., 2015). The purities can be improved by including additional separation through sucrose, iodixanol, or KBr density gradients, although these can further reduce the yield of EV preparations (Onódi et al., 2018). Specifically, ultracentrifugation separates low-density EVs (1.10–1.19 g/ml) from other particles with similar sedimentation coefficients, such as protein and RNA aggregates; however, HDLs (with densities 1.063–1.21 g/ml) can still be co-isolated (Lenassi et al., 2010; Yuana et al., 2014; Taylor and Shah, 2015; Konoshenko et al., 2018; Ramirez et al., 2018).

Extracellular Vesicle Isolation Based on Solubility

Polymer polyethylene glycol (PEG), protein organic solvent precipitation (PROSPR) plus cold acetone or acetate-based isolation can be used to precipitate EVs based on removal of water; alternatively, protamine is a positively charged molecule used to form aggregates with negatively charged EVs (Deregibus et al., 2016; Gámez-Valero et al., 2016). Such precipitation methods are very unspecific for EV isolation as they can co-precipitate protein and RNA aggregates, lipoproteins, and any other nanoparticles with similar properties to EVs (Helwa et al., 2017; Konoshenko et al., 2018). The precipitating agent also becomes another potential contaminant that remains in the EV preparations, and therefore this method has a limited choice for downstream analysis. Precipitation is mainly used coupled to other EV isolation methods in connection with RNA characterization, or on low sample volumes (Gardiner et al., 2016). However, care needs to be taken when interpreting the results (Ramirez et al., 2018; Zhang et al., 2020). Several commercial kits are available, with differences reported for the yields, size distributions, and purities of the precipitated EVs (Patel et al., 2019).

Extracellular Vesicle Isolation Based on Specific Markers

The presence of specific transmembrane proteins, receptors, and lipids on the surface of EVs allows for the isolation of EV

subpopulations using immunoaffinity, which is based on strong and specific interactions between EV-specific antigens and related antibodies (Witwer et al., 2013). Most commonly, antibodies are bound to beads or other matrices, and they recognize different tetraspanins (e.g., CD9, CD63, CD81) or phosphatidylserine on the surface of EVs (Geurickx et al., 2019; Martin-Jaular et al., 2021). Alternatively, negative selection for immune-depletion of unwanted components can be used before other EV isolation steps, which can remove the highly abundant blood proteins and lipoproteins (Mørk et al., 2017). Immunoaffinity separation is recommended for highly specific separation of EV subpopulations that carry characteristic markers (Witwer et al., 2013; Ramirez et al., 2018; Chen J. et al., 2019). Contaminants can be efficiently removed from such EV isolates, although broad knowledge of the EVs under study is needed, as otherwise information can be lost by the removal of EVs that do not carry the specific marker. The binding of antibodies to target EVs is also very strong, so their removal from the captured EVs is difficult, with the risk of adversely affecting the EVs in the process.

Emerging Methods for Extracellular Vesicle Isolation

The more recently emerged microfluidics-based techniques show promise for an essential role in isolation, detection, and analysis of plasma EVs in the future (Guo et al., 2018). These are based on trapping EVs in microchannels, where the isolation, detection, and analysis of the EVs occur on a single integrated circuit of only a few square centimeters of a chip (Han et al., 2020). Microfluidics allow the processing of samples with very low volumes, thus also reducing the consumption of reagents. More importantly, even though microfluidics methods integrate multiple functional modules, they can be automated, which can provide high throughput and precision with short processing times (Chen YS. et al., 2019). Despite these advantages, the isolation step in microfluidics is often based on the same EV characteristics as described for classical isolation methods (i.e., surface biomarkers, size), and therefore these methods share the same limitations in terms of contaminants.

To overcome this, new methods have been developed. The use of devices with external force, such as electrophoretic or acoustic forces, can provide label-free isolation of EVs with relatively high purities and yields (Wu et al., 2017; Shi et al., 2019). Same applies to devices without external force, such as dynamic lateral displacement with nanopillars and viscoelastic inertial flow. However, both approaches demand high costs and precise operating conditions (Salafi et al., 2019). Recently, aqueous two-phase systems with bulky centrifugation have been used to separate EVs with high yields and moderate purities (Han et al., 2020). For aqueous two-phase systems on-chip, these are formed by dissolving two incompatible polymers, or a polymer and a salt, in water in a microfluidic channel, which can provide quick separation of EVs from proteins, and can be followed by precise collection at the outlet by laminar flow separation (Han et al., 2020).

These on-chip EV isolation methods can be followed by conventional detection and analysis of the EV isolates, or can

be coupled to downstream microfluidics-based EV characterization (Guo et al., 2018; Lu et al., 2019), while retaining the same limitations in terms of the remaining contaminants. Integration of a microfluidics chip that lyses EVs using a surface acoustic wave, with a concentration and sensing microfluidics chip and an electrokinetic membrane sensor, recently showed potential for absolute quantification of both free-floating miRNAs and EV-miRNAs in plasma (Ramshani et al., 2019). Moreover, a microfluidics system that combines a membrane-based filtration module with a magnetic-bead-based immunoassay provided automated EV isolation and characterization directly from whole human blood (Chen YS. et al., 2019).

Integration of EV isolation with on-chip analysis also has great potential in cancer diagnosis and for monitoring of treatment responses, although standardized procedures for sample collection, storage, and pre-treatment, as well as the positive and negative criteria for the tested biomarkers, need to be determined before this can be fully translated into theranostics (Lu et al., 2019; Soekmadji et al., 2020). Moreover, the production of such chips is still very complex and expensive. At the moment, the lack of inexpensive, simple to use, scalable, and robust methods for the production of microfluidics devices is preventing the rapid mainstream adaptation of these technologies. However, in the future, microfluidics systems combined with viscoelastic fluids, optics, and plasmonics should provide opportunities for automated, transportable, precise, and high-throughput EV research, particularly when combined with the potential to be integrated with a smartphone or with machine-learning tools (Meng et al., 2021).

In summary, EVs can be isolated from blood using diverse techniques, each of which has its own advantages and disadvantages. A good understanding of possible contaminants in relation to EV isolation methods is thus needed for the appropriate selection of the downstream EV analysis methods and data interpretation. Combining two or more methods that can separate particles based on different EV characteristics is ideal to obtain pure EV preparations (Geeurickx et al., 2019; Zhang et al., 2020; Elgamal et al., 2021; Ji et al., 2021), although this can lead to increased losses of EVs, and is constrained by typically low starting volumes of biological samples. Advances in microfluidics hold great potential for rapid isolation of EVs from very small sample volumes; however, further improvements and validation are needed for clinical applications (Konoshenko et al., 2018; Chen J. et al., 2019; Ji et al., 2021; Pasetto et al., 2021).

INTERPRETATION OF DOWNSTREAM CHARACTERIZATION METHODS IN BLOOD BIOMARKER STUDIES DEPENDS ON THE PURITY OF THE EXTRACELLULAR VESICLE PREPARATIONS

Extracellular vesicle characterization methods can generally be divided into biochemical methods, which help to identify the nucleic acid, protein, lipid, and metabolite compositions of EVs,

and biophysical methods, which help to describe EVs according to size, concentration, charge, density, stiffness, and light scattering. Better characterization of EVs is important to understand their fundamental roles in physiological and pathological processes, and this knowledge also needs to be translated into the clinic to be used for improved diagnostics and therapies. To improve the interpretation of EV functional studies and the reliability and reproducibility of EV biomarker studies, an understanding of the influence of blood contaminants on EV characterization is needed. The first step is to understand the main principle of analysis for each technique and if it can account for the contaminants that are expected to be present in the samples after the preceding EV isolation method.

Biochemical Methods for Identification of the Molecular Composition of Extracellular Vesicles

Most EV studies are interested in their nucleic acid and protein compositions, although there is increasing interest also for their lipids and metabolites (Gassart et al., 2003; Lydic et al., 2016; Ditiatkovski et al., 2020). The molecular composition of EVs is dependent on the type of the cell or tissue of origin, and also reflects their (patho)physiological state. This can help to identify origin-specific subsets of EVs in the blood, and to detect EV molecular signatures related to diseases. Therefore, blood EVs are a very promising source of biomarkers for diverse diseases.

Extracellular vesicle-associated RNAs (most commonly miRNAs, but also long noncoding RNAs, viral RNAs) represent one of the most promising and frequently studied EV-related biomarkers, as these have important roles in disease etiology and pathogenesis, and are present in sufficient quantities for detection by established molecular methods (Amorim et al., 2017; Mateescu et al., 2017; Tang et al., 2017; Soekmadji et al., 2018; Profiling et al., 2019; Srinivasan et al., 2019; Driedonks et al., 2020; Hooten, 2020; Liang et al., 2020). Much less is known about EV-associated DNA, although it appears that the DNA can be located in the EV lumen or attached to the surface of EVs as single or double-stranded molecules that are protected from degradation by the bound histones. EV-associated DNA is also heterogeneous in origin (i.e., genomic, mitochondrial) and size (i.e., a few hundred base pairs in small EVs, up to >2 million base pairs in large EVs) (Goričar et al., 2021). EVs protect the nucleic acids from degradation by nucleases that are commonly present in biological fluids, making them remarkably stable under different storage conditions.

To gain unbiased knowledge, next-generation sequencing or PCR profiling arrays have been used to analyze nucleic acids extracted from EVs, while quantitative PCR is used to test interesting targets on larger cohorts (Soekmadji et al., 2018). As changes in EV-enclosed RNA compositions due to diseases can be relatively small, it is important to remove any extra-vesicular RNAs that might be bound to co-isolated contaminants (Gallo et al., 2012; Bracht et al., 2021). Protein and lipoprotein contaminants are known to carry miRNAs in the blood, and might therefore alter EV RNA analysis (Vickers et al., 2011; Vergauwen et al., 2017; Buzás et al., 2018). Before RNA analysis,

EV preparations can thus be processed using proteinases and RNases to remove extra-vesicular RNA (Bracht et al., 2021). If platelet-derived EVs are also present due to improper collecting and processing of the blood, then these can overshadow the pathology-dependent miRNA signals (Palviainen et al., 2020). Cell-free DNA analysis, on the other hand, is mostly constrained by the low quantity and fragmentation of EV-bound DNA, and is not particularly affected by blood contaminants (Zhang L. et al., 2019; Keller et al., 2021).

Extracellular vesicle-associated proteins are another promising source of biomarkers, as these can have many important roles in disease pathologies. Surface proteins can be used for enrichment of EVs that carry specific markers, or for direct quantification of specific EV subpopulations, which overcomes the problem of dilution of pathology-related EV signals in the blood. For quantification of specific protein targets, Western blotting or ELISA are most often used, while for unbiased characterization of EV-associated proteins, mass spectrometry and EV flow cytometry (described in **Section 4.2**) are generally used (Pocsfalvi et al., 2016; Görgens et al., 2019; Tian et al., 2020; Martin-Jaular et al., 2021). The presence of EVs in a sample can often be confirmed using Western blotting of common EV-related proteins, such as tetraspanins, Alix, Tsg101, and HSP70. As most EV markers are also detectable in whole cells, sample purity should be assessed by EV-negative controls, such as calnexin or histones (Ramirez et al., 2018). ELISA can provide relatively cheap detection of target proteins in a large number of samples, and it is therefore well established and widely used in research and medical applications (Pocsfalvi et al., 2016).

Commercial EV protein detection platforms and kits are now becoming available to simplify EV protein analysis. Commercial ELISA kits can help with quantification of generic EV-positive markers, such as tetraspanins, or disease-related membrane proteins, like PD-L1 or ICAM-1, or EV luminal cargo, like cytokines (Nardi et al., 2016; Hosseinkhani et al., 2017; Hosseinkhani et al., 2020; Venkatesan et al., 2017; Willis et al., 2017; Cordonnier et al., 2020). ELISA can also be used to measure the level of contaminants in a sample (e.g., apolipoproteins, albumin) (Nardi et al., 2016; Holcar et al., 2020). Other antibody-based methods include phenotyping of EVs using diverse antibody-coated bead technologies (e.g., flow cytometry bead-based multiplex analysis, such as 'MACSPlex' technology; multiplex bead-based immunoassays, such as 'Luminex' technology) or antibody-coated surfaces (e.g., EV protein arrays; multiplexed microarray chips for the immuno-capture of EVs, such as 'ExoView' technology) (Rausch et al., 2016; Bachurski et al., 2019; Brahmer et al., 2019; Štok et al., 2020). Users can usually decide between predetermined combinations of antigens included in a test (as usually CD9, CD63, CD81), or they can obtain a custom combination of chosen targets.

Rapid growth of mass-spectrometry-based strategies for proteome characterization has in recent years improved the level of molecular details that can be obtained from limited amounts of EVs isolated from blood (Rosa-Fernandes et al., 2017). The basic workflow for EV proteome determination using mass spectrometry is based on tryptic digestion of the

extracted protein mixture, followed by separation of proteins by nano high-pressure liquid chromatography and detection by tandem mass spectrometry (i.e., 'nanoHPLC-MS/MS'). This technique can identify thousands of proteins in a mixture, although distinguishing rarer proteins of interest from the background of highly abundant proteins can be a real challenge (Ruhen and Meehan, 2019). In all of these mentioned methods, protein and lipoprotein contaminants of EV isolates would directly affect the EV protein analysis, and therefore these need to be removed or allowed for. The latter can be achieved by co-analysis of lipoprotein- and protein-depleted samples during the method development, thus defining the contribution of the contaminants to the final results, or by quantifying the (lipo)protein concentrations in EV samples using simple methods such as ELISA.

According to MISEV 2018, albumin and apolipoproteins are the best markers of EV isolate contamination (Théry et al., 2019). To account for contamination with blood (lipo)proteins, albumin and ApoA1 (for HDLs) and ApoB (for LDLs, IDLs, VLDLs, Lp(a) and chylomicrons) levels can be determined by ELISA or Western blotting (Baranyai et al., 2015; Hong et al., 2016; Pietrowska et al., 2019; Gaspar et al., 2020; Holcar et al., 2020). To remove proteins, EV preparations can be processed using the proteases proteinase K, trypsin, and others, although this can impact upon the availability of any surface antigens, and thus impact upon the EV analysis (Muller et al., 2014; Shelke et al., 2014; Cvjetkovic et al., 2016; Stewart et al., 2016; Chettimada et al., 2018; Skliar et al., 2018; Choi et al., 2020).

Biophysical Methods for Characterization of Extracellular Vesicle Size and Concentration

Certain pathological factors can induce changes in EV size profiles and concentrations, such as hypoxia, autophagy, and stress (all typical of cancers). Thus, these biophysical characteristics of EVs can also be evaluated as biomarkers of disease states. Additionally, the sizes, charge, and membrane stiffness of EVs can affect their interactions with other particles or membranes, and are therefore important for the functional roles of EVs (Zhang et al., 2018).

Nanoparticle Tracking Analysis and Dynamic Light Scattering

These techniques both combine laser-light scattering of the particles in the sample for the measurement of the concentration and size distributions (Filipe et al., 2010). They are also both dependent on the Brownian movement of nanoparticles in solution. Nanoparticle tracking analysis (NTA) measures this movement through tracking and analysis of the particles under the microscope, and detection using a charge-coupled device camera for the amount of laser light refraction on a particle-by-particle basis. The movement of the nanoparticles is related to their size, with the refractive index of the nanoparticles as the limiting factor for detection. NTA can be used to detect particles with a hydrodynamic diameter of 30–1,000 nm (Chernyshev et al., 2015).

Dynamic light scattering (DLS) records time-dependent fluctuations in the scattered light intensity caused by interference from the nanoparticles in a sample, without visualizing the nanoparticles. It measures all of the particles in a suspension in the size range of 5–6,000 nm at the same time. The reported average particle size is thus biased toward the larger particles within a sample, as these scatter light more intensely than the smaller particles. DLS can also measure the charge that develops at the interface between the surface of EVs and their liquid medium, which is known as the zeta potential. The zeta potential of EVs is usually from -9 mV to -16 mV (Zhang et al., 2018). DLS is very effective for the analysis of homogeneous solutions of very small particles, whereas particle-by-particle measurement using the NTA approach is better for polydispersed samples, although larger particles can hinder detection of EVs in the sample by saturation of the camera, which will distort the results (Palmieri et al., 2014; Erdbrügger et al., 2016). As NTA and DLS detect particles only indirectly through refracted laser light, contaminants of similar sizes to the EVs will be indiscriminately detected and measured along with the EVs, such as protein aggregates, immune complexes or lipoproteins, thus effecting the analysis (Mørk et al., 2017). NTA and DLS should thus ideally follow EV isolation techniques that minimize contamination of EVs with particles in the same size range as EVs, or larger. Lipoproteins other than HDLs, which are too small to be detected by these methods, can be separated from EVs by ultracentrifugation combined with a density gradient/cushion (e.g., sucrose, iodixanol, KBr), based on the differences in their densities (Yuana et al., 2014; Sódar et al., 2016; Karimi et al., 2018). Larger protein aggregates can be removed with mild proteolytic treatments, although the potential effects of such treatments on the perceived EV size and bioactive EV-protein corona should be considered (Cvjetkovic et al., 2016; Skliar et al., 2018; Choi et al., 2020).

Resistive Pulse Sensing

Resistive pulse sensing (RPS) is a method that can be used to determine EV size distributions and concentrations, with detection here based on a transient change in an electric signal (Witwer et al., 2013). The system consists of two fluid cells that are filled with a conductive liquid, and that contain the sample. The fluid cells are separated by a nonconductive membrane with a single pore, which is under a baseline electric current. As a particle passes through the stretchable nanopore under the electric current, the current is transiently attenuated in proportion with the particle volume. As the detection range of specific particle sizes depends on the specific type of nanopore and the stretch that is applied to it, the use of various nanopore membranes is required to detect the entire EV size range when considering measurements of polydispersed EV populations with heterogeneous particle sizes (Witwer et al., 2013; Mørk et al., 2016; Ramirez et al., 2018). In some samples, pore-clogging can occur, which is another disadvantage of RPS (van der Pol et al., 2014). Like NTA, at the current stage, RPS does not provide particle phenotyping and discrimination between EVs and contaminants, such as protein aggregates or lipoproteins (Mørk et al., 2016).

Flow Cytometry

Flow cytometry is a technique that combines and uses principles of biochemical methods for the identification of the molecular compositions of EVs, and biophysical methods for characterization of EV sizes and concentrations. In classical flow cytometry, EVs can be labeled with fluorescent dyes and/or antibodies (e.g., against tetraspanins or a specific protein of interest). The samples are then passed through multiple lasers of differing wavelengths, with the scattered light detected. The amounts and directions of the forward scatter (i.e., 'FSC') or the fluorescence indicates the size of the EVs measured, while the side scatter (i.e., 'SSC') indicates the internal complexity of the observed particle (Gandham et al., 2020). Flow cytometry itself is a high-throughput, single-particle, multi-parameter analysis technique that can be used to analyze a large range of particle diameters (McVey et al., 2018).

The major problems and restrictions of flow cytometry for characterization of EVs do not arise as a result of contaminations of the EV samples; they are predominantly a consequence of technical limitations of the method itself. These include standardization of the light scattering and fluorescence data between different flow cytometers, limited resolution and detection of EVs <200 nm in diameter, the possibility of unspecific staining, and low brightness of the fluorochromes coupled to antibodies (Morales-Kastresana et al., 2017; McVey et al., 2018). This has led to the development of alternative approaches to improve small EV detection by flow cytometry, including nanoflow cytometry, high-resolution cytometry, and fluorescent triggering and imaging flow cytometry (Freitas et al., 2019; Görgens et al., 2019; Tian et al., 2020).

Similar to other techniques, another challenge of flow cytometry is the adequate and transparent reporting of the findings and the ambiguities inherent in the data interpretation (Welsh et al., 2017; Welsh et al., 2020a; Welsh et al., 2020b; Ramirez et al., 2018; Görgens et al., 2019; Tang et al., 2019; Ender et al., 2020). This was addressed in 2020 by a position paper on the minimum information that should be provided in an EV flow-cytometry-specific reporting framework (i.e., 'MIFlowCyt-EV'). This publication defined the critical information that should be reported relating to experimental design, sample staining, and EV detection and measurement in studies that report EV flow-cytometry data. Standardized reporting can improve quantitative comparisons of results from different laboratories, and support the development of new instruments and assays (Welsh et al., 2020a). For deeper insight into EV flow cytometry, please also consult the following articles (Arraud et al., 2016; Welsh et al., 2017; Welsh et al., 2020a; van der Pol et al., 2018; Görgens et al., 2019; Tian et al., 2020; Yang and Rhee, 2021).

Transmission Electron Microscopy and Scanning Electron Microscopy

These electron microscopy techniques can be used for morphological and structural characterization of EVs, and for EV purity evaluation and contaminant identification (Cvjetkovic et al., 2016; Gámez-Valero et al., 2016). The methods are based on

the detection of interactions between the electrons in fixed samples and those in the beam of accelerated electrons, which are transmitted through the sample in transmission electron microscopy (TEM), and over the surface of the sample in scanning electron microscopy (SEM). Differentiation of the edges and features of the sample depends primarily on the differences in the electron densities of different organic molecules in the samples. In electron microscopy, EVs can be observed as unstained, or can be stained to provide higher electron densities. Staining can be unspecific (e.g., osmium tetroxide, uranyl acetate, phosphotungstic acid) or specific (e.g., immunoTEM), with gold-labeled antibodies (Brisson et al., 2017). Protein aggregates have different electron densities than EVs after staining, which makes their presence in the sample very apparent (Muller et al., 2014; Hong et al., 2016; Karimi et al., 2018). Similarly, the presence of a clear phospholipid bilayer distinctly separates EVs from lipoproteins, even when they are of comparable sizes and shapes (Chernyshev et al., 2015; Sódar et al., 2016). However, the fixation and desiccation steps required for TEM and SEM can lead to nonuniform drying fronts, which can alter the sizes of EVs, and result in shape distortion (e.g., their characteristic ‘cup-shaped’ morphology) (Chernyshev et al., 2015). A special modification of classic TEM is provided by cryogenic (cryo-) TEM, which allows for direct observation of EVs without the dehydration, chemical fixation, and/or staining (Murata and Wolf, 2018). While cryo-TEM is particularly labor-intensive and requires a skilled operator, freezing of samples in their native hydrated state ensures the conservation of the physiological volume of the EVs. Capturing multiple dynamic states of EVs also allows three-dimensional tomography and spatial visualization of more complex structures in samples (Chernyshev et al., 2015; Murata and Wolf, 2018; Sharma et al., 2018). The maximum size of an observed object in cryo-TEM is limited by the thickness of the sample that can still be penetrated by the electron beam, which is ~500 nm in the case of 300 kV TEM (Murata and Wolf, 2018).

Atomic Force Microscopy

Atomic force microscopy (AFM) is an emerging alternative to optical and electron diffraction methods for studying EVs. In AFM, a very fine tip of a probe (i.e., a few nanometers) is scanned over a sample, in raster, line-by-line. The height of the tip is adjusted based on the instrument feedback, which translates into mechanical information and the topography of the sample. This feedback to the instrument can come from the frequency or amplitude of the tip oscillation, and the force exerted on the cantilever by the surface, or some combination of these three (Sharma et al., 2018). AFM primarily explores the mechanical properties of a sample. In the case of EVs, it analyzes the stiffness of the surface of the EVs, which has been shown to be dependent on their (patho)physiological state (Parisse et al., 2017). AFM can simultaneously measure the distribution of EV sizes and map their stiffness with nanometric precision. An antibody-coated tip and surfaces (e.g., mica, glass) can also be used to examine subsets of EVs, or to functionally analyze the morphology and structural heterogeneity of EVs (Parisse et al., 2017; Sharma et al., 2018).

Similar to electron microscopy, AFM is not a method that is suited for the analysis of large numbers of samples, or for conducting statistical analysis of an EV population; however, it provides accurate analysis of individual vesicles and their surfaces. Isolation methods that can significantly alter the surface or even sizes of EVs can affect these analyses. Evident protein contamination, aggregation of the sample, use of immunoprecipitation as the method of isolation, or any treatments with enzymes can all alter the outer surface of EVs (including the protein corona), and therefore these should be carefully considered when interpreting the results (Woo et al., 2016).

To summarize, biochemical and biophysical EV analysis methods are in general affected by any remaining nanosized contaminants in the EV isolates, although these effects can be minimized by pairing the EV characterization analysis with compatible EV isolation method(s) in terms of potential contaminants. Alternatively, EV analysis data should be interpreted carefully, taking into account any influence on the experiment outcomes of contaminants that might be present. Deeper understanding of the EV analysis methods and how they are influenced by specific contaminants will help to improve the interpretation of EV biomarker studies, and thereby their reliability and reproducibility.

CONCLUSION AND FUTURE PERSPECTIVES

Extracellular vesicles are membrane-bound nanometer-to micrometer-sized particles, which in humans are released from all cells and can accumulate in the blood and other body fluids. The sizes, concentrations and molecular compositions of EVs reflect the type and state of their cell of origin, making them intriguing candidates for research and biomarker discovery (Holcar et al., 2020; Palviainen et al., 2020). Peripheral blood is easily accessible, and is thus one of the most desirable sources of biological EVs. However, blood is a very complex fluid that has an abundance of nanoparticles that share molecular or/and biophysical characteristics with EVs, and can therefore co-isolate from blood plasma. This can obscure the biomarker potential or biological relevance of EVs, thus also distorting the outcomes of biomarker studies. However, most biomarker studies do not address the problem of contaminants in EV isolates, and how they can affect the downstream analysis.

To the best of our knowledge, this review is the first comprehensive description of the effects of different EV isolation methods on the co-isolation of major contaminants from blood plasma, which also covers the effects that these contaminants can have on downstream EV analysis. To account for co-isolated contaminants and improve the interpretation of these biomarker studies, a good understanding of the main principles of analysis for each technique is also needed. We believe that the details provided and discussed here will help to improve the rigor, reproducibility, and reliability of future EV biomarker studies.

Despite high expectations, investment of considerable effort, and promising results, EV-related biomarkers have not yet been routinely implemented in clinical practice, and much of the basic EV research remains difficult to reproduce (Popovic et al., 2018; Elgamal et al., 2021). An important step to overcome this is to consciously strive for rigor in the standardization of methods used in the field, with special care dedicated to the preanalytical stages in particular (Théry et al., 2019; Welsh et al., 2020a; Nieuwland et al., 2020). Consistent quantification of nonEV contaminants, thorough and transparent reporting of protocols used (preferably using ISEV-recommended platforms, such as EV-TRACK), and use of EV-reference materials will also facilitate better inter-study comparisons and help to provide more reliable and nuanced interpretations of results obtained (Van Deun et al., 2017; Geeurickx et al., 2019; Görgens et al., 2019; Tang et al., 2019). Furthermore, the inherent heterogeneity of EVs should be further addressed by improvements to the existing methods for EV isolation and characterization, and by development of new methods or creative combinations of those already established

(Chang et al., 2021; Ji et al., 2021; Martin-Jaular et al., 2021). Methods that can provide uniform subpopulations of EVs of high purities to which (sub)cellular origins can be attributed and for which functions can be defined will help to further the field and realize the full potential of EVs in the research and clinical settings.

AUTHOR CONTRIBUTIONS

MH and MK performed the literature searches. MH, MK, and ML participated in the writing and editing of the manuscript. All of the authors have read and approved the final manuscript.

FUNDING

This work was financially supported by the Slovenian Research Agency (ARRS Grant Nos. P1-0170 and J3-9255).

REFERENCES

- Alix-Panabières, C., and Pantel, K. (2017). Clinical Prospects of Liquid Biopsies. *Nat. Biomed. Eng.* 1, 1–3. doi:10.1038/s41551-017-0065
- Allen, R. M., Zhao, S., Ramirez Solano, M. A., Zhu, W., Michell, D. L., Wang, Y., et al. (2018). Bioinformatic Analysis of Endogenous and Exogenous Small RNAs on Lipoproteins. *J. Extracell. Vesicles* 7, 1506198–1506225. doi:10.1080/20013078.2018.1506198
- Amorim, M. G., Valieris, R., Drummond, R. D., Pizzi, M. P., Freitas, V. M., Sinigaglia-Coimbra, R., et al. (2017). A Total Transcriptome Profiling Method for Plasma-Derived Extracellular Vesicles: Applications for Liquid Biopsies. *Sci. Rep.* 7, 14395–14411. doi:10.1038/s41598-017-14264-5
- An, M., Wu, J., Zhu, J., and Lubman, D. M. (2018). Comparison of an Optimized Ultracentrifugation Method versus Size-Exclusion Chromatography for Isolation of Exosomes from Human Serum. *J. Proteome Res.* 17, 3599–3605. doi:10.1021/acs.jproteome.8b00479
- Anderson, N. L., and Anderson, N. G. (2002). The Human Plasma Proteome: History, Character, and Diagnostic Prospects. *Mol. Cel. Proteomics* 1, 845–867. doi:10.1074/mcp.R200007-MCP200
- Antich-Rosselló, M., Forteza-Genestra, M. A., Monjo, M., and Ramis, J. M. (2021). Platelet-derived Extracellular Vesicles for Regenerative Medicine. *Ijms* 22, 8580–8614. doi:10.3390/ijms22168580
- Arntz, O. J., Pieters, B. C. H., Thurlings, R. M., Wenink, M. H., van Lent, P. L. E. M., Koenders, M. I., et al. (2018). Rheumatoid Arthritis Patients with Circulating Extracellular Vesicles Positive for IgM Rheumatoid Factor Have Higher Disease Activity. *Front. Immunol.* 9, 2388–2411. doi:10.3389/fimmu.2018.02388
- Arraud, N., Gounou, C., Turpin, D., and Brisson, A. R. (2016). Fluorescence Triggering: A General Strategy for Enumerating and Phenotyping Extracellular Vesicles by Flow Cytometry. *Cytometry A* 89, 184–195. doi:10.1002/cyto.a.22669
- Bachurski, D., Schuldner, M., Nguyen, P. H., Malz, A., Reinert, K. S., Grenzi, P. C., et al. (2019). Extracellular Vesicle Measurements with Nanoparticle Tracking Analysis - an Accuracy and Repeatability Comparison between NanoSight NS300 and ZetaView. *J. Extracell. Vesicles* 8, 1596016–1596018. doi:10.1080/20013078.2019.1596016
- Badhwar, A., and Haqqani, A. S. (2020). Biomarker Potential of Brain-Secreted Extracellular Vesicles in Blood in Alzheimer's Disease. *Alzheimers Dement (Amst)* 12, e12001–14. doi:10.1002/dad2.12001
- Badovinac, D., Goričar, K., Zavrtanik, H., Petrič, M., Lavrin, T., Mavec, N., et al. (2021). Plasma Extracellular Vesicle Characteristics Correlate with Tumor Differentiation and Predict Overall Survival in Patients with Pancreatic Ductal Adenocarcinoma Undergoing Surgery with Curative Intent. *Jpm* 11, 77–12. doi:10.3390/jpm11020077
- Baranyai, T., Herczeg, K., Onódi, Z., Voszka, I., Módos, K., Marton, N., et al. (2015). Isolation of Exosomes from Blood Plasma: Qualitative and Quantitative Comparison of Ultracentrifugation and Size Exclusion Chromatography Methods. *PLoS One* 10, e0145686–13. doi:10.1371/journal.pone.0145686
- Berckmans, R. J., Lacroix, R., Hau, C. M., Sturk, A., and Nieuwland, R. (2019). Extracellular Vesicles and Coagulation in Blood from Healthy Humans Revisited. *J. Extracell. Vesicles* 8, 1688936–1689010. doi:10.1080/20013078.2019.1688936
- Böing, A. N., van der Pol, E., Grootemaat, A. E., Coumans, F. A., Sturk, A., Nieuwland, R., et al. (2014). Single-step Isolation of Extracellular Vesicles by Size-Exclusion Chromatography. *J. Extracell. Vesicles* 3, 1–11. doi:10.3402/jev.v3.23430
- Bracht, J. W. P., Mayo-de-las-Casas, C., Berenguer, J., Karachaliou, N., and Rosell, R. (2018). The Present and Future of Liquid Biopsies in Non-small Cell Lung Cancer: Combining Four Biosources for Diagnosis, Prognosis, Prediction, and Disease Monitoring. *Curr. Oncol. Rep.* 20, 70–10. doi:10.1007/s11912-018-0720-z
- Bracht, J. W. P., Gimenez-Capitan, A., Huang, C.-Y., Potie, N., Pedraz-Valdunciel, C., Warren, S., et al. (2021). Analysis of Extracellular Vesicle mRNA Derived from Plasma Using the nCounter Platform. *Sci. Rep.* 11, 1–12. doi:10.1038/s41598-021-83132-0
- Brahmer, A., Neuberger, E., Esch-Heisser, L., Haller, N., Jorgensen, M. M., Baek, R., et al. (2019). Platelets, Endothelial Cells and Leukocytes Contribute to the Exercise-Triggered Release of Extracellular Vesicles into the Circulation. *J. Extracell. Vesicles* 8, 1615820–1615919. doi:10.1080/20013078.2019.1615820
- Brisson, A. R., Tan, S., Linares, R., Gounou, C., and Arraud, N. (2017). Extracellular Vesicles from Activated Platelets: a Semiquantitative Cryo-Electron Microscopy and Immuno-Gold Labeling Study. *Platelets* 28, 263–271. doi:10.1080/09537104.2016.1268255
- Buzás, E. I., Tóth, E. Á., Sódar, B. W., and Szabó-Taylor, K. (2018). Molecular Interactions at the Surface of Extracellular Vesicles. *Semin. Immunopathol.* 40, 453–464. doi:10.1007/s00281-018-0682-0
- Chang, W. H., Cerione, R. A., and Antonyak, M. A. (2021). Extracellular Vesicles and Their Roles in Cancer Progression. *Methods Mol. Biol.* 2174, 143–170. doi:10.1007/978-1-0716-0759-6_10
- Charoenviriyakul, C., Takahashi, Y., Morishita, M., Nishikawa, M., and Takakura, Y. (2018). Role of Extracellular Vesicle Surface Proteins in the Pharmacokinetics of Extracellular Vesicles. *Mol. Pharm.* 15, 1073–1080. doi:10.1021/acs.molpharmaceut.7b00950
- Chen, J., Xu, Y., Wang, X., Liu, D., Yang, F., Zhu, X., et al. (2019a). Rapid and Efficient Isolation and Detection of Extracellular Vesicles from Plasma for Lung Cancer Diagnosis. *Lab. Chip* 19, 432–443. doi:10.1039/c8lc01193a
- Chen, Y. S., Ma, Y. D., Chen, C., Shiesh, S. C., and Lee, G. B. (2019b). An Integrated Microfluidic System for On-Chip Enrichment and Quantification of

- Circulating Extracellular Vesicles from Whole Blood. *Lab. Chip* 19, 3305–3315. doi:10.1039/c9lc00624a
- Chernyshev, V. S., Rachamadugu, R., Tseng, Y. H., Belnap, D. M., Jia, Y., Branch, K. J., et al. (2015). Size and Shape Characterization of Hydrated and Desiccated Exosomes. *Anal. Bioanal. Chem.* 407, 3285–3301. doi:10.1007/s00216-015-8535-3
- Chettimada, S., Lorenz, D. R., Misra, V., Dillon, S. T., Reeves, R. K., Manickam, C., et al. (2018). Exosome Markers Associated with Immune Activation and Oxidative Stress in HIV Patients on Antiretroviral Therapy. *Sci. Rep.* 8, 7227–7316. doi:10.1038/s41598-018-25515-4
- Chiva-Blanch, G., and Badimon, L. (2019). Cross-talk between Lipoproteins and Inflammation: the Role of Microvesicles. *J. Clin. Med.* 8, 1–12. doi:10.3390/jcm8122059
- Choi, D., Go, G., Kim, D. K., Lee, J., Park, S. M., Di Vizio, D., et al. (2020). Quantitative Proteomic Analysis of Trypsin-Treated Extracellular Vesicles to Identify the Real-Vesicular Proteins. *J. Extracell. Vesicles* 9, 1757209–1757212. doi:10.1080/20013078.2020.1757209
- Clotilde, T., Matias, O., Segura, E., Théry, C., Ostrowski, M., and Segura, E. (2009). Membrane Vesicles as Conveyors of Immune Responses. *Nat. Rev. Immunol.* 9, 581–593. doi:10.1007/s00018-018-2773-4
- Cocozza, F., Grisard, E., Martin-jaular, L., Mathieu, M., and Théry, C. (2020). SnapShot: Extracellular Vesicles. *Cell* 182, 262–e1. doi:10.1016/j.cell.2020.04.054
- Contado, C. (2017). Field Flow Fractionation Techniques to Explore the "Nano-World". *Anal. Bioanal. Chem.* 409, 2501–2518. doi:10.1007/s00216-017-0180-6
- Cordonnier, M., Nardin, C., Chanteloup, G., Derangere, V., Algros, M. P., Arnould, L., et al. (2020). Tracking the Evolution of Circulating Exosomal-PD-L1 to Monitor Melanoma Patients. *J. Extracell. Vesicles* 9, 1710899–1710911. doi:10.1080/20013078.2019.1710899
- Cvijetkovic, A., Jang, S. C., Konečná, B., Höög, J. L., Sihlbom, C., Lässer, C., et al. (2016). Detailed Analysis of Protein Topology of Extracellular Vesicles: Evidence of Unconventional Membrane Protein Orientation. *Sci. Rep.* 6, 1–12. doi:10.1038/srep36338
- Dang, X. T. T., Kavishka, J. M., Zhang, D. X., Pirisinu, M., and Le, M. T. N. (2020). Extracellular Vesicles as an Efficient and Versatile System for Drug Delivery. *Cells* 9, 1–36. doi:10.3390/cells9102191
- de Gassart, A., Géminard, C., Fèvre, B., Raposo, G., and Vidal, M. (2003). Lipid Raft-Associated Protein Sorting in Exosomes. *Blood* 102, 4336–4344. doi:10.1182/blood-2003-03-0871
- Deregibus, M. C., Figliolini, F., D'Antico, S., Manzini, P. M., Pasquino, C., De Lena, M., et al. (2016). Charge-based Precipitation of Extracellular Vesicles. *Int. J. Mol. Med.* 38, 1359–1366. doi:10.3892/ijmm.2016.2759
- Dickhout, A., and Koenen, R. R. (2018). Extracellular Vesicles as Biomarkers in Cardiovascular Disease: Chances and Risks. *Front. Cardiovasc. Med.* 5, 113–119. doi:10.3389/fcvm.2018.00113
- Ditiatkovski, M., Mukhamedova, N., Dragoljevic, D., Hoang, A., Low, H., Pushkarsky, T., et al. (2020). Modification of Lipid Rafts by Extracellular Vesicles Carrying HIV-1 Protein Nef Induces Redistribution of Amyloid Precursor Protein and Tau, Causing Neuronal Dysfunction. *J. Biol. Chem.* 295, 13377–13392. doi:10.1074/jbc.ra120.014642
- Dong, L., Zieren, R. C., Horie, K., Kim, C. J., Mallick, E., Jing, Y., et al. (2020). Comprehensive Evaluation of Methods for Small Extracellular Vesicles Separation from Human Plasma, Urine and Cell Culture Medium. *J. Extracellular Vesicles* 10, 1–20. doi:10.1002/jev2.12044
- Doyle, L. M., and Wang, M. Z. (2019). Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. *Cells* 8, 1–24. doi:10.3390/cells8070727
- Driedonks, T. A. P., Mol, S., de Bruin, S., Peters, A. L., Zhang, X., Lindenbergh, M. F. S., et al. (2020). Y-RNA Subtype Ratios in Plasma Extracellular Vesicles Are Cell Type-Specific and Are Candidate Biomarkers for Inflammatory Diseases. *J. Extracell. Vesicles* 9, 1764213–1764221. doi:10.1080/20013078.2020.1764213
- Duvvuri, B., and Lood, C. (2019). Cell-free DNA as a Biomarker in Autoimmune Rheumatic Diseases. *Front. Immunol.* 10, 502–521. doi:10.3389/fimmu.2019.00502
- Elgamal, S., Cocucci, E., Sass, E. J., Mo, X. M., Blissett, A. R., Calomeni, E. P., et al. (2021). Optimizing Extracellular Vesicles' Isolation from Chronic Lymphocytic Leukemia Patient Plasma and Cell Line Supernatant. *JCI Insight* 6, 1–19. doi:10.1172/jci.insight.137937
- Ender, F., Zamzow, P., Bubnoff, N. V., and Gieseler, F. (2020). Detection and Quantification of Extracellular Vesicles via FACS: Membrane Labeling Matters!. *Int. J. Mol. Sci.* 21, 1–13. doi:10.3390/ijms21010291
- Endzeliņš, E., Berger, A., Melne, V., Bajo-Santos, C., Sobolevska, K., Åbols, A., et al. (2017). Detection of Circulating miRNAs: Comparative Analysis of Extracellular Vesicle-Incorporated miRNAs and Cell-free miRNAs in Whole Plasma of Prostate Cancer Patients. *BMC Cancer* 17, 730–814. doi:10.1186/s12885-017-3737-z
- Erdbrügger, U., Lannigan, J., and Erdbr, U. (2016). Analytical Challenges of Extracellular Vesicle Detection: a Comparison of Different Techniques. *Cytometry A* 89, 123–134. doi:10.1002/cyto.a.22795
- Ezzat, K., Pernemalm, M., Pålsson, S., Roberts, T. C., Järver, P., Dondalska, A., et al. (2019). The Viral Protein corona Directs Viral Pathogenesis and Amyloid Aggregation. *Nat. Commun.* 10, 2331–2416. doi:10.1038/s41467-019-10192-2
- Feingold, K. R., and Grunfeld, C. (2010). The Acute Phase Response Inhibits Reverse Cholesterol Transport. *J. Lipid Res.* 51, 682–684. doi:10.1194/jlr.E005454
- Ference, B. A., Kastelein, J. J. P., and Catapano, A. L. (2020). Lipids and Lipoproteins in 2020. *JAMA* 324, 595–596. doi:10.1001/jama.2020.5685
- Filipe, V., Hawe, A., and Jiskoot, W. (2010). Critical Evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the Measurement of Nanoparticles and Protein Aggregates. *Pharm. Res.* 27, 796–810. doi:10.1007/s11095-010-0073-2
- Freitas, D., Balmaña, M., Poças, J., Campos, D., Osório, H., Konstantinidi, A., et al. (2019). Different Isolation Approaches lead to Diverse Glycosylated Extracellular Vesicle Populations. *J. Extracell. Vesicles* 8, 1621131–1621217. doi:10.1080/20013078.2019.1621131
- Frigerio, R., Musico, A., Brucala, M., Ridolfi, A., Galbiati, S., Vago, R., et al. (2021). Extracellular Vesicles Analysis in the COVID-19 Era: Insights on Serum Inactivation Protocols towards Downstream Isolation and Analysis. *Cells* 10, 544–612. doi:10.3390/cells10030544
- Furi, I., Momen-Heravi, F., and Szabo, G. (2017). Extracellular Vesicle Isolation: Present and Future. *Ann. Transl. Med.* 5, 263. doi:10.21037/atm.2017.03.95
- Gallo, A., Tandon, M., Alevizos, I., and Illei, G. G. (2012). The Majority of microRNAs Detectable in Serum and Saliva Is Concentrated in Exosomes. *PLoS One* 7, e30679–5. doi:10.1371/journal.pone.0030679
- Gámez-Valero, A., Monguió-Tortajada, M., Carreras-Planella, L., Franquesa, M., Beyer, K., and Borràs, F. E. (2016). Size-Exclusion Chromatography-Based Isolation Minimally Alters Extracellular Vesicles' Characteristics Compared to Precipitating Agents. *Sci. Rep.* 6, 33641–33649. doi:10.1038/srep33641
- Gandham, S., Su, X., Wood, J., Nocera, L. A., Alli, S. C., Milane, L., et al. (2020). Technologies and Standardization in Research on Extracellular Vesicles. *Trends Biotechnol.* 38, 1066–1098. doi:10.1016/j.tibtech.2020.05.012
- Gaspar, L. S., Santana, M. M., Henriques, C., Pinto, M. M., Ribeiro-Rodrigues, T. M., Girão, H., et al. (2020). Simple and Fast Sec-Based Protocol to Isolate Human Plasma-Derived Extracellular Vesicles for Transcriptional Research. *Mol. Ther. Methods Clin. Dev.* 18, 723–737. doi:10.1016/j.omtm.2020.07.012
- Geurickx, E., and Hendrix, A. (2020). Targets, Pitfalls and Reference Materials for Liquid Biopsy Tests in Cancer Diagnostics. *Mol. Aspects Med.* 72, 100828–100917. doi:10.1016/j.mam.2019.10.005
- Geurickx, E., Tulkens, J., Dhondt, B., Van Deun, J., Lippens, L., Vergauwen, G., et al. (2019). The Generation and Use of Recombinant Extracellular Vesicles as Biological Reference Material. *Nat. Commun.* 10, 3288–3312. doi:10.1038/s41467-019-11182-0
- Geyer, P. E., Voytik, E., Treit, P. V., Doll, S., Kleinhempel, A., Niu, L., et al. (2019). Plasma Proteome Profiling to Detect and Avoid Sample-Related Biases in Biomarker Studies. *EMBO Mol. Med.* 11, e10427–12. doi:10.15252/emmm.201910427
- Ghosh, A., Davey, M., Chute, I. C., Griffiths, S. G., Lewis, S., Chacko, S., et al. (2014). Rapid Isolation of Extracellular Vesicles from Cell Culture and Biological Fluids Using a Synthetic Peptide with Specific Affinity for Heat Shock Proteins. *PLoS One* 9, e110443–12. doi:10.1371/journal.pone.0110443
- González, E., and Falcón-Pérez, J. M. (2015). Cell-derived Extracellular Vesicles as a Platform to Identify Low-Invasive Disease Biomarkers. *Expert Rev. Mol. Diagn.* 15, 907–923. doi:10.1586/14737159.2015.1043272
- Görgens, A., Bremer, M., Ferrer-Tur, R., Murke, F., Tertel, T., Horn, P. A., et al. (2019). Optimisation of Imaging Flow Cytometry for the Analysis of Single Extracellular Vesicles by Using Fluorescence-Tagged Vesicles as Biological

- Reference Material. *J. Extracell. Vesicles* 8, 1587567–1587625. doi:10.1080/20013078.2019.1587567
- Goričar, K., Dolžan, V., and Lenassi, M. (2021). Extracellular Vesicles: a Novel Tool Facilitating Personalized Medicine and Pharmacogenomics in Oncology. *Front. Pharmacol.* 12, 1–16. doi:10.3389/fphar.2021.671298
- Guo, S. C., Tao, S. C., and Dawn, H. (2018). Microfluidics-based On-A-Chip Systems for Isolating and Analysing Extracellular Vesicles. *J. Extracell. Vesicles* 7, 1508271–1508316. doi:10.1080/20013078.2018.1508271
- Han, B. H., Kim, S., Seo, G., Heo, Y., Chung, S., and Kang, J. Y. (2020). Isolation of Extracellular Vesicles from Small Volumes of Plasma Using a Microfluidic Aqueous Two-phase System. *Lab. Chip* 20, 3552–3559. doi:10.1039/d0lc00345j
- Helwa, I., Cai, J., Drewry, M. D., Zimmerman, A., Dinkins, M. B., Khaled, M. L., et al. (2017). A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents. *PLoS One* 12, e0170628–22. doi:10.1371/journal.pone.0170628
- Holcar, M., Ferdin, J., Sitar, S., Tušek-Znidarič, M., Dolžan, V., Plemenitaš, A., et al. (2020). Enrichment of Plasma Extracellular Vesicles for Reliable Quantification of Their Size and Concentration for Biomarker Discovery. *Sci. Rep.* 10, 21346–21413. doi:10.1038/s41598-020-78422-y
- Hong, C. S., Funk, S., Muller, L., Boyiadzis, M., and Whiteside, T. L. (2016). Isolation of Biologically Active and Morphologically Intact Exosomes from Plasma of Patients with Cancer. *J. Extracell. Vesicles* 5, 29289–29311. doi:10.3402/jev.v5.29289
- Hoshino, A., Kim, H. S., Bojmar, L., Gyan, K. E., Cioffi, M., Hernandez, J., et al. (2020). Extracellular Vesicle and Particle Biomarkers Define Multiple Human Cancers. *Cell* 182, 1044, 2020. e18. doi:10.1016/j.cell.2020.07.009
- Hosseinkhani, B., van den Akker, N., D'Haen, J., Gagliardi, M., Struys, T., Lambrechts, I., et al. (2017). Direct Detection of Nano-Scale Extracellular Vesicles Derived from Inflammation-Triggered Endothelial Cells Using Surface Plasmon Resonance. *Nanomedicine* 13, 1663–1671. doi:10.1016/j.nano.2017.03.010
- Hosseinkhani, B., van den Akker, N. M. S., Molin, D. G. M., and Michiels, L. (2020). (Sub)populations of Extracellular Vesicles Released by TNF- α -triggered Human Endothelial Cells Promote Vascular Inflammation and Monocyte Migration. *J. Extracell. Vesicles* 9, 1801153–1801217. doi:10.1080/20013078.2020.1801153
- Jeppesen, D. K., Fenix, A. M., Franklin, J. L., Higginbotham, J. N., Zhang, Q., Zimmerman, L. J., et al. (2019). Reassessment of Exosome Composition. *Cell* 177, 428–e18. doi:10.1016/j.cell.2019.02.029
- Ji, X., Huang, S., Zhang, J., Bruce, T. F., Tan, Z., Wang, D., et al. (2021). A Novel Method of High-Purity Extracellular Vesicle Enrichment from Microliter-Scale Human Serum for Proteomic Analysis. *Electrophoresis* 42, 245–256. doi:10.1002/elps.202000223
- Johnsen, K. B., Gudbergsson, J. M., Andresen, T. L., and Simonsen, J. B. (2019). What Is the Blood Concentration of Extracellular Vesicles? Implications for the Use of Extracellular Vesicles as Blood-Borne Biomarkers of Cancer. *Biochim. Biophys. Acta Rev. Cancer* 1871, 109–116. doi:10.1016/j.bbcan.2018.11.006
- Jung, S., Jacobs, K. F. K., Shein, M., Schütz, A. K., Mohr, F., Stadler, H., et al. (2020). Efficient and Reproducible Depletion of Hepatitis B Virus from Plasma Derived Extracellular Vesicles. *J. Extracellular Vesicles* 10, 1–11. doi:10.1002/jev.2.12040
- Jureka, A. S., Silvas, J. A., and Basler, C. F. (2020). Propagation, Inactivation, and Safety Testing of SARS-CoV-2. *Viruses* 12, 1–13. doi:10.3390/v12060622
- Kamal, N. N. S. B. N. M., and Shahidan, W. N. S. (2020). Non-exosomal and Exosomal Circulatory microRNAs: Which Are More Valid as Biomarkers? *Front. Pharmacol.* 10, 1–22. doi:10.3389/fphar.2019.01500
- Karasu, E., Eisenhardt, S. U., Harant, J., and Huber-Lang, M. (2018). Extracellular Vesicles: Packages Sent with Complement. *Front. Immunol.* 9, 721–815. doi:10.3389/fimmu.2018.00721
- Karimi, N., Cvjetkovic, A., Jang, S. C., Crescitelli, R., Hosseinpour Feizi, M. A., Nieuwland, R., et al. (2018). Detailed Analysis of the Plasma Extracellular Vesicle Proteome after Separation from Lipoproteins. *Cell. Mol. Life Sci.* 75, 2873–2886. doi:10.1007/s00018-018-2773-4
- Keller, L., Belloum, Y., Wikman, H., and Pantel, K. (2021). Clinical Relevance of Blood-Based ctDNA Analysis: Mutation Detection and beyond. *Br. J. Cancer* 124, 345–358. doi:10.1038/s41416-020-01047-5
- Konoshenko, M. Y., Lekhnov, E. A., Vlassov, A. V., and Laktionov, P. P. (2018/2018). Isolation of Extracellular Vesicles: General Methodologies and Latest Trends. *Biomed. Res. Int.* 2018, 8545347–8545427. doi:10.1155/2018/8545347
- Laurenzana, I., Trino, S., Lamorte, D., Girasole, M., Dinarelli, S., De Stradis, A., et al. (2021). Analysis of Amount, Size, Protein Phenotype and Molecular Content of Circulating Extracellular Vesicles Identifies New Biomarkers in Multiple Myeloma. *Int. J. Nanomedicine* 16, 3141–3160. doi:10.2147/IJN.S303391
- Leeman, M., Choi, J., Hansson, S., Storm, M. U., and Nilsson, L. (2018). Proteins and Antibodies in Serum, Plasma, and Whole Blood-Size Characterization Using Asymmetrical Flow Field-Flow Fractionation (AF4). *Anal. Bioanal. Chem.* 410, 4867–4873. doi:10.1007/s00216-018-1127-2
- Lenassi, M., Cagney, G., Liao, M., Vaupotic, T., Bartholomeeusens, K., Cheng, Y., et al. (2010). HIV Nef Is Secreted in Exosomes and Triggers Apoptosis in Bystander CD4+ T Cells. *Traffic* 11, 110–122. doi:10.1111/j.1600-0854.2009.01006.x.HIV
- Liang, Y., Huang, S., Qiao, L., Peng, X., Li, C., Lin, K., et al. (2020). Characterization of Protein, Long Noncoding RNA and microRNA Signatures in Extracellular Vesicles Derived from Resting and Degranulated Mast Cells. *J. Extracell. Vesicles* 9, 1697583–1697621. doi:10.1080/20013078.2019.1697583
- Liangsapree, T., Multia, E., and Riekkola, M. L. (2021). Modern Isolation and Separation Techniques for Extracellular Vesicles. *J. Chromatogr. A* 1636, 461773. doi:10.1016/j.chroma.2020.461773
- Liu, C., Zhao, J., Tian, F., Cai, L., Zhang, W., Feng, Q., et al. (2019). Low-cost Thermophoretic Profiling of Extracellular-Vesicle Surface Proteins for the Early Detection and Classification of Cancers. *Nat. Biomed. Eng.* 3, 183–193. doi:10.1038/s41551-018-0343-6
- Lötvall, J., Skog, J., Vlassov, A. V., Sádico, A. A., Rohde, E., Gere, J., et al. (2014). Short Course in Extracellular Vesicles - the Transition from Tissue to Liquid Biopsies. *J. Circulating Biomarkers* 3, 8–5. doi:10.5772/60053
- Lu, J., Pang, J., Chen, Y., Dong, Q., Sheng, J., Luo, Y., et al. (2019). Application of Microfluidic Chips in Separation and Analysis of Extracellular Vesicles in Liquid Biopsy for Cancer. *Micromachines (Basel)* 10, 390. doi:10.3390/mi10060390
- Lydic, T. A., Townsend, S., Adda, C. G., Collins, C., Mathivanan, S., and Reid, G. E. (2016). Rapid and Comprehensive 'shotgun' Lipidome Profiling of Colorectal Cancer Cell Derived Exosomes. *Methods* 87, 83–95. doi:10.1016/j.jymeth.2015.04.014
- Maione, F., Cappellano, G., Bellan, M., Raineri, D., and Chiocchetti, A. (2020). Chicken-or-egg Question: Which Came First, Extracellular Vesicles or Autoimmune Diseases? *J. Leukoc. Biol.* 108, 601–616. doi:10.1002/JLB.3MR0120-232R
- Martin-Jaular, L., Nevo, N., Schessner, J. P., Tkach, M., Jouve, M., Dingli, F., et al. (2021). Unbiased Proteomic Profiling of Host Cell Extracellular Vesicle Composition and Dynamics upon HIV-1 Infection. *EMBO J.* 40, e105492–25. doi:10.15252/embj.2020105492
- Martins, S. d. T., and Alves, L. R. (2020). Extracellular Vesicles in Viral Infections: Two Sides of the Same coin? *Front. Cel. Infect. Microbiol.* 10, 1–14. doi:10.3389/fcimb.2020.593170
- Mateescu, B., Kowal, E. J., van Balkom, B. W., Bartel, S., Bhattacharyya, S. N., Buzás, E. I., et al. (2017). Obstacles and Opportunities in the Functional Analysis of Extracellular Vesicle RNA - an ISEV Position Paper. *J. Extracell. Vesicles* 6, 1286095–1286133. doi:10.1080/20013078.2017.1286095
- Mathieu, M., Martin-Jaular, L., Lavieu, G., and Théry, C. (2019). Specificities of Secretion and Uptake of Exosomes and Other Extracellular Vesicles for Cell-To-Cell Communication. *Nat. Cel Biol.* 21, 9–17. doi:10.1038/s41556-018-0250-9
- Matsumoto, A., Takahashi, Y., Chang, H. Y., Wu, Y. W., Yamamoto, A., Ishihama, Y., et al. (2020). Blood Concentrations of Small Extracellular Vesicles Are Determined by a Balance between Abundant Secretion and Rapid Clearance. *J. Extracell. Vesicles* 9, 1696517–1696616. doi:10.1080/20013078.2019.1696517
- McVey, M. J., Spring, C. M., and Kuebler, W. M. (2018). Improved Resolution in Extracellular Vesicle Populations Using 405 Instead of 488 Nm Side Scatter. *J. Extracell. Vesicles* 7, 1454776–1454812. doi:10.1080/20013078.2018.1454776
- Meng, Y., Asghari, M., Aslan, M. K., Yilmaz, A., Mateescu, B., Stavakis, S., et al. (2021). Microfluidics for Extracellular Vesicle Separation and Mimetic Synthesis: Recent Advances and Future Perspectives. *Chem. Eng. J.* 404, 126110. doi:10.1016/j.cej.2020.126110

- Monguió-Tortajada, M., Gálvez-Montón, C., Bayes-Genis, A., Roura, S., and Borràs, F. E. (2019). Extracellular Vesicle Isolation Methods: Rising Impact of Size-Exclusion Chromatography. *Cel. Mol. Life Sci.* 76, 2369–2382. doi:10.1007/s00018-019-03071-y
- Morales-Kastresana, A., Telford, B., Musich, T. A., McKinnon, K., Clayborne, C., Braig, Z., et al. (2017). Labeling Extracellular Vesicles for Nanoscale Flow Cytometry. *Sci. Rep.* 7, 1878–1910. doi:10.1007/978-3-540-71693-8_810.1038/s41598-017-01731-2
- Mork, M., Pedersen, S., Botha, J., Lund, S. M., and Kristensen, S. R. (2016). Preanalytical, Analytical, and Biological Variation of Blood Plasma Submicron Particle Levels Measured with Nanoparticle Tracking Analysis and Tunable Resistive Pulse Sensing. *Scand. J. Clin. Lab. Invest.* 76, 349–360. doi:10.1080/00365513.2016.1178801
- Mork, M., Handberg, A., Pedersen, S., Jørgensen, M. M., Bæk, R., Nielsen, M. K., et al. (2017). Prospects and Limitations of Antibody-Mediated Clearing of Lipoproteins from Blood Plasma Prior to Nanoparticle Tracking Analysis of Extracellular Vesicles. *J. Extracellular Vesicles* 6, 1308779–1308810. doi:10.1080/20013078.2017.1308779
- Morrison, G. J., and Goldkorn, A. (2018). Development and Application of Liquid Biopsies in Metastatic Prostate Cancer. *Curr. Oncol. Rep.* 20, 35–39. doi:10.1007/s11912-018-0683-0
- Muller, L., Hong, C. S., Stolz, D. B., Watkins, S. C., and Whiteside, T. L. (2014). Isolation of Biologically-Active Exosomes from Human Plasma. *J. Immunol. Methods* 411, 55–65. doi:10.1016/j.jim.2014.06.007
- Murata, K., and Wolf, M. (20181862). Cryo-electron Microscopy for Structural Analysis of Dynamic Biological Macromolecules. *Biochim. Biophys. Acta Gen. Subj* 1862, 324–334. doi:10.1016/j.bbagen.2017.07.020
- Nakajima, K., Hinman, J., Pfaffinger, D., Edelstein, C., and Scanu, A. M. (2001). Changes in Plasma Triglyceride Levels Shift Lipoprotein(a) Density in Parallel with that of LDL Independently of Apolipoprotein(a) Size. *Arterioscler Thromb. Vasc. Biol.* 21, 1238–1243. doi:10.1161/hq0701.092246
- Nardi, Fda. S., Michelon, T. F., Neumann, J., Manvailer, L. F., Wagner, B., Horn, P. A., et al. (2016). High Levels of Circulating Extracellular Vesicles with Altered Expression and Function during Pregnancy. *Immunobiology* 221, 753–760. doi:10.1016/j.imbio.2016.03.001
- Navarro, A., Molins, L., Marrades, R. M., Moises, J., Viñolas, N., Morales, S., et al. (2019). Exosome Analysis in Tumor-Draining Pulmonary Vein Identifies Nscl Patients with Higher Risk of Relapse after Curative Surgery. *Cancers (Basel)* 11, 1–11. doi:10.3390/cancers11020249
- Nguyen, V. H., and Lee, B. J. (2017). Protein corona: a New Approach for Nanomedicine Design. *Int. J. Nanomedicine* 12, 3137–3151. doi:10.2147/IJN.S129300
- Nieuwland, R., Falcón-Pérez, J. M., Théry, C., and Witwer, K. W. (2020). Rigor and Standardization of Extracellular Vesicle Research: Paving the Road towards Robustness. *J. Extracellular Vesicles* 10, 1–5. doi:10.1002/jev2.12037
- Nolte-’t Hoen, E., Cremer, T., Gallo, R. C., Margolis, L. B., Hoen, E. N., Cremer, T., et al. (2016). Extracellular Vesicles and Viruses: Are They Close Relatives? *Proc. Natl. Acad. Sci. U. S. A.* 113, 9155–9161. doi:10.1073/pnas.1605146113
- Noren Hooten, N. (2020). Extracellular Vesicles and Extracellular RNA in Aging and Age-Related Disease. *Transl. Med. Aging* 4, 96–98. doi:10.1016/j.tma.2020.07.006
- Notarangelo, M., Zucal, C., Modelska, A., Pesce, I., Scarducci, G., Potrich, C., et al. (2019). Ultrasensitive Detection of Cancer Biomarkers by Nickel-Based Isolation of Polydisperse Extracellular Vesicles from Blood. *EBioMedicine* 43, 114–126. doi:10.1016/j.ebiom.2019.04.039
- Nunez Lopez, Y. O., Casu, A., and Pratley, R. E. (2021). Investigation of Extracellular Vesicles from Sars-Cov-2 Infected Specimens: a Safety Perspective. *Front. Immunol.* 12, 1–8. doi:10.3389/fimmu.2021.617042
- Oeyen, E., Van Mol, K., Baggerman, G., Willems, H., Boonen, K., Rolfo, C., et al. (2018). Ultrafiltration and Size Exclusion Chromatography Combined with Asymmetrical-Flow Field-Flow Fractionation for the Isolation and Characterisation of Extracellular Vesicles from Urine. *J. Extracell. Vesicles* 7, 1490143–1490212. doi:10.1080/20013078.2018.1490143
- Onódi, Z., Pelyhe, C., Terézia Nagy, C., Brenner, G. B., Almási, L., Kittel, Á., et al. (2018). Isolation of High-Purity Extracellular Vesicles by the Combination of Iodixanol Density Gradient Ultracentrifugation and Bind-Elute Chromatography from Blood Plasma. *Front. Physiol.* 9, 1–11. doi:10.3389/fphys.2018.01479
- Palmieri, V., Lucchetti, D., Gatto, I., Maiorana, A., Marcantoni, M., Maulucci, G., et al. (2014). Dynamic Light Scattering for the Characterization and Counting of Extracellular Vesicles: a Powerful Noninvasive Tool. *J. Nanopart. Res.* 16, 1–8. doi:10.1007/s11051-014-2583-z
- Palviainen, M., Saari, H., Kärkkäinen, O., Pekkinen, J., Auriola, S., Yliperttula, M., et al. (2019). Metabolic Signature of Extracellular Vesicles Depends on the Cell Culture Conditions. *J. Extracell. Vesicles* 8, 1596669–1596713. doi:10.1080/20013078.2019.1596669
- Palviainen, M., Saraswat, M., Varga, Z., Kitka, D., Neuvonen, M., Puhka, M., et al. (2020). Extracellular Vesicles from Human Plasma and Serum Are Carriers of Extravesicular Cargo-Implications for Biomarker Discovery. *PLoS One* 15, e0236439–19. doi:10.1371/journal.pone.0236439
- Pang, B., Zhu, Y., Ni, J., Thompson, J., Malouf, D., Bucci, J., et al. (2020). Extracellular Vesicles: the Next Generation of Biomarkers for Liquid Biopsy-Based Prostate Cancer Diagnosis. *Theranostics* 10, 2309–2326. doi:10.7150/thno.39486
- Parisse, P., Rago, I., Ulloa Severino, L., Perissinotto, F., Ambrosetti, E., Paoletti, P., et al. (2017). Atomic Force Microscopy Analysis of Extracellular Vesicles. *Eur. Biophys. J.* 46, 813–820. doi:10.1007/s00249-017-1252-4
- Pasetto, L., Callegaro, S., Corbelli, A., Fiordaliso, F., Ferrara, D., Brunelli, L., et al. (2021). Decoding Distinctive Features of Plasma Extracellular Vesicles in Amyotrophic Lateral Sclerosis. *Mol. Neurodegener.* 16, 52–21. doi:10.1186/s13024-021-00470-3
- Pasquetto, V., Santonastaso, A., Grandi, S., Derosa, G., D’Angelo, A., and Scotti, C. (2016). Lipoprotein (A) Particles Characterization by Dynamic Light Scattering. *Bjmmr* 16, 1–8. doi:10.9734/BJMMR/2016/27076
- Patel, G. K., Khan, M. A., Zubair, H., Srivastava, S. K., Khushman, M., Singh, S., et al. (2019). Comparative Analysis of Exosome Isolation Methods Using Culture Supernatant for Optimum Yield, Purity and Downstream Applications. *Sci. Rep.* 9, 5335–5410. doi:10.1038/s41598-019-41800-2
- Pietrowska, M., Wlosowicz, A., Gawin, M., and Widlak, P. (2019). MS-based Proteomic Analysis of Serum and Plasma: Problem of High Abundant Components and Lights and Shadows of Albumin Removal. *Adv. Exp. Med. Biol.* 1073, 57–76. doi:10.1007/978-3-030-12298-0_3
- Pocsfalvi, G., Stanly, C., Vilasi, A., Fiume, I., Capasso, G., Turiák, L., et al. (2016). Mass Spectrometry of Extracellular Vesicles. *Mass. Spectrom. Rev.* 35, 3–21. doi:10.1002/mas.21457
- Popovic, M., Mazzega, E., Toffoletto, B., and de Marco, A. (2018). Isolation of Anti-extra-cellular Vesicle Single-Domain Antibodies by Direct Panning on Vesicle-Enriched Fractions. *Microb. Cel Fact.* 17, 6–13. doi:10.1186/s12934-017-0856-9
- Pugholm, L. H., Bæk, R., Søndergaard, E. K., Revenfeld, A. L., Jørgensen, M. M., and Varming, K. (2016). Phenotyping of Leukocytes and Leukocyte-Derived Extracellular Vesicles. *J. Immunol. Res.* 2016, 6391264–6391312. doi:10.1155/2016/6391264
- Puhm, F., Boilard, E., and MacHlus, K. R. (2021). Platelet Extracellular Vesicles: Beyond the Blood. *Arterioscler. Thromb. Vasc. Biol.* 41, 87–96. doi:10.1161/ATVBAHA.120.314644
- Ramirez, M. I., Amorim, M. G., Gadelha, C., Milic, I., Welsh, J. A., Freitas, V. M., et al. (2018). Technical Challenges of Working with Extracellular Vesicles. *Nanoscale* 10, 881–906. doi:10.1039/c7nr08360b
- Ramshani, Z., Zhang, C., Richards, K., Chen, L., Xu, G., Stiles, B. L., et al. (2019). Extracellular Vesicle microRNA Quantification from Plasma Using an Integrated Microfluidic Device. *Commun. Biol.* 2, 189–9. doi:10.1038/s42003-019-0435-1
- Rausch, T. K., Schillert, A., Ziegler, A., Lükking, A., Zucht, H. D., and Schulz-Knappe, P. (2016). Comparison of Pre-processing Methods for Multiplex Bead-Based Immunoassays. *BMC Genomics* 17, 601–610. doi:10.1186/s12864-016-2888-7
- Rosa-Fernandes, L., Rocha, V. B., Carregari, V. C., Urbani, A., and Palmisano, G. (2017). A Perspective on Extracellular Vesicles Proteomics. *Front. Chem.* 5, 1–19. doi:10.3389/fchem.2017.00102
- Rozowsky, J., Kitchen, R. R., Park, J. J., Galeev, T. R., Diao, J., Warrell, J., et al. (2019). exceRpt: A Comprehensive Analytic Platform for Extracellular Rna Profiling. *Cell Syst* 8, 352–e3. doi:10.1016/j.cels.2019.03.004
- Ruhen, O., and Meehan, K. (2019). Tumor-derived Extracellular Vesicles as a Novel Source of Protein Biomarkers for Cancer Diagnosis and Monitoring. *Proteomics* 19, e1800155. doi:10.1002/pmic.201800155

- Salafi, T., Zhang, Y., and Zhang, Y. (2019). A Review on Deterministic Lateral Displacement for Particle Separation and Detection. *Nano-micro Lett.* 11, 77. doi:10.1007/s40820-019-0308-7
- Scheffer, P. G., Bakker, S. J., Heine, R. J., and Teerlink, T. (1997). Measurement of Low-Density Lipoprotein Particle Size by High-Performance Gel-Filtration Chromatography. *Clin. Chem.* 43, 1904–1912. doi:10.1093/clinchem/43.10.1904
- Shamsi, K. S., Pierce, A., Ashton, A. S., Halade, D. G., Richardson, A., and Espinoza, S. E. (2012). Proteomic Screening of Glycoproteins in Human Plasma for Frailty Biomarkers. *J. Gerontol. A. Biol. Sci. Med. Sci.* 67, 853–864. doi:10.1093/gerona/glr224
- Shao, H., Im, H., Castro, C. M., Breakefield, X., Weissleder, R., and Lee, H. (2018). New Technologies for Analysis of Extracellular Vesicles. *Chem. Rev.* 118, 1917–1950. doi:10.1021/acs.chemrev.7b00534
- Sharma, S., Leclaire, M., and Gimzewski, J. K. (2018). Ascent of Atomic Force Microscopy as a Nanoanalytical Tool for Exosomes and Other Extracellular Vesicles. *Nanotechnology* 29, 132001–132014. doi:10.1088/1361-6528/aaab06
- Shelke, G. V., Lässer, C., Ghossein, Y. S., and Lötvall, J. (2014). Importance of Exosome Depletion Protocols to Eliminate Functional and RNA-Containing Extracellular Vesicles from Fetal Bovine Serum. *J. Extracell. Vesicles* 3, 1–8. doi:10.3402/jev.v3.24783
- Shi, L., Kuhnell, D., Borra, V. J., Langevin, S. M., Nakamura, T., and Esfandiari, L. (2019). Rapid and Label-free Isolation of Small Extracellular Vesicles from Biofluids Utilizing a Novel Insulator Based Dielectrophoretic Device. *Lab. Chip* 19, 3726–3734. doi:10.1039/c9lc00902g
- Sidhom, K., Obi, P. O., and Saleem, A. (2020). A Review of Exosomal Isolation Methods: Is Size Exclusion Chromatography the Best Option? *Int. J. Mol. Sci.* 21, 1–19. doi:10.3390/ijms21186466
- Simonsen, J. B. (2017). What Are We Looking at? Extracellular Vesicles, Lipoproteins, or Both? *Circ. Res.* 121, 920–922. doi:10.1161/CIRCRESAHA.117.311767
- Singh, P., Szegedy, I. C., Ricci, M., Zsila, F., Juhász, T., Mihály, J., et al. (2020). Membrane Active Peptides Remove Surface Adsorbed Protein corona from Extracellular Vesicles of Red Blood Cells. *Front. Chem.* 8, 1–10. doi:10.3389/fchem.2020.00703
- Sitar, S., Kejžar, A., Pahovnik, D., Kogej, K., Tušek-Žnidarič, M., Lenassi, M., et al. (2015). Size Characterization and Quantification of Exosomes by Asymmetrical-Flow Field-Flow Fractionation. *Anal. Chem.* 87, 9225–9233. doi:10.1021/acs.analchem.5b01636
- Sitar, S., Vezočnik, V., Maček, P., Kogej, K., Pahovnik, D., and Žagar, E. (2017). Pitfalls in Size Characterization of Soft Particles by Dynamic Light Scattering Online Coupled to Asymmetrical Flow Field-Flow Fractionation. *Anal. Chem.* 89, 11744–11752. doi:10.1021/acs.analchem.7b03251
- Skliar, M., Chernyshev, V. S., Belnap, D. M., Sergey, G. V., Al-Hakami, S. M., Bernard, P. S., et al. (2018). Membrane Proteins Significantly Restrict Exosome Mobility. *Biochem. Biophys. Res. Commun.* 501, 1055–1059. doi:10.1016/j.bbrc.2018.05.107
- Sódar, B. W., Kittel, Á., Pálóczi, K., Vukman, K. V., Osteikoetxea, X., Szabó-Taylor, K., et al. (2016). Low-density Lipoprotein Mimics Blood Plasma-Derived Exosomes and Microvesicles during Isolation and Detection. *Sci. Rep.* 6, 24316–24412. doi:10.1038/srep24316
- Sódar, B. W., Kovács, Á., Visnovitz, T., Pállinger, É., Vékey, K., Pocsfalvi, G., et al. (2017). Best Practice of Identification and Proteomic Analysis of Extracellular Vesicles in Human Health and Disease. *Expert Rev. Proteomics* 14, 1073–1090. doi:10.1080/14789450.2017.1392244
- Soekmadji, C., Hill, A. F., Wauben, M. H., Buzás, E. I., Di Vizio, D., Gardiner, C., et al. (2018). Towards Mechanisms and Standardization in Extracellular Vesicle and Extracellular RNA Studies: Results of a Worldwide Survey. *J. Extracell. Vesicles* 7, 1535745–1535746. doi:10.1080/20013078.2018.1535745
- Soekmadji, C., Li, B., Huang, Y., Wang, H., An, T., Liu, C., et al. (2020). The Future of Extracellular Vesicles as Theranostics - an ISEV Meeting Report. *J. Extracell. Vesicles* 9, 1809766. doi:10.1080/20013078.2020.1809766
- Srinivasan, S., Duval, M. X., Kaimal, V., Cuff, C., and Clarke, S. H. (2019). Assessment of Methods for Serum Extracellular Vesicle Small RNA Sequencing to Support Biomarker Development. *J. Extracell. Vesicles* 8, 1684425–1684512. doi:10.1080/20013078.2019.1684425
- Stewart, S., Gessler, F., Pluchino, S., and Moreau, K. (2016). Inside-out: Unpredicted Annexin A2 Localisation on the Surface of Extracellular Vesicles. *Matters (Zür.)*, 1–8. doi:10.19185/matters.201602000015
- Štok, U., Blokar, E., Lenassi, M., Holcar, M., Frank-bertoncelj, M., Erman, A., et al. (2020). Characterization of Plasma-Derived Small Extracellular Vesicles Indicates Ongoing Endothelial and Platelet Activation in Patients with Thrombotic Antiphospholipid Syndrome. *Cells* 9, 1–14. doi:10.3390/cells9051211
- Strojan, K., Leonardi, A., Bregar, V. B., Križaj, I., Svete, J., and Pavlin, M. (2017). Dispersion of Nanoparticles in Different media Importantly Determines the Composition of Their Protein corona. *PLoS One* 12, e0169552–21. doi:10.1371/journal.pone.0169552
- Takov, K., Yellon, D. M., and Davidson, S. M. (2019). Comparison of Small Extracellular Vesicles Isolated from Plasma by Ultracentrifugation or Size-Exclusion Chromatography: Yield, Purity and Functional Potential. *J. Extracell. Vesicles* 8, 1560809–1560818. doi:10.1080/20013078.2018.1560809
- Tang, V. A., Fritzsche, A. K., Renner, T. M., Burger, D., and Lannigan, J. A. (2019). Engineered Retroviruses as Fluorescent Biological Reference Particles for Nanoscale Flow Cytometry. *bioRxiv Prepr.*
- Tang, Y. T., Huang, Y. Y., Zheng, L., Qin, S. H., Xu, X. P., An, T. X., et al. (2017). Comparison of Isolation Methods of Exosomes and Exosomal RNA from Cell Culture Medium and Serum. *Int. J. Mol. Med.* 40, 834–844. doi:10.3892/ijmm.2017.3080
- Taus, F., Meneguzzi, A., Castelli, M., and Minuz, P. (2019). Platelet-derived Extracellular Vesicles as Target of Antiplatelet Agents. What Is the Evidence? *Front. Pharmacol.* 10, 1256–1312. doi:10.3389/fphar.2019.01256
- Taylor, D. D., and Shah, S. (2015). Methods of Isolating Extracellular Vesicles Impact Down-Stream Analyses of Their Cargoes. *Methods* 87, 3–10. doi:10.1016/j.jymeth.2015.02.019
- Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., et al. (2019). Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): a Position Statement of the International Society for Extracellular Vesicles and Update of the MISEV2014 Guidelines. *J. Extracell. Vesicles* 8, 1–43. doi:10.1080/20013078.2018.1535750
- Tian, Y., Gong, M., Hu, Y., Liu, H., Zhang, W., Zhang, M., et al. (2020). Quality and Efficiency Assessment of Six Extracellular Vesicle Isolation Methods by Nano-Flow Cytometry. *J. Extracell. Vesicles* 9, 1697028–1697117. doi:10.1080/20013078.2019.1697028
- Tripsiano, C., Weiss, R., Karuthedom George, S., Fischer, M. B., and Weber, V. (2020). Extracellular Vesicles Derived from Platelets, Red Blood Cells, and Monocyte-like Cells Differ Regarding Their Ability to Induce Factor Xii-dependent Thrombin Generation. *Front. Cel. Dev. Biol.* 8, 298–311. doi:10.3389/fcell.2020.00298
- Tulkens, J., De Wever, O., and Hendrix, A. (2020). Analyzing Bacterial Extracellular Vesicles in Human Body Fluids by Orthogonal Biophysical Separation and Biochemical Characterization. *Nat. Protoc.* 15, 40–67. doi:10.1038/s41596-019-0236-5
- van der Pol, E., Coumans, F. A., Grootemaat, A. E., Gardiner, C., Sargent, I. L., Harrison, P., et al. (2014). Particle Size Distribution of Exosomes and Microvesicles Determined by Transmission Electron Microscopy, Flow Cytometry, Nanoparticle Tracking Analysis, and Resistive Pulse Sensing. *J. Thromb. Haemost.* 12, 1182–1192. doi:10.1111/jth.12602
- van der Pol, E., de Rond, L., Coumans, F. A. W., Gool, E. L., Böing, A. N., Sturk, A., et al. (2018). Absolute Sizing and Label-free Identification of Extracellular Vesicles by Flow Cytometry. *Nanomedicine* 14, 801–810. doi:10.1016/j.nano.2017.12.012
- Van Deun, J., Van Deun, J., Mestdagh, P., Agostinis, P., Akay, Ö., Anand, S., et al. (2017). EV-TRACK: Transparent Reporting and Centralizing Knowledge in Extracellular Vesicle Research. *Nat. Methods* 14, 228–232. doi:10.1038/nmeth.4185
- Varga, Z., Yuana, Y., Grootemaat, A. E., van der Pol, E., Gollwitzer, C., Krumrey, M., et al. (2014). Towards Traceable Size Determination of Extracellular Vesicles. *J. Extracell. Vesicles* 3, 1–10. doi:10.3402/jev.v3.23298
- Vasconcelos, M. H., Caires, H. R., Abols, A., Xavier, C. P. R., and Linē, A. (2019). Extracellular Vesicles as a Novel Source of Biomarkers in Liquid Biopsies for Monitoring Cancer Progression and Drug Resistance. *Drug Resist. Updat* 47, 100647. doi:10.1016/j.drug.2019.100647
- Venkatesan, T., Choi, Y. W., Lee, J., and Kim, Y. K. (2017). Pinus Densiflora Needle Supercritical Fluid Extract Suppresses the Expression of Pro-inflammatory Mediators iNOS, IL-6 and IL-1β, and Activation of

- Inflammatory STAT1 and STAT3 Signaling Proteins in Bacterial Lipopolysaccharide-Challenged Murine Macrophages. *Daru* 25, 18–10. doi:10.1186/s40199-017-0184-y
- Vergauwen, G., Dhondt, B., Van Deun, J., De Smedt, E., Berx, G., Timmerman, E., et al. (2017). Confounding Factors of Ultrafiltration and Protein Analysis in Extracellular Vesicle Research. *Sci. Rep.* 7, 2704–2712. doi:10.1038/s41598-017-02599-y
- Vickers, K. C., Palmisano, B. T., Shoucri, B. M., Shamburek, R. D., and Remaley, A. T. (2011). MicroRNAs Are Transported in Plasma and Delivered to Recipient Cells by High-Density Lipoproteins. *Nat. Cell Biol.* 13, 423–433. doi:10.1038/ncb2210.MicroRNAs
- Wagner, M., Holzschuh, S., Traeger, A., Fahr, A., and Schubert, U. S. (2014). Asymmetric Flow Field-Flow Fractionation in the Field of Nanomedicine. *Anal. Chem.* 86, 5201–5210. doi:10.1021/ac501664t
- Welsh, J. A., Holloway, J. A., Wilkinson, J. S., and Englyst, N. A. (2017). Extracellular Vesicle Flow Cytometry Analysis and Standardization. *Front. Cell Dev. Biol.* 5, 78–87. doi:10.3389/fcell.2017.00078
- Welsh, J. A., Van Der Pol, E., Arkesteijn, G. J. A., Bremer, M., Brisson, A., Coumans, F., et al. (2020a). MIFlowCyt-EV: a Framework for Standardized Reporting of Extracellular Vesicle Flow Cytometry Experiments. *J. Extracell. Vesicles* 9, 1713526–1713617. doi:10.1080/20013078.2020.1713526
- Welsh, J. A., van der Pol, E., Bettin, B. A., Carter, D. R. F., Hendrix, A., Lenassi, M., et al. (2020b). Towards Defining Reference Materials for Measuring Extracellular Vesicle Refractive index, Epitope Abundance, Size and Concentration. *J. Extracell. Vesicles* 9, 1816641–1816717. doi:10.1080/20013078.2020.1816641
- Willis, G. R., Kourembanas, S., and Mitsialis, S. A. (2017). Toward Exosome-Based Therapeutics: Isolation, Heterogeneity, and Fit-For-Purpose Potency. *Front. Cardiovasc. Med.* 4, 1–13. doi:10.3389/fcvm.2017.00063
- Witwer, K. W., Buzás, E. I., Bemis, L. T., Bora, A., Lässer, C., Lötvall, J., et al. (2013). Standardization of Sample Collection, Isolation and Analysis Methods in Extracellular Vesicle Research. *J. Extracell. Vesicles* 2, 1–25. doi:10.3402/jev.v2i0.20360
- Woo, J., Sharma, S., and Gimzewski, J. (2016). The Role of Isolation Methods on a Nanoscale Surface Structure and its Effect on the Size of Exosomes. *J. Circ. Biomark* 5, 11–19. doi:10.5772/64148
- Wu, B., Chen, X., Wang, J., Qing, X., Wang, Z., Ding, X., et al. (2020). Separation and Characterization of Extracellular Vesicles from Human Plasma by Asymmetrical Flow Field-Flow Fractionation. *Anal. Chim. Acta* 1127, 234–245. doi:10.1016/j.aca.2020.06.071
- Wu, F., Yin, Z., Yang, L., Fan, J., Xu, J., Jin, Y., et al. (2019). Smoking Induced Extracellular Vesicles Release and Their Distinct Properties in Non-small Cell Lung Cancer. *J. Cancer* 10, 3435–3443. doi:10.7150/jca.30425
- Wu, M., Ouyang, Y., Wang, Z., Zhang, R., Huang, P. H., Chen, C., et al. (2017). Isolation of Exosomes from Whole Blood by Integrating Acoustics and Microfluidics. *Proc. Natl. Acad. Sci. U S A* 114, 10584–10589. doi:10.1073/pnas.1709210114
- Yang, H. C., and Rhee, W. J. (2021). Single Step *In Situ* Detection of Surface Protein and MicroRNA in Clustered Extracellular Vesicles Using Flow Cytometry. *Jcm* 10, 319–414. doi:10.3390/jcm10020319
- Yu, H., Sun, T., An, J., Wen, L., Liu, F., Bu, Z., et al. (2020). Potential Roles of Exosomes in Parkinson's Disease: From Pathogenesis, Diagnosis, and Treatment to Prognosis. *Front. Cell Dev. Biol.* 8, 86–15. doi:10.3389/fcell.2020.00086
- Yuana, Y., Böing, A. N., Grootemaat, A. E., van der Pol, E., Hau, C. M., Cizmar, P., et al. (2015). Handling and Storage of Human Body Fluids for Analysis of Extracellular Vesicles. *J. Extracell. Vesicles* 4, 29260–29312. doi:10.3402/jev.v4.29260
- Yuana, Y., Levels, J., Grootemaat, A., Sturk, A., and Nieuwland, R. (2014). Co-isolation of Extracellular Vesicles and High-Density Lipoproteins Using Density Gradient Ultracentrifugation. *J. Extracell. Vesicles* 3, 1–5. doi:10.3402/jev.v3.23262
- Zaborowski, M. P., Balaj, L., Breakefield, X. O., and Lai, C. P. (2015). Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study. *Bioscience* 65, 783–797. doi:10.1093/biosci/biv084
- Zhang, H., Freitas, D., Kim, H. S., Fabijanic, K., Li, Z., Chen, H., et al. (2018). Identification of Distinct Nanoparticles and Subsets of Extracellular Vesicles by Asymmetric Flow Field-Flow Fractionation. *Nat. Cell Biol.* 20, 332–343. doi:10.1038/s41556-018-0040-4
- Zhang, H., and Lyden, D. (2019). Asymmetric-flow Field-Flow Fractionation Technology for Exomere and Small Extracellular Vesicle Separation and Characterization. *Nat. Protoc.* 14, 1027–1053. doi:10.1038/s41596-019-0126-x
- Zhang, L., Liang, Y., Li, S., Zeng, F., Meng, Y., Chen, Z., et al. (2019a). The Interplay of Circulating Tumor DNA and Chromatin Modification, Therapeutic Resistance, and Metastasis. *Mol. Cancer* 18, 36–20. doi:10.1186/s12943-019-0989-z
- Zhang, Q., Higginbotham, J. N., Jeppesen, D. K., Yang, Y. P., Li, W., McKinley, E. T., et al. (2019b). Transfer of Functional Cargo in Exomeres. *Cell Rep* 27, 940–e6. doi:10.1016/j.celrep.2019.01.009
- Zhang, X., Borg, E. G. F., Liaci, A. M., Vos, H. R., and Stoorvogel, W. (2020). A Novel Three Step Protocol to Isolate Extracellular Vesicles from Plasma or Cell Culture Medium with Both High Yield and Purity. *J. Extracell. Vesicles* 9, 1791450–1791519. doi:10.1080/20013078.2020.1791450

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Holcar, Kandušer and Lenassi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Multi-Omics Analysis of Microglial Extracellular Vesicles From Human Alzheimer's Disease Brain Tissue Reveals Disease-Associated Signatures

Whitaker Cohn^{1†}, Mikhail Melnik^{2†}, Calvin Huang², Bruce Teter¹, Sujyoti Chandra¹, Chunni Zhu¹, Laura Beth McIntire³, Varghese John¹, Karen H. Gyls^{2‡} and Tina Bilousova^{1,2*‡}

OPEN ACCESS

Edited by:

Satish Balasaheb Nimse,
Hallym University, South Korea

Reviewed by:

Kevin Alvin Clayton,
Boston University, United States
Nilanjana Banerjee,
Manovikas Kendra, India

*Correspondence:

Tina Bilousova
tbilousova@mednet.ucla.edu

[†]These authors have contributed
equally to this work and share first
authorship

[‡]These authors share last authorship

Specialty section:

This article was submitted to
Experimental Pharmacology and Drug
Discovery,
a section of the journal
Frontiers in Pharmacology

Received: 28 August 2021

Accepted: 19 October 2021

Published: 02 December 2021

Citation:

Cohn W, Melnik M, Huang C, Teter B,
Chandra S, Zhu C, McIntire LB,
John V, Gyls KH and Bilousova T
(2021) Multi-Omics Analysis of
Microglial Extracellular Vesicles From
Human Alzheimer's Disease Brain
Tissue Reveals Disease-
Associated Signatures.
Front. Pharmacol. 12:766082.
doi: 10.3389/fphar.2021.766082

¹Drug Discovery Lab, Department of Neurology, University of California, Los Angeles, Los Angeles, CA, United States, ²School of Nursing, University of California, Los Angeles, Los Angeles, CA, United States, ³Taub Institute for Research on Alzheimer's Disease and the Aging Brain, Department of Pathology and Cell Biology, Columbia University Medical Center, New York, NY, United States

Alzheimer's disease (AD) is the most common cause of dementia, yet there is no cure or diagnostics available prior to the onset of clinical symptoms. Extracellular vesicles (EVs) are lipid bilayer-delimited particles that are released from almost all types of cell. Genome-wide association studies have linked multiple AD genetic risk factors to microglia-specific pathways. It is plausible that microglia-derived EVs may play a role in the progression of AD by contributing to the dissemination of insoluble pathogenic proteins, such as tau and A β . Despite the potential utility of EVs as a diagnostic tool, our knowledge of human brain EV subpopulations is limited. Here we present a method for isolating microglial CD11b-positive small EVs from cryopreserved human brain tissue, as well as an integrated multiomics analysis of microglial EVs enriched from the parietal cortex of four late-stage AD (Braak V-VI) and three age-matched normal/low pathology (NL) cases. This integrated analysis revealed 1,000 proteins, 594 lipids, and 105 miRNAs using shotgun proteomics, targeted lipidomics, and NanoString nCounter technology, respectively. The results showed a significant reduction in the abundance of homeostatic microglia markers P2RY12 and TMEM119, and increased levels of disease-associated microglia markers FTH1 and TREM2, in CD11b-positive EVs from AD brain compared to NL cases. Tau abundance was significantly higher in AD brain-derived microglial EVs. These changes were accompanied by the upregulation of synaptic and neuron-specific proteins in the AD group. Levels of free cholesterol were elevated in microglial EVs from the AD brain. Lipidomic analysis also revealed a proinflammatory lipid profile, endolysosomal dysfunction, and a significant AD-associated decrease in levels of docosahexaenoic acid (DHA)-containing polyunsaturated lipids, suggesting a potential defect in acyl-chain remodeling. Additionally, four miRNAs associated with immune and cellular senescence signaling pathways were significantly upregulated in the AD group. Our data suggest that loss of the homeostatic microglia signature in late AD stages may be accompanied by endolysosomal impairment and the release of undigested neuronal and myelin debris, including tau, through extracellular

vesicles. We suggest that the analysis of microglia-derived EVs has merit for identifying novel EV-associated biomarkers and providing a framework for future larger-scale multiomics studies on patient-derived cell-type-specific EVs.

Keywords: Alzheimer's disease, extracellular vesicles, exosomes, microglia, omics analysis

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia, yet there is no cure or diagnostics available prior to the onset of clinical symptoms (2021 Alzheimer's disease facts and figures, 2021). The main neuropathological hallmarks of AD include the accumulation of amyloid beta peptide (A β)-containing plaques, hyperphosphorylated tau protein (p-tau)-composed neurofibrillary tangles, extensive neuroinflammation, synaptic loss, and neuronal cell death (Serrano-Pozoe et al., 2011). Numerous anti-AD drug candidates have proven to be effective in AD animal models but subsequently failed in clinical trials (Mehta et al., 2017). These failures can be attributed to three factors: 1) limited knowledge of the complex cellular and molecular mechanisms causing disease onset; 2) late initiation of the experimental treatments; and 3) inadequate monitoring of the treatment effects due to the absence of a biomarker panel that provides accurate longitudinal information regarding disease progression (Yiannopoulou et al., 2019).

Small extracellular vesicles (EVs), originating either from the plasma membrane (microvesicles) or from multivesicular bodies (exosomes), play a role in many of the major pathological and physiological pathways altered in AD, including A β aggregation (Dinkins et al., 2014; Kokubo, et al., 2005; Rajendran, et al., 2006; Yuyama, et al., 2014), spread of tau and A β seeds (Asai, et al., 2015; Bilousova, et al., 2018; Bilousova, et al., 2020; Joshi, et al., 2014; Sardar Sinha, et al., 2018), neuroinflammation (Pascual et al., 2020), synaptic transmission (An, et al., 2013), cell death (G. Wang, et al., 2012; Winston, et al., 2019), and senescence (D'Anca, et al., 2019). Interestingly, the two greatest genetic risk factors for late-onset AD (LOAD), apolipoprotein E (apoE) and bridging integrator-1 (Bin1), are involved in EV biogenesis and/or cargo sorting (Crotti, et al., 2019; Peng, et al., 2019), suggesting direct involvement of EVs in the development AD pathophysiology. Moreover, the molecular composition of EVs reflects the state and makeup of their cells of origin, and thus, they may be an invaluable resource for identifying important biomarkers of the disease. Indeed, brain-derived EVs cross the blood-brain barrier (BBB) harboring disease-associated molecules such as p-tau and A β . In this context, they provide an accessible reservoir of biomarkers that might predict the development of AD at the asymptomatic stage, as well as the conversion from mild cognitive impairment (MCI)/prodromal stage to clinical AD (Fiandaca, et al., 2015; Goetzl et al., 2016; Goetzl et al., 2018; Hornung et al., 2020; Winston, et al., 2016).

Genome-wide association studies (GWAS) have identified multiple AD risk loci located in or near genes preferentially expressed in microglia, suggesting that microglial dysfunction may have a causative role in disease development (Takatori et al.,

2019). Moreover, single-cell and single-nuclei RNA sequencing analysis (RNA-seq) revealed the presence of disease-associated microglia (DAM) clusters near A β plaques in both animal models and in the human AD brain (Butovsky and Weiner, 2018; Del-Aguila, et al., 2019; Mathys, et al., 2019). DAM populations are characterized by the loss of a homeostatic transcriptional signature, including decreases in purinergic 2Y receptor 12 (P2RY12), P2RY13, transmembrane protein 119 (TMEM119), CX3C chemokine receptor 1 (CX3CR1), and others, and activation of genes responsible for either proinflammatory microglia activation (Stage 1 DAM) or a neurodegeneration restrictive phenotype (Stage 2 DAM). Upregulation of apoE, ferritin heavy chain-1 (FTH-1), beta-2-microglobulin (B2m), major histocompatibility complex class I (MHC class I), and DAPI2 are changes associated with the Stage 1 DAM transcriptome profile, while increases in the triggering receptor expressed on myeloid cells 2 (TREM 2) and exosomal markers CD63 and CD9 are part of the Stage 2 DAM program (Deczkowska, et al., 2018; Xue and Du, 2021). Upregulation of exosomal markers suggests that an increase in exosome production may help to resolve inflammation; on the other hand, microglial EVs, specifically the neutral sphingomyelinase 2 (nSMase2)-dependent exosomal population, take part in spreading tau pathology *in vitro* and in mouse models of tauopathy (Asai, et al., 2015; Maphis, et al., 2015).

Despite progress identifying cell-specific EVs in body fluids (Fiandaca, et al., 2015; Goetzl et al., 2016; Goetzl, Mustapic, et al., 2016; Wang, et al., 2012; Willis, et al., 2017), it is important to note that little is known about the composition of cell-type-specific EV subpopulations isolated from human brain tissue. Changes in the brain EV proteome in human AD cases and animal models suggest the enrichment of a neurodegenerative microglial signature in bulk brain EV preparations (Muraoka, et al., 2020; Muraoka, et al., 2021). Our knowledge of the miRNA transcriptome and lipidome of AD brain-derived EVs is even more limited (Cheng, et al., 2020; Su, et al., 2021). Here we specifically focus on human microglia-derived EVs isolated from cryopreserved human tissue from late AD (Braak V-VI) and normal/low pathology (NL) cases in order to characterize their molecular profiles utilizing an integrative approach combining proteomic, transcriptomic (miRNA), and lipidomic analyses.

MATERIALS AND METHODS

Purification of Small Microglial EVs From Cryopreserved Human Brain Tissue

Human brain parietal cortex samples were prepared as described (Gyls and Bilousova, 2017) using tissue from cases with postmortem interval (PMI) less than or equal to 7 h. Briefly,

following harvesting of the human brain from Brodmann area A7, A39, or A40, the tissue was finely minced (1–3 mm fragments) on ice and suspended in a solution of sucrose and protease inhibitors (0.32 M sucrose, 2 mM EDTA, 2 mM EGTA, 0.2 mM PMSF, 1 mM NaPP, 5 mM NaF, 10 mM Tris-HCL, pH 8.0), with 10 ml sucrose buffer/g tissue, and then slowly frozen to -80°C ; this protocol is based on a described method (Dodd, et al., 1986). On the day of EV isolation, the cryopreserved brain tissue was quickly thawed at 37°C , centrifuged (1,000 $\times$ g, 2 min, 4°C) to remove cryopreservation buffer, and weighed. The tissue was gently dissociated using an adult brain dissociation kit and a GentleMACS dissociator (Miltenyi Biotec) according to the instructions of the manufacturer (0.5 g of tissue per enzymatic reaction). In a recent study comparing six different enzyme protocols for cell dissociation from the rodent brain, this kit yielded the highest number of live cells (Hussain, et al., 2018). After the dissociation step, the cell suspension was passed through a MACS Smart strainer (70 μm ; Miltenyi Biotec). Small EV fractions were purified by sequential centrifugation steps including sucrose density gradient ultracentrifugation followed by washing steps as described (Vella, et al., 2017). This protocol for brain EV isolation has been extensively validated in our previous publications and by others, and enrichment of small EVs in the 0.6 M sucrose layer (F2) has been demonstrated (Bilousova, et al., 2020; Huang, et al., 2020; Vassileff, et al., 2020; Zhu, et al., 2021). Final F2 pellets were resuspended in 25 mM trehalose in PBS, pH 7.4 with a protease and phosphatase inhibitor cocktail (ThermoFisher); the volume of the resuspension solution was adjusted based on the original brain tissue weight (1 g of tissue/150 μl solution). Trehalose prevents EV aggregation and serves as a cryopreservant (Bosch, et al., 2016). Small portions of the F2 small EV fractions were frozen at -80°C for further characterization (see below), and the remainder was used for immunoprecipitation (IP) of microglial EVs using antibodies for the myeloid cell-specific marker CD11b (BioLegend; Clone M1/70) or for control IP reactions with isotype control rat IgG2b antibodies (BioLegend; Clone RTK4530). Covalent coupling of antibodies to Dynabeads M-270 epoxy beads was performed with the Dynabead antibody coupling kit (ThermoFisher; 14311D) according to the instructions of the manufacturer. F2 fractions were incubated with a human-specific FcR blocking reagent (Miltenyi biotec) for 5 min on ice (5 μl of the blocking reagent per 100 μl of F2), followed by 1:20 dilution with 1% bovine serum albumin (BSA) in PBS, pH 7.4. We used 1.25 mg of antibody-coupled Dynabeads per 2 ml of the 1:20 diluted F2 sample. IP reactions were incubated overnight at 4°C with rotation, followed by 4 consecutive 10-min washes with rotation: the first wash with 0.1% BSA/PBS, the second wash with 25 mM citrate-phosphate buffer, pH 5 to reduce the amount of nonspecifically bound material as previously described (Heinzelman et al., 2016), followed by two washes with PBS, pH 7.4. Dynabeads with bound microglial EVs were aliquoted based on the original tissue weight (microglial EVs from 1 g of tissue per aliquot) and frozen at -80°C for downstream analysis.

TABLE 1 | Antibodies used for immunoblot analysis of small EV (F2) fractions and microglial EVs isolated from human parietal cortex.

Target protein	Company	Clone name	Figure number
CD9	ThermoFisher	TS9	1C, E
CD81	ThermoFisher	M38	1C
Syntenin-1	Abcam	EPR8102	1C
Calnexin	Santa Cruz Biotech	AF18	1C
GM130	Cell Signaling	D6B1	1C
CD63	ThermoFisher	TS63	1D
Tau	ThermoFisher	HT7	1E
CD11b	BioLegend	M1/70	1E
TREM2	Abcam	EPR20243	2D
apoE	Abcam	E6D7	2D

Transmission Electron Microscopy (TEM)

For quality control purposes, small amounts of purified brain-derived EVs were loaded on the formvar/carbon 400 mesh copper EM grids (Ted Pella, Inc.), incubated for 30 min, then fixed in 2% paraformaldehyde solution (3 min), followed by washes with distilled water, and staining with 2% uranyl acetate solution (3 min). The grids were dried for at least 1 h at room temperature, and the negative-stained EVs were imaged on a JEOL 100CX electron microscope at 60 kV and $\times 29,000$ magnification.

Tunable Resistive Pulse Sensing Analysis

Size distribution and concentrations of EV samples were measured by the Tunable Resistive Pulse Sensing (TRPS) method using the qNano Gold instrument (Izon Science). NP100 nanopore (particle size range: 50–330 nm) and CPC100 calibration particles were used for the analysis. Data analysis was performed using the qNano instrument software.

Immunoblotting

Microglial EVs were separated from Dynabeads by incubation in a Tris-Glycine SDS sample buffer without reducing agents at 95°C for 5 min after which immunoblot analysis was performed as described above. Proteins from microglial and total small EV fractions were separated by 10–20% Tris-Glycine SDS-PAGE under reducing (50 mM dithiothreitol) or nonreducing (for tetraspanins) conditions, transferred to PVDF membrane, and probed with primary antibodies (Table 1) followed by HRP-conjugated secondary antibodies (Jackson ImmunoResearch). Chemiluminescent signals were obtained with Super Signal West Femto substrate (Thermo Scientific Pierce 34095), detected using the BioSpectrum 600 imaging system, and quantified using the VisionWorks version 6.6A software (UVP; Upland, CA). Data were analyzed by Student's t-test.

Proteomic Analysis

One aliquot of frozen microglial EVs isolated from 1 g of parietal cortex tissue from each case presented in Table 2 was used for the proteomic analysis. Microglial EV samples were diluted in lysis buffer [200 μL , 12 mM sodium lauroyl sarcosine, 0.5% sodium

TABLE 2 | Case information for human samples.

Case ID	Age, years	Sex	PMI	Diagnosis	A β plaque pathology in parietal cortex	Neurofibrillary degeneration stage (Braak)
NL1	65	F	6	MSA, Striatonigral degeneration	0	I
NL2	100	F	4	Normal (mild Braak changes)	0	III
NL3	91	F	7	Normal, multiple infarctions	0	II
AD1	86	M	6	AD, vascular dementia	Severe	V
AD2	95	F	6	AD, vascular dementia	Severe	VI
AD3	95	M	5	AD, LB in SN&LC, CAA	Moderate	V
AD4	88	M	7	AD, diffuse LB	Severe	VI

F, female; M, male; AD, Alzheimer disease; CAA, cerebral amyloid angiopathy; MSE, multiple system atrophy; SN, substantia nigra; PMI, postmortem interval; LB, Lewy bodies.

deoxycholate, 50 mM triethylammonium bicarbonate (TEAB)] and Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Waltham, MA), and then were treated with tris(2-carboxyethyl) phosphine (10 mM, 30 min, 60°C), alkylated (chloroacetamide 40 mM, 30 min, 25°C in the dark), and digested with Sequencing Grade Modified Trypsin (Promega, Madison, WI; 1 μ g, 37°C, overnight). The samples were then desalted on C18 StageTips according to Rappsilber's protocol (Rappsilber et al., 2007). The collected eluent was then chemically modified using a TMT10plex Isobaric Label Reagent Set (Thermo Fisher Scientific) per the protocol of the manufacturer. The samples were pooled according to the protein content (1 μ g of peptide from each sample) and desalted again according to Rappsilber's protocol (Rappsilber, et al., 2007). The eluants were injected onto a reverse-phase nanobore HPLC column (AcuTech Scientific, C18, 1.8- μ m particle size, 360 μ m x 20 cm, 150 μ m ID), equilibrated in solvent A, and eluted (300 nl/min) with an increasing concentration of solvent B (acetonitrile/water/FA, 98/2/0.1, v/v/v: min/% B; 0/0, 5/3, 18/7, 74/12, 144/24, 153/27, 162/40, 164/80, 174/80, 176/0, 180/0) using a Dionex UltiMate 3,000 RSLCnano System (Thermo Fisher Scientific). The effluent from the column was directed to a nanospray ionization source connected to an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) acquiring mass spectra utilizing the Synchronous Precursor Selection (SPS) MS3 method in which isolation and MS3 fragmentation of MS2 fragment ions eliminate isolation interference and dynamic range compression often observed in isobaric tandem mass tag-based proteomics experiments.

For statistical analysis, the raw data were searched against the Uniprot human reviewed protein database using SEQUEST-HT in Proteome Discoverer (Version 2.4, Thermo Scientific), which provided measurements of relative abundance of the identified peptides. Decoy database searching was used to generate high confidence tryptic peptides (FDR < 1%). Tryptic peptides containing amino acid sequences unique to individual proteins were used to identify and provide relative quantification between proteins in each sample. Between-group comparisons were analyzed using the abundance ratio *p*-value (Student's *t*-test). Gene set enrichment gene ontology (GO) and pathway analysis was performed by the STRING database (version 11.5), which was used for functional interpretation of the proteomics data and

provided *p*-values corrected by the FDR method (Szklarczyk, et al., 2019).

MicroRNA Transcriptomics

Microglial EV samples isolated from 1 g of parietal cortex tissue from each case presented in **Table 2** were used for RNA purification with the SerMir Exosome RNA Column Purification Kit (Systems Bioscience Inc). Purified RNA samples were run on nCounter human microRNA panel Human v3 miRNA according to the instructions of the manufacturer (NanoString). The panel contains 800 pairs of probes specific for a predefined set of biologically relevant miRNAs, which were combined with a series of internal controls to form a Human miRNA Panel CodeSet (NanoString Technologies). Raw counts for miRNA targets were analyzed using the Nanostring nCounter Digital Analyzer software according to the instructions of the manufacture.

Statistical analysis of NanoString nCounter data was performed as described (Brumbaugh et al., 2011). Only miRNAs that were above background in five out of seven samples from two groups (NL and AD) were selected for the analysis, which resulted in normalized counts and a list of differentially expressed microRNAs that significantly differ between NL and AD groups. Using the miRNet software, we then identified top pathways in the Reactome pathway database, which can be controlled by the miRNAs shown to be significantly altered in the AD group compared to NL by Student's *t*-test; *p*-values were corrected by the FDR method.

Lipidomic Analysis

We interrogated 34 classes, including free cholesterol (FC), cholesteryl ester (CE), acyl carnitine (AC), monoacylglycerol (MG), diacylglycerol (DG), triacylglycerol (TG), ceramide (Cer), sphingomyelin (SM), monohexosylceramide (MhCer), sulfatides (Sulf), lactosylceramide (LacCer), monosialodihexosylganglioside (GM3), globotriaosylceramide (GB3), phosphatidic acid (PA), phosphatidylcholine (PC), ether phosphatidylcholine (PCe), phosphatidylethanolamine (PE), plasmalogen phosphatidylethanolamine (Pep), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), bis(monoacylglycerol)phosphate (BMP), acyl phosphatidylglycerol (AcylPG), lysophosphatidylcholine (LPC), ether lysophosphatidylcholine

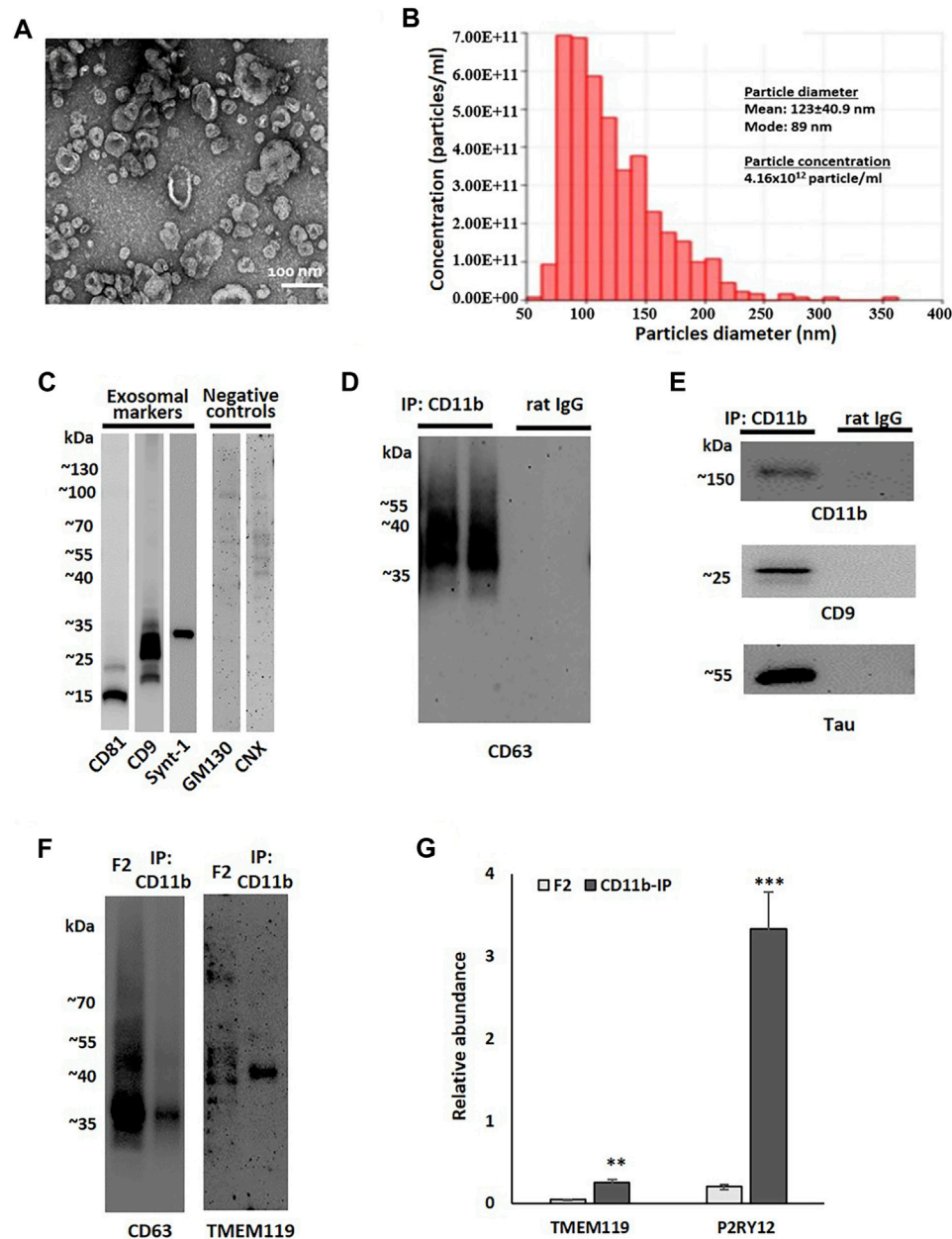


FIGURE 1 | Small EV fraction characterization and microglial EV purification. **(A)** Representative transmission electron microscopy (TEM) image of small EV fraction (F2) isolated from the human parietal cortex. **(B)** Tunable resistive pulse sensing (TRPS) analysis of the F2 fraction isolated from the human parietal cortex. **(C)** A representative image of immunoblot analysis of an F2 fraction using antibodies against exosomal markers (CD9, CD81, and syntenin-1) and negative control markers (GM130 and calnexin, CNX). **(D)** A representative image of immunoblot analysis of two immunoprecipitation (IP) samples pull-down either with antibodies against microglial marker CD11b or with corresponding isotype control antibodies (rat IgG2b). Membrane was probed with the exosomal marker CD63. **(E)** Immunoblotting of CD11b-IP samples and isotype control samples probed with antibodies against a microglial marker (CD11b), an exosomal marker (CD9), and tau. **(F)** Immunoblotting of the F2 fraction isolated from the human parietal cortex and corresponded CD11b-IP fraction with antibodies against the exosomal marker CD63 and the microglial marker TMEM119. The sample volume was equal between CD63 and TMEM119 immunoblots. **(G)** Relative abundances of microglial markers, TMEM119 and P2RY12, in F2 ($n = 6$) and CD11b-IP samples ($n = 7$) as indicated by LC-MS/MS (** $p < 0.001$; *** $p < 0.0001$).

(LPCe), lysophosphatidylethanolamine (LPE), plasmalogen lysophosphatidylethanolamine (LPEp), lysophosphatidylinositol (LPI), lysophosphatidylserine (LPS), N-Acyl phosphatidylethanolamine (NAPE), N-acyl phosphatidylserine (NAPS), and N-acyl serine (NSer), encompassing 593 individual

lipid species. Immuno-isolated microglial EV preparations were subjected to modified Bligh–Dyer lipid extraction (Bligh and Dyer, 1959), dried, resuspended, and subjected to liquid-chromatography tandem mass spectrometry (LC-MS/MS) targeted lipidomics using Agilent 1,260 Infinity HPLC

integrated to an Agilent 6490A QQQ mass spectrometer controlled by Masshunter v 7.0 (Agilent Technologies, Santa Clara, CA) in positive and negative ion modes as previously described (Chan, et al., 2012; Guan et al., 2007; Hsu et al., 2004). Quantification of lipid species was accomplished using multiple reaction monitoring (MRM) transitions (Chan, et al., 2012; Guan, et al., 2007; Hsu, et al., 2004) under both positive and negative ionization modes in conjunction with referencing of appropriate internal standards: PA 14:0/14:0, PC 14:0/14:0, PE 14:0/14:0, PG 15:0/15:0, PI 17:0/20:4, PS 14:0/14:0, BMP 14:0/14:0, APG 14:0/14:0, LPC 17:0, LPE 14:0, LPI 13:0, Cer d18:1/17:0, SM d18:1/12:0, dhSM d18:0/12:0, GalCer d18:1/12:0, GluCer d18:1/12:0, LacCer d18:1/12:0, D7-cholesterol, CE 17:0, MG 17:0, 4ME 16:0 diether DG, and D5-TG 16:0/18:0/16:0 (Avanti Polar Lipids, Alabaster, AL). Lipid levels for each sample were calculated by summing the total number of moles of all lipid species measured by all three LC-MS methodologies and then normalizing that total to mol%. The final data are presented as mean mol% with error bars showing mean $\pm$ S.E. Graph-pad Prism was used to analyze the lipidomics data.

For statistical analysis, two-way repeated measures (mixed model) ANOVA was completed on individual lipid classes among the lipid species using an unweighted means analysis due to the different *n* for LN and AD groups. Bonferroni post-test was used to compare replicate means by row (lipid species) in which microglia NL and microglia AD were compared.

RESULTS

Microglia-Derived Small EV Isolation and Characterization

Based on a previously described method of brain EV isolation (Vella, et al., 2017), brain tissue from two late AD cases (Braak V-VI) was used for the development and standardization of the microglial EV isolation protocol. Sucrose-gradient fractions enriched in EVs were isolated from cryopreserved tissue after enzymatic digestion and ultracentrifugation. TEM and TRPS analysis confirmed the abundance of small EVs (below 200 nm in diameter) in Fraction 2 (F2) (Figures 1A,B). This fraction was enriched in exosomal markers CD9, CD81, and synenin-1 and contained only trace amounts of negative control markers, such as Golgi complex protein GM130 and endoplasmic reticulum protein calnexin (CNX) (Thery, et al., 2018) (Figure 1C). The F2 fraction was further used for the isolation of microglial EVs by immunoprecipitation with antibodies against the microglia-specific marker CD11b. Immunoblot analysis demonstrated the enrichment of exosomal markers, CD63 and CD9, and the microglial marker, CD11b, in small EVs immunoprecipitated with CD11b-conjugated Dynabeads when compared to isotype control antibody-conjugated beads (Figures 1D,E). We also compared the levels of the microglial marker TMEM119 between the F2 fraction and the corresponding CD11b-positive microglial fraction (Figure 1F). The data demonstrate the enrichment of TMEM119 in the IP fraction despite much higher EV load in the F2 lane as documented by the CD63 signal (Figure 1F). We tested relative abundances of TMEM119 and

another microglial marker P2RY12 in F2 and CD11b-IP samples by proteomics. Proteomic analysis revealed the enrichment of microglia-associated proteins in CD11b-pos EVs when compared to F2 fractions from NL and AD brain tissue. Relative to other proteins in each sample, two microglia markers, TMEM119 and P2RY12, were significantly more abundant in CD11b-purified samples with 6.1- and 16.9-fold increase, respectively (Figure 1G). Tau was detected in some but not all microglial EV samples by immunoblotting (Figure 1E); however, A β peptide was not detected (data not shown) either because of low levels or difficulties relating to A β detection without prior delipidation (Perez-Gonzalez, et al., 2017).

For the omics analyses, microglial EVs enriched from 1 g of cryopreserved human parietal cortex tissue from three normal/low pathology cases (NL 1-3) and four late AD cases (AD 1-4) were used. Demographic information for all the cases is presented in Table 2.

Proteomic Analysis of Microglial EVs

Using Tandem Mass Tag (TMT)-based quantitative proteomics, a total of 1,000 unique proteins were identified in the microglia-derived small EV samples from the human parietal cortex. Among these, 985 were detected in both NL and AD groups. Three proteins, fatty acid binding protein 3, heart type (FABP3), mitochondrial copper transporter Solute Carrier Family 25 Member 3 (SLC25A3), and GTPase Atlantin-3(Alt3), were only detected in the NL samples. Twelve proteins were detected only in the AD samples. These included innate immune response proteins toll-like receptor 8 (TLR8) and CX3C chemokine receptor 1 (CX3CR1), neuron-specific proteins, cell cycle exit and neuronal differentiation 1 (CEND1), synaptosomal-associated protein 25 (SNAP25) and myelin-associated glycoprotein (MAG), and two known regulators of amyloid precursor protein (APP) metabolism, calpain-2 catalytic subunit (CAPN2) (Mahaman, et al., 2019) and integral membrane protein 2B (ITM2B/BRI2) (Matsuda et al., 2008) (Figure 2A). A total of 469 proteins were quantified in all analyzed samples, providing relative abundances for each protein. Differences in protein abundances are illustrated via a volcano plot, which shows that 4 proteins were significantly downregulated and 23 proteins were significantly upregulated in the AD group when compared to the NL group (Figure 2B). A heatmap showing differential expression of the significantly altered proteins across individual samples is presented in Figure 2C. The protein composition of microglia EVs from AD cases reflects the loss of the homeostatic signature of their cells of origin, with a significant decrease in the abundance of two main markers of homeostatic microglia, TMEM119 and P2RY12 (2.8-fold and 1.8-fold, respectively). Abundances of the phagocytic microglial marker FCGR1A (CD64) and a known TREM2 ligand, ApoA1, were also significantly lower in the AD group (Figures 2B,C). There was a significant upregulation of the Stage 1 DAM (proinflammatory phenotype) marker, ferritin heavy chain-1 (FTH1; 2.7-fold) (Figures 2B,C). Most proteins with significantly higher abundances in AD cases can be divided into four groups: 1) neuronal and synapse-enriched proteins: synaptotagmin-2 (SYT-2; 5.5-fold), synaptotagmin-11 (SYT11;

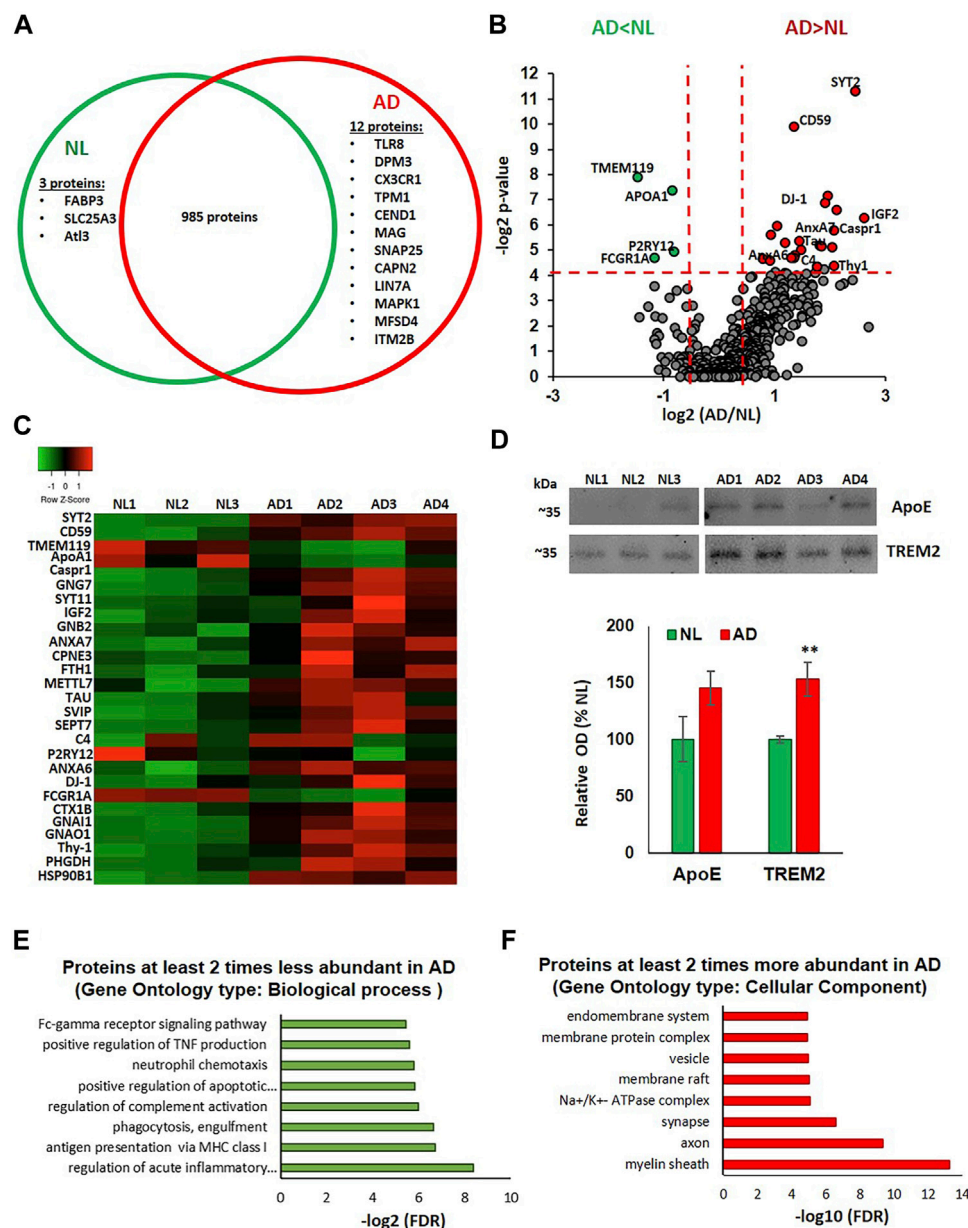


FIGURE 2 | Proteomic analysis of microglial EVs from normal/low pathology and late-stage AD cases. **(A)** Venn diagram representing microglial proteins differentially detected in NL and AD groups. **(B)** Volcano plot showing a degree of differential expression of proteins in NL and AD groups. Vertical red dotted lines separate proteins, which are two or more times less abundant in AD (far left segment) and two or more times more abundant in AD (far right segment). Horizontal red dotted line separates the top part of the plot containing dots representing proteins whose abundances are significantly different between NL and AD groups ($p < 0.05$) and the bottom part (no significant changes). Proteins that are significantly different between AD and NL groups are color-coded: two or more times more abundant in AD are presented in red, two or more times less abundant in green. **(C)** Heat map of proteins significantly up- and downregulated in AD compared to the NL group across seven evaluated human cases. **(D)** Immunoblotting of CD11b-IP samples with antibodies against ApoE and TREM2. Representative images are in the upper section and the composite data are in the lower section ($p < 0.05$). **(E)** Gene ontology enrichment bioinformatic analysis of proteins two or more times less abundant in AD compared to NL. **(F)** Gene ontology enrichment bioinformatic analysis of proteins two or more times more abundant in AD compared to NL.

4.4-fold), Syntaxin-1B (STX1B; 2.5-fold), Thy1 membrane glycoprotein (Thy1; 4.2-fold), and tau protein (3.5-fold); 2) complement regulators: complement component 4B (C4B; 2.8-fold) and CD59 (2.6-fold); 3) GTPases: septin-7 (SEPT7; 4.1-fold) and G-protein complex subunits, guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-7 (GNG7; 3.8-fold),

guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 (GNB2; 2-fold), guanine nucleotide-binding protein G(i) subunit alpha-1 (GNAI1; 1.7-fold), and guanine nucleotide-binding protein G(o) subunit alpha (GNAO1; 1.9-fold); and 4) members of the annexin family involved in vesicle traffic, aggregation, and membrane fusion: annexins A6 (ANXA6;

2.6-fold) and A7 (ANXA7; 4.2-fold) (Lizarbe et al., 2013). We also identified AD-related increases in toll-like receptor chaperone, heat shock protein 90B1 (HSP90B1; 3.4-fold) (Binder, 2014); antioxidant protein, DJ-1, which is coded by a gene causative for autosomal recessive Parkinson's disease (PARK7; 2.6-fold) (Hijioka et al., 2017) and has an emerging role in regulation of immune responses (Zhang, et al., 2020); neurotrophic insulin-like growth factor 2 (IGF2; 6.2-fold); contactin associated protein 1 (Caspr-1; 3.9-fold), which reduces A β production (Fan, et al., 2013) and promotes release of prosurvival secreted amyloid precursor protein (APP) domain sAPP α (Tang, et al., 2020); calcium-dependent phospholipid-binding protein copine-3 (CPNE3; 1.9-fold); and D-3-phosphoglycerate dehydrogenase (PHGDH; 3.4-fold), an enzyme essential for the synthesis of L-serine (**Figures 2B,C**). Interestingly, brain levels of the PHGDH are decreased in AD brain samples, and extracellular PHGDH mRNA was recently proposed to be an early presymptomatic blood marker for Alzheimer's disease (Yan, et al., 2020). Several proteins with mostly unknown functions, including small VCP/p97-interacting protein (SVIP; 3.6-fold) and methyltransferase-like protein 7A (METTL7A; 2.3-fold), were also significantly more abundant in microglial EVs from the AD brain (**Figures 2B,C**).

Notably, the abundance of ApoE, a Stage 1 DAM marker, was 1.4 times higher in microglial EVs from the AD brain when compared to NL, although this difference was not statistically significant. We subsequently confirmed this result using immunoblotting (**Figure 2D**), which also revealed an ~40% increase in ApoE levels in the AD group, validating the changes observed in the proteomics analysis. To our surprise, the mass spectrometric analysis did not detect the microglia marker TREM2. In contrast, TREM2 was detected in the same samples using immunoblotting, and average TREM2 levels were significantly higher in the AD group (**Figure 2D**). Interestingly, amyloid-beta precursor protein (APP) was detected in all samples with only one unique peptide (AA 578-589) that corresponds to a region of A β . The abundance of the APP/A β was 1.9 times higher in the AD group when compared to NL, but this difference was not statistically significant (data not shown).

The proteomic data set was further analyzed using the STRING database for enrichment gene ontology (GO) and pathway analysis. Proteins exhibiting a decrease of twofold or greater in the AD group were enriched in several immune regulation pathways, including acute inflammatory response, antigen presentation, phagocytosis, complement regulation, TNF production, and Fc-gamma receptor signaling (**Figure 2E**). Proteins exhibiting an increase of twofold or greater in the AD group showed enrichment in the following cellular components: myelin sheath, synaptic, and endosomal vesicular related proteins (**Figure 2F**).

miRNA Profiling of Microglial EVs

We identified 105 miRNAs present in 5 or more of the analyzed human cases using the nCounter miRNA expression panel. Fold changes (AD/NL) and *p*-values corresponding to each identified miRNA were illustrated by a volcano plot, which revealed that levels of four miRNA—miR-28-5p, miR-381-3p, miR-651-5p,

and miR-188-5p—were significantly higher in microglial EVs from AD cases when compared to NL cases (**Figure 3A**). Functional interpretation of these data was performed using miRNet (Fan, et al., 2016), which revealed SUMOylation, toll-like receptor (TLR), Fc epsilon receptor I (FCERI), and senescence pathways to be among those regulated by the four significantly increased miRNAs (**Figure 3B**).

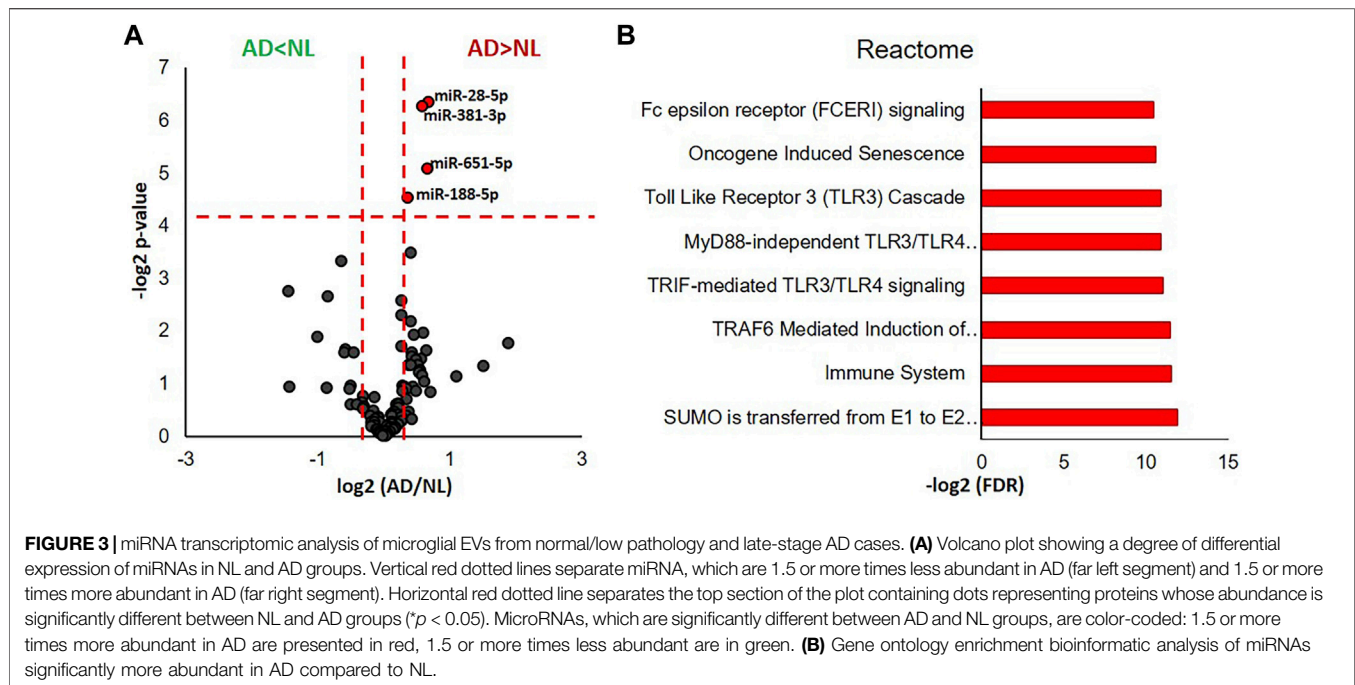
Lipidomics of Microglial EVs

Analysis across all lipid classes without regard to acyl chain composition indicated that only free cholesterol (FC) was increased in microglia-derived EVs from the AD brain (**Figure 4A**). Phospholipids showed acyl chain specific changes in mono- and polyunsaturated species. Microglia-derived EVs from the AD brain showed a significant deficit in phosphatidylethanolamine (PE) 38:0 and 38:1 (**Figure 4B**) and a concurrent loss of the PE metabolites—lysoPE 2:4 and N-acyl phosphatidylethanolamine (NAPE) 16:0/18:0/20:4 (**Figures 4C,D**). Interestingly, differences in microglia-derived EVs from the AD brain were seen on phospholipids likely to harbor docosahexaenoic acid (22:6) in the sn-2 position. Specifically, microglia-derived EVs showed significant deficits in phosphatidic acid (PA) 40:6 (**Figure 4E**) and phosphatidylserine (PS) 40:6 (**Figure 4F**). A trend toward depletion of 40:6 was also detected in PE (PE 40:6) (**Figure 4B**) and PEp (PEp40:6) (data not shown). Specific lipid changes observed in microglial EVs included upregulation of the most abundant lipid species of BMP and monohexosylceramides (mhCer). The most abundant BMP species BMP 36:2 was enriched in EVs derived from the AD brain (**Figure 4G**). Finally, the most abundant mhCer, mhCer d18:1/24:1, was significantly upregulated in microglial-derived EVs from the AD brain (**Figure 4H**).

DISCUSSION

We evaluated the changes across the proteome, lipidome, and miRNA transcriptome of small EVs of microglial origin (positive for myeloid cell marker, CD11B) isolated from the human parietal cortex of three normal/low pathology (NL) and four late-stage AD cases (Table 2).

We found a significant decrease in the abundance of known homeostatic microglial markers, P2RY12 and TMEM119, and a corresponding increase in DAM markers, FTH1 (Stage 1 DAM) and TREM2 (Stages 1 and 2 DAM), in microglial EVs from the AD brain when compared to NL. However, a homeostatic microglia marker, Cx3CR1, was detected in AD, but not in the NL group, and a classical Stage 1 DAM marker, ApoE, was only marginally increased in AD (**Figures 2A–D**). Overall, these changes better recapitulate the overall brain cell transcriptional signatures of DAM from mouse AD models (Deczkowska, et al., 2018), which were not reproduced in a recent human RNAseq study (Srinivasan, et al., 2020). A possible explanation for the discrepancy can be disproportional enrichment of DAM-derived EVs in the microglial secretome from the AD tissue. The burdens of cortical dense-core plaques in AD mouse models (Tg2576, APP_{Swe}PS1 Δ E9, and 5XFAD) greatly exceed the cortical plaque



burdens in human AD cases (P. Liu, et al., 2017). As a result, the proportion of DAM cells surrounding dense-core plaques (Keren-Shaul, et al., 2017) may be higher in the mouse models when compared with the human disease, and a DAM-specific transcriptomic signature may be easier to detect. It has been recently demonstrated that small EV secretion is highly upregulated in mouse DAM cells (Clayton, et al., 2021). Moreover, mouse and human DAM display a transcriptional signature characteristic of senescent cells (Hu, et al., 2021), which are known to secrete vast numbers of EVs as a part of the senescence-associated secretory phenotype (SASP). In agreement with this, our miRNA transcriptome data revealed senescence as one of the top pathways controlled by four miRNAs identified to be significantly upregulated in AD microglial EVs (Figure 3B). Thus, it is possible that analysis of human microglial EVs may more specifically address changes in the A β -plaque-associated DAM population when compared to cell RNAseq analysis.

Microglial EVs either contain low levels of tau protein or a composition of tau species that cannot be easily detected by immunoblotting analysis. Yet, tau was detected in all samples by mass spectrometry, and tau abundance was significantly higher in AD when compared to NL samples (Figures 2B,C). These data confirmed a potential role of microglia-derived EVs in the spread of tau pathology in the human AD brain, as it was previously described *in vitro* and in mouse tauopathy and AD models (Asai, et al., 2015; Clayton, et al., 2021; Maphis, et al., 2015). The specific microglial EV-associated tau species and their proteopathic seeding potential still need to be defined.

In addition to the increase in abundance of tau protein in AD brain-derived microglial EVs, we found that levels of neuron-specific and synapse-enriched proteins were either exclusively detected (CEND1 and SNAP25) or significantly upregulated

(SYT-1, SYT-11, STX1B, and Thy1) in the AD group (Figures 2A,B). Myelin-specific protein MAG was only found in microglial EVs from AD, but not in NL samples (Figure 2A). Moreover, myelin sheath was the cellular component most enriched in proteins that were increased twofold or greater in the AD group (Figure 2F). Increases in synaptic protein VGlut1 in microglial cells from the 5xFAD mouse model and in TDP43-depleted microglia with a hyperphagocytic phenotype have been described (Brioschi, et al., 2020), so it is likely that synaptic and myelin-specific proteins have been phagocytosed prior to entering microglial exosomes. In this regard, elevated levels of complement protein, C4, in microglial EVs from AD cases (Figure 2B) may suggest that the process of complement-dependent engulfment of synapses by microglia may be involved (Thion and Garel, 2018; Yilmaz, et al., 2021). On the other hand, C4-coated EVs may play a role as scavengers and thus may protect cells from complement attack (Karasu et al., 2018). Interestingly, we also observed an increase in membrane attack complex (MAC)-inhibitory protein, CD59, which is known to negatively regulate complement-mediated phagocytosis (Schartz and Tenner, 2020; Tenner, 2020).

Our lipidomic analysis revealed an increase in cholesterol in AD microglial EVs (Figure 4A), which is consistent with the phagocytosis of neuronal debris by microglial cells. Cholesterol metabolism has also been associated with immune activation (Deczkowska, et al., 2018; Orre, et al., 2014; Simon, 2014; Spann and Glass, 2013; Wong, et al., 2020). A recent study reported cholesteryl ester accumulation in TREM2-deficient microglia that fails to mount an immune response (Nugent, et al., 2020). However, we did not find significant changes in CE species levels in microglia-derived EVs in the AD cases reported here.

We also found a significant increase in the major bis(monoacylglycerol)phosphate (BMP) and

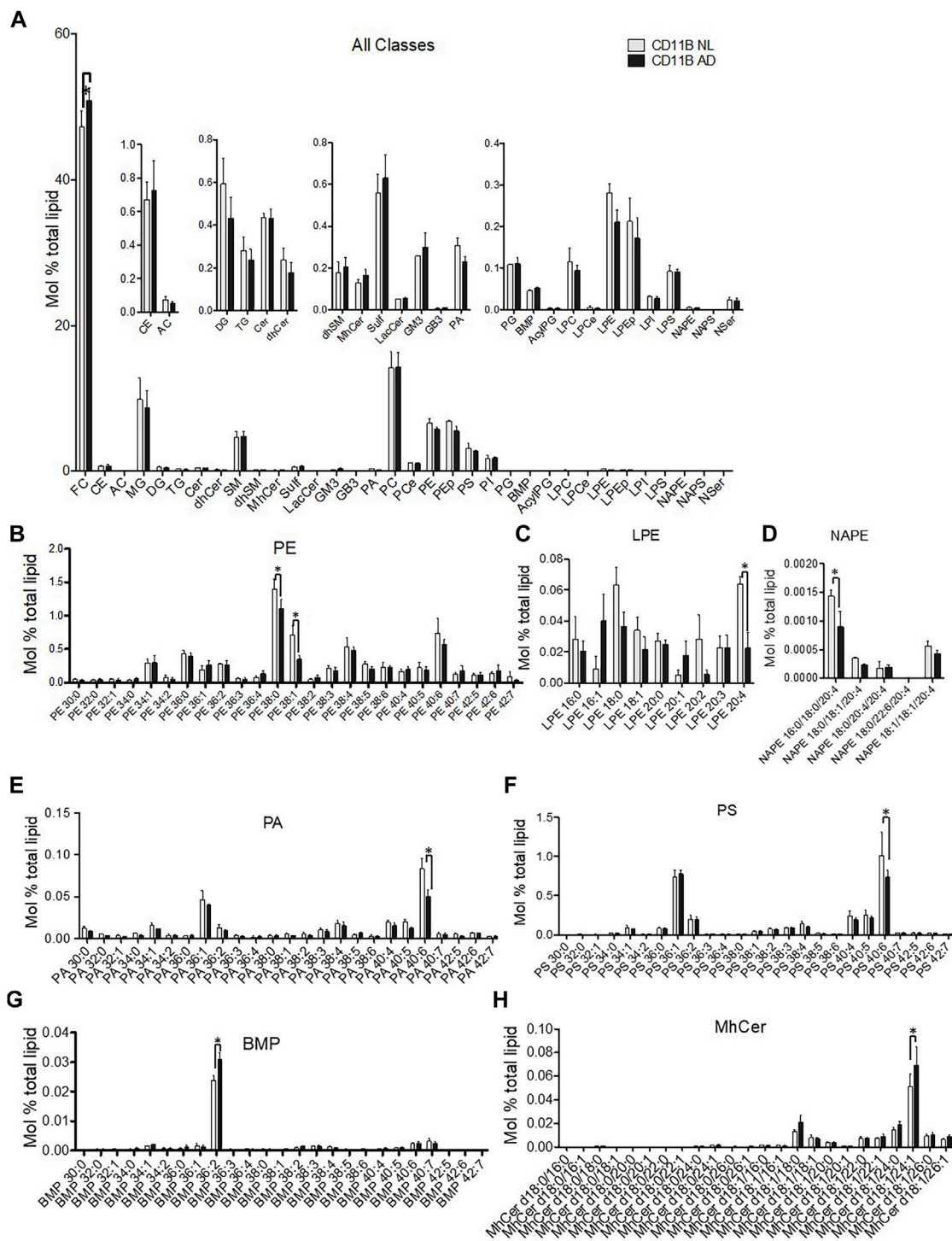


FIGURE 4 | Lipidomic analysis of microglial EVs from normal/low pathology and late-stage AD cases. **(A)** Relative quantification 34 lipid classes and 593 individual lipid species in microglial EVs. Lipids are quantified as mol% total lipid by normalizing the molar amount of each lipid species to the summed total moles of all lipid species in each sample. The inset panels show lower abundance species on the expanded y-axis. Abbreviations are free cholesterol (FC), cholesteryl ester (CE), acyl carnitine (AC), monoacylglycerol (MG), diacylglycerol (DG), triacylglycerol (TG), ceramide (Cer), sphingomyelin (SM), monohexosylceramide (MhCer), sulfatides (Sulf), lactosylceramide (LacCer), monosialodihexosylganglioside (GM3), globotriaosylceramide (GB3), phosphatidic acid (PA), phosphatidylcholine (PC), ether phosphatidylcholine (PCe), phosphatidylethanolamine (PE), plasmalogen phosphatidylethanolamine (Pep), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), bis(monoacylglycerol)phosphate (BMP), acyl phosphatidylglycerol (AcylPG), lysophosphatidylcholine (LPC), ether lysophosphatidylcholine (LPCe), lysophosphatidylethanolamine (LPE), plasmalogen lysophosphatidylethanolamine (LPEp), lysophosphatidylinositol (LPI), lysophosphatidylserine (LPS), N-Acyl (Continued)

FIGURE 4 | phosphatidylethanolamine (NAPE), N-acyl phosphatidylserine (NAPS), and N-acyl serine (NSer). Data are presented as mean mol% with error bars showing mean $\pm$ S.E. FC is significantly increased shown in the primary panel ($p < 0.01$). **(B)** PE species were detected using mass spectrometry and are shown using relative quantification as mol%. PE 38:0 ($p < 0.05$); PE 38:1 ($p < 0.01$). **(C)** Most abundant LPE [LPE20:4 ($p < 0.05$)] and **(D)** NAPE [NAPE 16:0/18:0/20:4 ($p < 0.05$)] lipid species are shown to significantly differ between LN- and AD-derived microglia EV. **(E)** PA40:6 lipid species is decreased in microglia-derived EV from the AD brain (AD) compared to control (LN) ($p < 0.0001$), while no other PA species are significantly affected. **(F)** The most abundant PS species, PS40:6 species, is decreased in EV derived from the AD brain (AD) compared to control (LN) ($p < 0.001$) while no other PS species are significantly altered. **(G)** The most abundant BMP 36:2 ($p < 0.0001$) and **(H)** MhCer d18:1/24:1 ($p < 0.01$) lipid species are increased in AD-derived microglia EV. All data are presented as mean mol% with error bars showing mean $\pm$ S.E.

monohexosylceramide (MhCer) lipid species (**Figures 4G,H**). An increase of this species of BMP, a lysosome-specific lipid, suggests increased lysosomal lipid content in AD microglia (Showalter, et al., 2020). Elevated levels of MhCer may play a role in microglial activation and immune response (Brennan, et al., 2017; Miltenberger-Miltenyi, et al., 2020; Niimura et al., 2010), as well as be a precursor of more complex gangliosides (Merrill, 2011). Interestingly, enrichment of BMP and MhCer in EVs has been previously linked to the impairment of endolysosomal function induced by Vsp34 kinase inhibition, which causes phosphatidylinositol-3-phosphate (PI3P) deficiency (Miranda, et al., 2018). Thus, endolysosomal impairment can explain the elevated levels of neuronal and myelin molecules in microglial EVs from the AD brain and potentially lead to the disruption of microglial pathways as suggested by the bioinformatic analysis in **Figure 2E**.

We observed that a reduction in phospholipids is likely to harbor docosahexaenoic acid (DHA, 22:6) in the sn-2 position in microglial EVs from the AD brain. One likely acyl chain configuration for PS40:6 and PA40:6 is sn-1 18:0, sn-2 22:6. These data echo the previously described DHA decrease in the bulk preparations of human AD brain EVs and global AD-related brain DHA deficiency, which has a proinflammatory effect and may also disrupt A β clearance (H. Su, et al., 2021). The selective loss of DHA among PA and PS (**Figures 4E,F**) as well as the trend in PE (**Figure 2B**) suggests the overactivation of DHA-selective phospholipase A 2 (PLA2) and a deficit in acyl chain remodeling (Abdullah, et al., 2017; Calon, et al., 2004; Fernandez, et al., 2018; Fernandez, et al., 2021; Fonteh, et al., 2013; Granger, et al., 2019).

The loss of LPE20:4 in EVs derived from AD microglia (**Figure 4C**) may represent exhaustion of acyl chain remodeling of PE. Since 20:4 is cleaved from PE20:4 to liberate the free fatty acid, arachidonate, it may represent loss of this proinflammatory fatty acid due to overactivation of PLA2, which has previously been reported in AD (Sanchez-Mejia, et al., 2008; Wang, et al., 2021). Interestingly, NAPE is an important precursor to endocannabinoid synthesis, which is dysregulated in AD (Bisogno and Di Marzo, 2008; Fonteh, et al., 2013; Liu, et al., 2006). Even though previous studies have reported global loss of PE and plasmalogen, our analyses show only a trend for PE depletion and no loss in plasmalogen from AD brain microglia-derived EVs (Han et al., 2001; Han et al., 2002).

We did not find AD-associated changes in key modulators of immune responses—miR-146a-5p, miR-155-5p, and miR-124-3p (Su et al., 2016); miR-124-3p was not detected in our samples, while miR-146a-5p and miR-155-5p levels were above background but did not show any significant differences between NL and AD groups (data not shown). Two out of

four miRNAs upregulated in the AD group, miR-188-5p and miR-381-3p (**Figure 3A**), are known to have neuroprotective effects. MiR-188-5p restores synaptic and cognitive deficits in 5xFAD mice (Lee, et al., 2016), and miR-381-3p promotes the recovery of spinal cord injury in rats (Chen et al., 2018). In addition to a neuroprotective miRNA signature, proteomics revealed increases in the abundance of IGF2, ITM2B and CASPR-1, and DJ-1 in microglial EVs from AD cases (**Figures 2A–B**). Neurotrophic factor IGF2 was shown to reduce A β amyloidosis, reverse synaptic deficits, and improve memory in AD animal models (Mellott et al., 2014; Pascual-Lucas, et al., 2014). ITM2B and CASPR-1 are known to regulate APP metabolism and suppress A β production (Fan, et al., 2013; Matsuda, et al., 2008; Tang, et al., 2020), and DJ-1 is an important antioxidant with newly discovered immune regulation functions (Zhang, et al., 2020). The presence of both neuroprotective and pathology-related miRNA and proteins in AD cases, a seemingly contradictory result, may represent both a consequence of, and a response to, disease pathogenesis. It is interesting to surmise that the response and consequence may be present as the cargo in different EV subpopulations, either derived from the same cells or from microglia at different stages of activation. Further evaluation of EV subpopulations and the molecular mechanisms dictating their release may provide a new avenue for AD therapeutic development with the aim to suppress subpopulations containing pathogenic cargo, while promoting the release of neuroprotective EVs.

In conclusion, our data suggest that loss of a homeostatic signature and the deterioration of functional microglia in late AD stages may accompany endolysosomal impairment and the release of undigested neuronal and myelin debris, including tau, through extracellular vesicles. We also found a significant AD-associated decrease in levels of DHA-containing polyunsaturated lipids of different classes, which may be associated with global DHA deficiency in AD and indicate a potential defect in the acyl-chain remodeling by PLA2 and lysophospholipid acyltransferases. On the other hand, the AD-specific microglial EV signature also includes increases in some miRNAs and proteins with neuroprotective properties. It is currently unknown if those “harmful” vs. “protective” molecular signatures represent different subpopulations of microglial EVs. Results from our study support the hypothesis that the molecular composition of EVs reflects functional changes in microglia consistent with a diseased state. To the best of our knowledge, this is the first study analyzing proteins, lipids, and miRNAs in cell-type specific EVs from human brain tissue. The main limitation of the study is the small number of cases, and our

results require further validation in larger cohorts, and when possible inclusion of samples from earlier disease stages. Despite these limitations, this proof-of-concept study clearly demonstrates the feasibility of using multiple omics analyses on small microglial EVs isolated from cryopreserved human brain tissue. Our results exemplify the superiority of an integrative approach when compared to individual proteomics, lipidomics, or microRNA analyses, and suggest that new AD biomarkers may arise from all three different classes of biomolecules.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: PRIDE, accession no: PXD028898.

AUTHOR CONTRIBUTIONS

LM, VJ, KG, and TB participated in the research design. WC, MM, CH, LM, BT, SC, CZ, and TB conducted experiments. WC, MM, LM, and TB performed data analysis. WC, MM, BT, LM,

SC, VJ, KG, and TB wrote or contributed to the writing of the manuscript.

FUNDING

The work was supported by the following grants: NIH AG063767 to TB and KG, NIH AG051386 and AFOSR FA9550-17-1-0399 to VJ, and NIH R56 High Priority, Short-Term Project Award, NIH 1R56AG062271-01A1 and Department of Defense, W81XWH-19-1-0817, GRANT12727289 for LM.

ACKNOWLEDGMENTS

We thank the AD Research Center Neuropathology Cores of USC, UCLA, and UC Irvine for human autopsy brain samples and neuropathology reports. We also would like to acknowledge the Brain Research Institute electron microscopy core facility, the UCLA-DOE Biochemistry core facility, and the UCLA Center for Systems Biomedicine. We would also like to acknowledge Kym Francis Faull and Julian Whitelegge from the UCLA Pasarow Mass Spectrometry Laboratory for the use of their instrumentation and their contributions in editing the manuscript.

REFERENCES

- 2021 Alzheimer's disease facts and figures, 2021 2021 Alzheimer's disease facts and figures (2021). *Alzheimers Dement* 17, 327–406. doi:10.1002/alz.12328
- Abdullah, L., Evans, J. E., Emmerich, T., Crynen, G., Shackleton, B., Keegan, A. P., et al. (2017). APOE ε4 Specific Imbalance of Arachidonic Acid and Docosahexaenoic Acid in Serum Phospholipids Identifies Individuals with Preclinical Mild Cognitive Impairment/Alzheimer's Disease. *Aging (Albany NY)* 9, 964–985. doi:10.18632/aging.101203
- An, K., Klyubin, I., Kim, Y., Jung, J. H., Mably, A. J., O'Dowd, S. T., et al. (2013). Exosomes Neutralize Synaptic-Plasticity-Disrupting Activity of Aβ Assemblies *In Vivo*. *Mol. Brain* 6, 47. doi:10.1186/1756-6606-6-47
- Asai, H., Ikezu, S., Tsunoda, S., Medalla, M., Luebke, J., Haydar, T., et al. (2015). Depletion of Microglia and Inhibition of Exosome Synthesis Halt Tau Propagation. *Nat. Neurosci.* 18, 1584–1593. doi:10.1038/nn.4132
- Bilousova, T., Elias, C., Miyoshi, E., Alam, M. P., Zhu, C., Campagna, J., et al. (2018). Suppression of Tau Propagation Using an Inhibitor that Targets the DK-Switch of nSMase2. *Biochem. Biophys. Res. Commun.* 499, 751–757. doi:10.1016/j.bbrc.2018.03.209
- Bilousova, T., Simmons, B. J., Knapp, R. R., Elias, C. J., Campagna, J., Melnik, M., et al. (2020). Dual Neutral Sphingomyelinase-2/Acetylcholinesterase Inhibitors for the Treatment of Alzheimer's Disease. *ACS Chem. Biol.* 15, 1671. doi:10.1021/acschembio.0c00311
- Binder, R. J. (2014). Functions of Heat Shock Proteins in Pathways of the Innate and Adaptive Immune System. *J. Immunol.* 193, 5765–5771. doi:10.4049/jimmunol.1401417
- Bisogno, T., and Di Marzo, V. (2008). The Role of the Endocannabinoid System in Alzheimer's Disease: Facts and Hypotheses. *Curr. Pharm. Des.* 14, 2299–3305. doi:10.2174/138161208785740027
- Bligh, E. G., and Dyer, W. J. (1959). A Rapid Method of Total Lipid Extraction and Purification. *Can. J. Biochem. Physiol.* 37, 911–917. doi:10.1139/o59-099
- Bosch, S., de Beaufort, L., Allard, M., Mosser, M., Heichette, C., Chrétien, D., et al. (2016). Trehalose Prevents Aggregation of Exosomes and Cryodamage. *Sci. Rep.* 6, 36162. doi:10.1038/srep36162
- Brennan, P. J., Cheng, T. Y., Pellicci, D. G., Watts, G. F. M., Veerapen, N., Young, D. C., et al. (2017). Structural Determination of Lipid Antigens Captured at the CD1d-T-Cell Receptor Interface. *Proc. Natl. Acad. Sci. U S A.* 114, 8348–8353. doi:10.1073/pnas.1705882114
- Brioschi, S., d'Errico, P., Amann, L. S., Janova, H., Wojcik, S. M., Meyer-Luehmann, M., et al. (2020). Detection of Synaptic Proteins in Microglia by Flow Cytometry. *Front. Mol. Neurosci.* 13, 149. doi:10.3389/fnmol.2020.00149
- Brumbaugh, C. D., Kim, H. J., Giovacchini, M., and Pourmand, N. (2011). NanoStriDE: Normalization and Differential Expression Analysis of NanoString nCounter Data. *BMC Bioinformatics.* 12, 479. doi:10.1186/1471-2105-12-479
- Butovsky, O., and Weiner, H. L. (2018). Microglial Signatures and Their Role in Health and Disease. *Nat. Rev. Neurosci.* 19, 622–635. doi:10.1038/s41583-018-0057-5
- Calon, F., Lim, G. P., Yang, F., Morihara, T., Teter, B., Ubeda, O., et al. (2004). Docosahexaenoic Acid Protects from Dendritic Pathology in an Alzheimer's Disease Mouse Model. *Neuron* 43, 633–645. doi:10.1016/j.neuron.2004.08.013
- Chan, R. B., Oliveira, T. G., Cortes, E. P., Honig, L. S., Duff, K. E., Small, S. A., et al. (2012). Comparative Lipidomic Analysis of Mouse and Human Brain with Alzheimer Disease. *J. Biol. Chem.* 287, 2678–2688. doi:10.1074/jbc.M111.274142
- Chen, W. C., Luo, J., Cao, X. Q., Cheng, X. G., and He, D. W. (2018). Overexpression of miR-381-3p Promotes the Recovery of Spinal Cord Injury. *Eur. Rev. Med. Pharmacol. Sci.* 22, 5429–5437. doi:10.26355/eurrev_201809_15802
- Cheng, L., Vella, L. J., Barnham, K. J., McLean, C., Masters, C. L., and Hill, A. F. (2020). Small RNA Fingerprinting of Alzheimer's Disease Frontal Cortex Extracellular Vesicles and Their Comparison with Peripheral Extracellular Vesicles. *J. Extracell. Vesicles* 9, 1766822. doi:10.1080/20013078.2020.1766822
- Clayton, K., Delpech, J. C., Herron, S., Iwahara, N., Ericsson, M., Saito, T., et al. (2021). Plaque Associated Microglia Hyper-Secrete Extracellular Vesicles and Accelerate Tau Propagation in a Humanized APP Mouse Model. *Mol. Neurodegener.* 16, 18. doi:10.1186/s13024-021-00440-9
- Crotti, A., Sait, H. R., McAvoy, K. M., Estrada, K., Ergun, A., Szak, S., et al. (2019). BIN1 Favors the Spreading of Tau via Extracellular Vesicles. *Sci. Rep.* 9, 9477. doi:10.1038/s41598-019-45676-0

- D'Anca, M., Fenoglio, C., Serpente, M., Arosio, B., Cesari, M., Scarpini, E. A., et al. (2019). Exosome Determinants of Physiological Aging and Age-Related Neurodegenerative Diseases. *Front. Aging Neurosci.* 11, 232. doi:10.3389/fnagi.2019.00232
- Deczkowska, A., Keren-Shaul, H., Weiner, A., Colonna, M., Schwartz, M., and Amit, I. (2018). Disease-Associated Microglia: A Universal Immune Sensor of Neurodegeneration. *Cell* 173, 1073–1081. doi:10.1016/j.cell.2018.05.003
- Del-Aguila, J. L., Li, Z., Dube, U., Mihindukulasuriya, K. A., Budde, J. P., Fernandez, M. V., et al. (2019). A Single-Nuclei RNA Sequencing Study of Mendelian and Sporadic AD in the Human Brain. *Alzheimers Res. Ther.* 11, 71. doi:10.1186/s13195-019-0524-x
- Dinkins, M. B., Dasgupta, S., Wang, G., Zhu, G., and Bieberich, E. (2014). Exosome Reduction *In Vivo* Is Associated with Lower Amyloid Plaque Load in the 5XFAD Mouse Model of Alzheimer's Disease. *Neurobiol. Aging* 35, 1792–1800. doi:10.1016/j.neurobiolaging.2014.02.012
- Dodd, P. R., Hardy, J. A., Baig, E. B., Kidd, A. M., Bird, E. D., Watson, W. E., et al. (1986). Optimization of Freezing, Storage, and Thawing Conditions for the Preparation of Metabolically Active Synaptosomes from Frozen Rat and Human Brain. *Neurochem. Pathol.* 4, 177–198. doi:10.1007/BF02834357
- Fan, L. F., Xu, D. E., Wang, W. H., Yan, K., Wu, H., Yao, X. Q., et al. (2013). Caspr Interaction with Amyloid Precursor Protein Reduces Amyloid- β Generation *In Vitro*. *Neurosci. Lett.* 548, 255–260. doi:10.1016/j.neulet.2013.05.055
- Fan, Y., Siklenka, K., Arora, S. K., Ribeiro, P., Kimmins, S., and Xia, J. (2016). miRNet - Sissecting miRNA-Target Interactions and Functional Associations Through Network-Based Visual Analysis. *Nucleic Acids Res.* 44, W135–W141. doi:10.1093/nar/gkw288
- Fernandez, R. F., Kim, S. Q., Zhao, Y., Foguth, R. M., Weera, M. M., Counihan, J. L., et al. (2018). Acyl-CoA Synthetase 6 Enriches the Neuroprotective omega-3 Fatty Acid DHA in the Brain. *Proc. Natl. Acad. Sci. U S A.* 115, 12525–12530. doi:10.1073/pnas.1807958115
- Fernandez, R. F., Pereyra, A. S., Diaz, V., Wilson, E. S., Litwa, K. A., Martínez-Garazabal, J., et al. (2021). Acyl-CoA Synthetase 6 Is Required for Brain Docosahexaenoic Acid Retention and Neuroprotection during Aging. *JCI Insight* 6. doi:10.1172/jci.insight.144351
- Fiandaca, M. S., Kapogiannis, D., Mapstone, M., Boxer, A., Eitan, E., Schwartz, J. B., et al. (2015). Identification of Preclinical Alzheimer's Disease by a Profile of Pathogenic Proteins in Neurally Derived Blood Exosomes: A Case-Control Study. *Alzheimers Dement* 11, 600–e1. doi:10.1016/j.jalz.2014.06.008
- Fonteh, A. N., Chiang, J., Cipolla, M., Hale, J., Diallo, F., Chirino, A., et al. (2013). Alterations in Cerebrospinal Fluid Glycerophospholipids and Phospholipase A2 Activity in Alzheimer's Disease. *J. Lipid Res.* 54, 2884–2897. doi:10.1194/jlr.M037622
- Goetzl, E. J., Goetzl, L., Karliner, J. S., Tang, N., and Pulliam, L. (2016). Human Plasma Platelet-Derived Exosomes: Effects of Aspirin. *FASEB J.* 30, 2058–2063. doi:10.1096/fj.201500150R
- Goetzl, E. J., Mustapic, M., Kapogiannis, D., Eitan, E., Lobach, I. V., Goetzl, L., et al. (2016). Cargo Proteins of Plasma Astrocyte-Derived Exosomes in Alzheimer's Disease. *FASEB J.* 30, 3853–3859. doi:10.1096/fj.201600756R
- Goetzl, E. J., Schwartz, J. B., Abner, E. L., Jicha, G. A., and Kapogiannis, D. (2018). High Complement Levels in Astrocyte-Derived Exosomes of Alzheimer Disease. *Ann. Neurol.* 83, 544–552. doi:10.1002/ana.25172
- Granger, M. W., Liu, H., Fowler, C. F., Blanchard, A. P., Taylor, M. W., Sherman, S. P. M., et al. (2019). Distinct Disruptions in Land's Cycle Remodeling of Glycerophosphocholines in Murine Cortex Mark Symptomatic Onset and Progression in Two Alzheimer's Disease Mouse Models. *J. Neurochem.* 149, 499–517. doi:10.1111/jnc.14560
- Guan, Z., Li, S., Smith, D. C., Shaw, W. A., and Raetz, C. R. (2007). Identification of N-Acylphosphatidylserine Molecules in Eukaryotic Cells. *Biochemistry* 46, 14500–14513. doi:10.1021/bi701907g
- Gylys, K. H., and Bilousova, T. (2017). Flow Cytometry Analysis and Quantitative Characterization of Tau in Synaptosomes from Alzheimer's Disease Brains. *Methods Mol. Biol.* 1523, 273–284. doi:10.1007/978-1-4939-6598-4_16
- Han, X., Holtzman, D. M., and McKeel, D. W., Jr. (2001). Plasmalogen Deficiency in Early Alzheimer's Disease Subjects and in Animal Models: Molecular Characterization Using Electrospray Ionization Mass Spectrometry. *J. Neurochem.* 77, 1168–1180. doi:10.1046/j.1471-4159.2001.00332.x
- Han, X., M Holtzman, D., McKeel, D. W., Jr., Kelley, J., and Morris, J. C. (2002). Substantial Sulfatide Deficiency and Ceramide Elevation in Very Early Alzheimer's Disease: Potential Role in Disease Pathogenesis. *J. Neurochem.* 82, 809–818. doi:10.1046/j.1471-4159.2002.00997.x
- Heinzelman, P., Bilousova, T., Campagna, J., and John, V. (2016). Nanoscale Extracellular Vesicle Analysis in Alzheimer's Disease Diagnosis and Therapy. *Int. J. Alzheimers Dis.* 2016, 8053139. doi:10.1155/2016/8053139
- Hijioka, M., Inden, M., Yanagisawa, D., and Kitamura, Y. (2017). DJ-1/PARK7: A New Therapeutic Target for Neurodegenerative Disorders. *Biol. Pharm. Bull.* 40, 548–552. doi:10.1248/bpb.b16-01006
- Hornung, S., Dutta, S., and Bitan, G. (2020). CNS-derived Blood Exosomes as a Promising Source of Biomarkers: Opportunities and Challenges. *Front. Mol. Neurosci.* 13, 38. doi:10.3389/fnmol.2020.00038
- Hsu, F. F., Turk, J., Shi, Y., and Groisman, E. A. (2004). Characterization of Acylphosphatidylglycerols from *Salmonella typhimurium* by Tandem Mass Spectrometry with Electrospray Ionization. *J. Am. Soc. Mass. Spectrom.* 15, 1–11. doi:10.1016/j.jasms.2003.08.006
- Hu, Y., Fryatt, G. L., Ghorbani, M., Obst, J., Menassa, D. A., Martin-Estebane, M., et al. (2021). Replicative Senescence Dictates the Emergence of Disease-Associated Microglia and Contributes to A β Pathology. *Cell Rep* 35, 109228. doi:10.1016/j.celrep.2021.109228
- Huang, Y., Cheng, L., Turchinovich, A., Mahairaki, V., Troncoso, J. C., Pletniková, O., et al. (2020). Influence of Species and Processing Parameters on Recovery and Content of Brain Tissue-Derived Extracellular Vesicles. *J. Extracell Vesicles* 9, 1785746. doi:10.1080/20013078.2020.1785746
- Hussain, R. Z., Miller-Little, W. A., Doelger, R., Cutter, G. R., Loof, N., Cravens, P. D., et al. (2018). Defining Standard Enzymatic Dissociation Methods for Individual Brains and Spinal Cords in EAE. *Neurol. Neuroimmunol Neuroinflamm* 5, e437. doi:10.1212/NXI.0000000000000437
- Joshi, P., Turola, E., Ruiz, A., Bergami, A., Libera, D. D., Benussi, L., et al. (2014). Microglia Convert Aggregated Amyloid- β into Neurotoxic Forms through the Shedding of Microvesicles. *Cell Death Differ* 21, 582–593. doi:10.1038/cdd.2013.180
- Karasu, E., Eisenhardt, S. U., Harant, J., and Huber-Lang, M. (2018). Extracellular Vesicles: Packages Sent With Complement. *Front Immunol* 9, 721. doi:10.3389/fimmu.2018.00721
- Keren-Shaul, H., Spinrad, A., Weiner, A., Matcovitch-Natan, O., Dvir-Szternfeld, R., Ulland, T. K., et al. (2017). A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell* 169, 1276–e17. doi:10.1016/j.cell.2017.05.018
- Kokubo, H., Saido, T. C., Iwata, N., Helms, J. B., Shinohara, R., and Yamaguchi, H. (2005). Part of Membrane-Bound Abeta Exists in Rafts within Senile Plaques in Tg2576 Mouse Brain. *Neurobiol. Aging* 26, 409–418. doi:10.1016/j.neurobiolaging.2004.04.008
- Lee, K., Kim, H., An, K., Kwon, O. B., Park, S., Cha, J. H., et al. (2016). Replenishment of microRNA-188-5p Restores the Synaptic and Cognitive Deficits in 5XFAD Mouse Model of Alzheimer's Disease. *Sci. Rep.* 6, 34433. doi:10.1038/srep34433
- Liu, J., Wang, L., Harvey-White, J., Osei-Hyiaman, D., Razdan, R., Gong, Q., et al. (2006). A Biosynthetic Pathway for Anandamide. *Proc. Natl. Acad. Sci. U S A.* 103, 13345–13350. doi:10.1073/pnas.0601832103
- Liu, P., Reichl, J. H., Rao, E. R., McNellis, B. M., Huang, E. S., Hemmy, L. S., et al. (2017). Quantitative Comparison of Dense-Core Amyloid Plaque Accumulation in Amyloid- β Protein Precursor Transgenic Mice. *J. Alzheimers Dis.* 56, 743–761. doi:10.3233/JAD-161027
- Lizarbe, M. A., Barrasa, J. I., Olmo, N., Gavilanes, F., and Turnay, J. (2013). Annexin-phospholipid Interactions. Functional Implications. *Int. J. Mol. Sci.* 14, 2652–2683. doi:10.3390/ijms14022652
- Mahaman, Y. A. R., Huang, F., Kessete Afewerky, H., Maibouge, T. M. S., Ghose, B., and Wang, X. (2019). Involvement of Calpain in the Neuropathogenesis of Alzheimer's Disease. *Med. Res. Rev.* 39, 608–630. doi:10.1002/med.21534
- Maphis, N., Xu, G., Kokiko-Cochran, O. N., Jiang, S., Cardona, A., Ransohoff, R. M., et al. (2015). Reactive Microglia Drive Tau Pathology and Contribute to the Spreading of Pathological Tau in the Brain. *Brain* 138, 1738–1755. doi:10.1093/brain/awv081
- Mathys, H., Davila-Velderrain, J., Peng, Z., Gao, F., Mohammadi, S., Young, J. Z., et al. (2019). Single-cell Transcriptomic Analysis of Alzheimer's Disease. *Nature* 570, 332–337. doi:10.1038/s41586-019-1195-2
- Matsuda, S., Giliberto, L., Matsuda, Y., McGowan, E. M., and D'Adamio, L. (2008). BRI2 Inhibits Amyloid Beta-Peptide Precursor Protein Processing by

- Interfering with the Docking of Secretases to the Substrate. *J. Neurosci.* 28, 8668–8676. doi:10.1523/JNEUROSCI.2094-08.2008
- Mehta, D., Jackson, R., Paul, G., Shi, J., and Sabbagh, M. (2017). Why Do Trials for Alzheimer's Disease Drugs Keep Failing? A Discontinued Drug Perspective for 2010–2015. *Expert Opin. Investig. Drugs* 26, 735–739. doi:10.1080/13543784.2017.1323868
- Mellott, T. J., Pender, S. M., Burke, R. M., Langley, E. A., and Blusztajn, J. K. (2014). IGF2 Ameliorates Amyloidosis, Increases Cholinergic Marker Expression and Raises BMP9 and Neurotrophin Levels in the hippocampus of the APPswePS1dE9 Alzheimer's Disease Model Mice. *PLoS One* 9, e94287. doi:10.1371/journal.pone.0094287
- Merrill, A. H., Jr. (2011). Sphingolipid and Glycosphingolipid Metabolic Pathways in the Era of Sphingolipidomics. *Chem. Rev.* 111, 6387–6422. doi:10.1021/cr2002917
- Miltenberger-Miltenyi, G., Cruz-Machado, A. R., Saville, J., Conceição, V. A., Calado, A., Lopes, I., et al. (2020). Increased Monoheoxylceramide Levels in the Serum of Established Rheumatoid Arthritis Patients. *Rheumatology (Oxford)* 59, 2085–2089. doi:10.1093/rheumatology/kez545
- Miranda, A. M., Lasiecka, Z. M., Xu, Y., Neufeld, J., Shahriar, S., Simoes, S., et al. (2018). Neuronal Lysosomal Dysfunction Releases Exosomes Harboring APP C-Terminal Fragments and Unique Lipid Signatures. *Nat. Commun.* 9, 291. doi:10.1038/s41467-017-02533-w
- Muraoka, S., DeLeo, A. M., Sethi, M. K., Yukawa-Takamatsu, K., Yang, Z., Ko, J., et al. (2020). Proteomic and Biological Profiling of Extracellular Vesicles from Alzheimer's Disease Human Brain Tissues. *Alzheimers Dement* 16, 896–907. doi:10.1002/alz.12089
- Muraoka, S., Jedrychowski, M. P., Iwahara, N., Abdullah, M., Onos, K. D., Keezer, K. J., et al. (2021). Enrichment of Neurodegenerative Microglia Signature in Brain-Derived Extracellular Vesicles Isolated from Alzheimer's Disease Mouse Models. *J. Proteome Res.* 20, 1733–1743. doi:10.1021/acs.jproteome.0c00934
- Niimura, Y., Moue, T., Takahashi, N., and Nagai, K. (2010). Modification of Sphingoglycolipids and Sulfolipids in Kidney Cell Lines under Heat Stress: Activation of Monoheoxylceramide Synthesis as a Ceramide Scavenger. *Glycobiology* 20, 710–717. doi:10.1093/glycob/cwq018
- Nugent, A. A., Lin, K., van Lengerich, B., Lianoglou, S., Przybyla, L., Davis, S. S., et al. (2020). TREM2 Regulates Microglial Cholesterol Metabolism upon Chronic Phagocytic Challenge. *Neuron* 105, 837–e9. doi:10.1016/j.neuron.2019.12.007
- Orre, M., Kamphuis, W., Osborn, L. M., Jansen, A. H. P., Kooijman, L., Bossers, K., et al. (2014). Isolation of Glia from Alzheimer's Mice Reveals Inflammation and Dysfunction. *Neurobiol. Aging* 35, 2746–2760. doi:10.1016/j.neurobiolaging.2014.06.004
- Pascual, M., Ibáñez, F., and Guerri, C. (2020). Exosomes as Mediators of Neuron-Glia Communication in Neuroinflammation. *Neural Regen. Res.* 15, 796–801. doi:10.4103/1673-5374.268893
- Pascual-Lucas, M., Viana da Silva, S., Di Scala, M., Garcia-Barroso, C., González-Aseguinolaza, G., Mulle, C., et al. (2014). Insulin-like Growth Factor 2 Reverses Memory and Synaptic Deficits in APP Transgenic Mice. *EMBO Mol. Med.* 6, 1246–1262. doi:10.15252/emmm.201404228
- Peng, K. Y., Pérez-González, R., Alldred, M. J., Goulbourne, C. N., Morales-Corralliza, J., Saito, M., et al. (2019). Apolipoprotein E4 Genotype Compromises Brain Exosome Production. *Brain* 142, 163–175. doi:10.1093/brain/awy289
- Pérez-González, R., Gauthier, S. A., Kumar, A., Saito, M., Saito, M., and Levy, E. (2017). A Method for Isolation of Extracellular Vesicles and Characterization of Exosomes from Brain Extracellular Space. *Methods Mol. Biol.* 1545, 139–151. doi:10.1007/978-1-4939-6728-5_10
- Rajendran, L., Honsho, M., Zahn, T. R., Keller, P., Geiger, K. D., Verkade, P., et al. (2006). Alzheimer's Disease Beta-Amyloid Peptides Are Released in Association with Exosomes. *Proc. Natl. Acad. Sci. U S A* 103, 11172–11177. doi:10.1073/pnas.0603838103
- Rappsilber, J., Mann, M., and Ishihama, Y. (2007). Protocol for Micro-purification, Enrichment, Pre-fractionation and Storage of Peptides for Proteomics Using StageTips. *Nat. Protoc.* 2, 1896–1906. doi:10.1038/nprot.2007.261
- Sanchez-Mejia, R. O., Newman, J. W., Toh, S., Yu, G. Q., Zhou, Y., Halabisky, B., et al. (2008). Phospholipase A2 Reduction Ameliorates Cognitive Deficits in a Mouse Model of Alzheimer's Disease. *Nat. Neurosci.* 11, 1311–1318. doi:10.1038/nn.2213
- Sardar Sinha, M., Ansell-Schultz, A., Civitelli, L., Hildesjö, C., Larsson, M., Lannfelt, L., et al. (2018). Alzheimer's Disease Pathology Propagation by Exosomes Containing Toxic Amyloid-Beta Oligomers. *Acta Neuropathol.* 136, 41–56. doi:10.1007/s00401-018-1868-1
- Schartz, N. D., and Tenner, A. J. (2020). The Good, the Bad, and the Opportunities of the Complement System in Neurodegenerative Disease. *J. Neuroinflammation* 17, 354. doi:10.1186/s12974-020-02024-8
- Serrano-Pozo, A., Frosch, M. P., Masliah, E., and Hyman, B. T. (2011). Neuropathological Alterations in Alzheimer Disease. *Cold Spring Harb Perspect. Med.* 1, a006189. doi:10.1101/cshperspect.a006189
- Showalter, M. R., Berg, A. L., Nagourney, A., Heil, H., Carraway, K. L., 3rd, and Fiehn, O. (2020). The Emerging and Diverse Roles of Bis(monoacylglycerol) Phosphate Lipids in Cellular Physiology and Disease. *Int. J. Mol. Sci.* 21. doi:10.3390/ijms21218067
- Simon, A. (2014). Cholesterol Metabolism and Immunity. *N. Engl. J. Med.* 371, 1933–1935. doi:10.1056/NEJMcibr1412016
- Spann, N. J., and Glass, C. K. (2013). Sterols and Oxysterols in Immune Cell Function. *Nat. Immunol.* 14, 893–900. doi:10.1038/ni.2681
- Srinivasan, K., Friedman, B. A., Etzeberria, A., Huntley, M. A., van der Brug, M. P., Foreman, O., et al. (2020). Alzheimer's Patient Microglia Exhibit Enhanced Aging and Unique Transcriptional Activation. *Cel Rep* 31, 107843. doi:10.1016/j.celrep.2020.107843
- Su, H., Rustam, Y. H., Masters, C. L., Makalic, E., McLean, C. A., Hill, A. F., et al. (2021). Characterization of Brain-Derived Extracellular Vesicle Lipids in Alzheimer's Disease. *J. Extracell. Vesicles* 10, e12089. doi:10.1002/jev2.12089
- Su, W., Aloï, M. S., and Garden, G. A. (2016). MicroRNAs Mediating CNS Inflammation: Small Regulators with Powerful Potential. *Brain Behav. Immun.* 52, 1–8. doi:10.1016/j.bbi.2015.07.003
- Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., et al. (2019). STRING v11: Protein-Protein Association Networks With Increased Coverage, Supporting Functional Discovery in Genome-Wide Experimental Datasets. *Nucleic Acids Res.* 47, D607–D13. doi:10.1093/nar/gky1131
- Takatori, S., Wang, W., Iguchi, A., and Tomita, T. (2019). Genetic Risk Factors for Alzheimer Disease: Emerging Roles of Microglia in Disease Pathomechanisms. *Adv. Exp. Med. Biol.* 1118, 83–116. doi:10.1007/978-3-030-05542-4_5
- Tang, S.-Y., Liu, D.-X., Li, Y., Wang, K.-J., Wang, X.-F., Su, Z.-K., et al. (2020). Caspr1 Facilitates sAPP α Production by Regulating α -Secretase ADAM9 in Brain Endothelial Cells. *Front. Mol. Neurosci.* 13, 23. doi:10.3389/fnmol.2020.00023
- Tenner, A. J. (2020). Complement-Mediated Events in Alzheimer's Disease: Mechanisms and Potential Therapeutic Targets. *J. Immunol.* 204, 306–315. doi:10.4049/jimmunol.1901068
- Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsohaina, R., et al. (2018). Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): a Position Statement of the International Society for Extracellular Vesicles and Update of the MISEV2014 Guidelines. *J. Extracell. Vesicles* 7, 1535750. doi:10.1080/20013078.2018.1535750
- Thion, M. S., and Garel, S. (2018). Microglia Under the Spotlight: Activity and Complement-Dependent Enrichment of Synapses. *Trends Neurosci* 41, 332–34. doi:10.1016/j.tins.2018.03.017
- Vassileff, N., Vella, L. J., Rajapaksha, H., Shambrook, M., Kenari, A. N., McLean, C., et al. (2020). Revealing the Proteome of Motor Cortex Derived Extracellular Vesicles Isolated from Amyotrophic Lateral Sclerosis Human Postmortem Tissues. *Cells* 9, 1709. doi:10.3390/cells9071709
- Vella, L. J., Scicluna, B. J., Cheng, L., Bowden, E. G., Masters, C. L., Ang, C. S., et al. (2017). A Rigorous Method to Enrich for Exosomes from Brain Tissue. *J. Extracell. Vesicles* 6, 1348885. doi:10.1080/20013078.2017.1348885
- Wang, B., Wu, L., Chen, J., Dong, L., Chen, C., Wen, Z., et al. (2021). Metabolism Pathways of Arachidonic Acids: Mechanisms and Potential Therapeutic Targets. *Signal. Transduct. Target. Ther.* 6, 94. doi:10.1038/s41392-020-00443-w
- Wang, G., Dinkins, M., He, Q., Zhu, G., Poirier, C., Campbell, A., et al. (2012). Astrocytes Secrete Exosomes Enriched with Proapoptotic Ceramide and Prostate Apoptosis Response 4 (PAR-4): Potential Mechanism of Apoptosis Induction in Alzheimer Disease (AD). *J. Biol. Chem.* 287, 21384–21395. doi:10.1074/jbc.M112.340513

- Willis, C. M., Ménoret, A., Jellison, E. R., Nicaise, A. M., Vella, A. T., and Crocker, S. J. (2017). A Refined Bead-free Method to Identify Astrocytic Exosomes in Primary Glial Cultures and Blood Plasma. *Front. Neurosci.* 11, 335. doi:10.3389/fnins.2017.00335
- Winston, C. N., Goetzl, E. J., Akers, J. C., Carter, B. S., Rockenstein, E. M., Galasko, D., et al. (2016). Prediction of Conversion from Mild Cognitive Impairment to Dementia with Neuronally Derived Blood Exosome Protein Profile. *Alzheimers Dement (Amst)* 3, 63–72. doi:10.1016/j.dadm.2016.04.001
- Winston, C. N., Romero, H. K., Ellisman, M., Nauss, S., Julovich, D. A., Conger, T., et al. (2019). Assessing Neuronal and Astrocyte Derived Exosomes from Individuals with Mild Traumatic Brain Injury for Markers of Neurodegeneration and Cytotoxic Activity. *Front. Neurosci.* 13, 1005. doi:10.3389/fnins.2019.01005
- Wong, M. Y., Lewis, M., Doherty, J. J., Shi, Y., Cashikar, A. G., Amelianchik, A., et al. (2020). 25-Hydroxycholesterol Amplifies Microglial IL-1 β Production in an apoE Isoform-dependent Manner. *J. Neuroinflammation* 17, 192. doi:10.1186/s12974-020-01869-3
- Xue, F., and Du, H. (2021). TREM2 Mediates Microglial Anti-inflammatory Activations in Alzheimer's Disease: Lessons Learned from Transcriptomics. *Cells* 10, 321. doi:10.3390/cells10020321
- Yan, Z., Zhou, Z., Wu, Q., Chen, Z. B., Koo, E. H., and Zhong, S. (2020). Presymptomatic Increase of an Extracellular RNA in Blood Plasma Associates with the Development of Alzheimer's Disease. *Curr. Biol.* 30, 1771–e3. doi:10.1016/j.cub.2020.02.084
- Yiannopoulou, K. G., Anastasiou, A. I., Zachariou, V., and Pelidou, S. H. (2019). Reasons for Failed Trials of Disease-Modifying Treatments for Alzheimer Disease and Their Contribution in Recent Research. *Biomedicine* 7, 97. doi:10.3390/biomedicine7040097
- Yilmaz, M., Yalcin, E., Presumey, J., Aw, E., Ma, M., Whelan, C. W., et al. (2021). Overexpression of Schizophrenia Susceptibility Factor Human Complement C4A Promotes Excessive Synaptic Loss and Behavioral Changes in Mice. *Nat. Neurosci.* 24, 214–224. doi:10.1038/s41593-020-00763-8
- Yuyama, K., Sun, H., Sakai, S., Mitsutake, S., Okada, M., Tahara, H., et al. (2014). Decreased Amyloid- β Pathologies by Intracerebral Loading of Glycosphingolipid-Enriched Exosomes in Alzheimer Model Mice. *J. Biol. Chem.* 289, 24488–24498. doi:10.1074/jbc.M114.577213
- Zhang, L., Wang, J., Wang, J., Yang, B., He, Q., and Weng, Q. (2020). Role of DJ-1 in Immune and Inflammatory Diseases. *Front. Immunol.* 11, 994. doi:10.3389/fimmu.2020.00994
- Zhu, C., Bilousova, T., Focht, S., Jun, M., Elias, C. J., Melnik, M., et al. (2021). Pharmacological Inhibition of nSMase2 Reduces Brain Exosome Release and α -synuclein Pathology in a Parkinson's Disease Model. *Mol. Brain* 14, 70. doi:10.1186/s13041-021-00776-9

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Cohn, Melnik, Huang, Teter, Chandra, Zhu, McIntire, John, Gyls and Bilousova. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



The Aquatic Invertebrate *Hydra vulgaris* Releases Molecular Messages Through Extracellular Vesicles

OPEN ACCESS

Edited by:

Suman Dutta,
University of California, Los Angeles,
United States

Reviewed by:

Subhra Prakash Hui,
University of Calcutta, India
Syed Feroj Ahmed,
University of Glasgow,
United Kingdom

*Correspondence:

Claudia Tortiglione
claudia.tortiglione@cnr.it

[†]These authors have contributed
equally to this work and share first
authorship

[†]Present address:

Alfredo Ambrosone,
Department of Pharmacy, Biomedical
Division, University of Salerno, Salerno,
Italy
Beata Miedziak,
Faculty of Biotechnology, University of
Rzeszów, Rzeszów, Poland

Specialty section:

This article was submitted to
Membrane Traffic,
a section of the journal
Frontiers in Cell and Developmental
Biology

Received: 01 October 2021

Accepted: 15 November 2021

Published: 20 December 2021

Citation:

Moros M, Fergola E, Marchesano V,
Mutarelli M, Tommasini G, Miedziak B,
Palumbo G, Ambrosone A, Tino A and
Tortiglione C (2021) The Aquatic
Invertebrate *Hydra vulgaris* Releases
Molecular Messages Through
Extracellular Vesicles.
Front. Cell Dev. Biol. 9:788117.
doi: 10.3389/fcell.2021.788117

**Maria Moros^{1,2†}, Eugenio Fergola^{2†}, Valentina Marchesano², Margherita Mutarelli²,
Giuseppina Tommasini², Beata Miedziak^{2†}, Giuliana Palumbo², Alfredo Ambrosone^{2†},
Angela Tino² and Claudia Tortiglione^{2*}**

¹Instituto de Nanociencia y Materiales de Aragón(INMA), CSIC-Universidad de Zaragoza, Zaragoza, Spain, ²Istituto di Scienze
Applicate e Sistemi Intelligenti "E. Caianiello", Consiglio Nazionale Delle Ricerche, Pozzuoli, Italy

Recent body of evidence demonstrates that extracellular vesicles (EVs) represent the first language of cell-cell communication emerged during evolution. In aquatic environments, transferring signals between cells by EVs offers protection against degradation, allowing delivering of chemical information in high local concentrations to the target cells. The packaging of multiple signals, including those of hydrophobic nature, ensures target cells to receive the same EV-conveyed messages, and the coordination of a variety of physiological processes across cells of a single organisms, or at the population level, i.e., mediating the population's response to changing environmental conditions. Here, we purified EVs from the medium of the freshwater invertebrate *Hydra vulgaris*, and the molecular profiling by proteomic and transcriptomic analyses revealed multiple markers of the exosome EV subtype, from structural proteins to stress induced messages promoting cell survival. Moreover, positive and negative regulators of the Wnt/ β -catenin signaling pathway, the major developmental pathway acting in body axial patterning, were identified. Functional analysis on amputated polyps revealed EV ability to modulate both head and foot regeneration, suggesting bioactivity of the EV cargo and opening new perspectives on the mechanisms of developmental signalling. Our results open the path to unravel EV biogenesis and function in all cnidarian species, tracing back the origin of the cell-cell, cross-species or cross-kingdom communication in aquatic ecosystems.

Keywords: extracellular vesicles, cell-cell communication, exosome, RNA-seq, *Hydra vulgaris*, aquatic invertebrate model

INTRODUCTION

Cell-cell communication is a mandatory condition for all the multicellular organisms as we know them; the possibility to exchange multiple and different signals between cells, at great distances or even between different organisms, supports the organism homeostasis and allows interacting with the surrounding environment. Intercellular cross-talk is achieved by various means, with the most straightforward one being the direct contact between communicating cells, also known as the juxtacrine signalling. Gap junctions are a well-known example of this form of communication and their functional characterization has been widely documented both in vertebrates and invertebrates

(Takaku et al., 2014; Misu et al., 2016; Zong et al., 2016). Conversely, paracrine or endocrine signalling, namely the release and subsequent uptake of signalling factors, does not require a direct contact between donor and recipient cells, allowing for short- and long-distance intercellular communication. In addition to communication routes based on the secretion of soluble molecules in the extracellular space, the discovery of signalling factors embedded in bilayer lipid membrane structures, termed extracellular vesicles (EVs), has gradually expanded our view on how cellular cross-talk works. Despite the lack of general consensus about their classification and nomenclature (Thery et al., 2018) EVs are broadly divided into three main categories: apoptotic bodies, microvesicles and exosomes, mainly based on their different diameter and biogenesis route (Kalra et al., 2012; Veziroglu and Mias, 2020). First discovered in sheep reticulocyte cultures (Pan and Johnstone, 1983) (Harding et al., 1984) (Johnstone et al., 1987), exosomes are spheroidal nano-sized vesicles (30–150 nm) which originate during early-to-late endosome maturation. Multiple events of inward budding along the endosome membrane selectively gather proteins and nucleic acids from the cytosol into intraluminal vesicles (ILV). The late endosome is henceforth termed multivesicular body (MVB) and its fusion with the cell plasma membrane releases these vesicles into the extracellular space (Colombo et al., 2014; Kalluri and Lebleu, 2020; Skryabin et al., 2020). The protein content of exosomes partly reflects the biogenesis and maturation pathways that these vesicles undertake; consequently, specific proteins can be used as biochemical markers in order to confirm the presence of such vesicles in the isolated fractions. A fair number of tetraspanin transmembrane proteins, such as CD9, CD63 and CD81, are enriched in exosomes, reflecting the spatial reorganization that endosomal proteins go through before the formation of the MVB (Kowal et al., 2016; Anand et al., 2019). Similarly, lysosomal-associated membrane proteins LAMP1 and LAMP2 are late endosomal markers and can usually be found in exosomes (Colombo et al., 2014). However, different populations of exosomes may have distinct molecular signatures (Kowal et al., 2016; Juan and Furthauer, 2018). In fact, one fascinating feature of this type of EVs is that their content, comprising proteins, lipids and nucleic acids, is determined not only by the cell type they originate from, but also by the physiological state of the organism, resulting in major differences of these vesicles and their cargo in terms of quantity and quality (Ludwig et al., 2019). Such diversity is in agreement with the many processes exosomes have been implicated into, such as parasitic host-pathogen interaction (Coakley et al., 2015), viral infection, immune response, cardiovascular diseases, central nervous system disease, and cancer progression, shuttling into the receiving cells nucleic acids, metabolites, lipid and proteins (Kalluri and Lebleu, 2020). Of particular interest in cancer biology is their role in the “education” of distant cells in order to create a pre-metastatic niche, thus

promoting metastasis (Peinado et al., 2012; Ghajar et al., 2013; Peinado et al., 2017). The intrinsic properties of exosomes in regulating complex intracellular pathways offer a new paradigm for their application as disease biomarkers, or as nanocarriers of therapeutic agents in cell-free therapies (Lu and Huang, 2020).

The wide portfolio of possible applications makes even more appealing the evolutionary study of EVs in model organisms, to increase our understanding in how they work and how they exert their functions in different contexts and environments. For instance, exosomes derived from *Drosophila melanogaster* have been identified as partly responsible for the establishment of Hedgehog (HH) and Wingless morphogenic gradients during the wing imaginal disc development (Gross et al., 2012; Gradilla et al., 2014). In *Caenorhabditis elegans* HH-related proteins are released on apical exosomes in a MVB-dependent manner involving V0-ATPase and P-4 ATPase activities (Liegeois et al., 2006; Wehman et al., 2011). However, very little is known about the role of EVs in aquatic environment. By virtue of their ability to transport abundant biological signals, to protect them from the highly heterogeneous and possibly damaging environment, and to increase the local concentration of one or more co-acting biomolecules that reach the target cells, EVs can have a profound impact on aquatic community structure and trophic level-interactions (Schatz and Vardi, 2018), providing important clues on interspecies and interkingdom relationships. Due to the conservation of key physiological mechanisms throughout the animal kingdom, evolutionary approaches to understanding EV biogenesis and bioactivity may profoundly impact on both fundamental and applied biology in vertebrates.

In this paper we report for the first time the presence of EVs in the freshwater cnidarian polyp *Hydra vulgaris* and characterized main morphological, biochemical and functional features. Classically used as a model organism in developmental biology, the structural simplicity of the *Hydra* body offers many advantages for a variety of investigations, from environmental ecotoxicology to nanobiotechnology, allowing to study the impact of exogenous nanostructured compounds (Quinn et al., 2012; Ambrosone et al., 2017; Tortiglione et al., 2017; Allocca et al., 2019) together with intracellular biotransformations and molecular responses (Veronesi et al., 2019; Moros et al., 2020a; Moros et al., 2020b) at animal, cell and molecular levels. The body wall is organized as a hollow tube, with an apical mouth opening surrounded by a ring of tentacles and a foot to anchor to a substrate. It is structured as a bilayer of epithelia, an ectoderm with epitheliomuscular cells facing the outer medium and an inner endoderm with all cells facing the body cavity, separated by an acellular mesoglea (**Figures 1A,B**). This tissue-like organization, with no organs and body fluids, is maintained by the continuous cell self-renewal and differentiation of three distinct stem cell lineages of endoderm, ectoderm, and interstitial stem cells, giving rise to a few differentiated cell type, i.e., neurons, gland cells, nematocytes and gametes (Bosch, 2009; Hobmayer et al., 2012). The plasticity and remodelling capacity of the body reaches the highest expression during the regeneration

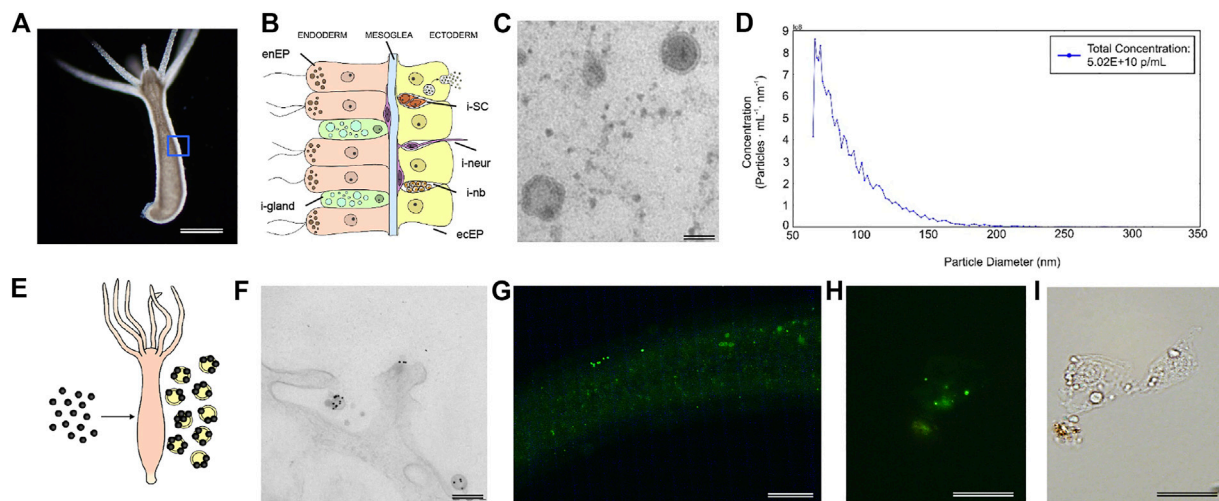


FIGURE 1 | Morphological characterization of *Hydra* EV. **(A)** Dark-field image of a living *Hydra vulgaris* showing the simple body plan and **(B)** a schematic representation of the diploblastic tissue organization in a cross-section view. Epitheliomuscular cells (ecEP, yellow) face the outer medium and the inner cavity (enEP, pink). Interstitial stem cells (ISC, orange) lay interspersed into the ectoderm giving rise to a few differentiated cell types such as nematoblasts (i-nb), neurons (i-neur) and gland cells (i-gland). EV release into the medium is shown for a single ectodermal cell. **(C)** TEM image of isolated vesicles exhibiting round morphology and a bilayered membrane. Scale bar, 50 nm. **(D)** MRPS measurements show the distribution of the EV size, presenting two major peaks at 66 and 70.5 nm (using a TS-400 cartridge). **(E)** Schematic illustration of *in vivo* assembling of AuNP decorated EVs, by treatment of *Hydra* with AuNPs. **(F)** TEM analysis 48 h post treatment shows AuNPs on the surface of EVs. Scale bar 200 nm. **(G)** PKH67 labelled-EVs were incubated 2 h with living *Hydra*. The picture shows the body column with green fluorescent granular signals, clearly detectable also on fixed single cells **(H)** obtained by maceration of treated polyps. **(I)** Bright field image of the same cell. Scale bar 200 μ m in **(G)**, 20 μ m in **(H,I)**.

process (Steele, 2002; Bode, 2003; Bosch, 2009; Vogg et al., 2019b). Indeed, the ability to regrow the whole body from tissue pieces or even cell aggregates has made *Hydra* a one-of-a-kind platform to study regeneration processes and to shed some light on why some organisms have partially lost this ability (Li et al., 2015). The Wnt/ β catenin signalling pathway represents the major developmental pathway acting in adult *Hydra* to maintain the oro-aboral axis, and it is reactivated during injury and throughout the regeneration to rebuild missing structures from amputated polyps (Hobmayer et al., 2000; Chera et al., 2009b; Lengfeld et al., 2009). Functional and transcriptomic evidences have recently showed the early activation of the Wnt/ β catenin signalling pathway as part of a generic transcriptional response to injury, which initiates an oral patterning cascade independent from the tissue context (Petersen et al., 2015; Cazet et al., 2021). The outcome of this activation appears regulated by long range signals generated by tissue organizers located at both animal poles (Bode, 2009; Petersen et al., 2015; Gufler et al., 2018; Wenger et al., 2019; Cazet et al., 2021), whose existence was proposed 40 years ago, by means of classical tissue manipulation techniques (Macwilliams, 1983). To date, in *Hydra*, no vehicles have been isolated for lipid-modified and poorly soluble signalling molecules. The EV function in developmental signalling, promoting the release and spread of canonical ligands and/or mediating the transfer of a variety of biologically active molecules from one cell to another is well documented (Gross et al., 2012; Zhang and Wrana, 2014; McGough and Vincent, 2016). This prompted us to search for similar EV mediated

signalling mechanisms also in *Hydra*, and to shed light on the early evolution of signalling mechanisms in metazoans. We developed a strategy for EV purification from the polyp culture medium, characterized their morphology and their molecular content at protein and RNA levels, and provide evidence on the bioactivity of their cargo. The shuttling of EV messages in target cells shows that this mechanism of cell-cell communication evolved early in animal evolution and may mediate not only physiological processes but also cross-species or cross-kingdom communication in aquatic ecosystems.

MATERIALS AND METHODS

Animal Culture

H. vulgaris (strain Zurich) was asexually cultured in Hydra medium (HM: 1 mM CaCl₂ and 0.1 mM NaHCO₃) at pH 7. The animals were kept at 18 $\pm$ 1°C and fed thrice a week with freshly hatched *Artemia salina* nauplii.

Collection of Extracellular Vesicles

For EV collection, 250 polyps were placed in 10 ml of HM in 6 cm Petri dishes. After 4 days starvation, *Hydra* conditioned medium was collected and centrifuged using a Beckman Coulter Avanti J-25 centrifuge (rotor JA-25.15) at 10,000 g for 30 min to remove tissue patches and at 20,000 g for 30 min to pellet any cellular debris. The resulting supernatant was then centrifuged at 74,200 g for 150 min at 4°C and the pelleted

EVs were washed in HM followed by one last centrifugation. Pelleted EVs were resuspended in either PBS or Hydra medium.

Transmission Electron Microscopy

EVs were visualized using TEM following a slightly modified protocol to that reported by Thery et al. (2006). Briefly, EVs were fixed with an equal volume of 4% paraformaldehyde and deposited onto a Formvar-carbon coated electron microscopy grid. Once dried, the grids were washed with PBS, fixed with 1% glutaraldehyde for 5 min and washed with distilled water 8 times. The samples were stained with 1% ammonium molybdate for 2 min, dried and observed using a FEI Tecnai 12,120 kV or JEOL United States, Inc., United States) at 80 kV. *Hydra* polyps were dissected into appropriate pieces to fit into cup-shaped HPF specimen carriers. Tissue pieces were pipetted into a 0.2- or 0.3-mm deep carrier and covered with an additional carrier. Finally, the obtained sandwiches were cryo-immobilized by HPF. Frozen sandwiches were transferred into appropriate containers for storage in Liquid N₂ for later use or subjected to freeze substitution (FS). The frozen samples were transferred under liquid N₂ into cryo-vials containing frozen FS cocktails (anhydrous acetone plus 1% OsO₄ and 0.1–0.2% uranyl acetate). Subsequently, the lids were screwed loosely onto the vials to permit safe evaporation of excess N₂ gas. The vials were placed into the precooled FS device and after about 1 h the lids tightened and FS was performed for at least 8 h at –80 to –90°C; warming up to –55°C at a rate of 5–10°C per hour, subsequent post-fixation and staining at –55°C for 6 h, followed by warming up to –30°C at a rate of 5–10°C per hour where samples were left for an additional 3 h. Finally, samples were washed three times with acetone (10 min each), 16 h in 10% epoxy resin in acetone, 6 h in 30% epoxy resin in acetone, 16 h 70% epoxy resin in acetone, 6 h 100% epoxy resin in acetone, 16 h 100% epoxy resin in acetone. Then the samples were placed in fresh resin and placed in oven at 60°C overnight.

Sample serial thin (70 nm) section of animals were cut with a diamond knife and mounted onto 150–200 mesh Hex grids. Ultrathin sections were examined with transmission EM Libra 120 EFTEM (Zeiss, Oberkochen, Germany) at 80 eV.

EV Sizing and Counting

Microfluidic Resistive Pulse Sensing (MRPS) (Cimorelli et al., 2021): measurements of isolated extracellular vesicle size and concentration were performed using the nCS1™ instrument (Spectradyne LLC, United States), which is based on the microfluidic resistive pulse sensing technology. EVs isolated from 250 polyps and resuspended in Hydra medium were diluted in equal volume with Tween/PBS buffer in order to meet the instrument requirements regarding medium conductivity. EV samples were analysed with TS-400 and TS-300 cartridges (measurement range 60–400 nm and 50–300 nm, respectively).

Dynamic light scattering (DLS) was performed with a Malvern Zetasizer using EVs diluted in PBS at pH 7.4. Three runs were made at 25°C and at 90° scattering angle.

Western Blot Analysis

Protein concentration was determined using a colorimetric assay based on the Bradford Assay (ThermoFisher) and samples were diluted with PBS to obtain equal protein concentration (EV and whole *Hydra* homogenates). Samples were mixed with 4x Laemmli Sample Buffer containing 2-mercaptoethanol (Biorad) and boiled for 5 min at 95°C. Proteins were resolved by SDS-PAGE (mini protean TGX precast any KDa gel (Biorad)), blotted onto PVDF membranes (Amersham) and blocked o/n at 4°C in 5% non-fat dry milk in PBS-T (0.5% Tween-20). Membranes were then incubated with the primary antibody for 2 h at room temperature, washed with PBS-T 0.1%, and incubated with goat anti-rabbit IgG Antibody, (H + L) HRP-conjugated secondary antibody (1:4,000, BioRad) for 1 h at room temperature. Finally, protein bands were detected using an enhanced chemiluminescence reagent (Clarity ECL Substrate (Biorad)) and imaged with ChemiDoc XRS System (BioRad) using Image Lab software (BioRad). Antibodies: rabbit anti-*Hydra* Annexin B12 1:1,000 (kindly gifted by Dr. M. Isas, Keck School of Medicine of USC Los Angeles, CA), mouse anti- β -Actin–Peroxidase (1:40,000, Sigma).

EV Staining and Uptake

EVs obtained as described before were labelled with PKH67 Green Fluorescent Cell Linker (Sigma Aldrich) following manufacturer's protocol, with minor modifications. Briefly, EVs obtained from 500 polyps in HM were added to 1 ml of diluent C. As a negative control, 1 ml of diluent C was mixed with the same volume of HM. Afterwards, 6 μ L of PKH67 dye was added and mixed for 30 sec by gently pipetting. After 5 min incubation at room temperature, 2 ml of 10% BSA was added to quench the unbound dye. EVs were washed and isolated by ultracentrifugation as described before. *Hydra* were incubated with PKH67- labelled EVs and with the negative control up to 5 h, washed with HM and imaged under a fluorescence microscope (Zeiss).

Regeneration Experiments

All the experiments were carried out using adult polyps starved for 24 h. For the head regeneration experiments, batches of 25 budless polyps were bisected at 80% body length (sub-hypostomal cut) or 50% body length (mid-gastric cut) and left regenerating for up to 72 h in presence of EVs isolated from 250 polyps (100 μ L) in a total volume of 300 μ L. The regeneration process was monitored at different time points using an optical microscope and polyps were grouped into four stages according to their tentacle morphogenetic features.

For the foot regeneration experiments, groups of 15 animals were bisected at 20% body length and transferred in HM containing EVs isolated from 250 polyps (100 μ L) in a total volume of 300 μ L. After 24 h, polyps were relaxed in 2% urethane in HM for 2 min and pre-fixed by adding 4% PFA/HM for 15 min. After removing the pre-fixation medium, animals were fixed for 1–3 h in 4% PFA/HM at 18°C and washed three times in PBS to remove any residual fixative. For the peroxidase assay, 2 ml of DAB solution was prepared

according to the manufacturer's instructions (Sigma-Aldrich tablets) and used for 15 min on the fixed animals. In order to stop the colorimetric reaction, the polyps were washed five times with ddH₂O and then mounted with PBS/Glycerol 1:1 on microscope slides for imaging.

Stained animals were observed by an inverted microscope (Axiovert 100, Zeiss) and images of each foot were taken with a digital camera (Olympus, DP 70) under the same conditions of acquisition (light and exposure time). The colorimetric analysis was performed using the HSB format on the acquired images in the ImageJ software version 1.53e. Three staining categories were arbitrarily set according to DAB positive area extension as low (0–9,000 μm^2), medium (9,000–19,000 μm^2) and high (19,000–28,000 μm^2).

EV Pre-treatment With Proteinase and RNase

Prior to total RNA extraction, EVs isolated by ultracentrifugation were enzymatically treated to eliminate the free RNA derived from the ribonucleoprotein complexes that could be present in the conditioned medium. Isolated vesicles were treated with 1 mg/ml of proteinase K for 30 min at 37°C. Afterward, the samples were incubated 10 min at 65°C to inactivate the proteinase K and then treated with 10 $\mu\text{g}/\text{ml}$ of RNase A for 15 min at 37°C.

qRT-PCR Analysis

After treating polyps for 24 and 48 h with EVs isolated from 750 *Hydra*, total RNA was extracted from animals using Trizol or from EVs using Total RNA Extraction Kit (Norgen Biotek, Corp.) following manufacturer's instructions and its concentration was determined using a SmartSpec plus spectrophotometer (Biorad). The first-strand cDNA was synthesized by High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). qRT-PCR was performed using a Fast SYBRTM Green Master Mix with premixed ROX (Applied Biosystems), in a StepOne Real-Time PCR System (Applied Biosystem) under the following fast cycling steps: initial denaturation for 20 s at 95°C, followed by 40 cycles at 95°C for 3 s, 60°C for 30 s. In addition, melting curves (20 min, from 59° to 90°C) were generated to check any undesired amplification products. *EF1 α* was used as internal control.

In order to validate RNA-seq analysis, a presence/absence experiment of selected targets was performed using an EV sample isolated from 2000 polyps. Specific primers of *Hydra* genes *Wnt3a*, *β -catenin*, *actin* and *Hsp70* were designed using the Primer3 software (<http://frodo.wi.mit.edu/primer3/>) and are listed in **Supplementary Table S3**, together with the corresponding GenBank accession numbers. Due to the qualitative nature of the presence/absence analysis, no internal control was used for the amplification reaction. The presence of such transcripts in the EV sample was confirmed by their cDNA amplification compared to negative controls, where no amplification occurred. Additional analysis by generation of melting curves (from 60 to 95, 0.5°C increment) confirmed sequence specific amplification in EVs and the absence of

amplification in No Template Control (NTC). Mean C_t values for each gene and its NTC are shown in **Supplementary Table S2**.

RNA Seq

EVs were collected at different times and stored at –80°C. Total RNA extraction was performed on EV samples collected from 10,000 polyps overall and in two replicates using “Total RNA Purification Kit” (Norgen Biotek Corp.) and following the manufacturer's supplementary protocol for exosomal RNA purification, with one modification: the optional DNA removal step was carried out using RQ1 RNase-Free DNase (Promega) at 0.1 U/mL. The two samples were sent to the Sequentia Biotech S.L. (Barcelona, Spain) company for quality check, cDNA library preparation using “Ovation[®] SoLo RNA-Seq Systems” (NuGEN Technologies, Inc.), and sequencing via a paired-end chemistry on an Illumina platform. After quality inspection of the produced reads with fastqc (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore), reads were cleaned to remove the first five bases of read1, the Illumina adapter sequence “AGATCGGAAGAGC” and the bases with quality below 20 from the 3' end using the program TrimGalore! (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore); the cleaned reads shorter than 35 bp were removed from further analysis (9.3% of the produced read pairs). Trimmed reads were then aligned using the program STAR (version 2.7.1a)⁷⁸ against the NCBI *Hydra vulgaris* reference genome (assembly Hydra_RP_1.0), while for gene expression quantification, trimmed reads were also aligned on the corresponding transcript sequences GCF_000004095.1_Hydra_RP_1.0_rna.fna.gz using the program kallisto (Dobin et al., 2013; Bray et al., 2016). Kallisto was also used to quantify genes using the reference transcriptome used in Siebert et al. (2019) in order to directly map the genes with the cell types where they were identified.

Protein Identification by MALDI-TOF/TOF

To perform the MALDI-TOF/TOF EV samples were first run into a SDS-Page gel. EV total proteins were quantified using a Bradford assay, 4x Laemmli Sample Buffer with 2-mercaptoethanol (Biorad) was added, samples were heated to 95°C for 5 min and they were run in a mini protean TGX precast 7.5% gel (Biorad). The gel was silver stained following standard procedures.

In-gel digestion- Protein bands were manually excised with a cutter, and in-gel digested with an automatic digester (Intavis, Bioanalytical Instruments, Cologne, Germany). Briefly, spots were washed with water, ammonium bicarbonate (100 mM), and acetonitrile. Next, samples were reduced by incubation with DTT (10 mM) at 60°C for 45 min and alkylated by incubation with iodoacetamide (50 mM) at room temperature for 30 min. Finally, proteins were digested with trypsin overnight at 37°C (5 ng/ μL , Trypsin Gold, Promega, WI, United States). Digestion was stopped by adding 0.5% TFA (trifluoroacetic acid), and tryptic peptides were extracted sequentially with increasing concentrations of acetonitrile in water. Peptides were concentrated and desalted by passing it through ZipTip C18 tips (Millipore) following the manufacturer's instructions and eluted with 50%ACN 0.1%TFA.

Peptides were spotted into an Opti-ToF 384 well insert plate (Sciex) with a saturated solution of alpha-cyano-4-hydroxycinnamic acid (CHCA) prepared in 50% ACN/0.1% TFA. Proteins were identified using a 4800plus MALDI-TOF/TOF (Sciex) in the reflector mode with accelerating voltage of 20 kV, mass range of 800–4000 Da and 1,000 shots/spectrum. MS/MS spectra were acquired automatically on the 20 most intense precursors. Spectra were calibrated externally using a standard protein mixture (4,700 Calmix, ABSciex).

Protein Identification

Proteins were identified with the search engine Mascot (Matrix Science, London, United Kingdom) against the NCBI prot database of *Hydra* (29,584 sequences). Search parameters used were: missed cleavage 1, fixed modifications carbamidomethyl (cysteines), variable modifications oxidation (methionine) and peptide and fragment mass tolerance 0.2 and 0.3 Da, respectively. Proteins with a score above 23 were considered a positive hit.

Protein Identification by LC-ESI-MS/MS

In solution digestion- Samples were evaporated and resuspended in 10 μ L of denaturing buffer (6 M urea, 100 mM Tris buffer pH 7.8). Next, cysteines were reduced with 1.5 μ L DTT (200 mM) for 30 min at 37°C and alkylated with 6 μ L of iodoacetamide (200 mM) for 30 min in the dark. Unreacted iodoacetamide was consumed adding 6 μ L of the reducing agent (200 mM DTT) for 30 min at room temperature. Samples were diluted with 50 mM ammonium bicarbonate to a final concentration less than 1 M of urea. Digestion was carried out overnight with the enzyme trypsin (Gold Trypsin, Promega) at 37°C and a 1:10 ratio (enzyme/protein). Reaction was stopped adding concentrated formic acid (Sigma). Samples were evaporated, resuspended in 2% acetonitrile, 0.1% formic acid and filtered through 0.45 μ m filters. Protein identification was performed on a nano-LC system (Tempo MDLC, Eksigent, Dublin, CA, United States) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (4000 QTRAP, Sciex). After precolumn desalting, tryptic digests (1 μ g) were separated on a C18 column (Acclaim PepMap100, 75 μ m id, 15 cm, 3 μ m particle size, Thermo Scientific, United States) at a flow rate of 300 nL/min and a 120 min linear gradient from 5 to 35% ACN in 0.1% formic acid. The mass spectrometer was interfaced with a nanospray source equipped with uncoated fused silica emitter tip (20 μ m inner diameter, 10 μ m tip, NewObjective, Woburn, MA) and was operated in the positive ion mode. MS source parameters were as follows: capillary voltage 2800 V, source temperature 150°C, declustering potential (DP) 85 V, curtain and ion source gas (nitrogen) 20 psi, and collision gas (nitrogen) high. Analyses were performed using an information dependent acquisition (IDA) method with the following steps: single enhanced mass spectra (EMS, 400–1,400 m/z) from which the eight most intense peaks were subjected to an enhanced product ion [EPI (MS/MS)] scan. Proteins were identified with the search engine Mascot (v2.3 MatrixScience,

United Kingdom) using NCBIprot database of *Hydra* (29,584 sequences). Search parameters used were: missed cleavages 1, fixed modifications carbamidomethyl (cysteines), variable modification oxidation (methionine) and peptide and fragment mass tolerance 0.5 and 0.3 Da, respectively. Only proteins with an overall Mascot score higher than 32 and at least two matching spectra were considered in the final list.

RESULTS

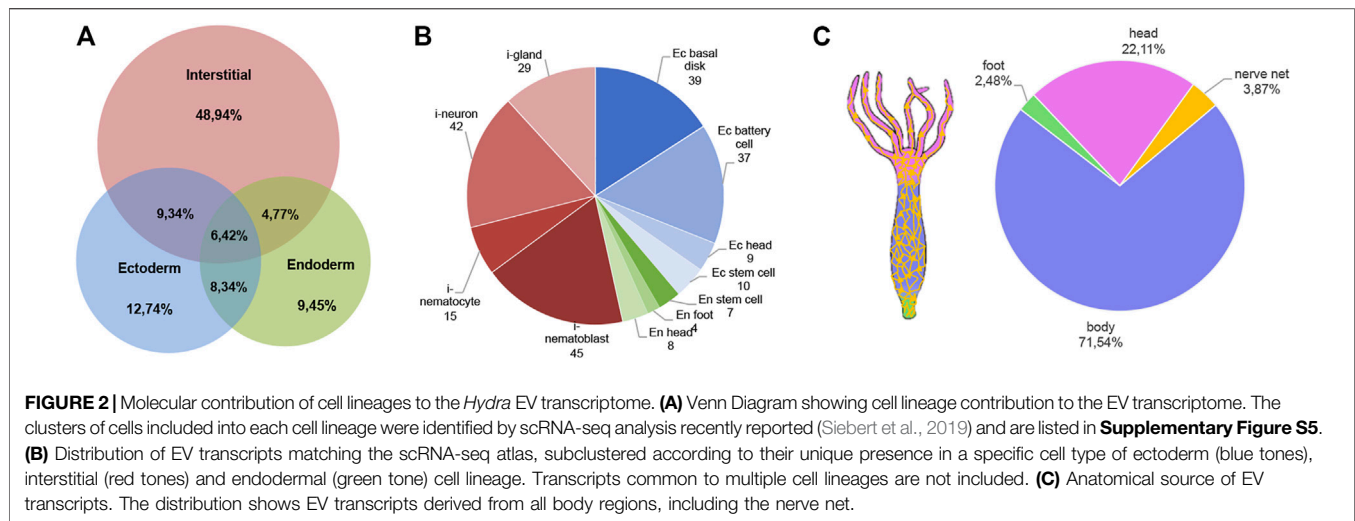
Hydra vulgaris Releases Round-Shaped Extracellular Vesicles Into the Aquatic Environment

EVs were isolated from the medium of starved polyps by differential centrifugation, following a well-established protocol (C Thery et al., 2006), slightly modified. Pelleted EVs suspended in Phosphate buffered saline (PBS) were then characterized by Transmission Electron Microscopy (TEM). **Figure 1C** shows the round shape of isolated EV and the presence of double membrane around the electron dense vesicle (**Supplementary Figure S1**). By microfluidic resistive pulse sensing (MRPS) a yield of 5×10^{10} particles/mL was estimated from a typical preparation (250 polyps into 10 ml of medium), while the distribution of the particle size showed two peaks at 66 and 70.5 nm (**Figure 1D** and **Supplementary Figure S1**). A good correlation with these measurements was obtained through other approaches, such as Dynamic Light Scattering (DLS) analysis and TEM (**Supplementary Figure S1**), both confirming the average size of 66 nm, and suggesting an enrichment in exosome-like vesicles. The presence of EVs on *Hydra* ectodermal cell surface was previously supposed during an ultrastructural study describing several inward and outward mechanisms negotiating the entrance, trafficking, and clearance of gold nanoparticles in animal tissues (Marchesano et al., 2013). To confirm this evidence, *Hydra* polyps were pulsed (24 h) with positively charged AuNPs, synthesised as previously described (Marchesano et al., 2013) and 48 h later processed for TEM (**Supplementary Figure S2**). Semi-thin slices showed the presence of round nanovesicles decorated with AuNPs on the external membrane of ectodermal cells, laying into the glycocalyx (**Figures 1E,F**), confirming *in vivo* assembly of AuNP-EV structures as part of the secretory route (Marchesano et al., 2013). This assembly demonstrates a strong interaction between EV membranes and positively charged AuNPs, likely due to electrostatic interaction.

The ability of EVs to be internalized into *Hydra* tissues was investigated by labelling freshly isolated EVs with PKH67 and incubating living polyps up to 5 h. Distinct green fluorescent signals were found on the body column and tentacles 2 h post incubation and in single cells obtained by maceration of treated polyps, indicating an efficient uptake of EVs into *Hydra* cells (**Figures 1G–I**; **Supplementary Figure S3**). These signals became

TABLE 1 | Name and putative function of selected proteins identified by ESI-MS/MS analysis.

Accession No.	Protein name	Putative function	Matched peaks	Matched peptide	Mascot score	ExoCarta
Cytoskeletal proteins						
CDG70628	Actin, cytoplasmic 1	Cytoskeleton component	18	16	235	Yes
XP_002161913	Tubulin beta chain	Cytoskeleton component	10	8	169	Yes
XP_004208788	Tubulin alpha-1D chain	Cytoskeleton component	5	4	41	Yes
Extracellular matrix constituents						
XP_012558692	Fibrillin-1	Microfibril assembly	11	10	125	Yes
XP_002166874	Fibrillin-2 isoform X1	Microfibril assembly	9	7	107	Yes
XP_002154154	Latent-transforming growth factor beta-binding protein 4-like	Extracellular matrix constituent	12	12	80	Yes
Membrane proteins						
NP_001296699	Annexin B12	Calcium-dependent membrane binding	7	4	100	No
XP_004212135	Protocadherin Fat 4, partial	Cell-cell adhesion	7	7	89	Yes
XP_004212674	Protocadherin Fat 1, partial	Cell-cell adhesion	17	17	71	Yes
XP_012553809	Annexin A4-like	Calcium-dependent membrane binding	14	13	61	Yes
XP_002165348	CD151 antigen	Exosome marker	5	5	56	Yes
XP_004211099	Usherlin-like, partial	Integral component membrane	2	2	47	No
Cell-cell communication and signal transduction						
AAN87350	14-3-3 protein B	Growth adaptation to food supply	2	2	54	No
XP_002156827	Ras-like GTP-binding protein RHO	Signal transduction	2	2	52	Yes
AEW90237	Voltage-gated sodium channel Nav2.1, partial	Neuronal signaling in Hydra	15	15	34	No
XP_012554672	cAMP-dependent protein kinase type II regulatory subunit-like isoform X2	cAMP-mediated signaling pathway	5	4	42	Yes
XP_004209147	Cell division control protein 42 homolog	Small GTPase-mediated signal transduction	2	2	36	No
AAW82079	Thyphedin	Foot formation stimulator	5	5	57	No
Others						
XP_002159321	Syntenin-1-like isoform X2	Biogenesis of exosomes	9	8	99	Yes
XP_002162060	Programmed cell death 6-interacting protein	Multivesicular body biogenesis	10	9	43	Yes
XP_002155023	Polyubiquitin-B	Vesicle cargo sorting	11	7	43	Yes
XP_002156816	Uncharacterized protein LOC100207118	Metalloproteinase with Thrombospondin motifs	5	5	130	—
CAJ33888	Putative serine protease inhibitor	Protective function against excessive autophagy	5	5	54	No



hardly detectable after 5 h, possibly suggesting the processing of the EVs into the recipient cells.

Hydra EVs Shuttle Typical Exosome-Associated Proteins and Key Components of Axial Patterning

Initially, a MALDI-TOF/TOF analysis performed on EV protein bands excised from an SDS-PAGE gel identified Annexin B12 and actin, whose presence was confirmed by immunoblot analyses (**Supplementary Figure S4**). Then a large scale EV isolation was achieved from 8,000 *Hydra*, and protein extracts were used for LC-ESI-MS/MS analysis and protein identification. The analysis from two biological replicates identified 52 proteins with a Mascot score >32 (**Supplementary Table S1**). **Table 1** shows selected unique *Hydra* proteins grouped for function. Remarkably, 29 proteins were present also in the Exocarta protein database (<http://www.exocarta.org/>), suggesting the exosome-like nature of the isolated EVs. The exosome-specific proteins include two annexins, a synthenin1-like protein involved in the biogenesis of exosomes, a programmed cell death 6-interacting protein involved in the MVB formation and the CD151 antigen belonging to the Tetraspanin family. Among others, chaperones (Heat Shock Proteins), cytoskeletal proteins (Actin, Tubulin), extracellular matrix constituents (Fibrillin 1, Fibrillin 2), and important components of cell-cell adhesion (Protocadherin fat 4) and vesicle biogenesis (Synthenin, Programmed cell death 6-interacting protein, Polyubiquitin-B) were found. Altogether the variety of putative proteins identified in the *Hydra* EVs, either structural or involved in cell-cell communication and signal transduction, suggests multiple functional roles played by EVs in adult *Hydra*, and their correlation with known exosome proteomes.

A comprehensive transcriptomic analysis was performed on total RNA from purified EVs (from two biological replicates) generating two cDNA libraries and conducting pair-end next-generation sequencings. The sequenced reads were first aligned against the NCBI *Hydra vulgaris* reference genome (assembly

Hydra_RP_1.0), where a percentage of 32 and 22% could be mapped. For gene expression quantification, the reads were also directly aligned using the program kallisto on the Hydra_RP_1.0 transcript sequences, where the total mapped reads on transcripts were 50 and 45% of the reads mapped on the genomic sequence. The presence of a subset of transcripts was confirmed in EV-derived RNA by using reverse transcription polymerase chain reaction PCR (RT-PCR). The selection was based on manual screening of genes already identified in EVs and exosomes (*hsp70* and *actin*) or belonging to the Wnt/ β -catenin signalling pathway (β -catenin, and *Wnt3*). We then used the minimum expression level in TPM (Transcripts Per Kilobase Million) of the validated genes to define an empirical detection threshold (0.4 TPM in at least one library). Using this definition, we selected 6,100 detected genes, among which 5,499 were protein-coding, 525 long non-coding and 31 tRNA genes. The detected genes were functionally annotated with the known associated Gene Ontology (GO) terms using OrthoDB to highlight the most important biological processes encoded by EV mRNAs (Kriventseva et al., 2019). The full list of the GO categorized genes is provided in Supplementary file 1. Interestingly, this analysis revealed that the most abundant transcripts within the Molecular Functions GO category are related to catalytic activity (alcohol dehydrogenase, sulfite oxidase), proteases and proteinase inhibitors (cathepsin, several members of the astacin family, antistasin), hydrolase and transferase activity. Within the Cellular Component GO category the genes encoding for intracellular and cytoplasmic anatomical structures were highly abundant, such as actin, cophylin, α -tubulin chain, β tubulin chain, talin-2, secreted glycoproteins (mucin 2-like, mucin 5AC-like), and LAMP, a well-known exosome marker. The transcripts categorized into the Biological processes GO class included the classes of protein metabolism (tRNA synthase, eIF3, eIF4, eEF1 α , tRNAs), signal transduction pathways (G-protein coupled receptors, Rho family, guanidine nuclear exchange factors, Wnt3, gremlin) and response to stress (Hsp70, Hsp90, Superoxide dismutase, Glutamate dehydrogenase, thioredoxin-like).

TABLE 2 | List of EV transcripts involved in the Wnt/ β -catenin signalling pathway matching the scRNA-seq database.

Gene id	Genes involved in Wnt/ β -catenin signaling pathway					
	Gene id (NCBI)	Gene name	Wnt modulator	Lineage	Cell type	Anatomical region
t8678aep	100,192,274	Dickkopf 1/2/4-A	Negative regulator	Interstitial	i_zimogen gland cell	Body
t14102aep	100,192,275	dickkopf-like protein Dlp-1	Negative regulator	Interstitial	i_zimogen gland cell	Body
t1688aep	100,192,284	β -catenin	Positive regulator	Ectoderm, Interstitial	Ec_battery cell, i_neuron/gland_cell_progenitor	Tentacle, Body
t29291aep	101,237,470	transcription factor Sp5-like	Negative regulator	Ectoderm, Endoderm	Ec_battery cell, En_head, En_tentacle	Head
t11826aep	100,199,257	Tcf	Positive regulator	Endoderm, Interstitial	En_head, i_neuron_ec	Head, Body
t34763aep	100,205,238	Naked cuticle	Negative regulator	Endoderm	En_head	Head
t13357aep	100,199,272	β -catenin-like protein 1	Positive regulator	Interstitial	i_female germline1, i_male germline, i_nb1, i_neuron/gland cell progenitor, i_SC, i_stem cell/progenitor	Body
t14194aep	100,203,050	Wnt3	Positive regulator	Endoderm	En_head	Head
t18735aep	100,213,948	FoxA/Budhead	Positive regulator	Interstitial	i_neuron/gland_cell_progenitor	Body
t31094aep	100,215,335	Foxd2-like		Endoderm	En_foot	Foot
t16296aep	100,199,630	HAS-7	Negative regulator	Interstitial	i_granular mucous gland cell, i_ymogen gland cell	Head, Body
t474aep	100,214,250	HmTSP	Negative regulator	Endoderm	En_tent-nem (pd), En_tentacle	Tentacle

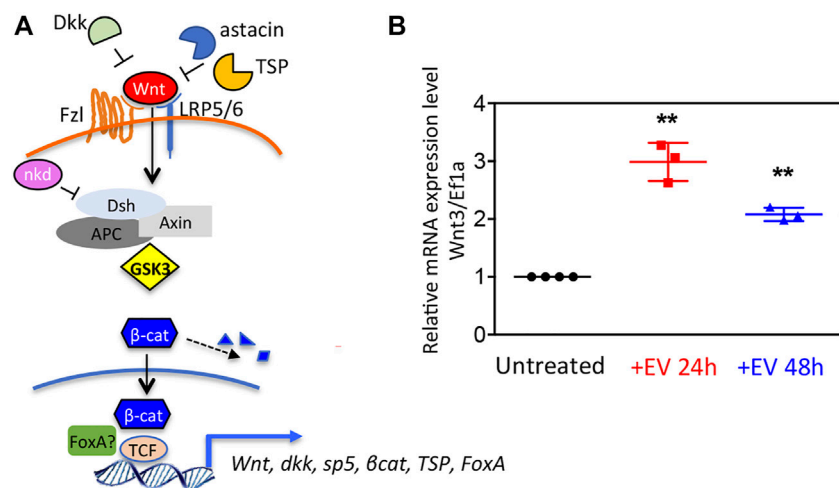


FIGURE 3 | Wnt signaling activation via *Hydra* EV. **(A)** Schematic view of the canonical Wnt/ β -catenin pathway at cellular level, in the activated state, showing extracellular and intracellular regulators identified in the *Hydra* EV transcriptome. **(B)** Relative mRNA expression levels of *Wnt3/Ef1a*. Whole polyps were treated for the indicated period with EVs freshly isolated from *Hydra* medium, then processed for RNA extraction and qRT-PCR. Data represent the average of three biological replicates ($n = 60$), each performed in triplicate and are presented as mean $\pm$ SD. Statistics was tested by two-sided unpaired t test. ** $p < 0.005$ ($p = 0.0015$ for 24 h analysis and $p = 0.0037$ for 48 h analysis).

Beside transcripts identification, lncRNA species were detected from the RNA-seq analysis. In one case the lncRNA was mapped adjacent to the *mucin5AC* gene locus, detected both at mRNA and protein levels, suggesting the possibility that EVs

may shuttle all molecular components for immediate and late availability of key information in target cells. The association of lncRNA with exosomes was first demonstrated by the analysis of human plasma-derived exosomes by RNA-seq (Huang et al., 2013),

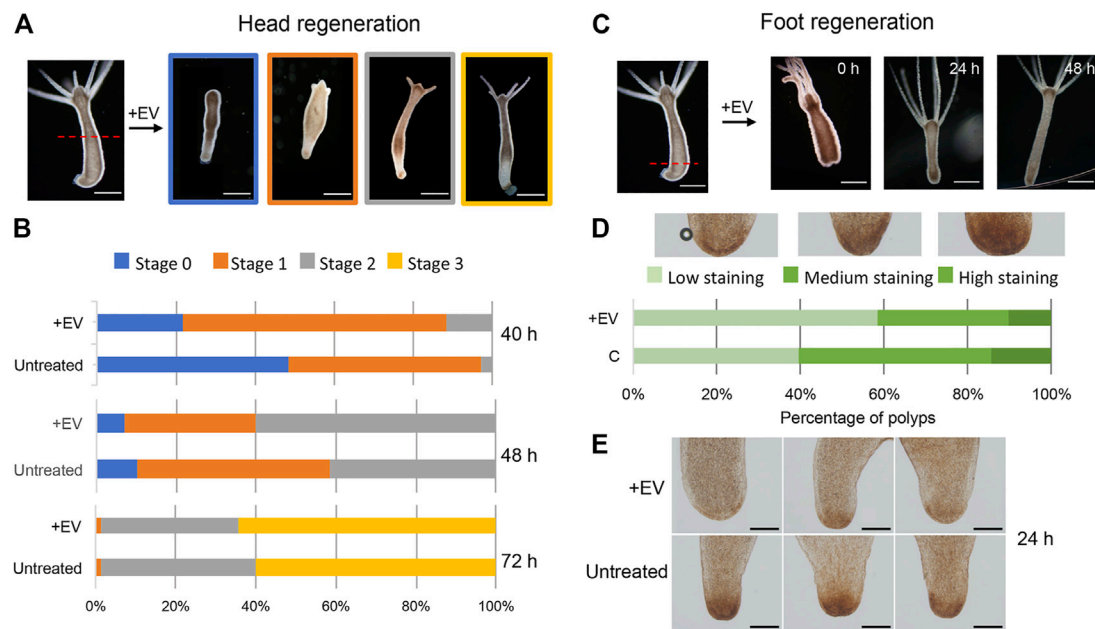


FIGURE 4 | *Hydra* head and foot regeneration are modulated by EVs. **(A)** Light microscopy images of regenerating polyps, grouped in four categories according to progressive developmental stage. **(B)** Histograms report the distribution of developmental stages at 40, 48 and 72 h post midgastric amputation (shown by the red dotted line). The bar color corresponds to the regenerative stages shown in **(A)**. Data represent the average of three independent experiments ($n = 70$). Differences in the distributions of untreated and treated polyps were statistically evaluated using Chi squared test. At 40 h $\chi^2 = 39.353$ with 2 degrees of freedom. Two-tailed *** p value = 0.000057; at 48 h $\chi^2 = 9.958$ with 2 degrees of freedom. Two-tailed * p value = 0.0069; not significant differences resulted at 72 h: $\chi^2 = 0.548$ with 2 degrees of freedom. The two-tailed p value = 0.7605 **(C)** Light microscopy images of foot regenerates at different time post amputation (red dotted line). **(D)** Distribution of foot regenerating polyps grouped in three categories: light, medium and dark green, corresponding to low, medium and high peroxidase staining, determined as DAB positive area by using the ImageJ software. Experiments were performed in triplicate ($n = 45$). $\chi^2 = 7.062$ with 2 degrees of freedom. two-tailed * p value: 0.0293. **(E)** Representative images of foot regenerating polyps (untreated and treated with EVs) 24 h p.a.

and has been recently functionally characterized and linked to disease, including a list of cancers, where they act *via* epigenetic regulation of key target genes. Their presence in *Hydra* EV might suggest a regulatory function in the receiving cells.

In order to map the EV cellular source in the animal body we used a recently-published single-cell RNA-seq (scRNA-seq) atlas (Siebert et al., 2019) to match *Hydra* EV-associated genes with molecular signatures of specific cell clusters, which were grouped according to cell lineage (ectodermal, endodermal and interstitial cell lineage), or anatomical location (head, body column and foot) (Figure 2). An alignment rate of 12% was obtained and 4,997 transcripts were detected, of which 2,975 were found in at least one cell type in the scRNA annotated on Swiss-prot (TPM 0.2 in at least one library, Supplementary file 2). A large percentage of transcripts (48%) was clustered as specific of the interstitial cell lineage, while those derived from the ectodermal (12.7%) and endodermal cell lineage (9.45%) were less abundant. By subclustering the EV transcripts according to unique cell type expression, using the same cell categories and differentiation trajectories identified in the scRNA-seq (Siebert et al., 2019), a remarkable contribute of basal disk cells, battery cells, neurons and nematoblasts emerged (Figures 2B,C). A detailed analysis subclustering EV transcripts into cell types of each cell lineage is shown in Supplementary Figure S5. Overall, this indicates the mixture of precise molecular information from multiple cell types in *Hydra* EVs. The list of the

five top genes in each cell cluster is provided in Supplementary Table S4 and the list of the 10 top genes identified matching the scRNAseq is shown in Supplementary Table S5. Interestingly, many genes belonging to the Wnt/ β catenin signalling pathway were identified (Table 2), including *Wnt3*, β -catenin, *naked cuticle* (Petersen et al., 2015) together with some inhibitors such as *dkk1/2/4* (Augustin et al., 2006; Guder et al., 2006), *astacin* (Ziegler et al., 2021), *thrombospondin* (Lommel et al., 2018), and *sp5* (Vogg et al., 2019a). Remarkably, *dkk1/2/4* and *astacin* were found among the most abundant transcripts specific of zymogen and granular mucous gland cell classes, strongly indicating their role in restricting the head organizer activity in the hypostome. By comparing the EV transcriptome to the proteome many annotated proteins (found also on exosome proteomes) were identified also at transcript level (*actin*, *tubulin*, *annexinB12*, *hsp70*), indicating the concomitant transfer of molecular information for immediate and programmed response in the target cells.

EVs Modulate Head and Foot Regeneration in *Hydra*

The finding in the EV transcriptome of both positive and negative regulators of the Wnt/ β -catenin pathway (Figure 3A) prompted us to evaluate the EV bioactivity in receiving polyps. *Wnt3* gene

transcript levels were shown upregulated in polyps treated with EVs, both at 24 and 48 h post treatment (**Figure 3B**), suggesting for the first time the involvement of *Hydra* EVs in the exogenous activation of Wnt signalling.

Next, the bioactivity of the EV molecular cargo was evaluated in the context of the regeneration process. Freshly prepared EVs were added to polyps amputated at midgastric level (50% of body column length), and the head regeneration efficiency was evaluated by morphometric analysis. Stumps of progressive developmental stage (stages 0, 1, 2 and 3) were monitored at regular intervals, starting at 24 h post amputation (p.a.) (**Figure 4A**). At each time point significant differences were observed in the distribution of developmental stages, with the EV-treated stumps always presenting more advanced stages compared to the controls (**Figure 4B**). Similar results were also obtained when the amputation was sub-hypostomal (80% of upper body length) (**Supplementary Figure S6**). These data indicate a clear enhancement effect played by EVs in the regeneration of the head, suggesting that the EV cargo may act as an exogenous source of molecular signals. The impact of EVs on the ability of the lower body cells to regenerate a new foot was evaluated by bisecting whole polyps at 20% of the body length (**Figure 4C**). The absence of distinctive morphological changes that characterize this process led us to use biochemical assays to monitor the differentiation of foot specific cells, based on the detection of peroxidase activity. This enzymatic activity is used as specific marker of basal disk cell differentiation, producing a strong signal at 36–48 h p.a. (Hoffmeister and Schaller, 1985). While control regenerates showed a clear peroxidase-positive basal disk at 36 h p.a., there was only a faint staining in the basal disk region at this time, indicating an inhibitory effect played by EVs on foot regeneration process. The observed modulation of the regeneration confirms the presence of important messages into the EVs, as detected by the transcriptome analysis, and suggest a possible regulatory role in *Hydra* biology.

DISCUSSION

The production and release of EVs has been documented in all three domains of life, both in unicellular and multicellular organisms. Functional studies have demonstrated their involvement in a large number of processes, including morphogenesis and development, while increasing evidences suggest that they may also participate in intra- and inter-species communication (Buck et al., 2014; Cruz et al., 2018; Cai et al., 2019). The evolutionary conservation of this form of communication poses important questions about the role it might have had on the generation of complex life as we know it today (Woith et al., 2019; Gill et al., 2019). Apart from our work detecting EVs on *Hydra* ectodermal cell surface (Marchesano et al., 2013), so far no evidences documented their presence in cnidarians, where they might act as signals for cell-cell communication, or as long range signals across species, or even kingdoms of aquatic life. We purified and characterized EVs from *Hydra* culture medium by differential centrifugation,

characterized their round-shaped and double-layered morphology via TEM and estimated the size in the range consistent with exosome EV subtype (Doyle and Wang, 2019). Proteomic analysis revealed numerous exosome markers, often related to their biogenesis route and cargo sorting mechanisms (Jeppesen et al., 2019; Juan and Furthauer, 2018). This evidence, together with the morphology, size and mRNA loading strongly suggests that the EVs isolated are highly enriched in this vesicle subtype. Most of the identified proteins correlate with the transcripts, including proteinases, catalytic proteins and transcription factors, suggesting the delivery of key molecular messages for early and late responses in the receiving cells. Stress induced molecular chaperons were found at the protein level (i.e., Hsp70, Hsp71, **Supplementary Table S1**) and among the most abundant transcripts of endodermal- and interstitial cell-specific transcripts (*hsp90* and *hsp70* respectively (**Supplementary Table S4**). Their presence, together with other stress related transcripts (i.e., stress induced phosphoprotein), superoxide dismutase and thioredoxin protein (disulphide isomerase) reveals an important role played by EVs in the stress response, conveying long distance messages to support survival and adaptation as described in literature. Similarly, detection of proteins and transcripts of the serine protease inhibitor kazal (Chera et al., 2009a) suggests a possible involvement of EVs in the starvation response, by preventing excessive autophagy. Indeed, induction of autophagy during starvation is the main survival strategy in *Hydra* and kazal was shown to support cell survival by preventing excessive autophagy (Chera et al., 2006). As EV harvesting occurs under starvation, kazal may support the animal survival to this condition, mirroring natural defence mechanisms acting through EVs.

In addition to the above-mentioned factors, a multitude of signalling components belonging to BMP, Notch and Wnt/ β -catenin pathways, were identified among mRNA transcripts. Our focus on the Wnt/ β -catenin signalling pathway, searching for positive and negative regulators of it in EVs, is due to the major role played by this pathway in body axial patterning. In *Hydra* two stable tissue organizers located at the oral and aboral poles are responsible for the tissue polarity (Bode, 2009). While the molecular bases of foot organizer are still largely unknown, the head organizer relies on the canonical Wnt/ β -catenin pathway and other integrating signalling pathways, such as the Notch-Delta (Broun et al., 2005; Gee et al., 2010; Munder et al., 2013). Using classical tissue manipulation techniques (Macwilliams, 1983) and more recently gene transcript profiling (Chera et al., 2009b; Wenger et al., 2019; Cazet et al., 2021) the presence of long-range inhibitory signals in *Hydra* head organizer has been supposed, acting in adults polyps to maintain the oral-aboral polarity and preventing the formation of ectopic organizers elsewhere in the body. Amputated organizers may transiently increase the ability of wounded tissue throughout the body column to form secondary axes, while during foot regeneration these inhibitory signals would prevent ectopic head formation at aboral-facing amputations (Cazet et al., 2021). To date, these signals have not been identified and in the present work we foresee the possibility that they may rely on

EVs released in the extracellular medium. We demonstrated that treating amputated polyps with EVs at an empirical dosage, likely much higher when compared to the physiological EV production, impacts on *Hydra* regeneration, and this effect may be due to the peculiar molecular cargo of EV collected under these conditions. While under physiological condition EVs may contribute to tissue organizer activity (Macwilliams, 1983; Cazet et al., 2021) by enhancing the head regeneration at the oral facing amputation region, and preventing the formation of multiple heads at the opposite end, under our condition EVs may inhibit foot regeneration, as a result of a unique blend of molecular signals. Indeed in addition to mRNA, tRNA and lncRNA here detected, EVs are known to vehicle small RNA, lipids, DNA and other metabolites (Veziroglu and Mias, 2020), opening the path to further analyses for a deeper characterization of *Hydra* EVs and unravel their role in developmental signalling and many other aspects of *Hydra* biology.

The alignment of EV transcriptome with the scRNA-seq atlas recently produced (Siebert et al., 2019) allowed to extract important information not only on the molecular composition of the EV cargo but also on the cell sources of EV transcripts. Under our condition of EV purification multiple cell types appeared involved in the loading and release of EVs. In addition to ectodermal cells expected to release EVs, as facing the external medium, a large contribute to the EV transcriptome matched transcripts specific of the interstitial stem cell lineage, dispersed throughout the body column, especially neurons. This suggests that EVs may mediate the information flow within the *Hydra* non overlapping neural networks (Dupre and Yuste, 2017). Interestingly, the sub-clustering analysis (Supplementary Figure S5) reveals that within the interstitial cell lineage a large fraction (18.7%) of the transcripts are germ line specific, suggesting the possibility that under the mild stressing condition used for EV collection the cells may release signals to induce gametogenesis and guarantee species survival. Finally, a minor fraction of transcripts was specific of endodermal cells, especially gland cells, which is consistent with their location, facing the gastric cavity, and their molecular repertoire of enzymatic activities and signalling factors, such as those restricting the Wnt signal in the hypostome (Guder et al., 2006; Lommel et al., 2018; Ziegler et al., 2021). A comparative analysis between EVs produced by transgenic lines tagged in each cell lineage would help in the future to confirm this *in silico* evidence.

Concerning the possible role *in vivo*, the EV information toolbox may be used for cell-cell communication to maintain homeostatic condition, i.e., axis polarity, regulate animal size and tissue growth. In other conditions, i.e., during budding, regeneration or stress response, they may act to orchestrate coordinated cell responses between distant tissue regions, or among different polyps. In this work we purified EVs from the medium of healthy polyps mirroring the condition used to enhance EV production from cell culture, i.e. starvation and crowding (Théry et al., 2006) and our omics and functional analyses are the outcomes of this particular state. EV may vary their molecular cargo under certain conditions, such as stress, reproduction, temperature changes, adapting the

molecular information to deliver diverse messages to the cells, from hydrophobic signals to lipids, second messengers, or nucleic acids. We believe that by comparing the EV content produced under different physiological states different outputs might be produced, leading to decipher the “code” used by cells to communicate to distant cells or organisms and to coordinate precise responses to environmental challenges, which is of outmost importance in aquatic environments.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD027774. RNA-seq data were deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE180886.

AUTHOR CONTRIBUTIONS

CT conceived the project, designed, and supervised research. MMo, EF, GT, GP, and AA performed EV isolation and *Hydra* experiments. VM performed TEM characterization. BM performed qRT-PCR. EF performed functional analyses. MMu performed bioinformatic analyses. AT and MMo analysed data and supervised research. CT wrote the manuscript with contributes from all authors.

ACKNOWLEDGMENTS

Proteomic analyses were performed in the Proteomics Platform of Servicios Científico Técnicos del CIBA (IACS-Universidad de Zaragoza), ProteoRed ISCIII member. RNA-seq analyses were performed by Sequentia Biotech S.L. (Barcelona, Spain). The authors acknowledge Mario Isas, Keck School of Medicine of USC Los Angeles, CA, for the kind gift of anti *Hydra* annexin B12 antibody. GT thanks the MIUR project SHARID-ARS01-01270 for financial support. MMo acknowledges financial support from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (Grant Agreement No 853468), from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 660228 and from the Spanish Ministry of Science and Innovation through the Ramón y Cajal grant RyC 2019-026860-I.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.788117/full#supplementary-material>

REFERENCES

- Allocca, M., Mattera, L., Bauduin, A., Miedziak, B., Moros, M., De Trizio, L., et al. (2019). An Integrated Multilevel Analysis Profiling Biosafety and Toxicity Induced by Indium- and Cadmium-Based Quantum Dots *In Vivo*. *Environ. Sci. Technol.* 53, 3938–3947. doi:10.1021/acs.est.9b00373
- Ambrosone, A., Roopin, M., Pelaz, B., Abdelmonem, A. M., Ackermann, L.-M., Mattera, L., et al. (2017). Dissecting Common and Divergent Molecular Pathways Elicited by CdSe/ZnS Quantum Dots in Freshwater and marine sentinel Invertebrates. *Nanotoxicology* 11, 289–303. doi:10.1080/17435390.2017.1295111
- Anand, S., Samuel, M., Kumar, S., and Mathivanan, S. (2019). Ticket to a Bubble Ride: Cargo Sorting into Exosomes and Extracellular Vesicles. *Biochim. Biophys. Acta (Bba) - Proteins Proteomics* 1867, 140203. doi:10.1016/j.bbapap.2019.02.005
- Augustin, R., Franke, A., Khalturin, K., Kiko, R., Siebert, S., Hemmrich, G., et al. (2006). Dickkopf Related Genes Are Components of the Positional Value Gradient in hydra. *Develop. Biol.* 296, 62–70. doi:10.1016/j.ydbio.2006.04.003
- Bode, H. R. (2009). Axial Patterning in hydra. *Cold Spring Harbor Perspect. Biol.* 1, a000463. doi:10.1101/cshperspect.a000463
- Bode, H. R. (2003). Head Regeneration in Hydra. *Dev. Dyn.* 226, 225–236. doi:10.1002/dvdy.10225
- Bosch, T. C. G. (2009). Hydra and the Evolution of Stem Cells. *Bioessays* 31, 478–486. doi:10.1002/bies.200800183
- Bray, N. L., Pimentel, H., Melsted, P., and Pachter, L. (2016). Near-optimal Probabilistic RNA-Seq Quantification. *Nat. Biotechnol.* 34, 525–527. doi:10.1038/nbt.3519
- Broun, M., Gee, L., Reinhardt, B., and Bode, H. R. (2005). Formation of the Head Organizer in hydra Involves the Canonical Wnt Pathway. *Development* 132, 2907–2916. doi:10.1242/dev.01848
- Buck, A. H., Coakley, G., Simbari, F., Mcsorley, H. J., Quintana, J. F., Le Bihan, T., et al. (2014). Exosomes Secreted by Nematode Parasites Transfer Small RNAs to Mammalian Cells and Modulate Innate Immunity. *Nat. Commun.* 5, 5488. doi:10.1038/ncomms6488
- Cai, Q., He, B., Weiberg, A., Buck, A. H., and Jin, H. (2019). Small RNAs and Extracellular Vesicles: New Mechanisms of Cross-Species Communication and Innovative Tools for Disease Control. *Plos Pathog.* 15, e1008090. doi:10.1371/journal.ppat.1008090
- Cazet, J. F., Cho, A., and Juliano, C. E. (2021). Generic Injuries Are Sufficient to Induce Ectopic Wnt Organizers in Hydra. *Elife* 10, e60562. doi:10.7554/elife.60562
- Chera, S., Buzgariu, W., Ghila, L., and Galliot, B. (2009a). Autophagy in Hydra: A Response to Starvation and Stress in Early Animal Evolution. *Biochim. Biophys. Acta (Bba) - Mol. Cel Res.* 1793, 1432–1443. doi:10.1016/j.bbamcr.2009.03.010
- Chera, S., De Rosa, R., Miljkovic-Licina, M., Dobretz, K., Ghila, L., Kaloulis, K., et al. (2006). Silencing of the hydra Serine Protease inhibitor Kazal1 gene Mimics the human SPINK1 pancreatic Phenotype. *J. Cel Sci.* 119, 846–857. doi:10.1242/jcs.02807
- Chera, S., Ghila, L., Dobretz, K., Wenger, Y., Bauer, C., Buzgariu, W., et al. (2009b). Apoptotic Cells Provide an Unexpected Source of Wnt3 Signaling to Drive hydra Head Regeneration. *Develop. Cel* 17, 279–289. doi:10.1016/j.devcel.2009.07.014
- Cimorelli, M., Nieuwland, R., Varga, Z., and van der Pol, E. (2021). Standardized Procedure to Measure the Size Distribution of Extracellular Vesicles Together with Other Particles in Biofluids with Microfluidic Resistive Pulse Sensing. *PLOS ONE* 16 (4), e0249603. doi:10.1371/journal.pone.0249603
- Coakley, G., Maizels, R. M., and Buck, A. H. (2015). Exosomes and Other Extracellular Vesicles: The New Communicators in Parasite Infections. *Trends Parasitology* 31, 477–489. doi:10.1016/j.pt.2015.06.009
- Colombo, M., Raposo, G., and Théry, C. (2014). Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles. *Annu. Rev. Cel Dev. Biol.* 30, 255–289. doi:10.1146/annurev-cellbio-101512-122326
- Cruz, L., Romero, J. A. A., Iglesia, R. P., and Lopes, M. H. (2018). Extracellular Vesicles: Decoding a New Language for Cellular Communication in Early Embryonic Development. *Front. Cel Dev. Biol.* 6, 94. doi:10.3389/fcell.2018.00094
- Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al. (2013). STAR: Ultrafast Universal RNA-Seq Aligner. *Bioinformatics* 29, 15–21. doi:10.1093/bioinformatics/bts635
- Doyle, L. M., and Wang, M. Z. (2019). Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. *Cells* 8, 727. doi:10.3390/cells8070727
- Dupre, C., and Yuste, R. (2017). Non-overlapping Neural Networks in *Hydra vulgaris*. *Curr. Biol.* 27, 1085–1097. doi:10.1016/j.cub.2017.02.049
- Gee, L., Hartig, J., Law, L., Wittlieb, J., Khalturin, K., Bosch, T. C. G., et al. (2010). β -Catenin Plays a central Role in Setting up the Head Organizer in hydra. *Develop. Biol.* 340, 116–124. doi:10.1016/j.ydbio.2009.12.036
- Ghajar, C. M., Peinado, H., Mori, H., Matei, I. R., Evason, K. J., Brazier, H., et al. (2013). The Perivascular Niche Regulates Breast Tumour Dormancy. *Nat. Cel Biol.* 15, 807–817. doi:10.1038/ncb2767
- Gill, S., Catchpole, R., and Forterre, P. (2019). Extracellular Membrane Vesicles in the Three Domains of Life and beyond. *FEMS Microbiol. Rev.* 43, 273–303. doi:10.1093/femsre/fuy042
- Gradilla, A.-C., González, E., Seijo, I., Andrés, G., Bischoff, M., González-Mendez, L., et al. (2014). Exosomes as Hedgehog Carriers in Cytoneme-Mediated Transport and Secretion. *Nat. Commun.* 5, 5649. doi:10.1038/ncomms6649
- Gross, J. C., Chaudhary, V., Bartscherer, K., and Boutros, M. (2012). Active Wnt Proteins Are Secreted on Exosomes. *Nat. Cel Biol* 14, 1036–1045. doi:10.1038/ncb2574
- Guder, C., Pinho, S., Nacak, T. G., Schmidt, H. A., Hobmayer, B., Niehrs, C., et al. (2006). An Ancient Wnt-Dickkopf Antagonism in Hydra. *Development* 133, 901–911. doi:10.1242/dev.02265
- Gufler, S., Artes, B., Bielen, H., Krainer, I., Eder, M.-K., Falschlunger, J., et al. (2018). β -Catenin Acts in a Position-independent Regeneration Response in the Simple Eumetazoan Hydra. *Develop. Biol.* 433, 310–323. doi:10.1016/j.ydbio.2017.09.005
- Harding, C., Heuser, J., and Stahl, P. (1984). Endocytosis and Intracellular Processing of Transferrin and Colloidal Gold-Transferrin in Rat Reticulocytes: Demonstration of a Pathway for Receptor Shedding. *Eur. J. Cel Biol.* 35, 256–263.
- Hobmayer, B., Jenewein, M., Eder, D., Eder, M.-K., Glasauer, S., Gufler, S., et al. (2012). Stenness in Hydra - a Current Perspective. *Int. J. Dev. Biol.* 56, 509–517. doi:10.1387/ijdb.113426bh
- Hobmayer, B., Rentzsch, F., Kuhn, K., Happel, C. M., Von Laue, C. C., Snyder, P., et al. (2000). WNT Signalling Molecules Act in axis Formation in the Diploblastic Metazoan Hydra. *Nature* 407, 186–189. doi:10.1038/35025063
- Hoffmeister, S., and Schaller, H. C. (1985). A New Biochemical Marker for Foot-specific Cell Differentiation in hydra. *Wilhelm Roux' Archiv* 194, 453–461. doi:10.1007/bf00868146
- Huang, X. Y., Yuan, T. Z., Tschannen, M., Sun, Z. F., Jacob, H., Du, M. J., et al. (2013). Characterization of Human Plasma-Derived Exosomal RNAs by Deep Sequencing. *Bmc Genomics* 14, 319. doi:10.1186/1471-2164-14-319
- Jeppesen, D. K., Fenix, A. M., Franklin, J. L., Higginbotham, J. N., Zhang, Q., Zimmerman, L. J., et al. (2019). Reassessment of Exosome Composition. *Cell* 177, 428–445. doi:10.1016/j.cell.2019.02.029
- Johnstone, R. M., Adam, M., Hammond, J. R., Orr, L., and Turbide, C. (1987). Vesicle Formation during Reticulocyte Maturation. Association of Plasma Membrane Activities with Released Vesicles (Exosomes). *J. Biol. Chem.* 262, 9412–9420. doi:10.1016/s0021-9258(18)48095-7
- Juan, T., and Fürthauer, M. (2018). Biogenesis and Function of ESCRT-dependent Extracellular Vesicles. *Semin. Cel Develop. Biol.* 74, 66–77. doi:10.1016/j.semcd.2017.08.022
- Kalluri, R., and Lebleu, V. S. (2020). The Biology, Function and Biomedical Applications of Exosomes. *Science* 367, eaau6977. doi:10.1126/science.aau6977
- Kalra, H., Simpson, R. J., Ji, H., Aikawa, E., Altevogt, P., Askenase, P., et al. (2012). Vesiclepedia: A Compendium for Extracellular Vesicles with Continuous Community Annotation. *Plos Biol.* 10, e1001450. doi:10.1371/journal.pbio.1001450
- Kowal, J., Arras, G., Colombo, M., Jouve, M., Morath, J. P., Prindal-Bengtson, B., et al. (2016). Proteomic Comparison Defines Novel Markers to Characterize Heterogeneous Populations of Extracellular Vesicle Subtypes. *Proc. Natl. Acad. Sci. U S A* 113, E968–E977. doi:10.1073/pnas.1521230113
- Kriventseva, E. V., Kuznetsov, D., Tegenfeldt, F., Manni, M., Dias, R., Simao, F. A., et al. (2019). OrthoDB V10: Sampling the Diversity of Animal, Plant, Fungal, Protist, Bacterial and Viral Genomes for Evolutionary and Functional Annotations of Orthologs. *Nucleic Acids Res.* 47, D807–D811. doi:10.1093/nar/gky1053

- Lengfeld, T., Watanabe, H., Simakov, O., Lindgens, D., Gee, L., Law, L., et al. (2009). Multiple Wnts Are Involved in Hydra Organizer Formation and Regeneration. *Dev. Biol.* 330, 186–199. doi:10.1016/j.ydbio.2009.02.004
- Li, Q., Yang, H., and Zhong, T. P. (2015). Regeneration across Metazoan Phylogeny: Lessons from Model Organisms. *J. Genet. Genomics* 42, 57–70. doi:10.1016/j.jgg.2014.12.002
- Liegeois, S., Benedetto, A., Garnier, J. M., Schwab, Y., and Labouesse, M. (2006). The V0-ATPase Mediates Apical Secretion of Exosomes Containing Hedgehog-Related Proteins in *Caenorhabditis elegans*. *J. Cell Biol.* 173, 949–961. doi:10.1083/jcb.200511072
- Lommel, M., Strompen, J., Hellewell, A. L., Balasubramanian, G. P., Christofidou, E. D., Thomson, A. R., et al. (2018). Hydra Mesoglea Proteome Identifies Thrombospondin as a Conserved Component Active in Head Organizer Restriction. *Scientific Rep.* 8, 11753. doi:10.1038/s41598-018-30035-2
- Lu, M., and Huang, Y. (2020). Bioinspired Exosome-like Therapeutics and Delivery Nanoplatfoms. *Biomaterials* 242, 119925. doi:10.1016/j.biomaterials.2020.119925
- Ludwig, N., Whiteside, T. L., and Reichert, T. E. (2019). Challenges in Exosome Isolation and Analysis in Health and Disease. *Int. J. Mol. Sci.* 20, 4684. doi:10.3390/ijms20194684
- Macwilliams, H. K. (1983). Hydra Transplantation Phenomena and the Mechanism of Hydra Head Regeneration .1. Properties of the Head Inhibition. *Develop. Biol.* 96, 217–238. doi:10.1016/0012-1606(83)90324-x
- Marchesano, V., Hernandez, Y., Salvenmoser, W., Ambrosone, A., Tino, A., Hobmayer, B., et al. (2013). Imaging Inward and Outward Trafficking of Gold Nanoparticles in Whole Animals. *ACS Nano* 7, 2431–2442. doi:10.1021/nn305747e
- McGough, I. J., and Vincent, J.-P. (2016). Exosomes in Developmental Signalling. *Development* 143, 2482. doi:10.1242/dev.126516
- Misu, A., Yamanaka, H., Aramaki, T., Kondo, S., Skerrett, I. M., Iovine, M. K., et al. (2016). Two Different Functions of Connexin43 Confer Two Different Bone Phenotypes in Zebrafish. *J. Biol. Chem.* 291, 12601–12611. doi:10.1074/jbc.m116.720110
- Moros, M., Di Maria, F., Dardano, P., Tommasini, G., Castillo-Michel, H., Kovtun, A., et al. (2020a). Vivo Bioengineering of Fluorescent Conductive Protein-Dye Microfibers. *iScience* 23, 101022. doi:10.1016/j.isci.2020.101022
- Moros, M., Lewinska, A., Merola, F., Ferraro, P., Wnuk, M., Tino, A., et al. (2020b). Gold Nanorods and Nanoprisms Mediate Different Photothermal Cell Death Mechanisms *In Vitro* and in *Vivo*. *ACS Appl. Mater. Inter.* 12, 13718–13730. doi:10.1021/acsami.0c02022
- Munder, S., Tischer, S., Grundhuber, M., Buchels, N., Bruckmeier, N., Eckert, S., et al. (2013). Notch-signalling Is Required for Head Regeneration and Tentacle Patterning in Hydra. *Dev. Biol.* 383, 146–157. doi:10.1016/j.ydbio.2013.08.022
- Pan, B. T., and Johnstone, R. M. (1983). Fate of the Transferrin Receptor during Maturation of Sheep Reticulocytes *In Vitro*: Selective Externalization of the Receptor. *Cell* 33, 967–978. doi:10.1016/0092-8674(83)90040-5
- Peinado, H., Alečković, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Moreno-Bueno, G., et al. (2012). Melanoma Exosomes Educate Bone Marrow Progenitor Cells toward a Pro-metastatic Phenotype through MET. *Nat. Med.* 18, 883–891. doi:10.1038/nm.2753
- Peinado, H., Zhang, H., Matei, I. R., Costa-Silva, B., Hoshino, A., Rodrigues, G., et al. (2017). Pre-metastatic Niches: Organ-specific Homes for Metastases. *Nat. Rev. Cancer* 17, 302–317. doi:10.1038/nrc.2017.6
- Perez-Riverol, Y., Csordas, A., Bai, J., Bernal-Llinares, M., Hewapathirana, S., Kundu, D. J., et al. (2019). The PRIDE Database and Related Tools and Resources in 2019: Improving Support for Quantification Data. *Nucleic Acids Res.* 47 (D1), D442–D450.
- Petersen, H. O., Hoyer, S. K., Looso, M., Lengfeld, T., Kuhn, A., Warnken, U., et al. (2015). A Comprehensive Transcriptomic and Proteomic Analysis of Hydra Head Regeneration. *Mol. Biol. Evol.* 32, 1928–1947. doi:10.1093/molbev/msv079
- Quinn, B., Gagnè, F., and Blaise, C. (2012). Hydra, a Model System for Environmental Studies. *Int. J. Dev. Biol.* 56, 613–625. doi:10.1387/ijdb.113469bq
- Schatz, D., and Vardi, A. (2018). Extracellular Vesicles — New Players in Cell–Cell Communication in Aquatic Environments. *Curr. Opin. Microbiol.* 43, 148–154. doi:10.1016/j.mib.2018.01.014
- Siebert, S., Farrell, J. A., Cazet, J. F., Abeykoon, Y., Primack, A. S., Schnitzler, C. E., et al. (2019). Stem Cell Differentiation Trajectories in Hydra Resolved at Single-Cell Resolution. *Science* 365. doi:10.1126/science.aav9314
- Skryabin, G. O., Komelkov, A. V., Savelyeva, E. E., and Tchevkina, E. M. (2020). Lipid Rafts in Exosome Biogenesis. *Biochemistry (Mosc)* 85, 177–191. doi:10.1134/s0006297920020054
- Steele, R. E. (2002). Developmental Signaling in Hydra: what Does it Take to Build a "simple" Animal? *Dev. Biol.* 248, 199–219. doi:10.1006/dbio.2002.0744
- Takaku, Y., Hwang, J. S., Wolf, A., Bottger, A., Shimizu, H., David, C. N., et al. (2014). Innexin gap Junctions in Nerve Cells Coordinate Spontaneous Contractile Behavior in Hydra Polyps. *Sci. Rep.* 4, 3573. doi:10.1038/srep03573
- Thery, C., Amigorena, S., Raposo, G., and Clayton, A. (2006). Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids. *Curr. Protoc. Cell Biol.* Chapter 3, Unit 3.22. doi:10.1002/0471143030.cb0322s30
- Thery, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., and Anderson, J. D. (2018). Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): a Position Statement of the International Society for Extracellular Vesicles and Update of the MISEV2014 Guidelines. *J. Extracell. Vesicles* 7, 1535750. doi:10.1080/20013078.2018.1535750
- Tortiglione, C., Antognazza, M. R., Tino, A., Bossio, C., Marchesano, V., Bauduin, A., et al. (2017). Semiconducting Polymers Are Light Nanotransducers in Eyeless Animals. *Sci. Adv.* 3, e1601699. doi:10.1126/sciadv.1601699
- Veronesi, G., Moros, M., Castillo-Michel, H., Mattera, L., Onorato, G., Wegner, K. D., et al. (2019). Vivo Biotransformations of Indium Phosphide Quantum Dots Revealed by X-Ray Microspectroscopy. *ACS Appl. Mater. Inter.* 11, 35630–35640. doi:10.1021/acsami.9b15433
- Veziroglu, E. M., and Mias, G. (2020). Characterizing Extracellular Vesicles and Their Diverse RNA. *Contents Front. Genet.* 11, 700. doi:10.3389/fgene.2020.00700
- Vogg, M. C., Beccari, L., Olle, L. I., Rampon, C., Vriza, S., Perruchoud, C., et al. (2019a). An Evolutionarily-Conserved Wnt3/beta-catenin/Sp5 Feedback Loop Restricts Head Organizer Activity in Hydra. *Nat. Commun.* 10, 312. doi:10.1038/s41467-018-08242-2
- Vogg, M. C., Galliot, B., and Tsiairis, C. D. (2019b). Model Systems for Regeneration: Hydra. *Development* 146, dev177212. doi:10.1242/dev.177212
- Wehman, A. M., Poggioli, C., Schweinsberg, P., Grant, B. D., and Nance, J. (2011). The P4-ATPase TAT-5 Inhibits the Budding of Extracellular Vesicles in *C. elegans* Embryos. *Curr. Biol.* 21, 1951–1959. doi:10.1016/j.cub.2011.10.040
- Wenger, Y., Buzgariu, W., Perruchoud, C., Loichot, G., and Galliot, B. (2019). Generic and Context-dependent Gene Modulations during Hydra Whole Body Regeneration. *bioRxiv*, 587147. doi:10.1101/587147
- Woith, E., Fuhrmann, G., and Melzig, M. F. (2019). Extracellular Vesicles-Connecting Kingdoms. *Int. J. Mol. Sci.* 20, 5695. doi:10.3390/ijms20225695
- Zhang, L., and Wrana, J. L. (2014). The Emerging Role of Exosomes in Wnt Secretion and Transport. *Curr. Opin. Genet. Dev.* 27, 14–19. doi:10.1016/j.gde.2014.03.006
- Ziegler, B., Yiallouris, I., Trageser, B., Kumar, S., Mercker, M., Kling, S., et al. (2021). The Wnt-specific Astacin Proteinase HAS-7 Restricts Head Organizer Formation in Hydra. *Bmc Biol.* 19, 120. doi:10.1186/s12915-021-01046-9
- Zong, L., Zhu, Y., Liang, R., and Zhao, H. B. (2016). Gap junction Mediated miRNA Intercellular Transfer and Gene Regulation: A Novel Mechanism for Intercellular Genetic Communication. *Sci. Rep.* 6, 19884. doi:10.1038/srep19884

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Moros, Fergola, Marchesano, Mutarelli, Tommasini, Miedziak, Palumbo, Ambrosone, Tino and Tortiglione. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



OPEN ACCESS

Edited by:

Laura Maggi,
Università degli Studi di Firenze,
Italy

Reviewed by:

Lucio Barile,
University of Zurich, Switzerland
Theresa L. Whiteside,
University of Pittsburgh, United States

***Correspondence:**

Renata Grifantini
grifantini@ingm.org
Elisa Pesce
pesce@ingm.org

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Cytokines and Soluble
Mediators in Immunity,
a section of the journal
Frontiers in Immunology

Received: 29 September 2021

Accepted: 16 December 2021

Published: 17 January 2022

Citation:

Pesce E, Manfrini N,
Cordiglieri C, Santi S, Bandera A,
Gobbini A, Gruarin P, Favalli A,
Bombaci M, Cuomo A, Collino F,
Crici G, Ungaro R, Lombardi A,
Mangioni D, Muscatello A, Aliberti S,
Blasi F, Gori A, Abrignani S,
De Francesco R, Biffo S and
Grifantini R (2022) Exosomes
Recovered From
the Plasma of COVID-19 Patients
Expose SARS-CoV-2 Spike-Derived
Fragments and Contribute to the
Adaptive Immune Response.
Front. Immunol. 12:785941.
doi: 10.3389/fimmu.2021.785941

Exosomes Recovered From the Plasma of COVID-19 Patients Expose SARS-CoV-2 Spike-Derived Fragments and Contribute to the Adaptive Immune Response

Elisa Pesce^{1*†}, Nicola Manfrini^{1,2†}, Chiara Cordiglieri¹, Spartaco Santi^{3,4},
Alessandra Bandera^{5,6,7}, Andrea Gobbini¹, Paola Gruarin¹, Andrea Favalli¹,
Mauro Bombaci¹, Alessandro Cuomo⁸, Federica Collino^{9,10}, Giulia Crici^{9,10},
Riccardo Ungaro⁵, Andrea Lombardi^{5,6}, Davide Mangioni⁵, Antonio Muscatello⁵,
Stefano Aliberti^{6,11}, Francesco Blasi^{6,11}, Andrea Gori^{5,6,7}, Sergio Abrignani^{1,12},
Raffaele De Francesco^{1,13}, Stefano Biffo^{1,2} and Renata Grifantini^{1*}

¹ Istituto Nazionale Genetica Molecolare (INGM), Istituto Nazionale Genetica Molecolare "Romeo ed Enrica Invernizzi", Milan, Italy, ² Department of Biosciences, Università degli Studi di Milano, Milan, Italy, ³ Unit of Bologna, Consiglio Nazionale delle Ricerche (CNR) Institute of Molecular Genetics "Luigi Luca Cavalli-Sforza", Bologna, Italy, ⁴ Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Istituto Ortopedico Rizzoli, Bologna, Italy, ⁵ Infectious Diseases Unit, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, ⁶ Department of Pathophysiology and Transplantation, Università degli Studi di Milano, Milan, Italy, ⁷ Centre for Multidisciplinary Research in Health Science (MACH), Università degli Studi di Milano, Milan, Italy, ⁸ Department of Experimental Oncology, Istituto Europeo di Oncologia (IEO), European Institute of Oncology Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Milan, Italy, ⁹ Laboratory of Translational Research in Paediatric Nephro-Urology, Fondazione Ca' Granda IRCCS Ospedale Maggiore Policlinico, Milan, Italy, ¹⁰ Department of Clinical Sciences and Community Health, University of Milano, Milan, Italy, ¹¹ Respiratory Unit and Cystic Fibrosis Adult Center, Respiratory Unit and Cystic Fibrosis Adult Center, Milan, Italy, ¹² Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milan, Italy, ¹³ Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milan, Italy

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by beta-coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that has rapidly spread across the globe starting from February 2020. It is well established that during viral infection, extracellular vesicles become delivery/presenting vectors of viral material. However, studies regarding extracellular vesicle function in COVID-19 pathology are still scanty. Here, we performed a comparative study on exosomes recovered from the plasma of either MILD or SEVERE COVID-19 patients. We show that although both types of vesicles efficiently display SARS-CoV-2 spike-derived peptides and carry immunomodulatory molecules, only those of MILD patients are capable of efficiently regulating antigen-specific CD4⁺ T-cell responses. Accordingly, by mass spectrometry, we show that the proteome of exosomes of MILD patients correlates with a proper functioning of the immune system, while that of SEVERE patients is associated with increased and chronic inflammation. Overall, we show that exosomes recovered from the plasma of COVID-19 patients possess SARS-CoV-2-derived protein

material, have an active role in enhancing the immune response, and possess a cargo that reflects the pathological state of patients in the acute phase of the disease.

Keywords: COVID-19, SARS-CoV-2, exosomes, immune activation, antigen-presenting cells (APCs), soluble mediators in immunity

INTRODUCTION

Coronavirus disease 2019 (COVID-19) primarily affects the lung epithelium, and infection may lead to pneumonia, respiratory distress syndromes, acute lung injury, and death. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a spherical or pleomorphic enveloped virus with a typical size range of 80–120 nm in diameter. It contains a positive single-stranded RNA of 30 kb, surrounded by a membrane embedded with several viral proteins. Of fundamental importance for viral internalization is the Spike (S) protein (1). During viral entry, the S protein binds, through its receptor-binding domain (RBD), to angiotensin-converting enzyme 2 (ACE2) receptors of host cells. Next, the serine protease TMPRSS2 primes the S protein for internalization (2). Once internalized, the virus matures, replicates, and, lastly, leaves the host cells in order to spread in the surrounding tissues. Many viruses are known to enter extracellular double-membrane vesicles (EDMVs) during intra-host spreading (3, 4). To date, however, literature covering this topic for SARS-CoV-2 is scanty.

Exosomes are small vesicles (30–100 nm in diameter) of endocytic origin and are released from cells into the extracellular environment during both normal and pathological conditions (5). They are formed by the inward budding of late endosomal membranes that give rise to intracellular multivesicular bodies (MVBs) that then fuse with the plasma membrane releasing the intraluminal exosomes into the extracellular space. They are secreted by all cell types and are present in bodily fluids, such as blood, urine, and saliva, breast milk, and bronchial and nasal lavage (6). Although the protein composition of exosomes reflects that of the parent cell, exosomes have common and peculiar components. They are in fact generally rich in tetraspanins (CD9, CD63, CD81) and heat shock and Rab proteins, which are routinely used as exosomal markers. Exosomes are an important tool for intercellular communication, as they act as shuttles for the transfer of biologically active proteins, lipids, and RNAs (7). *In vitro* studies have demonstrated that exosomes play a dual role: they promote pathogen transmission and exacerbate infection in some cases, while they contribute to host defense and control infection in others. Currently, very little is known about the characteristics, behavior, and contribution to viral infection of *in vivo* generated exosomes (8).

In this study, we purified exosomes from the plasma of COVID-19 patients using immuno-isolation methods and highlighted their function in the context of COVID-19 infection. In addition, we analyzed the differences in protein composition (internal cargo and membrane surface components) of exosomes recovered from healthy donors

(HDs) and patients experiencing COVID-19 in a MILD or SEVERE form and elucidated possible functional roles.

MATERIALS AND METHODS

Reagent or Resource	Source	Identifier
Antibodies and Chemicals		
Anti-SARS-CoV-2 Spike RBD	Sino Biological	40592-T62
4- μ m-diameter latex beads	Invitrogen	A37304
Anti-human CD9 FITC	BD	555371
	Biosciences	
Anti-human CD63 PE	BD	556020
	Biosciences	
Anti-SARS-CoV-2 Spike S1 (Mab Clone #007)	Sino Biological	40150-R007
Anti-HSP70	Cell Signaling	4872
Anti-human CD19 APC-Cy7	BD	557791
	Biosciences	
Anti-human CD68 FITC	BD	562117
	Biosciences	
Anti-human CD86 PE-Cy7	BD	561128
	Biosciences	
Anti-human HLA-DR APC-Cy7	BioLegend	307617
Anti-human HLA-DR PE-Cy5	BD	555813
	Biosciences	
Anti-human CD11b FITC	BD	562793
	Biosciences	
Anti-human CD11c PE-Cy5	BD	551077
	Biosciences	
Anti-human CD25 PE	BD	555432
	Biosciences	
Anti-human CD3 APC	BD	555342
	Biosciences	
Anti-human CD56 APC	BD	555518
	Biosciences	
Anti-human CD4 APC-Cy7	BD	557871
	Biosciences	
Purified mouse anti-human HLA-DR, DP, DQ	BD	555557
	Pharmingen™	
Anti-human TNF α FITC	BD	552889
	Biosciences	
Anti-human IL-2 APC	BD	555434
	Biosciences	
Anti-human IFN γ PerCP-Cy5.5	BD	560704
	Biosciences	
EZ-Link Sulfo-NHS-LC-Biotinylation Kit	Thermo Scientific	21435
Streptavidin-horseradish peroxidase (HRP) conjugate	Invitrogen	SA10001
Dynabeads™ Protein G	Invitrogen	10009D
Alexa Fluor™ 647 Antibody Labeling Kit	Invitrogen	A20186
Abberior® STAR RED	Abberior	STRED -1002
CD14 MicroBeads	Miltenyi Biotec	cat 130-050-201

(Continued)

Continued

Reagent or Resource	Source	Identifier
CD4 ⁺ T Cell Isolation Kit	Miltenyi Biotec	cat 130-096-533
CellTrace™ Violet Cell Prolif. Kit	Invitrogen	C34557
Interleukin-2, human	Roche	10799068001
Anti-human CD4 BUV395	BD	564724
Anti-human HLA-DR BUV 650	BD	564724
qEV/35nm columns SEC Columns for Exosomes Separation and Purification	IZON	–
PreOmics “IST” Kit	PreOmics GmbH	P.O. 00001
High pH Reversed-Phase Peptide Fractionation Kit	Pierce™	84868
EasySpray PEPMAAP RSLC C18	Thermo Fisher Scientific	ES801
Intracellular Fixation and Permeabilization Buffer Set	eBioscience	88-8824-00
Anti-human Syntenin (EPR8102)	abcam	ab236071
5-nm Gold-anti-Rabbit Fabs	BBI International	
Formvar/Carbon Film 10 nm/1 nm thick on Square 200 mesh Copper Grid	Electron Microscopy Sciences	FCF200CU50
Anti-human CD63 (Ts63)	Invitrogen	10628D
Anti-human CD9	Abcam	92726
Anti-human TSG101 (4A10)	Abcam	Ab83
Anti-ApoA (EPR2949)	Santa Cruz	sc-376818
Critical commercial assays		
ExoTEST Ready to Use Kit for Overall Exosome capture and quantification from human plasma	Hansa BioMed	–
ExoView™ Tetraspanin chip	Nanoview Biosciences	
Software		
FACSDiva	BD Biosciences	
FlowJO		v.10.6.2
ExoViewer		
MaxQuant (MQ)		v.1.6.10.43
NTA 3.4 software	Malvern Instruments	v.3.4
Perseus		
Heatmapper		
FunRich		v.3.1.4
R Studio	GNU	v4.0.3
topGO49	R package	v2.42.0
ggplot	R package	v3.3.3
Cytoscape		v3.8.2
STRINGapp		
Omics visualizer app		
ImageJ	NIH	
ITEM		

FTC, Fluorescein isothiocyanate; PE, Phycoerythrin; APC-Cy7, Allophycocyanin-Cy7; PE-Cy7, Phycoerythrin-Cy7; PE-Cy5, Phycoerythrin-Cy5; APC, Allophycocyanin; PerCP-Cy5.5, Peridinin chlorophyll-Cy5.5; BUV395, Brilliant Ultraviolet 395; BUV650, Brilliant Ultraviolet 650; Hsp70, Heat shock protein 70; HLA-DR, Human Leukocyte Antigen-DR -DP -DQ isotype; TNF α , Tumor necrosis factor alpha; IL-2, Interleukin-2; IFN γ , Interferon gamma; SEC, Size Exclusion Chromatography; TSG101, tumour susceptibility gene 101; ApoA, Apolipoprotein A.

Sample Collection

Plasma samples from COVID-19 patients were collected by the Ospedale Maggiore Policlinico (Milan, Italy) between March and June 2020 in the acute phase of infection from venous blood samples using Ethylenediaminetetraacetic acid (EDTA) as

anticoagulant. The clinical classification of MILD and SEVERE patients was done following the recommended WHO clinical progression scale score (MILD patient scores ranged between 1 and 4, whereas SEVERE patients had scores between 5 and 10) (9). Detailed information is shown in **Supplementary Table S1**. For the SEVERE group, samples were collected during patients' hospitalization in COVID-19 high dependency units. COVID-19 patients were diagnosed by RT-PCR nasopharyngeal swab. HDs were chosen to be representative of both sexes and of varied ages (26–65 years). While gender was matched between COVID-19 patients and healthy controls in this cohort, the average age of the SEVERE and MILD group was not, the first being much higher than the second, following an intrinsic characteristic of COVID-19 epidemiology that shows a worse prognosis in elderly individuals.

Human Plasma Preparation

In this study, 100 μ l of plasma samples were prepared by 3 centrifugation steps (Step 1: 10' at 500 g; Step 2: 20' at 2,000 g; Step 3: 30' at 14,000 g) to eliminate red blood cells and cellular debris. After each step, the supernatant was transferred to a new tube and the pellet was discarded. After the last centrifugation, the supernatant was diluted in Phosphate-buffered saline (PBS). To exclude cell-free virions in the exosome preparations, we always used immune affinity purification method using anti-tetraspanin antibody-conjugated magnetic or latex beads.

Immunocapture-Based ELISA

Circulating exosomes from plasma of MILD and SEVERE patients or HDs were captured using the ExoTEST Kit (Hansa BioMed) according to the manufacturer's instructions. The ELISA plate with 100- μ l test samples loaded per well was incubated at room temperature with shaking for 30 min. After washing 3 times with washing buffer, 100 μ l of biotinylated anti-SARS-CoV-2-S RBD primary antibody (1:1,000 ratio) were added to each well and incubated at room temperature while shaking for 2 h (2–3 rotations per second). The plate was washed again with the washing buffer, and 100 μ l of diluted streptavidin HRP-conjugated were added to each well. The plate was incubated at room temperature while shaking for 1 h (2–3 rotations per second). Then, 100 μ l of Substrate Chromogenic Solution was added to each well and incubated uncovered at room temperature in the dark for 5–10 min. The plate was monitored until a blue color was visible. At this point, the reaction was stopped by adding 100 μ l of stop solution to each well. The absorbance was recorded at 450 nm within 10 min with an Infinite F200 (Thermo Fisher Scientific, USA).

Flow Cytometry Analysis

For immunoisolation of exosomes recovered from plasma, 10 μ l of 4- μ m-diameter latex beads were incubated with 20 μ l purified anti-CD63 mAb for 30 min at room temperature in a final volume of 50 μ l. After 15 min, the volume was made up to 200 μ l with PBS, and samples were incubated overnight at 4°C with gentle agitation. For Fluorescence activated cell sorting (FACS) analysis, exosomes recovered from blood plasma were incubated in 60 μ l for 30 min at 4°C with anti-CD63-latex beads. The

volume was made up to 400 μ l with PBS and incubated for 2 h at 4°C. To eliminate the unspecific antibody binding, beads resulting after plasma incubation were blocked with 5% bovine serum albumin (BSA) solution for 30 min at room temperature. Vesicle-coated beads were washed twice in washing buffer (1% BSA in PBS) and resuspended in 400 μ l of washing buffer, stained with the indicated fluorescent antibodies, and analyzed on a FACSCantoI flow cytometer (BD Biosciences) and FACSDiva software. Flow cytometry analysis was done interchangeably using an isotype antibody or a secondary antibody control.

For exosome integrity analysis, we also assessed syntenin compartmentalization in the exosome cargo. The Intracellular Fixation and Permeabilization Buffer Set (eBioscience) was used to fix and permeabilize exosomes for intravesicular staining. Exosomes were fixed in 100 μ l of IC Fixation Buffer and incubated in the dark at room temperature for 20 min. After incubation, the samples were washed twice with 500 μ l of permeabilization buffer and then resuspended in 100 μ l of the same buffer. The samples were then incubated with PE anti-human syntenin for 30 min at room temperature. After incubation, the samples were washed twice with 500 μ l of permeabilization buffer and resuspended in 200 μ l of 1% BSA-PBS and analyzed.

Nanoparticle Tracking Analysis

The size distribution and concentration of plasma-derived exosomes were analyzed by nanoparticle tracking analysis (NTA) using NanoSight model NS300 equipped with a Blue488 laser and a sCMOS camera (Malvern Instruments, Malvern, UK). Here, 2 μ l of sample was diluted in 1 ml of PBS and then injected into the laser chamber. The following settings were used for data acquisition: camera level, 13; acquisition time, 60 s; and detection threshold, 5. Data were analyzed using the NTA 3.4 software (Malvern Instruments). Three recordings were performed for each sample. The evaluation of the particle size distribution (PSD) was performed through the following parameters: Mean, Mode, SD, D10, D50 (Median), and D90, which indicate, respectively, the average, most frequent particle class size, standard deviation, and the 10%, 50%, and 90% percentiles of the analyzed particles.

Exosome Coimmunoprecipitation Followed by Western Blotting

In this study, 5 μ g of anti-CD63, anti-CD9, and anti-CD81 mAbs, diluted in 200 μ l PBS, were added to 1.5 mg (50 μ l) of the protein G-coated Dynabeads® suspension and incubated with rotation for 20 min at room temperature. The Abs-coated beads were separated from the non-bound antibodies by placing the tube on a magnet for 1 min and removing the supernatant. Beads–Ab complexes were resuspended in 200 μ l PBS with Tween®-20 and washed by gentle pipetting. Plasma samples (100 μ l) were added to the Dynabeads®–Ab complexes, and the beads were incubated with rotation Overnight (O/N) at 4°C. The Dynabeads®–Ab–antigen complexes were washed 3 times using 200 μ l PBS and were resuspended in 100 μ l PBS. The bead suspension was then transferred to a clean tube to avoid

co-elution of proteins bound to the tube wall. The tube was then placed on a magnet for 1 min, and the beads were recovered. Next, 50 μ l of premixed NuPAGE® LDS Sample Buffer and NuPAGE Sample Reducing Agent (mixed as per manufacturer's instructions) were added to the Dynabeads®–Ab–antigen complex and heated for 10 min at 70°C. Then, the tube was placed on a magnet for 1 min, and the supernatant/sample was loaded onto a NuPAGE 4%–12% Bis–Tris gel (Invitrogen, Waltham, MA, USA) and transferred onto a Polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Burlington, MA, USA). Membranes were blocked in 5% non-fat powdered milk in TBS-T (0.5% Tween-20) and probed with the indicated antibodies for 2 h at room temperature. Membranes were washed, and HRP-conjugated antibodies were added for 1 h at room temperature. Detection was performed using enhanced chemiluminescence (ECL) reagents (Invitrogen) according to the manufacturer's guidelines.

Exoview Analysis

Exosomes recovered from plasma of COVID-19 patients and HDs were analyzed using ExoView Tetraspanin chips (NanoView Biosciences, Boston, MA, USA) arrayed with antibodies against the CD81, CD63, CD9, and CD41a proteins. Mouse IgG1 was used as a negative control. In brief, 35 μ l of each sample were dropped onto the chip surface (placed in a sealed 24-well plate) and incubated for 16 h at room temperature. Each chip was then washed once on an orbital shaker with PBST (PBS supplemented with 0.05% Tween-20) for 3 min, then washed three additional times with PBS for 3 min. After washing, the chips were incubated with anti-SARS-CoV-2-S RBD, conjugated with Alexa Fluor 647, in PBST supplemented with 2% BSA in a volume of 250 μ l for 2 h at room temperature without shaking. Next, each chip was washed once with PBST, 3 times with PBS, once in filtered deionized water, and then dried at room temperature for 1 h. The chips were then imaged with the ExoView R100 reader (ExoView) and analyzed using the ExoViewer software with a sizing threshold set to 50–200-nm diameter. The resulting size and fluorescence intensity information for each individual exosome was exported to Excel for statistical analyses. Fluorescence values are reported in arbitrary units.

Stimulated Emission Depletion Microscopy

For SARS-CoV-2-S immunolabeling and stimulated emission depletion (STED) microscopy evaluation, latex bead-isolated exosomes were used, similarly to what was previously described for FACS immunostaining. Exosomes prepared from blood plasma of HD and MILD and SEVERE COVID patients were incubated with anti-CD63-latex beads, and beads were then blocked with 5% BSA solution to eliminate unspecific antibody binding. Vesicle-coated beads were washed and seeded onto glass coverslips (n. 1.5 thickness; electron microscopy), then stained with anti-SARS-CoV-2-S RBD (1:500), followed by hybridization with secondary antibodies conjugated with the Star®RED fluorophore (Abberior; 1:200), and finally mounted onto glass slides with ProlongGlass mounting reagent for super-resolution (Molecular Probes, Thermo Fisher Scientific). Samples were

acquired using a demo version of the Abberior STEDYCON microscope for simultaneous confocal and STED microscopy (kind collaboration with Abberior Instrument) with 4 excitation laser lines and a 775-nm depletion STED laser. Star*RED fluorophore excitation was kept at 10% power of the 640-nm laser power, whereas Star*RED fluorophore depletion was obtained at 100% laser power in order to achieve 30-nm resolution, at pixel size 15 nm, with 7 lines of STED acquisition over different Z-plan, spanning throughout the whole z-dimension of the latex beads. A total of $n = 9$ beads were acquired in STED imaging for all conditions (HD, MILD, SEVERE) with duplicate independent biological replicates, reaching a total of $n = 18$ for each condition. STED-resolved microscopy images were quantified upon precise photon counting. Data were normalized with subtraction of photon counts of background signal, as evaluated by anti-SARS-CoV-2-S RBD–Star*RED in HD samples, with mean photon count in raw images =44, whereas mean photon count in raw images from MILD and SEVERE samples were =152 and =80, respectively.

Transmission Electron Microscopy

Transmission electron microscopy evaluation of RBD immunogold labeling was performed on HD, MILD COVID-19, and SEVERE COVID-19 plasma-derived exosomes purified *via* lattice-bead immunocapture as described in the previous sections and stained in suspension with rabbit anti-SARS-CoV-2-S RBD (1:500) followed by 5-nm Gold-anti-Rabbit Fabs (BBI International) (1:200). After staining, samples were fixed in 1% glutaraldehyde and finally kept in PBS pending TEM observation. TEM sample preparation was performed similarly to what has been recently described (10). Here, 5 μ l of the pooled EV sample was layered onto a formvar/carbon-coated 200 mesh grids and allowed to settle for 20 min. No further negative stain was performed in order to preserve a proper contrast for immunogold labeling. The grids were blotted and allowed to air-dry at room temperature. The observations were carried out with a JEOLJEM-1011 (Jeol Jem, Peabody, MA, USA) transmission electron microscope operated at 100 kV. Images were captured using a Morada G2 TEM digital camera (Olympus Soft Imaging Solutions, Münster, Germany) and iTEM software. Negative controls (not shown) were performed in the absence of primary antibodies. Here, 10–15 images were captured from each of three randomly selected areas of each grid at $\times 50,000$ and $\times 100,000$ lens magnification. The camera magnifications were calibrated using a grid with a grating replica (EMS cata #80050) with line spacing of 463 nm (2,160 lines/ μ m). Scale bars reflect the magnification at the camera. TEM micrographs were analyzed manually. Rounded or “cup-shaped” particles with high-contrast edges were considered exosomes and measured using Fiji-ImageJ (with Java 1.8.0_172, 64-bit).

STOchastic Reconstruction Microscopy

Single-molecule super-resolution microscopy for RBD fluorescent immunolabeling on MILD COVID plasma-derived exosomes was performed with direct stochastic optical reconstruction microscopy (D-STORM) modality (i.e., spontaneous particle blinking with only excitation in

fluorophore reporter wavelength). Exosomes were purified *via* lattice-bead immunocapture as previously described and stained in suspension with rabbit anti-SARS-CoV-2-S RBD (1:500) followed by AlexaFluor-647 Goat anti-Rabbit IgGs (Molecular Probes, Thermo Fisher) at 1:200. After staining, samples were fixed in 1% Paraformaldehyde (PFA) for fluorophore preservation and finally kept in PBS pending STORM assay. For acquisition, 5 μ l of the labeled exosome pool were carefully layered at the center of a glass-inserted 35-mm petri dish (CellView) and air-dried under the laminar hood for 30 min to achieve a correct glass-surface deposition of labeled exosomes before adding 250 μ l of freshly prepared STORM buffer (Abbelight) and topping the sample with a 22-mm square glass. The achieved volume of buffer of acquisition surface was optimized for correct evanescence wave formation within total internal reflection fluorescent (TIRF) illumination. Acquisitions were performed on an N-STORM instrument (Nikon Instruments) mounted on a Nikon Ti widefield with DU-897 EM-CCD camera (Andor Technology) with TIRF illuminator and $\times 100$ TIRF (NA 1.49) objective (Nikon Instrument), coupled with 10-mW 647 excitation/reported laser (CrystaLaser) used at 70% power for D-STORM for 10,000 frames/acquisition over a constant TIRF-plane angle, with 1 frame exposure detection (at 10–20 ms range) at 17 Mhz, for both 2D and 3D STORM. After acquisitions, particle-detection data reconstruction was obtained with the *ad hoc* STORM analysis module in NIS-Elements v.5.31 (Lim-Nikon Instruments) with the following parameters for blinking molecule detection over acquired frames: standard Gaussian fitting for the localization of specific molecules characterized by minimum bit Height 500, maximum bit Height 65535, over a CCD bit baseline 100, for specific counting of molecules with minimum width 20 nm, maximum width 200 nm, initial fit width 100 nm. For 2D STORM, the x and y coordinates from each blinking molecule were retrieved for molecule localization; for 3D STORM, the 3 coordinates (x, y, z) were retrieved and 3D volume viewing was performed.

CD4⁺ T-Cell Activation

CD14 monocytes purified from peripheral blood mononuclear cells (PBMCs) by positive immunomagnetic sorting were loaded with 10×10^7 of purified MILD patient-recovered exosomes for 6 h with rotation at 37°C. After incubation, monocytes were washed with RPMI containing 10% serum (exosome free), irradiated, cocultured with autologous negative immunomagnetic purified CD4⁺ T cells, and labeled with cell trace. Interleukin (IL)-2 was added after 48 h of culture. After 4 days, cells were stained with anti-CD4/BUV395 and anti-HLA-DR/BUV 650 and analyzed by flow cytometry (BD Symphony).

Exosome Purification for Mass Spectrometry

Exosomes were purified from the plasma of 3 HDs, 4 patients presenting MILD COVID-19 symptoms, and 4 patients presenting SEVERE COVID-19 symptoms using qEV SMART Size Exclusion Chromatography (SEC) columns following manufacturer's instructions. Briefly, 150 μ l of plasma were centrifuged at 1,500 g for 10 min in order to remove cells and

large contaminant particles. After a subsequent centrifugation at 10,000 g for 10 min, supernatants were loaded on IZON qEV/35nm columns, previously washed and equilibrated with 3 volumes of PBS. After column loading, samples were eluted with PBS using a void volume of 1 ml and an elution volume of 600 μ l. Purified exosomes were then concentrated and processed for mass spectrometry analysis.

Mass Spectrometry of Purified Exosomes

Quantitative proteome profiling from exosomes was achieved by a library-based approach combined with MS boxcar acquisition method in a label-free experiment as reported before (11). We constructed three different libraries by pooling proportional quantities of different exosome preparations from each sample type (MILD, SEVERE, and HD) assessed by Bicinchoninic acid (BCA) protein quantitation assay. Samples were run independently and then aligned to their respective libraries. In brief, proteins were denatured, reduced, alkylated, and digested, and peptides were purified on StageTips (12) using reagents from the PreOmics “iST” Kit (P.O. 00001, PreOmics GmbH). Only samples for the library were fractionated using a commercial High pH Reversed-Phase Peptide Fractionation Kit (Thermo Scientific). In all cases, dried peptides were reconstituted in 5 μ l of LC-LOAD buffer (P.O. 00001, PreOmics GmbH). Samples were measured using liquid chromatography–mass spectrometry (LC-MS) instrumentation consisting of an EASY-nLC 1200 system coupled to a nano-electrospray ion source and a Q Exactive HF Orbitrap (all Thermo Fisher Scientific). Purified peptides were separated on an EasySpray PEPMAF RSLC C18 (Thermo Fisher Scientific) kept at 45°C constant to reduce column back pressure. Solvent A was 0.1% formic acid (FA), and solvent B was 0.1% FA in 80% Acetonitrile (ACN). Samples were loaded in aqueous 0.1% (FA) solution at constant pressure of 980 Bar. Peptides were separated with a gradient of 3%–30% solvent B over 39 min followed by a gradient of 30%–60% for 5 min and 60%–95% over 1 min at a flow rate of 300 nl/min. For the library, standard DDA experiments were performed with a data-dependent top15 method. In brief, MS spectra (from m/z 375–1,550) were analyzed in the Orbitrap detector with resolution $R = 60,000$ at m/z 200. The 10 most intense peptide ions with charge states ≥ 2 were sequentially isolated to a target value of 3e6 and fragmented by Higher Energy Collision Dissociation (HCD) with a normalized collision energy setting of 28%. The maximum allowed ion accumulation times were 20 ms for full scans and 80 ms for MSMS, and the target value for MSMS was set to 1e5. The dynamic exclusion time was set to 20 s. Unfractionated peptides corresponding to the study samples were injected in single-shot analysis (number of replicates equal to 4) with a BoxCar scan method where instrument acquisition was controlled by MaxQuant Live software (version 1.2) keeping default scan protocol parameters (13).

Quantification and Statistical Analysis

Acquired raw data obtained by mass spectrometry analysis were analyzed using the MaxQuant (MQ) (14) version 1.6.10.43, and peptide lists were searched against the human Uniprot FASTA

database (74470 Entries) with the Andromeda search engine (15). The main search was performed with an initial mass tolerance of 7 ppm. False discovery rate (FDR) for both protein and peptide identifications was set to a maximum of 1% with enzyme specificity set to Trypsin/P. A maximum of 2 missed cleavages was allowed, and the minimum peptide length was fixed at 7 amino acids and Carbamidomethylation of Cysteine was specified as a fixed modification. Peptides were identified with an initial precursor mass deviation of 7 ppm and a fragment mass deviation of 20 ppm. “Match between run algorithm” (MBR) in MaxQuant (16) was performed after constructing a matching library consisting of pooled samples. For label-free protein quantitation (LFQ), we required a minimum ratio count of 2 (17). All proteins and peptides matching to the reversed database were filtered out. ProteinGroups.txt table from MQ output was analyzed using Perseus platform version (18). In brief, two-sample Student’s *t*-test was used to determine the significantly changed proteins between disease and control groups with a permutation-based FDR of 5%. For significant hits, minimal fold changes together with *p* values (controlled by the *s0* parameter in Perseus) were used with a permutation-based FDR of 0.05 resulting from an *s0* set to 0.1. Heatmap was generated using Heatmapper (19). Proteins were hierarchically clustered by setting Euclidean distance and average linkage method as parameters. Specific biological process enrichment and their plotting were generated in R Studio using R v4.0.3 and topGO (20) v2.42.0 passing the weight algorithm and Fisher test to the runTest function. A cutoff of 0.05 was applied, and the top 18 enriched terms were visualized with ggplot v3.3.3 (21). Protein–protein interaction (PPI) networks of unique upregulated and downregulated proteins are retrieved using the online version of Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database version 11.0, setting the maximum number of interactions for the first and second shell to not more than 10 interactors. Generated networks are imported in Cytoscape v3.8.2, where functional enrichment was performed using STRINGapp enrichment. Gene Ontology (GO) term sorting is performed by combining strength and number of input proteins retrieved for the term. Omics visualizer app (22) is used to show biologically relevant terms. For the other comparative analyses, two-tailed *t*-test and ANOVA test were used.

RESULTS

Exosomes Recovered From the Plasma of MILD COVID-19 Patients Carry a Higher Amount of SARS-CoV-2-S-Derived Peptides Compared to Those of SEVERE COVID-19 Patients

In order to characterize exosomes of different COVID-19 patients, plasma from 20 individuals who tested positive for SARS-CoV-2 infection by nasopharyngeal swab real-time PCR was collected in the acute phase of the disease (within 21 days

from the diagnosis). Overall, we enrolled 11 males and 9 females with a median age of 57.5 years [interquartile range (IQR) 27.5–70] and a median time from diagnosis of 14 days (IQR 14–16). Patients with a maximal WHO clinical progression score [https://doi.org/10.1016/S1473-3099(20)30483-7] between 1 and 4 were classified as MILD, whereas those with a maximal score between 5 and 10 were classified as SEVERE (9) (**Supplementary Table S1**). The selected MILD patients never transitioned to a SEVERE state during the course of the disease. As a control group, we enrolled 20 COVID-negative HDs. At first, we tested whether exosomes recovered from plasma of COVID-19 patients exposed any SARS-CoV-2-derived peptide. By using ExoTEST™, a platform for exosome quantification and characterization (23–25), and commercial anti-SARS-CoV-2-S antibodies for the detection of the S protein, we found that the SARS-CoV-2-S protein or derived fragments were clearly present in exosomes of COVID-19 patients but not in those of HDs, as expected (**Figure 1A**). Next, we subgrouped COVID-19 patient exosomes into MILD and SEVERE and analyzed whether SARS-CoV-2-S was more abundant in one of the two classes. Intriguingly, MILD patients had a higher amount of circulating SARS-CoV-2-S⁺ exosomes compared to patients with SEVERE symptoms (**Figure 1B**). To confirm this unexpected result, we recovered extracellular vesicles (EVs) from the plasma of either COVID-19 patients or HDs through sequential centrifugation and, after validating their size through NTA (**Figure S1A**), we used two alternative exosome isolation/characterization approaches: one based on anti-CD63-conjugated bead purification, followed by flow cytometry using anti-SARS-CoV-2-S-RBD antibodies for S protein detection (**Figure S1B**) and the other based on immunoprecipitation using anti-tetraspanins (CD63, CD9, CD81), followed by Western blotting using both anti-SARS-CoV-2-S RBD and anti-SARS-CoV-2-S1 antibodies (the latter specific for S protein subunit 1) in order to confirm the origin of the fragments detected. Flow cytometry confirmed that anti-SARS-CoV-2-S RBD antibodies bound exosomes of MILD COVID-19 patients with higher affinity compared to those of SEVERE patients, while they showed negligible binding to HD exosomes (**Figure 1C** and **Figure S1C**). Integrity of the analyzed exosomes was assessed by probing for cytosolic exosomal marker syntenin before and after permeabilization. Syntenin was detectable only after permeabilization, as expected for fully intact exosomes (**Figure S1D**). Western blotting revealed that both anti-SARS-CoV-2-S RBD and anti-SARS-CoV-2-S1 antibodies recognized protein bands predominantly in exosomes of MILD patients. These bands had molecular weights (MWs) lower than 130 kDa (**Figure 1D**), thus corresponding to degradation fragments of the S protein, whose full-length monomer is 180 kDa. Purity of exosomal preparations was confirmed by positive detection of canonical exosomal markers, namely, CD63, CD9, TSG101, and syntenin, and lack of contaminant plasma proteins (i.e., ApoA) (**Figure 1E**).

Taken together, these results suggest that SARS-CoV-2-S-derived fragments are present in exosomes of all COVID-19 patients but are specifically and preferentially enriched in those of patients classified as MILD.

SARS-CoV-2-S⁺ Exosomes Are Enriched in CD9 and CD41a

Next, we addressed whether the presence of SARS-CoV-2-S-derived peptides was associated to specific exosomal subpopulations by using the ExoView™ platform, which allows for single particle interferometric imaging measurements and analysis of differential coexpression of exosomal markers (26, 27). Exosomes were captured using antibodies against the canonical CD9, CD63, and CD81 exosome markers and platelet-specific CD41a (**Figure 1F**). CD41a was added to our strategy, since platelets function as an important exosomal source and are known to be hyperactivated in COVID-19 patients (28). By label-free detection, we observed that most of the exosomes were captured by anti-CD9 and anti-CD41a antibodies, and intriguingly, exosomes of MILD COVID-19 patients were remarkably more abundant compared to those of both SEVERE patients and HDs (**Figure 1G**). In addition, we found that exosomes from COVID-19 patients had an increased size distribution compared to HD exosomes, independently of the specific marker analyzed (**Figure S2**). Interestingly, CD41a⁺ exosomes of MILD COVID-19 patients were larger (50–160 nm) than those of SEVERE COVID-19 patients (50–100 nm) (**Figure S2**, top), possibly indicating a different cargo for the two exosome groups. Next, we wanted to assess whether the SARS-CoV-2-S-derived fragments were evenly distributed in exosomes or enriched in one of the four exosome subpopulations. We found that SARS-CoV-2-S was detected in all the exosomes analyzed, except for the CD81⁺ subpopulation (**Figure 1H**). When comparing MILD and SEVERE COVID-19 patient-recovered exosomes, we found that SARS-CoV-2-S peptides were drastically more enriched in the CD41a⁺ and CD9⁺ subpopulations of those recovered from MILD patients (**Figure 1H**).

This result was confirmed using STED microscopy, a fast-performing super-resolution technique for resolving objects smaller than the light diffraction limit. By visualizing and quantifying the levels of SARS-CoV-2-S fragments on bead-purified CD9⁺ exosomes, we confirmed a significant enrichment of SARS-CoV-2-S peptides in MILD COVID-19 patient compared to SEVERE COVID-19 patient exosomes (**Figure 2A** and **Figure S3**).

Next, to further validate this result at nanometer levels, we performed TEM using a suspension immunogold labeling protocol optimized to detect SARS-CoV-2-S peptides on immunocaptured exosomes (**Figure 2B**). No gold deposition (namely, anti-SARS-CoV-2-S RBD labeling) was detectable in HD micrographs (**Figure 2C** upper panel), whereas an increased level of gold deposition was evident on the ringlike surface of exosomes of MILD compared to SEVERE patient samples (**Figure 2C** middle and lower panel). To better visualize SARS-CoV-2-S-derived peptides at the single-molecule level and define their spatial localization on the surface of exosomes, we performed single-molecule localization microscopy *via* TIRF/Direct-STORM with stochastic blinking reconstruction, as previously reported (29). Given the high density of blinking fluorophores needed for this approach, the experiment was performed only on MILD patient exosomes. Single-molecule

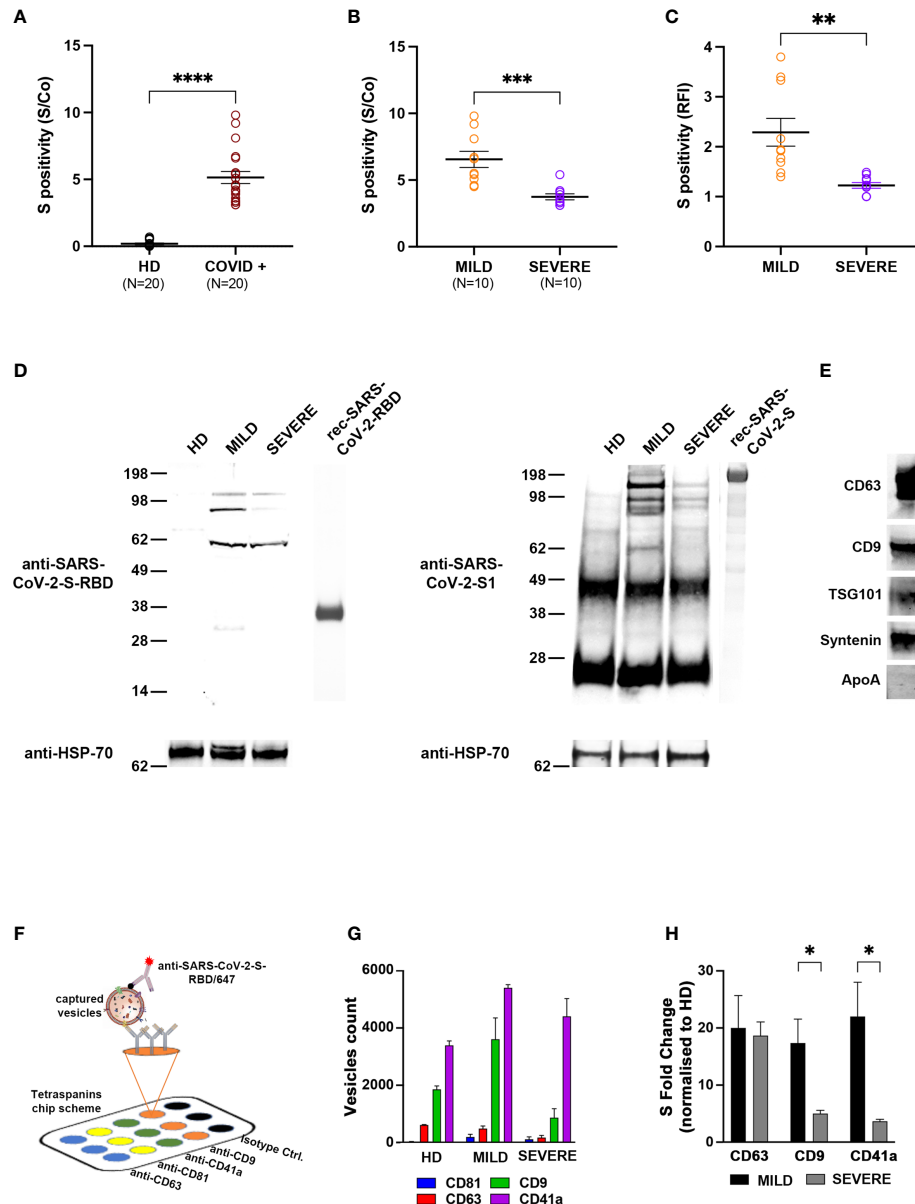
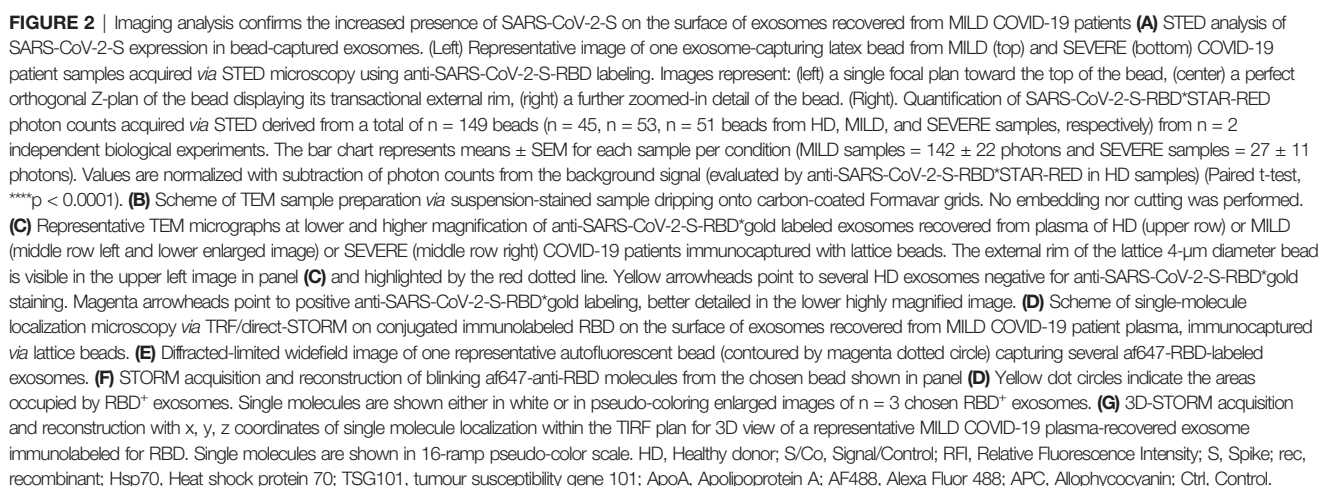


FIGURE 1 | Circulating exosomes of MILD COVID-19 patients carry more SARS-CoV-2-S-derived peptides than those of SEVERE COVID-19 patients and are enriched in CD9 and CD41a exosomal markers. **(A, B)** Tetraspanin ELISA assay performed on 20 COVID-19 patients (10 with MILD symptoms and 10 with SEVERE symptoms) and 20 COVID-19-negative HDs. Patient-recovered samples were first analyzed as a whole **(A)** and subsequently separated based on class **(B)**. Positivity for S protein was calculated as the presence of the signal compared to a control well (S/Co) (Two-tailed t-test **** $p < 0.0001$). **(C)** Flow cytometry analysis. Latex beads coated with the anti-CD63 antibodies were incubated with plasma-recovered exosomes. Bead-bound exosomes were subjected to flow cytometry. Anti-CD9/AF488 antibodies were used to define and gate the specific exosome population. The percentage of exosomes positive for anti-SARS-CoV-2-S-RBD/APC is reported in RFI (value vs. isotype control) (One-way ANOVA with Tukey test **** $p < 0.0001$). **(D)** Western blot showing anti-SARS-CoV-2-S-RBD (left) and anti-SARS-CoV-2-S1 immunoblotting (right) in exosomes of COVID-19 patients or HDs. Exosomes were immunoprecipitated with anti-tetraspanin (CD63, CD9, CD81) antibodies and immunoblotted for the indicated proteins. Exosomal marker HSP70 was used as a loading control. Blots are representative of three independent experiments. **(E)** Western blots showing the presence of exosomal markers CD63, CD9, TSG101, and syntenin and absence of contaminant plasma-protein ApoA. **(F)** Scheme of the ExoView™ tetraspanin chip. EVs from plasma of COVID-19 patients or HDs were immobilized on ExoView™ chips by affinity capture against CD81, CD63, CD9, and CD41a exosomal transmembrane proteins. Once affinity-captured, the samples were incubated with fluorescent anti-SARS-CoV-2-S-RBD antibodies and analyzed using ExoView™ R100. **(G)** Difference in the average particle count from each antibody referred to in panel (A). **(H)** Colocalization of SARS-CoV-2-S on the surface of the vesicles captured on the chip with the indicated antibodies. Histograms represent the count number (expressed as protein fold change) of SARS-CoV-2-S⁺ exosomes (t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). HD, Healthy donor; S/Co, Signal/Control; RFI, Relative Fluorescence Intensity; S, Spike; rec, recombinant; Hsp70, Heat shock protein 70; TSG101, tumour susceptibility gene 101; ApoA, Apolipoprotein A; AF488, Alexa Fluor 488; APC, Allophycocyanin; Ctrl, Control.



imaging of MILD patient exosomes revealed that SARS-CoV-2-S-derived fragments were widespread and highly abundant across the entire exosomal surface (**Figures 2D–F**). Moreover, we also performed 3D-STORM on best-emitting samples in order to retrieve within the TIRF plan a correct volumetric localization of blinking molecules. Such analysis further confirmed the localization of RBD at the level of the exosome surface, with no signal localizing within the inner vesicle compartment (**Figure 2G** and **Supplementary Video 1**).

Exosomes of MILD COVID-19 Patients Induce CD4⁺ T-Cell Activation More Efficiently Than Those of SEVERE Patients

The data obtained so far suggested that the higher abundance of SARS-CoV-2-S⁺ exosomes could be somewhat beneficial to MILD patients and contribute to their better outcome during viral infection. Among various possibilities, we hypothesized that exosomes could contribute to the adaptive immune response against SARS-CoV-2 infection by possibly exposing SARS-CoV-2-S-derived particles. To verify this assumption, at first, we analyzed the origin of SARS-CoV-2-S⁺ exosomes. Since all exosomes contain proteins derived from the parent cell and can partly maintain parent cell functionality, we characterized exosome origin using antibodies against major immune cell surface receptors. We found that SARS-CoV-2-S⁺ exosomes of MILD COVID-19 patients were mostly of B cell, dendritic cell, and monocyte/macrophage origin, as they displayed B-cell marker CD19, integrin CD11b, costimulatory molecule CD86, and MHC-class II HLA-DR (**Figure 3A**). This result indicated that most MILD patient exosomes containing SARS-CoV-2-S fragments are derived from antigen-presenting cells (APCs) and could possibly maintain antigen-presenting functions. Since proper antigen-presenting capability requires the presence of both adhesion molecules and costimulatory factors, we quantified the levels of adhesion factor Intercellular Adhesion Molecule 1 (ICAM-1) and of costimulatory molecules Human Leukocyte Antigen-DR isotype (HLA-DR), B7-2, and B7-H1 (30–32) on the surface of patient-recovered exosomes. We found that all MILD patient exosomes were in general more enriched in all the markers analyzed (**Figure 3B**). These data suggested that SARS-CoV-2-S⁺ exosomes recovered from MILD COVID-19 patients could act *per se* as antigen-presenting vehicles and modulate antigen-specific T-cell responses. To verify this hypothesis, at first, we incubated *in vitro* exosomes of COVID-19 patients and HDs with autologous CD4⁺ T cells and analyzed cell proliferation. We found that COVID-19 patient-recovered exosomes were capable of stimulating CD4⁺ T-cell growth, while HD-recovered exosomes were not (**Figure 3C**). Most interestingly, exosomes of MILD COVID-19 patients were more efficient in stimulating CD4⁺ T-cell growth compared to those of SEVERE COVID-19 patients (**Figure 3C**). To assess whether exosomes of MILD COVID-19 patients induced T-cell growth by actual T-cell activation, we tested their capability to modulate the expression of middle, CD25, and late, HLA-DR, T-lymphocyte activation markers cocultured in matched CD4⁺ T cells. We found that both markers were actually drastically upregulated only in the samples cocultured with exosomes of MILD COVID-19 patients (**Figure 3D**), suggesting that T-cell proliferation was indeed a consequence of efficient T-cell

activation triggered by exosomes. Comparable results were obtained when we repeated the assay using allogeneic T cells derived from HDs, thereby excluding the possibility that the lower immunostimulatory activity of SEVERE patient exosomes could be due to dysfunctional T cells (data not shown).

Next, we checked whether antigen presentation was driving exosome-dependent T-cell activation. We replicated the previous experiment but blocking MHC-II with specific antibodies. We found that MHC-II blockade significantly reduced exosome-dependent T-cell activation (**Figure 3E**), confirming that an antigen-presenting activity of MILD COVID-19 patient exosomes was the actual driving force of CD4⁺ T-cell activation. This evidence was further confirmed by the fact that MILD patient exosomes stimulated CD4⁺ T cells to produce high levels of IL-2, a cytokine whose secretion is known to be triggered early during antigen presentation (**Figure 3F**). Interestingly, two other cytokines secreted during antigen presentation, interferon (IFN) γ and tumor necrosis factor (TNF) α , were not enriched possibly due to their different induction kinetics (**Figure 3F**).

Next, we investigated if MILD COVID-19 patient-recovered exosomes were capable of favoring CD4⁺ T-cell activation driven by other APC sources. We checked if MILD COVID-19 patient-recovered exosomes could enhance CD4⁺ T-cell activation. Coculturing MILD COVID-19 patient exosomes with CD4⁺ T cells and autologous monocytes as primary APC source caused a significant increase in CD4⁺ T-cell HLA-DR protein levels (**Figure 3G**), an effect that was totally abolished when we blocked MHC-II, confirming the requirement of cross-presentation for efficient CD4⁺ T-cell activation and indicating a peculiar antigen cross-presentation activity of MILD COVID-19 exosomes.

Our findings suggest that MILD COVID-19 patient-recovered exosomes are capable of favoring CD4⁺ T-cell activation by functioning as an antigen-presenting source and by promoting T-cell activation.

The Protein Repertoire of Exosomes Recovered From The Plasma of MILD COVID-19 Patients Correlates With a Signature of Immune Response Enhancement

With the intent to corroborate our results and better define the functional roles of COVID-19 patient-recovered exosomes, we defined their proteomes. For each patient class (MILD and SEVERE), we selected the 4 exosome samples with the highest SARS-CoV-2-S positivity (indicated by an asterisk in **Supplementary Table S1**), while for HDs, exosome samples were selected randomly. Mass spectrometry was performed on whole exosome protein content. Comparing the proteomes of the three sets of samples, we identified 130 differentially abundant proteins (**Supplementary Table S2**), 92% of which were previously annotated in public EV databases [EXOCARTA (33)] as EV/exosome components, confirming the efficacy of our exosome purification. As expected, and in line by previously reported studies regarding conventional exosome purification methods (34, 35), highly abundant apolipoproteins were detected in all three sample types analyzed (**Supplementary Table S2**) but did

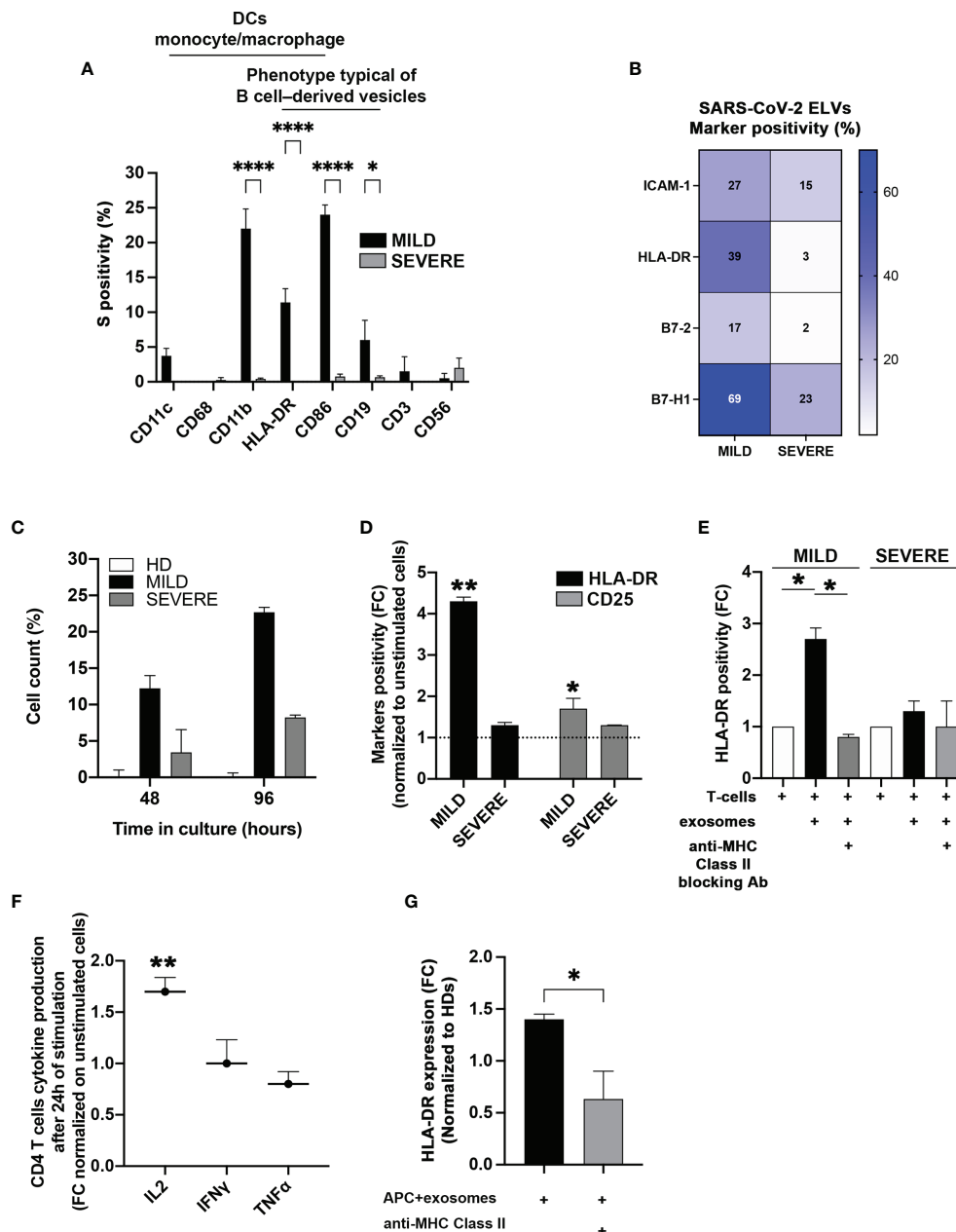
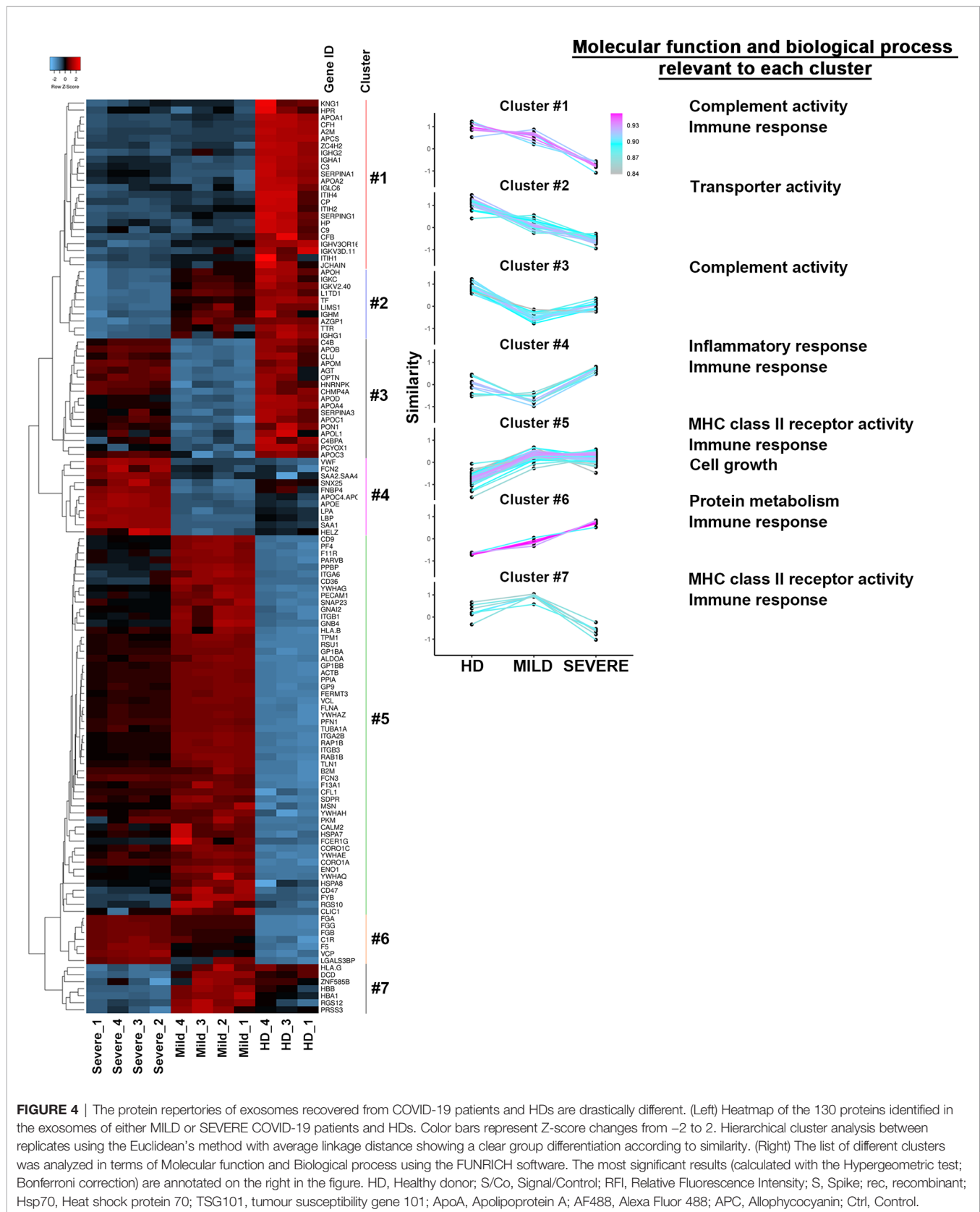


FIGURE 3 | Exosomes recovered from the plasma of MILD COVID-19 patients stimulate CD4⁺ T-cell immune responses. **(A)** Cytofluorimetric analysis of immune cell markers. SARS-CoV-2-S⁺ exosomes recovered from the plasma of COVID-19 patients and immunocaptured with anti-CD63/CD9 latex beads were analyzed by flow cytometry with the indicated surface markers (Two-way ANOVA with Tukey test, * $p < 0.03$; **** $p < 0.0001$). **(B)** Cytofluorimetric analysis of costimulatory molecules and ICAM-1 on patient-recovered exosomes. Exosomes recovered from patients ($N = 6$) were captured with anti-CD63 beads and gated for CD9 positivity. The heatmap shows expression levels of the indicated markers based on a color scale from white (no expression) to blue (highest expression). **(C)** Stimulation of CD4⁺ T cells. Coculture of sorted CD4⁺ T cells with exosomes isolated from the plasma of MILD and SEVERE patients or HDs for up 96 h. Trypan blue was used to discriminate between live and dead cells. Results are representative of three independent experiments performed in triplicate. **(D, E)** Activation of CD4⁺ T cells. **(D)** The stimulatory effect of exosomes recovered from plasma of COVID-19 patients on CD4⁺ T cells was measured by flow cytometry, detecting the expression of HLA-DR (black bars) and CD25 (gray bars) after 2 days of culture. Unstimulated cells were used as a threshold to calculate marker positivity (dotted line). **(E)** The same assay as in panel **(C)** was conducted with or without HLA-DR blocking antibodies (t-test, * $p < 0.05$, ** $p < 0.01$ and one-way ANOVA with Tukey test, * $p < 0.03$). **(F)** Cytokine analysis. Production of the indicated cytokines by CD4⁺ T cells was measured after 24 h of incubation with exosomes. **(G)** Flow cytometry analysis of HLA-DR induction by exosomes. The graphs show the expression of HLA-DR on isolated CD4⁺ T cells following incubation with matched monocytes pulsed with exosomes recovered from MILD COVID-19 patients. HLA-DR blockade was performed by treating the cells with anti-HLA-DR antibodies (Double-tailed t-test, * $p < 0.05$, ** $p < 0.01$). HD, Healthy donor; S/Co, Signal/Control; RFI, Relative Fluorescence Intensity; S, Spike; rec, recombinant; Hsp70, Heat shock protein 70; TSG101, tumour susceptibility gene 101; ApoA, Apolipoprotein A; AF488, Alexa Fluor 488; APC, Allophycocyanin; Ctrl, Control.



not interfere with downstream analyses. Pairwise correlation analysis of protein contents clearly revealed that samples of the same class displayed a high internal correlation mirrored by lower correlation with samples of other classes (**Figure S4A**), indicating a high homogeneity among exosomes recovered from different donors. This result was further confirmed by hierarchical clustering that, by gathering the 130 proteins into 7 groups, clearly separated MILD COVID-19-, SEVERE COVID-19-, and HD-recovered exosomes (**Figure 4**, left). To assess the characteristics of each cluster found, we carried out a comparative analysis using FunRich (36). Our results revealed that the clusters linked to HD exosomes correlated with complement activity and immune response activation, those linked to SEVERE COVID-19 patient exosomes correlated with immune/inflammatory responses and protein metabolism, while those mainly related to MILD COVID-19 patient exosomes were related to immune response, cell growth, signal transduction, and MHC class II receptor functionality (**Figure 4**, right), well confirming our *in vitro* results that depicted MILD COVID-19 patient exosomes as strong stimulators of CD4⁺ T-cell activation and growth.

Next, we focused on differentially abundant proteins. We identified 102 proteins differentially present in MILD COVID-19- vs. HD-recovered exosomes and 87 proteins differentially abundant in SEVERE COVID-19- vs. HD-recovered exosomes. Here, 72 proteins were shared between the two groups, while a total of 45 proteins were specific for one group or the other (**Figure 5A** and **Figure S4B**). By focusing on the latter 45 proteins, we selected the 22 with the highest enrichment. These included 16 proteins for the MILD and 6 for the SEVERE patient group (**Figure 5B**). GO term analysis indicated that the proteins recovered from MILD COVID-19 patient exosomes were mainly involved in immune cell activation, while those recovered from SEVERE COVID-19 patient exosomes were mainly involved in stress and inflammatory responses (**Figure 5C**). Such results were also confirmed by interrogating the STRING (37) database. Exosomes recovered from MILD COVID-19 patients showed an enrichment in proteins involved in pathways related to antigen processing, presentation of exogenous peptides (FDR <1.56E-07), and myeloid cell activation (FDR <6.65E-08) (**Figure 5D**, left), while those recovered from SEVERE COVID-19 patients showed an enrichment in proteins involved in pathways regulating acute inflammatory responses (FDR <3.58E-13) and complement activation (FDR <3.07E-14) (**Figure 5D**, right), further strengthening the notion that MILD and SEVERE patient exosomes are drastically different and possess different immunomodulatory functions, the first mainly involved in immune cell activation and the second principally involved in inflammation.

DISCUSSION

Exosomes and other types of EVs play a number of critical roles in cell-to-cell communication. Their composition and biological activities change depending on their origin and can be drastically modified by bacterial, fungal, and viral infections (38). It has been reported that EVs may incorporate pathogen proteins and/or

fragments of viral RNA from infected cells in order to shuttle the material to target cells, an event which plays important roles in viral infection responses (39–41). In addition, there is evidence that viruses can use endocytic routes to enter uninfected cells and/or hijack EV secretory pathways to exit infected cells, indicating that EVs and viruses share common cell entry and biogenesis mechanisms (42, 43). Through the years, several studies analyzed the role of EVs and exosomes in viral infection, in particular in HIV, hepatitis C virus (HCV), and SARS pathologies (39, 40). Recently, a few works also characterized EVs of COVID-19 patients (44, 45), but an actual role in adaptive immunity was never assessed.

Here, we characterized the protein composition of exosomes of COVID-19 patients who experienced MILD or SEVERE symptoms, collecting samples around 14 days after diagnosis.

Unfortunately, for each sample analyzed, we had availability to a very limited amount of material, and this posed some methodological restrictions. For instance, we could not perform conventional collection of exosomes by ultracentrifugation. Exosomes were instead isolated and visualized by adopting different commercial kits and experimental approaches more suitable for low sample volumes but still reliable and well accepted by the scientific community (e.g., EXOTEST and ExoView).

We found that MILD COVID-19 patient exosomes had higher levels of the MHC class II receptor, which is responsible for antigen presentation to CD4⁺ T helper cells and high levels of both CD11b, a differentiation marker for cells of the myeloid-monocytic lineage (46, 47), and CD86, a type I transmembrane protein originally identified as a CD28/CTLA-4 ligand, which are both associated with T-cell activation (48). Strikingly, by using different approaches, we found that exosomes of MILD patients bear on their surface detectable SARS-CoV-2-S-derived fragments that could derive from either an active phagocytic activity or a transient viral infection of paternal cells. The isolation technique we used, based on antibody purification of the vesicles, excluded the possibility that SARS-CoV-2-S-derived fragments could originate from virus particle contaminants co-purifying with our exosomes.

MILD COVID-19 patient exosomes are able to activate both autologous and heterologous CD4⁺ T helper cells and induce IL-2 secretion *in vitro*, suggesting that *in vivo*, they could enhance the immune responses elicited against SARS-CoV-2 antigens, thereby possibly contributing to a better outcome or more rapid resolution of the infection. The fact that this ability was observed in both matched or unmatched PBMC samples excludes any potential bias given by T cell dysfunctionality in SEVERE COVID-19 patients and could suggest that the immunomodulatory effect is due at least in part to the presence of SARS-CoV-2-S-derived fragments exposed on the exosomal surface.

By characterizing patient-recovered exosomes, we generally observed that those of SEVERE patients harbored proteins involved in metabolism, inflammation, and stress responses, while those recovered from MILD patients showed an enrichment in proteins involved in immune activation, effector activity, and migration/chemotaxis, possibly reflecting a more efficient functioning of the immune system. In particular, our results identified specific features of MILD and SEVERE COVID-

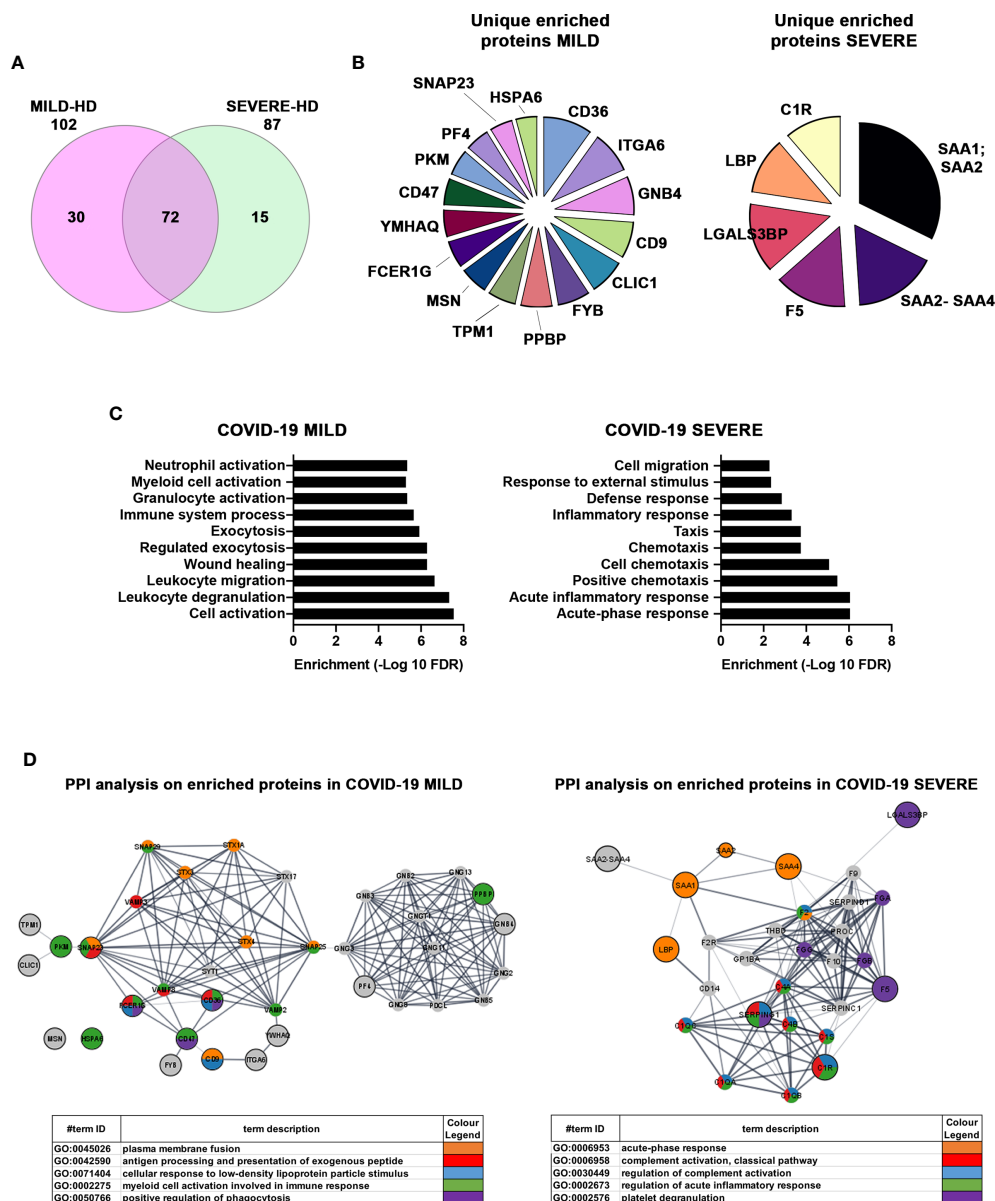


FIGURE 5 | Proteins enriched in exosomes of MILD COVID-19 patients are mainly involved in the immune response, while those enriched in the exosomes of SEVERE COVID-19 patients correlate with stress responses and acute inflammation. **(A)** Venn diagram representing the differentially abundant proteins residing in the exosomes of MILD (pink) and SEVERE (green) COVID-19 patients compared to the HD control group [cutoff value: Log fold change >3 and <-0.5; p value (t test) < 0.05]. Here, 102 and 87 proteins were identified as differentially expressed in MILD and SEVERE patients, respectively. The overlap among the two groups of COVID-19-infected patients shows that 72 proteins are present in both samples, while 45 are differentially abundant. **(B)** Pie charts of unique and enriched proteins derived from the exosomes of MILD (16 proteins) and SEVERE (6 proteins) COVID-19 patients. **(C)** The detected proteins were analyzed by GO term enrichment analysis (ShinyGO v0.61) using a p-value cutoff of (FDR) <0.01. Significantly enriched categories are shown. **(D)** Network interaction analysis for the enriched proteins related to exosomes of COVID-19 patients. Known and predicted protein-protein interactions (PPIs) were extracted from the STRING database (Version 11.0). Proteins are depicted as nodes, and the biological relationships between nodes are represented as edges (lines). The inputs are represented in gray, while other colors indicate the different pathways to which the inputs belong to. Tables summarize the GO enrichment analysis for biological processes. HD, Healthy donor; S/ Co, Signal/Control; RFI, Relative Fluorescence Intensity; S, Spike; rec, recombinant; Hsp70, Heat shock protein 70; TSG101, tumour susceptibility gene 101; ApoA, Apolipoprotein A; AF488, Alexa Fluor 488; APC, Allophycocyanin; Ctrl, Control.

19 patient exosomes. Here, 16 proteins were unequivocally associated with MILD COVID-19 patient exosomes, while 6 were found enriched exclusively in those of SEVERE COVID-19 patients (Figure 5B). Among the enriched MILD patient exosome proteins

we found are the following: CLIC1, CD9, FYB, CD36, CD47, and SNAP23, which are involved in antigen processing and cross-presentation (49–53) and trigger T-cell activation; PF4 and PPBP, which act as chemoattractants and activate, respectively,

neutrophils and monocytes (54); MSN, which acts on both T- and B-cell homeostasis by regulating lymphocyte egress from lymphoid organs (55); and ITGA6, which negatively impacts virus transcription (56). Exosomes of patients with SEVERE disease were instead enriched in complement factors, members of the coagulation system, inflammation modulators, and regulators of IL-6 pro-inflammatory signaling. Specifically, we found C1R, which is known to initiate complement activation (57, 58) and was shown to play a major role in acute and chronic inflammation, endothelial cell dysfunction, thrombus formation, and intravascular coagulation in SARS-CoV-2 patients (59); SAA2-SAA4 and SAA1-SAA2, which are markers of inflammatory response and tissue injury (60) and are induced by IL-6 (61); LGALS3BP, a pro-inflammatory factor (62) that is known to induce IL-6 expression (63); and LBP, whose upregulation contributes to inflammation (64) and correlates with immune response dysregulation in both pneumonia (65) and SARS-CoV-2 patients (64).

Collectively, our study confirms that plasma-recovered exosomes reproduce the molecular patterns of their cells of origin and reflect the different pathological states of COVID-19 patients. In agreement with published data (45, 66), our proteomic analysis of patient-recovered exosomes identified several molecules involved in immune responses, inflammation, and activation of both coagulation and complement pathways, which are the main mechanisms of COVID-19-associated tissue damage. We also highlighted that exosomes are peculiar indicators of the functional state of patients' immune cells, which are generally found to be better performing in individuals with MILD symptoms. This specific feature provides the rationale for future studies on alternative exosome-based preventive or prophylactic approaches to treat SARS-CoV-2 infection.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD029007.

ETHICS STATEMENT

The study was approved by the Institutional Review Board Milano Area 2 (#331_2020). The patients/participants provided their written informed consent to participate in this study.

REFERENCES

- Bayati A, Kumar R, Francis V, McPherson PS. SARS-CoV-2 Infects Cells After Viral Entry *via* Clathrin-Mediated Endocytosis. *J Biol Chem* (2021) 296:100306. doi: 10.1016/j.jbc.2021.100306
- Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* (2020) 181(2):271–80.e8. doi: 10.1016/j.cell.2020.02.052
- Margolis L, Sadovsky Y. The Biology of Extracellular Vesicles: The Known Unknowns. *PLoS Biol* (2019) 17(7):e3000363. doi: 10.1371/journal.pbio.3000363

AUTHOR CONTRIBUTIONS

Conceptualization, EP. Investigation, EP, NM, CC, SS, PG, AC, FC, GC, and AF. Writing—original draft, EP, NM, and RG. Resources, AB, RU, AL, DM, AM, SAI, FB, and AGor. Formal analysis, AGor and MB. Supervision, SAb, RDF, SB, and RG. Funding acquisition, RG. All authors reviewed and edited the drafts. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by the project COiMMUNITY (ID 1842163) to RG funded by Regione Lombardia, funded under POR FESR 2014-2020 resources, and by the project “END-COVID” (B/2020/0076) to AGor funded by Intesa San Paolo “Fondo di beneficenza ed opere di carattere sociale e culturale,” and by an unrestricted grant from Fondazione “Romeo ed Enrica Invernizzi”.

ACKNOWLEDGMENTS

The authors wish to thank Frédéric Eghiaian from Abberior Instruments and Alessandro Rossi from Crisel Instruments, from bringing and installing in INGM Imaging Facility the DEMO instrument Abberior STEDYCON, without which a complete set of results here presented and discussed would not have been obtained. The authors wish to acknowledge Andrea Pigozzo and Roberto Santoliquido from AlfatestLAB s.r.l. for the Exoview analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2021.785941/full#supplementary-material>

Supplementary Video 1 | (linked to **Figure 2G**): Video rendering of volumetric 3D image from stochastically reconstructed blinking single molecules on 3D STORM TIRF plan, executed on one representative EV, immunolabeled for RBD (with secondary labeling in AlexaFluor-647) from MILD COVID-19 patient. Video projections clearly highlight the majority of RBD-labeled molecules on the surface of the EV, with an inner part devoid of RBD signal, thus suggesting a specific 3D surface localization of RBD protein on plasma-derived EVs. Localized molecules are shown in pseudo-color 16-ramp scale to better perceive differences in signal intensities.

- Dogrammatzis C, Waisner H, Kalamvoki M. Cloaked Viruses and Viral Factors in Cutting Edge Exosome-Based Therapies. *Front Cell Dev Biol* (2020) 8. doi: 10.3389/fcell.2020.00376
- Stahl A, Johansson K, Mossberg M, Kahn R, Karpman D. Exosomes and Microvesicles in Normal Physiology, Pathophysiology, and Renal Diseases. *Pediatr Nephrol* (2019) 34(1):11–30. doi: 10.1007/s00467-017-3816-z
- Zhang Y, Liu Y, Liu H, Tang WH. Exosomes: Biogenesis, Biologic Function and Clinical Potential. *Cell Biosci* (2019) 9(1). doi: 10.1186/s13578-019-0282-2
- Doyle L, Wang M. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. *Cells* (2019) 8(7):727. doi: 10.3390/cells8070727

8. Bedford JG, Infusini G, Dagley LF, Villalon-Letelier F, Zheng MZM, Bennett-Wood V, et al. Airway Exosomes Released During Influenza Virus Infection Serve as a Key Component of the Antiviral Innate Immune Response. *Front Immunol* (2020) 11. doi: 10.3389/fimmu.2020.00887
9. Marshall JC, Murthy S, Diaz J, Adhikari NK, Angus DC, Arabi YM, et al. A Minimal Common Outcome Measure Set for COVID-19 Clinical Research. *Lancet Infect Dis* (2020) 20(8):e192–7. doi: 10.1016/S1473-3099(20)30483-7
10. Cavallo C, Merli G, Borzi RM, Zini N, D'Adamo S, Guescini M, et al. Small Extracellular Vesicles From Adipose Derived Stromal Cells Significantly Attenuate *In Vitro* the NF- κ B Dependent Inflammatory/Catabolic Environment of Osteoarthritis. *Sci Rep* (2021) 11(1):1053. doi: 10.1038/s41598-020-80032-7
11. Meier F, Geyer PE, Virreira Winter S, Cox J, Mann M. BoxCar Acquisition Method Enables Single-Shot Proteomics at a Depth of 10,000 Proteins in 100 Minutes. *Nat Methods* (2018) 15(6):440–8. doi: 10.1038/s41592-018-0003-5
12. Kulak NA, Pichler G, Paron I, Nagaraj N, Mann M. Minimal, Encapsulated Proteomic-Sample Processing Applied to Copy-Number Estimation in Eukaryotic Cells. *Nat Methods* (2014) 11(3):319–24. doi: 10.1038/nmeth.2834
13. Wichmann C, Meier F, Virreira Winter S, Brunner A-D, Cox J, Mann M. MaxQuant.Live Enables Global Targeting of More Than 25,000 Peptides. *Mol Cell Proteomics* (2019) 18(5):982–94. doi: 10.1074/mcp.TIR118.001131
14. Tyanova S, Temu T, Cox J. The MaxQuant Computational Platform for Mass Spectrometry-Based Shotgun Proteomics. *Nat Protoc* (2016) 11(12):2301–19. doi: 10.1038/nprot.2016.136
15. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. Andromeda: A Peptide Search Engine Integrated Into the MaxQuant Environment. *J Proteome Res* (2011) 10(4):1794–805. doi: 10.1021/pr101065j
16. Nagaraj N, Alexander Kulak N, Cox J, Neuhauser N, Mayr K, Hoerning O, et al. System-Wide Perturbation Analysis With Nearly Complete Coverage of the Yeast Proteome by Single-Shot Ultra HPLC Runs on a Bench Top Orbitrap. *Mol Cell Proteomics* (2012) 11(3):M111.013722. doi: 10.1074/mcp.M111.013722
17. Cox J, Hein MY, Luber CA, Paron I, Nagaraj N, Mann M. Accurate Proteome-Wide Label-Free Quantification by Delayed Normalization and Maximal Peptide Ratio Extraction, Termed MaxLFQ. *Mol Cell Proteomics* (2014) 13(9):2513–26. doi: 10.1074/mcp.M113.031591
18. Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, et al. The Perseus Computational Platform for Comprehensive Analysis of (Prote) Omics Data. *Nat Methods* (2016) 13(9):731–40. doi: 10.1038/nmeth.3901
19. Babicki S, Arndt D, Marcu A, Liang Y, Grant JR, Maciejewski A, et al. Heatmapper: Web-Enabled Heat Mapping for All. *Nucleic Acids Res* (2016) 44(W1):W147–53. doi: 10.1093/nar/gkw419
20. Shannon P. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome Res* (2003) 13(11):2498–504. doi: 10.1101/gr.1239303
21. Doncheva NT, Morris JH, Gorodkin J, Jensen LJ. Cytoscape StringApp: Network Analysis and Visualization of Proteomics Data. *J Proteome Res* (2019) 18(2):623–32. doi: 10.1021/acs.jproteome.8b00702
22. Legeay M, Doncheva NT, Morris JH, Jensen LJ. Visualize Omics Data on Networks With Omics Visualizer, a Cytoscape App. *F1000Research* (2020) 9:157. doi: 10.12688/f1000research.22280.1
23. Musante L, Tataruch-Weinert D, Kerjaschki D, Henry M, Meleady P, Holthofer H. Residual Urinary Extracellular Vesicles in Ultracentrifugation Supernatants After Hydrostatic Filtration Dialysis Enrichment. *J Extracellular Vesicles*. (2017) 6(1):1267896. doi: 10.1080/20013078.2016.1267896
24. Kosaka N, Kogure A, Yamamoto T, Urabe F, Usuba W, Prieto-Vila M, et al. Exploiting the Message From Cancer: The Diagnostic Value of Extracellular Vesicles for Clinical Applications. *Exp Mol Med* (2019) 51(3):1–9. doi: 10.1038/s12276-019-0219-1
25. Urabe F, Kosaka N, Ito K, Kimura T, Egawa S, Ochiya T. Extracellular Vesicles as Biomarkers and Therapeutic Targets for Cancer. *Am J Physiology-Cell Physiol* (2020) 318(1):C29–39. doi: 10.1152/ajpcell.00280.2019
26. Arab T, Mallick ER, Huang Y, Dong L, Liao Z, Zhao Z, et al. Characterization of Extracellular Vesicles and Synthetic Nanoparticles With Four Orthogonal Single-Particle Analysis Platforms. *J Extracellular Vesicles* (2021) 10(6). doi: 10.1002/jev.2.12079
27. Bachurski D, Schuldner M, Nguyen P-H, Malz A, Reinert KS, Grenzi PC, et al. Extracellular Vesicle Measurements With Nanoparticle Tracking Analysis – An Accuracy and Repeatability Comparison Between NanoSight NS300 and ZetaView. *J Extracellular Vesicles* (2019) 8(1):1596016. doi: 10.1080/20013078.2019.1596016
28. Caby M-P, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-Like Vesicles are Present in Human Blood Plasma. *Int Immunol* (2005) 17(7):879–87. doi: 10.1093/intimm/dxh267
29. Lennon KM, Wakefield DL, Maddox AL, Brehove MS, Willner AN, Garcia-Mansfield K, et al. Single Molecule Characterization of Individual Extracellular Vesicles From Pancreatic Cancer. *J Extracellular Vesicles* (2019) 8(1):1685634. doi: 10.1080/20013078.2019.1685634
30. Théry C, Duban L, Segura E, Véron P, Lantz O, Amigorena S. Indirect Activation of Naïve CD4⁺ T Cells by Dendritic Cell–Derived Exosomes. *Nat Immunol* (2002) 3(12):1156–62. doi: 10.1038/ni854
31. Segura E, Nicco C, Lombard B, Véron P, Raposo G, Batteux F, et al. ICAM-1 on Exosomes From Mature Dendritic Cells is Critical for Efficient Naïve T-Cell Priming. *Blood* (2005) 106(1):216–23. doi: 10.1182/blood-2005-01-0220
32. Hu W, Huang F, Ning L, Hao J, Wan J, Hao S. Enhanced Immunogenicity of Leukemia-Derived Exosomes via Transfection With Lentiviral Vectors Encoding Costimulatory Molecules. *Cell Oncol* (2020) 43(5):889–900. doi: 10.1007/s13402-020-00535-3
33. Mathivanan S, Fahner CJ, Reid GE, Simpson RJ. ExoCarta 2012: Database of Exosomal Proteins, RNA and Lipids. *Nucleic Acids Res* (2012) 40(D1):D1241–4. doi: 10.1093/nar/gkr828
34. Karimi N, Cvjetkovic A, Jang SC, Crescitelli R, Hosseinpour Feizi MA, Nieuwland R, et al. Detailed Analysis of the Plasma Extracellular Vesicle Proteome After Separation From Lipoproteins. *Cell Mol Life Sci* (2018) 75(15):2873–86. doi: 10.1007/s00018-018-2773-4
35. Brennan K, Martin K, Fitzgerald SP, O'Sullivan J, Wu Y, Blanco A, et al. A Comparison of Methods for the Isolation and Separation of Extracellular Vesicles From Protein and Lipid Particles in Human Serum. *Sci Rep* (2020) 10(1):1039. doi: 10.1038/s41598-020-57497-7
36. Pathan M, Keerthikumar S, Ang C-S, Gangoda L, Quek CYJ, Williamson NA, et al. FunRich: An Open Access Standalone Functional Enrichment and Interaction Network Analysis Tool. *PROTEOMICS* (2015) 15(15):2597–601. doi: 10.1002/pmic.201400515
37. Jensen LJ, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J, et al. STRING 8—a Global View on Proteins and Their Functional Interactions in 630 Organisms. *Nucleic Acids Res* (2009) 37(Database):D412–6. doi: 10.1093/nar/gkn760
38. Schorey JS, Cheng Y, Singh PP, Smith VL. Exosomes and Other Extracellular Vesicles in Host–Pathogen Interactions. *EMBO Rep* (2015) 16(1):24–43. doi: 10.15252/embr.201439363
39. Badierah RA, Uversky VN, Redwan EM. Dancing With Trojan Horses: An Interplay Between the Extracellular Vesicles and Viruses. *J Biomolecular Structure Dynamics*. (2021) 39(8):3034–60. doi: 10.1080/07391102.2020.1756409
40. Giannesi F, Aiello A, Franchi F, Percario ZA, Affabris E. The Role of Extracellular Vesicles as Allies of HIV, HCV and SARS Viruses. *Viruses* (2020) 12(5):571. doi: 10.3390/v12050571
41. Raab-Traub N, Dittmer DP. Viral Effects on the Content and Function of Extracellular Vesicles. *Nat Rev Microbiol* (2017) 15(9):559–72. doi: 10.1038/nrmicro.2017.60
42. Théry C, Ostrowski M, Segura E. Membrane Vesicles as Conveyors of Immune Responses. *Nat Rev Immunol* (2009) 9(8):581–93. doi: 10.1038/nri2567
43. Urbanelli L, Buratta S, Tancini B, Sagini K, Delo F, Porcellati S, et al. The Role of Extracellular Vesicles in Viral Infection and Transmission. *Vaccines* (2019) 7(3):102. doi: 10.3390/vaccines7030102
44. Balbi C, Burrello J, Bolis S, Lazzarini E, Biemmi V, Pianezzi E, et al. Circulating Extracellular Vesicles are Endowed With Enhanced Procoagulant Activity in SARS-CoV-2 Infection. *EBioMedicine* (2021) 67:103369. doi: 10.1016/j.ebiom.2021.103369
45. Lam SM, Zhang C, Wang Z, Ni Z, Zhang S, Yang S, et al. A Multi-Omics Investigation of the Composition and Function of Extracellular Vesicles Along the Temporal Trajectory of COVID-19. *Nat Metab* (2021) 3(7):909–22. doi: 10.1038/s42255-021-00425-4
46. Kim O, Monsel A, Bertrand M, Coriat P, Cavaillon J-M, Adib-Conquy M. Differential Down-Regulation of HLA-DR on Monocyte Subpopulations During Systemic Inflammation. *Crit Care* (2010) 14(2):R61. doi: 10.1186/cc8959

47. van Lochem EG, van der Velden VHJ, Wind HK, te Marvelde JG, Westerdal NAC, van Dongen JJM. Immunophenotypic Differentiation Patterns of Normal Hematopoiesis in Human Bone Marrow: Reference Patterns for Age-Related Changes and Disease-Induced Shifts. *Cytometry* (2004) 60B (1):1–13. doi: 10.1002/cyto.b.20008
48. Janakiram M, Pareek V, Cheng H, Narasimhulu DM, Zang X. Immune Checkpoint Blockade in Human Cancer Therapy: Lung Cancer and Hematologic Malignancies. *Immunotherapy* (2016) 8(7):809–19. doi: 10.2217/imt-2016-0001
49. Salao K, Jiang L, Li H, Tsai VW-W, Husaini Y, Curmi PMG, et al. CLIC1 Regulates Dendritic Cell Antigen Processing and Presentation by Modulating Phagosome Acidification and Proteolysis. *Biol Open* (2016) 5(5):620–30. doi: 10.1242/bio.018119
50. Blander JM. Regulation of the Cell Biology of Antigen Cross-Presentation. *Annu Rev Immunol* (2018) 36(1):717–53. doi: 10.1146/annurev-immunol-041015-055523
51. Rocha-Perugini V, Martínez del Hoyo G, González-Granado JM, Ramírez-Huesca M, Zorita V, Rubinstein E, et al. CD9 Regulates Major Histocompatibility Complex Class II Trafficking in Monocyte-Derived Dendritic Cells. *Mol Cell Biol* (2017) 37(15). doi: 10.1128/MCB.00202-17
52. Geng L, Pfister S, Kraeft S-K, Rudd CE, Adaptor FYB. (Fyn-Binding Protein) Regulates Integrin-Mediated Adhesion and Mediator Release: Differential Involvement of the FYB SH3 Domain. *Proc Natl Acad Sci* (2001) 98 (20):11527–32. doi: 10.1073/pnas.191378198
53. Wang H, Franco F, Tsui Y-C, Xie X, Trefny MP, Zappasodi R, et al. CD36-Mediated Metabolic Adaptation Supports Regulatory T Cell Survival and Function in Tumors. *Nat Immunol* (2020) 21(3):298–308. doi: 10.1038/s41590-019-0589-5
54. Flad H-D, Brandt E. Platelet-Derived Chemokines: Pathophysiology and Therapeutic Aspects. *Cell Mol Life Sci* (2010) 67(14):2363–86. doi: 10.1007/s00018-010-0306-x
55. Hunter MC, Teixeira A, Halin C. T Cell Trafficking Through Lymphatic Vessels. *Front Immunol* (2016) 7. doi: 10.3389/fimmu.2016.00613
56. Bowles JB, Steain M, Slobedman B, Abendroth A. Inhibition of Integrin $\alpha 6$ Expression by Cell-Free Varicella-Zoster Virus. *J Gen Virol* (2012) 93(8). doi: 10.1099/vir.0.039917-0
57. Hajishengallis G, Reis ES, Mastellos DC, Ricklin D, Lambris JD. Novel Mechanisms and Functions of Complement. *Nat Immunol* (2017) 18 (12):1288–98. doi: 10.1038/ni.3858
58. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: A Key System for Immune Surveillance and Homeostasis. *Nat Immunol* (2010) 11(9):785–97. doi: 10.1038/ni.1923
59. Noris M, Benigni A, Remuzzi G. The Case of Complement Activation in COVID-19 Multiorgan Impact. *Kidney Int* (2020) 98(2):314–22. doi: 10.1016/j.kint.2020.05.013
60. Sack GH. Serum Amyloid A – A Review. *Mol Med* (2018) 24(1). doi: 10.1186/s10020-018-0047-0
61. Hagihara M, Hasegawa T, Sato S, Yoshikawa S, Ohkubo K, Morii T. Ribonucleopeptides: Functional RNA-Peptide Complexes. *Biopolymers* (2004) 76(1):66. doi: 10.1002/bip.10576
62. Xu G, Xia Z, Deng F, Liu L, Wang Q, Yu Y, et al. Inducible LGALS3BP/90K Activates Antiviral Innate Immune Responses by Targeting TRAF6 and TRAF3 Complex. *PloS Pathog* (2019) 15(8):e1008002. doi: 10.1371/journal.ppat.1008002
63. Silverman MN, Sternberg EM. Glucocorticoid Regulation of Inflammation and its Functional Correlates: From HPA Axis to Glucocorticoid Receptor Dysfunction. *Ann New York Acad Sci* (2012) 1261(1):55–63. doi: 10.1111/j.1749-6632.2012.06633.x
64. Messner CB, Demichev V, Wendisch D, Michalick L, White M, Freiwald A, et al. Ultra-High-Throughput Clinical Proteomics Reveals Classifiers of COVID-19 Infection. *Cell Syst* (2020) 11(1):11–24.e4. doi: 10.1016/j.cels.2020.05.012
65. Gucht Sv, Reeth Kv, Nauwynck H, Pensaert M. Porcine Reproductive and Respiratory Syndrome Virus Infection Increases CD14 Expression and Lipopolysaccharide-Binding Protein in the Lungs of Pigs. *Viral Immunol* (2005) 18(1):116–26. doi: 10.1089/vim.2005.18.116
66. Barberis E, Vanella VV, Falasca M, Caneperio V, Cappellano G, Raineri D, et al. Circulating Exosomes Are Strongly Involved in SARS-CoV-2 Infection. *Front Mol Biosci* (2021) 8. doi: 10.3389/fmolb.2021.632290

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Pesce, Manfrini, Cordiglieri, Santi, Bandera, Gobbini, Gruarin, Favalli, Bombaci, Cuomo, Collino, Cricri, Ungaro, Lombardi, Mangioni, Muscatello, Aliberti, Blasi, Gori, Abrignani, De Francesco, Biffo and Grifantini. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Biogenesis and Biological Functions of Extracellular Vesicles in Cellular and Organismal Communication With Microbes

Yuan Fang^{1,2,3}, Zhiwen Wang¹, Xili Liu^{1*} and Brett M. Tyler^{3*}

¹ Department of Plant Pathology, College of Plant Protection, China Agricultural University, Beijing, China, ² College of Landscape and Ecological Engineering, Hebei University of Engineering, Handan, China, ³ Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR, United States

OPEN ACCESS

Edited by:

Suman Dutta,
International Institute of Innovation
and Technology, India

Reviewed by:

Kenneth Perry Hough,
Cytogenetics, Inc., United States
Josefa Badia,
University of Barcelona, Spain
Ravinder Malik,
University of California, Los Angeles,
United States

*Correspondence:

Xili Liu
seedling@cau.edu.cn
Brett M. Tyler
tyler2908@gmail.com

Specialty section:

This article was submitted to
Infectious Agents and Disease,
a section of the journal
Frontiers in Microbiology

Received: 18 November 2021

Accepted: 31 January 2022

Published: 18 February 2022

Citation:

Fang Y, Wang Z, Liu X and
Tyler BM (2022) Biogenesis
and Biological Functions
of Extracellular Vesicles in Cellular
and Organismal Communication With
Microbes.
Front. Microbiol. 13:817844.
doi: 10.3389/fmicb.2022.817844

Extracellular vesicles (EVs) represent a prominent mechanism of transport and interaction between cells, especially microbes. Increasing evidence indicates that EVs play a key role in the physiological and pathological processes of pathogens and other symbionts. Recent research has focused on the specific functions of these vesicles during pathogen-host interactions, including trans-kingdom delivery of small RNAs, proteins and metabolites. Much current research on the function of EVs is focused on immunity and the interactions of microbes with human cells, while the roles of EVs during plant-microbe interactions have recently emerged in importance. In this review, we summarize recent research on the biogenesis of these vesicles and their functions in biology and pathology. Many key questions remain unclear, including the full structural and functional diversity of EVs, the roles of EVs in communication among microbes within microbiomes, how specific cargoes are targeted to EVs, whether EVs are targeted to specific destinations, and the full scope of EVs' transport of virulence effectors and of RNA and DNA molecules.

Keywords: extracellular vesicles (EVs), biogenesis, pathology, pathogen-plant interaction, cell to cell communication

CHARACTERISTICS AND COMPOSITION OF EXTRACELLULAR VESICLES

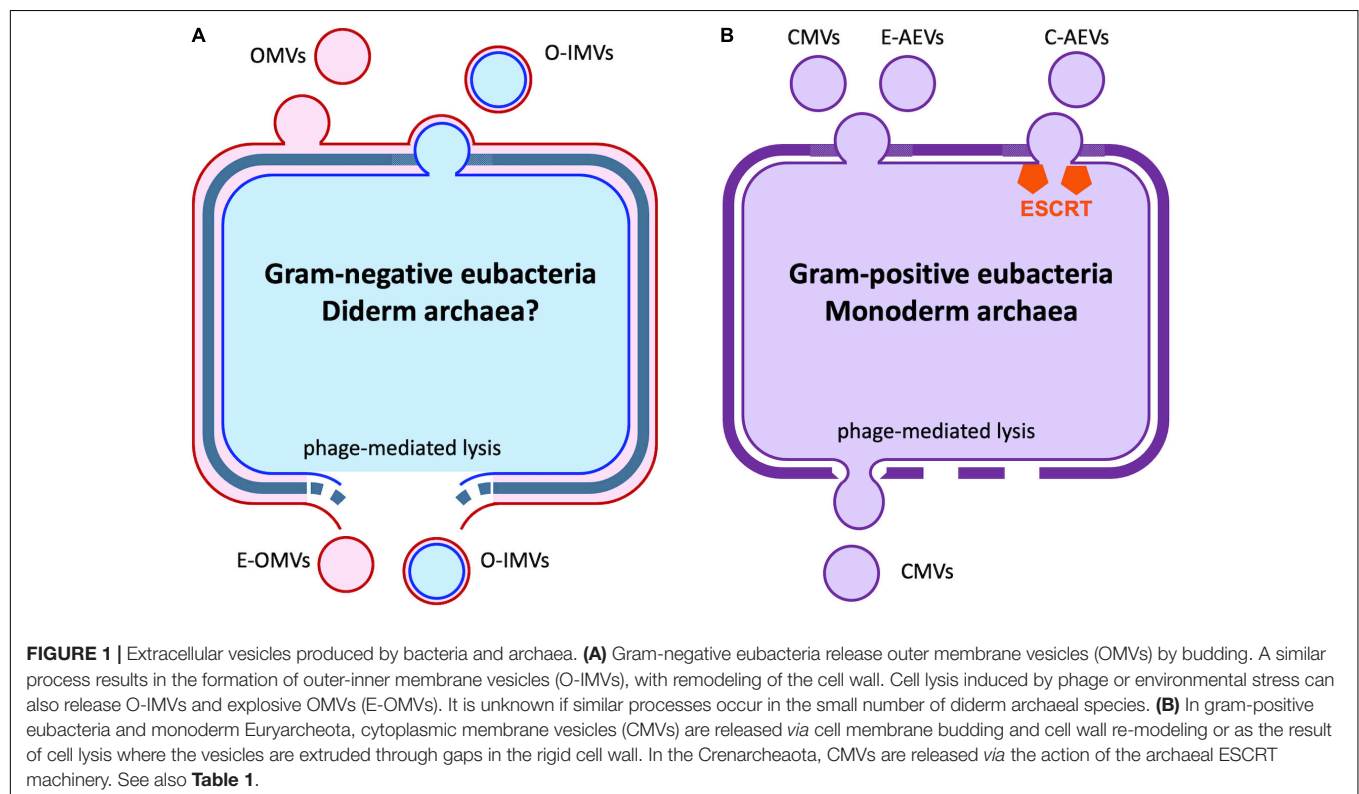
Extracellular vesicles (EVs) are a heterogeneous group of spheroid or cup-shaped membranous structures released by living cells that function in extracellular spaces. EVs have been observed in a wide diversity of prokaryotes and eukaryotes (Yoon et al., 2014; Woith et al., 2019; Díaz-Garrido et al., 2021a,b; Liu et al., 2021a). EVs could transfer a wide range of bioactive molecules including enzymes, sterols, phospholipids, polysaccharides, pigments, toxins and nucleic acids (Oliveira et al., 2013; Brown et al., 2015; Peres da Silva et al., 2015; Woith et al., 2019; Munhoz da Rocha et al., 2020). Studies in bacteria found that both Gram-negative and Gram-positive bacteria can produce EVs, usually called bacteria-derived EVs (BEVs). Gram-negative bacteria-produced BEVs can be divided into three types, namely outer membrane vesicles (OMVs), outer-inner membrane vesicles (O-IMVs), and explosive outer membrane vesicles (E-OMVs) (Table 1 and Figure 1A). Unlike Gram-negative bacteria, Gram-positive bacteria have a single cell membrane surrounded

TABLE 1 | Characteristics and biogenesis of different extracellular vesicles (EVs).

	EV type ^a	Size ^a	Origin ^a	References ^a
Archaeal EVs (AEVs)	Crenarchaeotal AEVs (C-AEVs)	90–230 nm	Archaeal ESCRT machinery	Liu et al., 2021a
	Euryarchaeotal AEVs (E-AEVs)	50–150 nm	Budding of the cell membrane	Liu et al., 2021a
Bacterial EVs (BEVs)	Outer membrane vesicles (OMVs)	20–300 nm	Blebbing of the outer membrane of gram negative bacteria	Díaz-Garrido et al., 2021a
	Outer-inner membrane vesicles (O-IMVs)	60–160 nm	Blebbing of the inner and outer membrane of gram negative bacteria	Pérez-Cruz et al., 2015
	Explosive outer membrane vesicles (E-OMVs)	110–800 nm	Phage-mediated cell lysis of gram negative bacteria	Turnbull et al., 2016; Toyofuku et al., 2017
	Cytoplasmic membrane vesicles (CMVs)	20–400 nm	Budding or extrusion of the cell membrane and release through cell wall pores or holes of gram-positive bacteria	Toyofuku et al., 2019; Briaud and Carroll, 2020
	Exosomes	30–150 nm	Released by multi-vesicular bodies fusing with plasma membrane	Joffe et al., 2016
Traditional subtypes of eukaryotic EVs	Microvesicles	100–1,000 nm	Outward budding of the plasma membrane	Joffe et al., 2016
	Apoptotic bodies	800–1,000 nm	Programmed cell death	Joffe et al., 2016
Additional subtypes of EVs in plants	Tetraspanin-positive EVs	Unclear ^b	Released by multi-vesicular bodies fusing with plasma membrane	Cai et al., 2018
	Penetration1-positive EVs	Unclear	Unclear	Rutter and Innes, 2017
	Exocyst-positive organelle-derived EVs	200–500 nm	Unclear	Wang et al., 2010
	Pollensomes	28–60 nm	Pollen-released secretory nanovesicles	Prado et al., 2014

^aDetails in each column (from left to right) describe: the classes of EVs, the size range of EVs, the biogenesis mechanism of EVs, and the primary literature references.

^bPublished data do not provide a clear answer.



by a thick, rigid cell wall, and EVs produced by Gram-positive bacteria consist of cytoplasmic membrane vesicles (CMVs) (Díaz-Garrido et al., 2021a; **Table 1** and **Figure 1B**). Archaeal

EVs have been classified by differences in biogenesis and taxa of origin, crenarchaeotal AEVs (C-AEVs) produced *via* the archaeal ESCRT machinery, and euryarchaeotal AEVs (E-AEVs)

produced *via* cell membrane blebbing (Liu et al., 2021a; **Table 1** and **Figure 1**). Eukaryotic EVs have been divided into three classes: exosomes, microvesicles (MVs) and apoptotic cell-derived vesicles (Woith et al., 2019; Munhoz da Rocha et al., 2020; Cai et al., 2021; **Figure 2** and **Table 1**). Exosomes are the smallest eukaryotic EVs with approximate dimensions ranging from 30 nm to 150 nm. Exosomes form intracellularly through inward budding of endosomal membranes resulting in the formation of intraluminal vesicles (ILVs) within multivesicular bodies (MVBs). Mature MVBs then fuse with the plasma membrane to release the ILVs as exosomes into the extracellular space. Microvesicles, sometimes called ectosomes, are formed directly by outward budding of the plasma membrane. They are generally larger than exosomes, and range in size from 100 to 1,000 nm. Apoptotic bodies are the largest EVs, with sizes usually ranging from 800 to 1,000 nm. They are formed from cells during programmed cell death (Joffe et al., 2016). These three classes of EVs have overlapping dimensions and components, and are similar in shape and density. Thus it remains difficult to cleanly isolate and distinguish the different classes of EVs. Furthermore, it remains unclear whether there are additional subtypes of each class of EVs, perhaps with distinctive contents, functions or destinations. For example, in plants at least three additional classes of EVs have been identified (Cai et al., 2021): low-density EVs lacking tetraspanins but containing the plant-specific syntaxin, PEN1; EVs derived from exocyst-positive organelles (EXPOs); and specialized secretory nanovesicles, called pollensomes, released by germinating pollen (**Table 1**). Thus, rapid and effective technologies to isolate and purify different classes and sub-types of EVs, perhaps by affinity

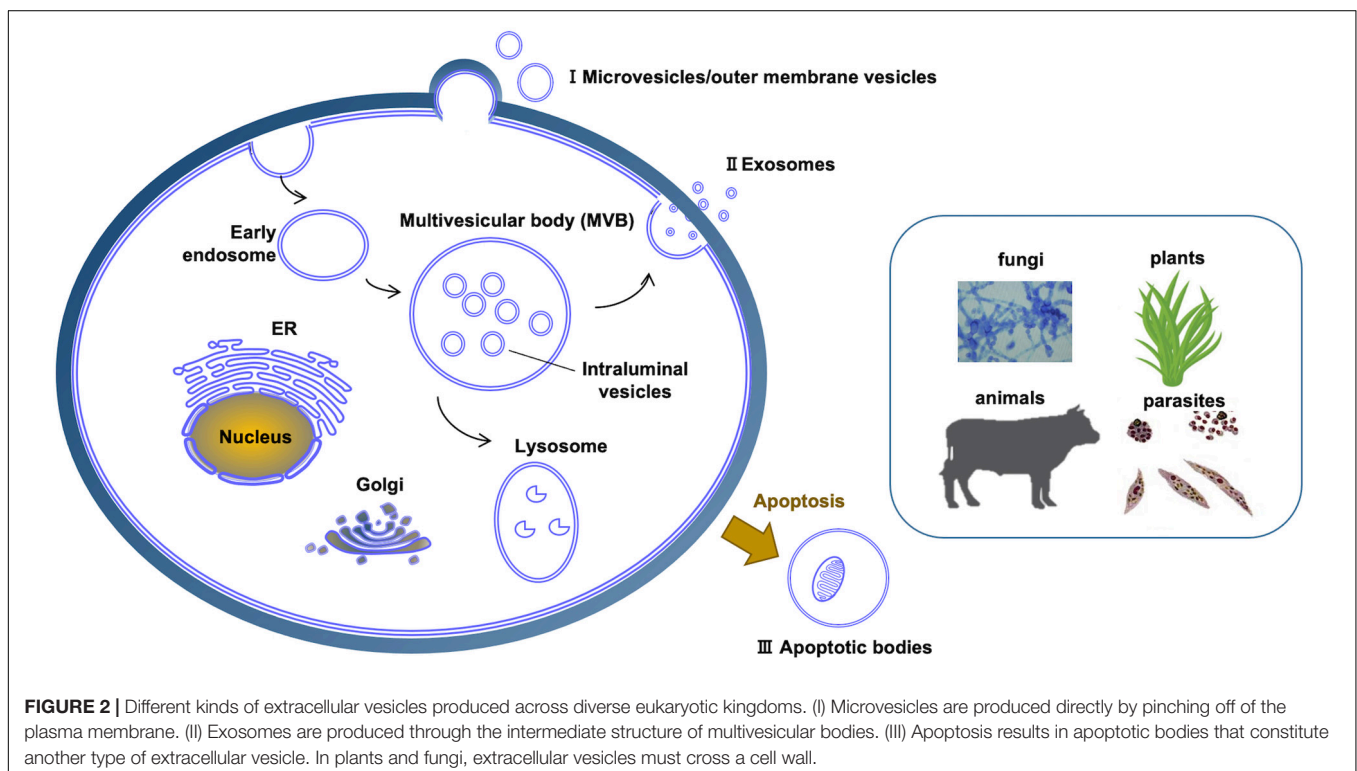
methods targeting specific proteins or lipids (Cai et al., 2021), will be important to advance the study of the formation and function of EVs.

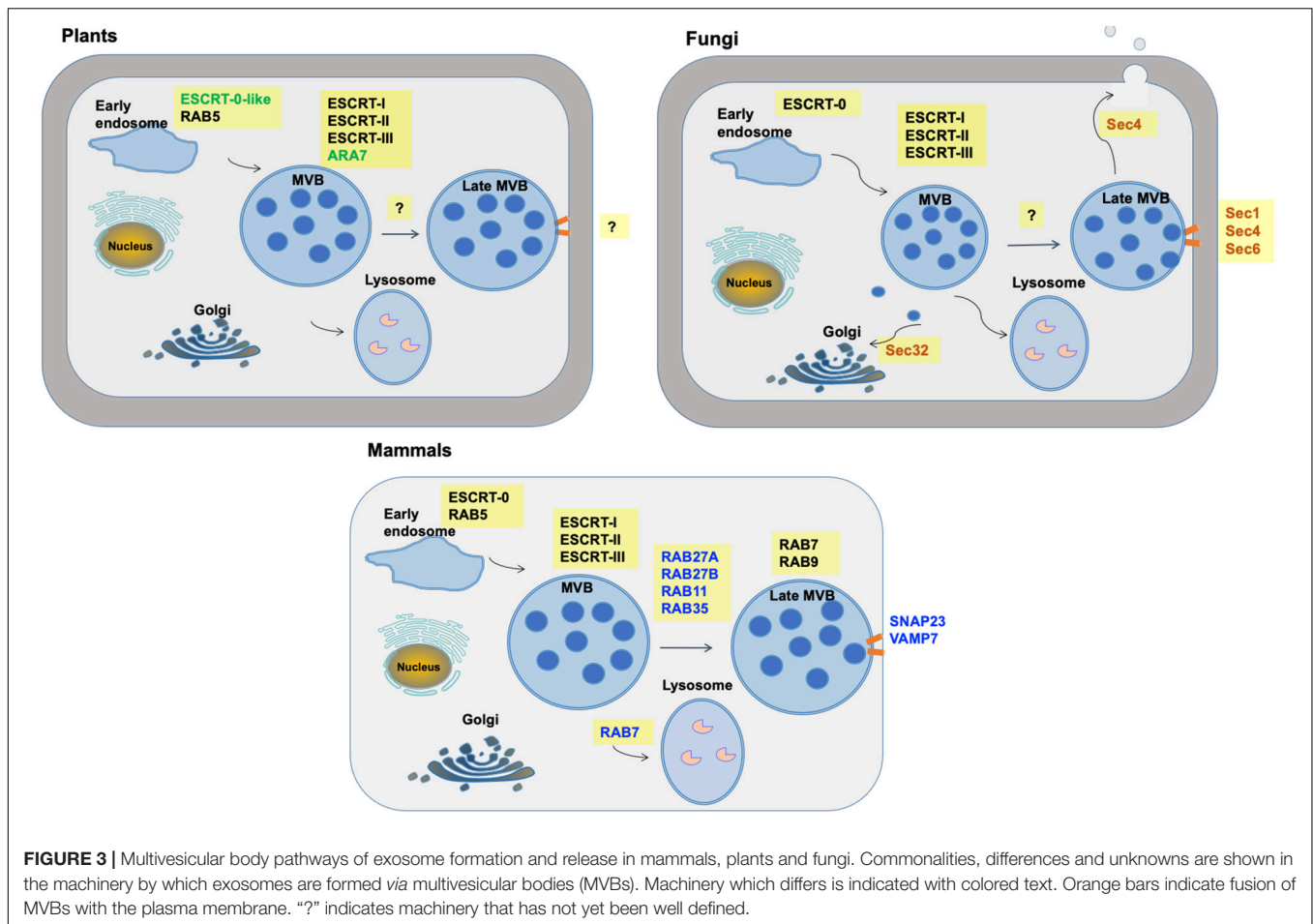
MULTIVESICULAR BODY-MEDIATED EXOSOME FORMATION IN MAMMALS AND PLANTS

The mechanisms of exosome biogenesis have been extensively characterized (**Figure 3**), especially in mammalian cells, where at least four pathways of exosome biogenesis have been identified. They include pathways dependent on tetraspanins, metabolism of specific lipids, the Endosomal Sorting Complex Required for Transport (ESCRT) machinery, and pathways that require all three of these mechanisms (Colombo et al., 2014; Vietri et al., 2020).

Tetraspanins are a family of transmembrane proteins with four transmembrane domains and two extracellular domains. The larger extracellular domain, EC2, contains a stretch of highly conserved amino acids. There are 34 tetraspanins in mammals and 17 in the model plant *Arabidopsis thaliana*. Of these, five in mammals (CD9, CD63, CD37, CD81, and CD82) and two in *Arabidopsis* (TET8 and TET9) have been associated with exosomes (Cai et al., 2021).

The mammalian ESCRT machinery is composed of 4 protein complexes, namely ESCRT-0, -I, -II, and -III plus several accessory proteins, such as Alix, VPS4, and VTA-1 (Colombo et al., 2014; **Figure 4**). The ESCRT machinery is both important to deliver cargo into the MVB prior to exosome formation,





as well as formation of the MVBs. ESCRT-0 consists of the protein STAM1 and the phosphatidylinositol-3-phosphate (PI3P)-binding protein HRS (hepatocyte growth factor-regulated tyrosine kinase substrate). PI3P-binding recruits ESCRT-0 to the endosomal membrane, where HRS recruits the ESCRT-I complex *via* its TSG101 subunit. ESCRT- II is then recruited and together with ESCRT-I act to deform the endosomal membrane inward to form buds. ESCRT- II or associated protein Alix then recruits ESCRT-III which is responsible for vesicle scission and formation of ILVs within the MVBs. Finally, the AAA-ATPase VPS4 mediates the dissociation of the ESCRT machinery from the membrane. The interactions between the ESCRT proteins and the soluble cargo proteins they are responsible for sorting into ILVs and exosomes are still not fully understood. ESCRT-0 carries 10 binding sites for polyubiquitinated proteins and therefore efficiently recruits ubiquitinated cargo proteins to the endosomal membrane during ILV formation (Vietri et al., 2020). ESCRT-I and ESCRT-II also include ubiquitin-binding subunits. Some evidence suggests that the chaperone HSC70 can bind to proteins with a KFERQ motif and also to phosphatidylserine on the outer membrane of the maturing MVBs, providing a ubiquitin-independent recruitment pathway (Sahu et al., 2011). Also, the ESCRT-II accessory protein Alix can bind some cargoes directly (Vietri et al., 2020).

After MVB maturation, fusion of MVBs with the plasma membrane is regulated by the RAB family of small GTPase proteins. The RAB family is composed of over 60 members and it is important in vesicle transport between multiple cellular compartments (Stenmark, 2009). Of these, RAB11, RAB27, and RAB35 play key roles in targeting of MVBs to the plasma membrane (Kowal et al., 2014; Blanc and Vidal, 2018). MVB fusion with the membrane resulting in the release of exosomes is mediated by SNARE (soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor) proteins, namely VAMP7 (vesicle-associated membrane protein 7) located on the MVBs and SNAP23 on the plasma membrane (D'Souza-Schorey and Schorey, 2018).

Homologs of most ESCRT proteins are found in plants (Figure 4), with the exception of ESCRT-0 proteins (Gao et al., 2017). The plant-specific ESCRT protein FREE1 (FYVE domain protein required for endosomal sorting 1) may replace the role of the ESCRT-0 complex. Like ESCRT-0, FREE1 could bind both PI3P and ubiquitinated proteins. Furthermore, FREE1 could bind directly to the ESCRT-I complex *via* its VPS23 subunit (Gao et al., 2014). Silencing FREE1 (Gao et al., 2014) or mutations in subunits of ESCRT-II (Wang H. J. et al., 2017), ESCRT-III (Shahriari et al., 2010; Cai et al., 2014; Yang et al., 2016), or ESCRT accessory proteins (Cai et al., 2014; Wang et al., 2015)

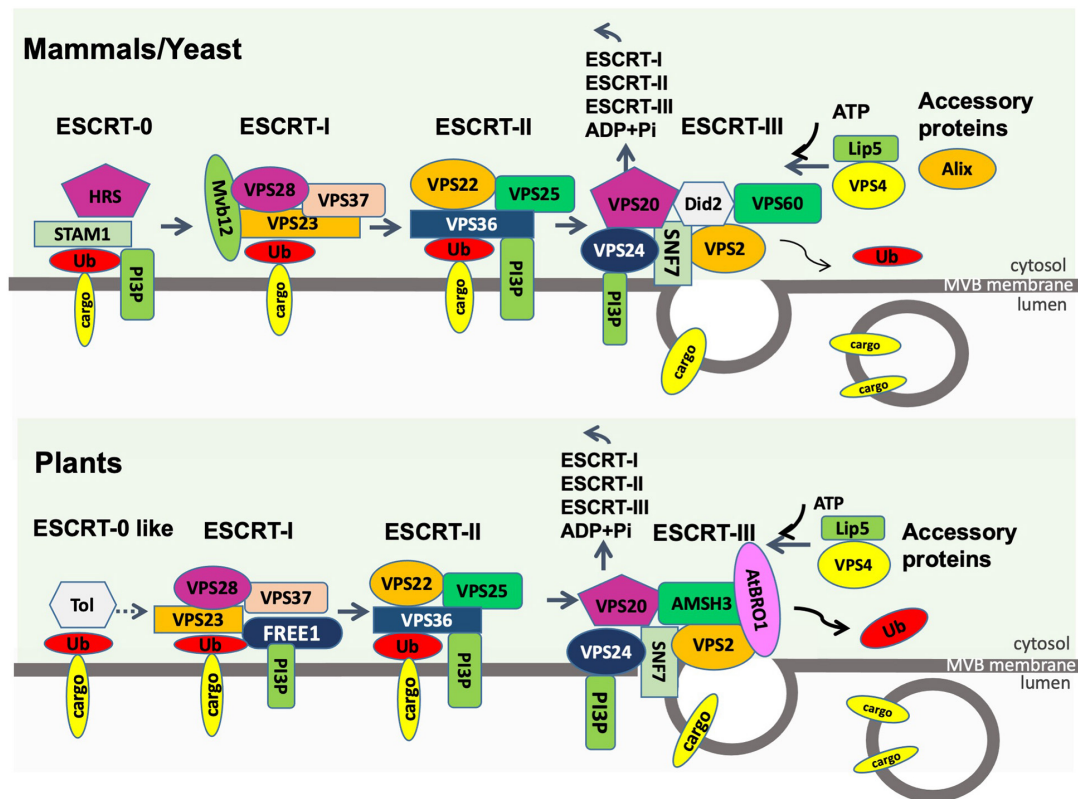


FIGURE 4 | Endosomal sorting complex required for transport (ESCRT) machinery in plants compared to that in mammals and fungi. The process of loading ubiquitinated membrane cargoes into exosomes is shown. Ub, ubiquitin; PI3P, phosphatidylinositol-3-phosphate; ADP, adenosine diphosphate; Pi, phosphate. All other entities shown are protein components of the ESCRT machinery. The first step in the plant pathway is not well described.

in *A. thaliana* all affected normal formation of MVBs. A further difference from mammalian cells was that the ESCRT-III complex appeared more important to MVB formation than ESCRT-II in mammals (Gao et al., 2017). The mechanism of MVB targeting to the plasma membrane for release of exosomes is not well understood in plants. It has been hypothesized that, during plant infection, MVBs destined for vacuolar fusion are redirected to the site of infection to deliver exosomes containing defense molecules (Hansen and Nielsen, 2018). Tao et al. (2019) observed that the plasma membrane-localized ARM-E3 ubiquitin ligase, SAUL1, and its paralog AtPUB43, could mediate tethering of the MVBs to the plasma membrane during infection of *Nicotiana benthamiana* by *Phytophthora capsici*.

EXTRACELLULAR VESICLE BIOGENESIS AND RELEASE IN FUNGI

Fungal EVs were first isolated and characterized from culture supernatants of the human pathogen *Cryptococcus neoformans* (Rodrigues et al., 2007). Since then, related research has been reported in 11 additional fungal species (Rodrigues and Casadevall, 2018). EVs of *Cryptococcus gatti* effectuate long-distance coordination of virulence between fungal cells engulfed in different macrophages, while at the population level, secretion

of EVs in *Candida albicans* is important for biofilm formation and antifungal resistance (Kwon et al., 2021). The mechanisms of biogenesis and release of fungal EVs are not as well elucidated as in mammals (Figure 3). Genetic studies have implicated at least three different pathways in the release of EVs in fungi, including the conventional post-Golgi secretory pathway, ESCRT-mediated release of exosomes via MVBs (Figure 4), and an unconventional secretory pathway involving Golgi reassembly stacking proteins (GRASP).

Conventional Post-golgi Secretory Pathway

Several proteins related to components of this pathway have been knocked down to study the biogenesis mechanism of EV formation in *Saccharomyces cerevisiae* and *C. neoformans*. In *S. cerevisiae*, mutations in Sec1 and Sec4, which are small GTPases involved in membrane fusion and vesicle targeting, respectively, either altered EV composition or the kinetics of release, or both (Oliveira et al., 2010). In *S. cerevisiae* and *C. neoformans*, Sec32 mutations affected EV composition; Sec32 is involved in trafficking from the ER to the Golgi (Oliveira et al., 2010). Furthermore, mutations in Sec6 reduced EV release by *C. neoformans*; Sec6 is a component of the exocyst which is responsible for delivery of post-Golgi exocytic vesicles

to the plasma membrane prior to exocytic membrane fusion (Mei and Guo, 2018).

Endosomal Sorting Complex Required for Transport-Mediated Release of Exosomes *via* Multivesicular Bodies

In line with the involvement of the ESCRT machinery in the release of EVs from metazoan cells, disruption of fungal genes encoding ESCRT subunits impacted the secretion and composition of EVs. In *S. cerevisiae*, mutations in the genes encoding the VPS2, VPS23, and VPS36 subunits affected EV abundance and proteomic profiles (Zhao et al., 2019) and similar results were observed in *C. albicans* (Zarnowski et al., 2018). In *C. neoformans*, mutations in the VPS27 gene resulted in abnormal MVB and EV morphology (Park et al., 2020). However, in no cases could the mutations individually block the secretion of the vesicles, and indirect effects of the mutations on EV biogenesis could not be ruled out. These results are consistent with there being multiple alternative mechanisms for formation of EVs, and experiments with many combinations of mutations may be needed to dissect EV production in fungi.

Unconventional Secretory Pathway Involving Golgi Reassembly Stacking Proteins

The principal function of GRASP proteins is to maintain the stacks of flat cisternal membranes that constitute the Golgi (Ahat et al., 2019), and to regulate autophagosome-lysosome fusion. However, in mammals, the GRASP55 protein (Ahat et al., 2019) and in yeast, the *GRH1*-encoded GRASP protein (Oliveira et al., 2010), have been implicated in unconventional secretion of several proteins lacking secretory leaders, including acyl-CoA binding protein (ACBP) (Ahat et al., 2019). Relevant to this review, Oliveira et al. (2010) observed that *grh1* mutants exhibited decreased release of EVs, though they could not distinguish between a direct role in EV biogenesis and indirect effects on the cellular distribution of sphingolipids which are an important component of EVs. GRASP was also shown to participate in EV-mediated export of mRNA in *C. neoformans* (Peres da Silva et al., 2018). In yeast, unconventional secretion of ACBP was shown to involve packaging of the protein into autophagosomes, fusion of the autophagosomes with early endosomes, maturation of the endosomes into MVBs, and fusion of the MVBs with the plasma membrane, likely releasing ACBP-containing EVs (Duran et al., 2010). However, it remains unclear if this is a general pathway for release of EVs, or one specific to ACBP.

In addition to transport across the plasma membrane, EVs must traverse the cell wall in the case of organisms that have them, such as plants, bacteria, fungi and oomycetes (Woith et al., 2019). The ability of liposomes containing amphotericin B to enter fungal cells (Walker et al., 2018) and the evidence for cross-kingdom communication *via* EVs (Cai et al., 2018) strongly implies that EVs can also traverse cell walls from the outside inward. Despite extensive observations in *C. neoformans* (e.g., Wolf et al., 2014), the mechanisms of EV cell wall transit remains unclear. Current hypotheses include: the presence of

guide channels, the actions of cell-wall-remodeling enzymes, and intrinsic visco-elasticity of cell walls (Rizzo et al., 2020). It also remains unclear if the directional transit of EVs requires specific transport mechanisms as is observed for the trafficking of intracellular vesicles.

PRODUCTION OF EXTRACELLULAR VESICLES BY BACTERIA AND ARCHAEA

The release of BEVs by bacteria was first observed in *Vibrio cholerae* in 1967 (Chatterjee and Das, 1967), where they were described as round membranous structures produced by budding and detachment from the bacterial outer membrane. The mechanisms underlying BEV biogenesis remain poorly understood. Processes associated with BEV release include cell wall turnover; physical, salt, or antibiotic-induced stress; lipopolysaccharide and phospholipid remodeling of the outer membrane; and the action of quorum-sensing signal molecules (Díaz-Garrido et al., 2021a). OMVs have been characterized as containing cell wall components, lipopolysaccharides, enzymes and other proteins, and secondary metabolites, as well as DNA molecules and an array of RNA molecules including tRNAs, mRNAs, non-coding RNAs, and fragments of rRNAs (Woith et al., 2019; Munhoz da Rocha et al., 2020; Cai et al., 2021; Díaz-Garrido et al., 2021a). Gram-negative bacteria also produce “outer-inner membrane vesicles” (O-IMVs), likely by similar mechanisms as OMVs. O-IMVs exhibit both an outer and an inner membrane, and enclose both periplasmic and cytoplasmic contents, including DNA (Pérez-Cruz et al., 2015). In gram-positive bacteria, genetic analysis suggests that formation of BEVs is a normal physiological process; vesicles with cytoplasmic contents (CMVs) are pinched off from the cell membrane *via* lipid-remodeling, and then released through the cell wall *via* remodeling proteins that include autolysins and penicillin-binding proteins (Briaud and Carroll, 2020). Phage-mediated lysis can also produce “explosive OMVs” (E-OMVs) in gram-negative bacteria (Turnbull et al., 2016). In gram-positive bacteria, a similar process can result in CMVs being extruded through gaps in the cell wall (Toyofuku et al., 2017).

Archaea also produce EVs (AEVs). The majority of archaea have a single membrane and a flexible external cell wall-like structure, though a minority of species resemble gram-negative bacteria in having an outer membrane (Liu et al., 2021a). The mechanisms of AEV production differ markedly across phyla. Several archaea phyla encode orthologs of the eukaryotic ESCRT machinery, including the Crenarchaeota. In the Crenarchaeota, proteomic and genetic analysis, especially in *Sulfolobus islandicus* (Liu et al., 2021b), has demonstrated the importance of the ESCRT machinery in C-AEV production (Liu et al., 2021a). The Euryarchaeota, however, lack ESCRT homologs and E-AEV production appears to occur by budding from the cell membrane *via* an unknown mechanism (Liu et al., 2021a). Like CMVs, AEVs enclose diverse cytoplasmic contents. However, both C-AEVs and E-AEVs appear to have a particular role in the encapsulation, stabilization, and cell-to-cell transmission of DNA fragments, including chromosomal

fragments and plasmid molecules (Liu et al., 2021a), a process termed vesiduction (Erdmann et al., 2017).

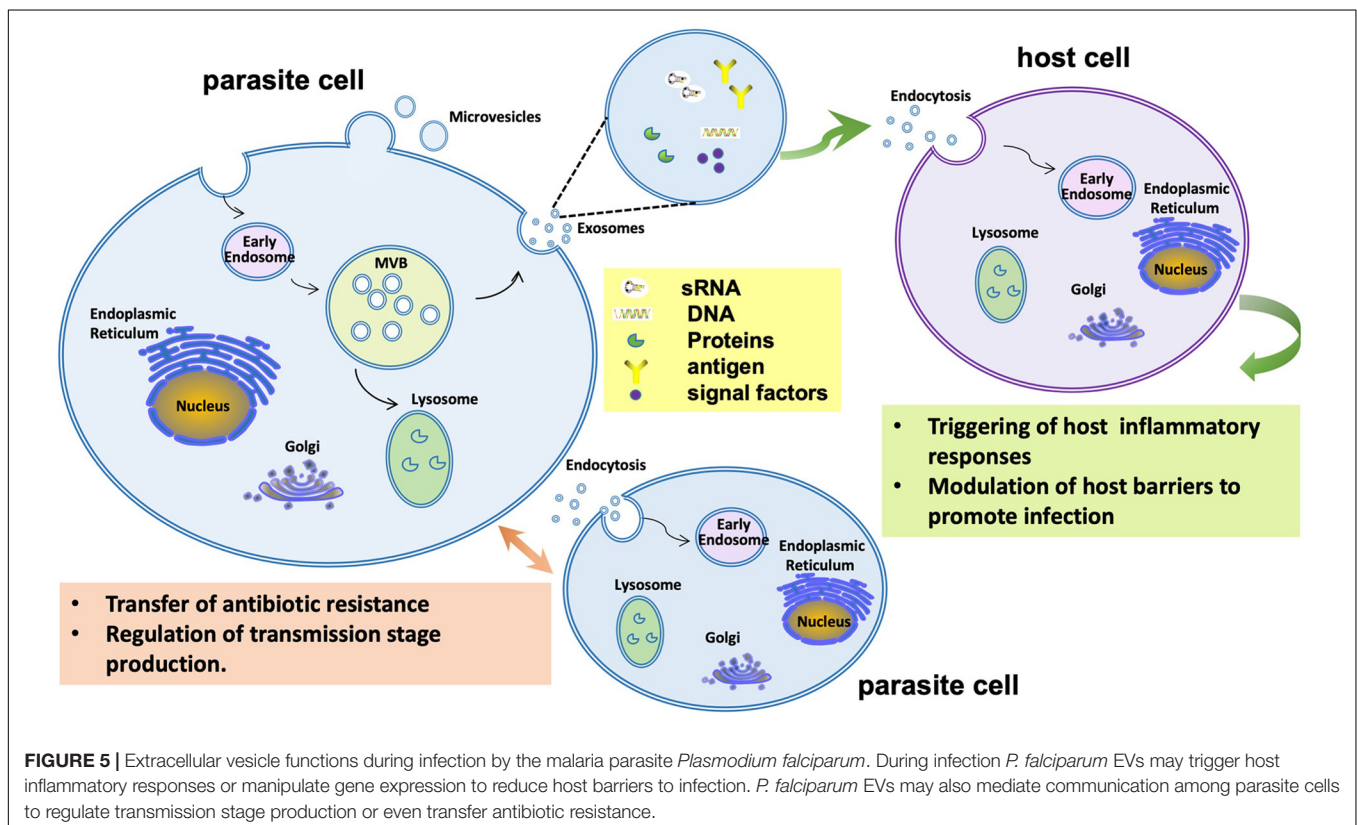
BIOLOGICAL FUNCTIONS OF EXTRACELLULAR VESICLES IN HOST-MICROBE INTERACTIONS

Multi-cellular hosts such as animals and plants host rich communities of microbes (microbiomes). These communities are typically dominated by eubacteria, but also may include archaea, fungi, oomycetes, protozoa, algae, viruses, and microfauna. The relationships among these organisms and with their hosts can range across all forms of symbiosis from parasitism, competition and commensalism to mutualism (Tyler, 2009). EVs are rapidly emerging as key players in the communications and physiological interactions among these microbes and their hosts (Woith et al., 2019; Caruana and Walper, 2020; Munhoz da Rocha et al., 2020; Cai et al., 2021; Díaz-Garrido et al., 2021a,b; Fucarino et al., 2021; Qiao et al., 2021; **Figures 5, 6** and **Table 2**). These roles can include nutrition, virulence, predation, host defense, immuno-regulation, developmental signaling and environmental modification. While much work has focused on pathogenic microbes, there is also accumulating evidence that symbiotic microbes may also employ EVs to modulate host interactions (Roth et al., 2019; Rizzo et al., 2020; Cai et al., 2021; Díaz-Garrido et al., 2021a,b). In particular, EVs have emerged as a mechanism for the transport of RNA molecules from the microbe to the host

in order to modulate host metabolism and immune responses (Munhoz da Rocha et al., 2020; Cai et al., 2021; Qiao et al., 2021). Likewise, the delivery of host RNA molecules to microbes has emerged as an important function of host EVs (Munhoz da Rocha et al., 2020; Cai et al., 2021; Qiao et al., 2021).

Extracellular Vesicle Function in Plant and Animal Pathogens and Symbionts

Bacteria-derived extracellular vesicles produced by Gram-negative bacteria have been implicated in many aspects of bacterial pathogenicity and proliferation, including nutrient acquisition, stress responses, virulence factor delivery, biofilm formation, and development of antibiotic resistance (Kuehn and Kesty, 2005; Schwechheimer and Kuehn, 2015; Joffe et al., 2016; Toyofuku et al., 2019; Woith et al., 2019; Díaz-Garrido et al., 2021a). Virulence factors delivered by BEVs include alkaline phosphatase, hemolytic phospholipase C and Cif toxin in the case of *Pseudomonas aeruginosa* (Bomberger et al., 2009) and Shiga toxin 2a, cytolethal distending toxin V and EHEC-hemolysin in the case of enterohemorrhagic *Escherichia coli* (Bielaszewska et al., 2017). After being internalized by host cells, BEVs can release these virulence cargos and cause cell death (Bielaszewska et al., 2017). Mammalian host responses to BEVs can also trigger pathological immune responses, such as sepsis (Park et al., 2018). Although BEVs have been reported in bacterial plant pathogens, there is little information yet about their role in pathogenesis. One analysis of BEV-enriched fractions from the grape and citrus pathogen *Xylella fastidiosa* revealed the presence of virulence



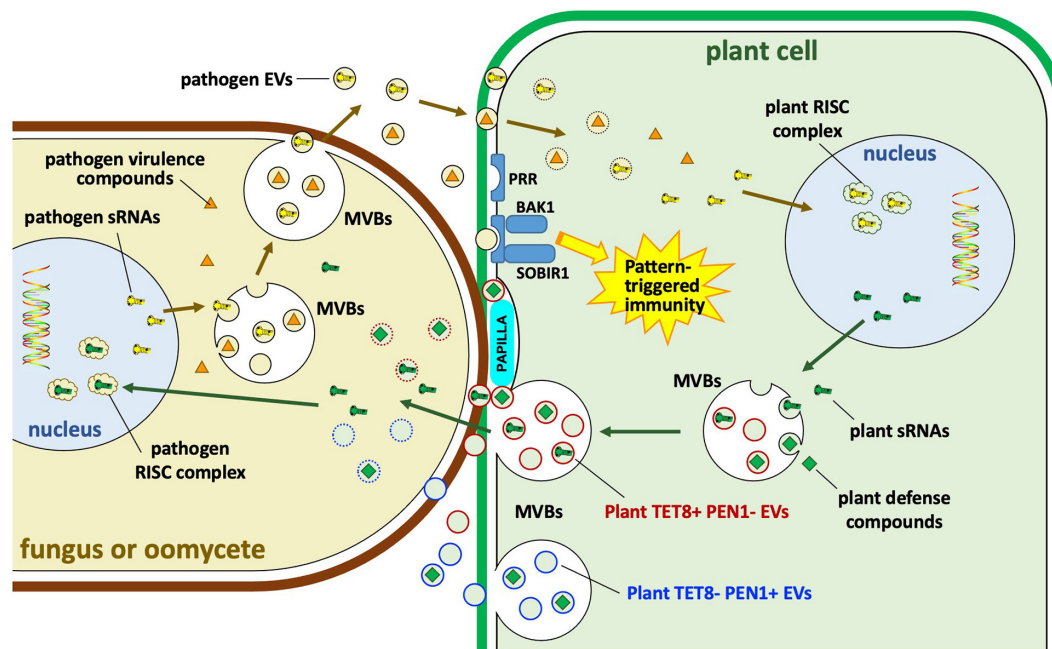


FIGURE 6 | Extracellular vesicle roles in interactions of plants with fungi and oomycetes. Plants and their eukaryotic pathogens exchange EVs during infection. Plant EVs carrying Tetraspanin8 (Tet8) but lacking syntaxin PEN1 (TET8+ PEN1– EVs) carry small RNAs (sRNAs) and other defense compounds including small molecules, enzymes and other proteins. The specific role of plant TET8-negative PEN1-positive (TET– PEN1+) EVs remains unclear. Some of those molecules may enter the pathogen cytoplasm or the nucleus where sRNAs may bind to pathogen RNA-induced silencing complexes (RISC); some enzymes such as callose synthase may be targeted to papillae to strengthen cell wall defenses against pathogen invasion. Pathogen EVs may carry sRNAs as well as other virulence compounds, possibly including some effectors. sRNAs may target plant RISC complexes. MVBs, multivesicular bodies; PRR, pattern recognition receptor; BAK1, BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1; SOBIR1, suppressor of BRASSINOSTEROID INSENSITIVE 1 (BRI1)-associated kinase (BAK1)-interacting receptor kinase 1.

TABLE 2 | Virulence factors delivered by EVs and function during pathogen infection.

Microbe	Virulence factors	Function	References
Bacteria			
<i>Pseudomonas aeruginosa</i>	Alkaline phosphatase, hemolytic phospholipase C, toxin Cif	Trigger pathological immune responses	Bomberger et al., 2009
<i>Escherichia coli</i>	Toxin V, EHEC- hemolysin	Trigger pathological immune responses	Bielaszewska et al., 2017
<i>Xylella fastidiosa</i>	Lipases, esterases, proteases, porins, pectin lyase-like protein, and signaling factors	Unclear	Feitosa-Junior et al., 2019
<i>Bradyrhizobium japonicum</i>	Small RNAs	Unclear	Ren et al., 2019
Fungi			
<i>Candida albicans</i>	Agglutinins, lysophospholipases, and secreted aspartic proteases		Konečná et al., 2019
<i>Cryptococcus gatti</i>	Unclear	Promote infection of pathogen in host	Bielska et al., 2018
<i>Sporothrix brasiliensis</i>	Unclear	Promote infection pathogen in host	Ikeda et al., 2018
<i>Beauveria bassiana</i>	Small RNAs, mRNAs	Modulate host immune responses	Cui et al., 2019
<i>Botrytis cinerea</i>	Small RNAs, mRNAs	Modulate host immune responses	Weiherg et al., 2013
<i>Verticillium dahliae</i>	Small RNAs, mRNAs	Modulate host immune responses	Wang et al., 2016
<i>Puccinia striiformis</i> f. sp. <i>tritici</i>	Small RNAs, mRNAs	Modulate host immune responses	Wang M. et al., 2017
<i>Ustilago maydis</i>	Small RNAs, mRNAs	Modulate host immune responses	Kwon et al., 2021
Oomycete			
<i>Hyaloperonospora arabidopsidis</i>	Small RNAs, mRNAs	Modulate host immune responses	Dunker et al., 2020
Nematode			
<i>Heligmosomoides polygyrus</i>	microRNAs, Y RNAs, Argonaute protein	Suppress innate immune responses in mice	Buck et al., 2014
Apicomplexan parasite <i>Plasmodium falciparum</i>	miRNAs	Trigger host systemic inflammatory responses and promote infection by pathogen in host	Mantel et al., 2016

factors including lipases, esterases, proteases, porins, and a pectin lyase-like protein, as well as diffusible signaling factors, suggesting that BEVs might have a role in delivering these factors (Feitosa-Junior et al., 2019). The identification of bacterial small RNAs as host-signaling molecules during nodulation of soybean by *Bradyrhizobium japonicum* (Ren et al., 2019) also suggests the involvement of BEVs as potential carriers of those RNAs.

The contributions of EVs to microbiome-host interactions have been studied in particular detail in the context of the bacterial members of mammalian microbiomes (Ñahui Palomino et al., 2021), especially those of the mammalian gut (reviewed in detail by Díaz-Garrido et al., 2021a,b) and airways (reviewed by Fucarino et al., 2021). In the gut, a dense inner layer of mucus separates the host epithelial cells from the microbial community, while a looser outer layer of mucus serves as a matrix that is colonized by the microbes. While these mucus layers restrict the movement of microbial cells including bacteria, BEVs can readily pass these barriers, physiologically connecting the cells with each other and with the host. One role played by the BEVs is a dietary one. BEV cargoes are rich in enzymes such as glycosidases, sulfatases, proteases, and inositol phosphatases that can break down host-indigestible dietary glycans and also host mucins, releasing nutrients such as carbohydrates, short chain fatty acids, and phosphates to both host and microbes. BEVs are also important effectors of the role of the gut microbiome in stimulating the maintenance of the epithelial barrier and providing a healthy ongoing priming of the innate immune system (Díaz-Garrido et al., 2021a). BEVs can also cross the epithelial layer into the body, likely *via* paracellular diffusion, transcytosis and/or *via* phagocytotic cells in the mucosal epithelium (Jones et al., 2020; Díaz-Garrido et al., 2021a), with physiological effects that are still being explored.

In mammalian airways, as in the gut, host EVs play critical roles in maintaining a healthy balance of immunological activities within the mucosa and its associated mucus layer (Fucarino et al., 2021). This balance can be disrupted by external factors such as allergens and tobacco smoke, resulting in asthma and chronic pulmonary obstructive disease (COPD). These disruptions can result in microbial dysbioses that include release of BEVs rich in pro-inflammatory signals, creating a positive feedback loop that exacerbates disease. One well-studied example is the interaction between airway epithelial cells and *Pseudomonas aeruginosa*, a gram-negative bacterium that causes dangerous infections in patients with COPD and cystic fibrosis. *P. aeruginosa* BEVs deliver a 23-nucleotide tRNA fragment (sRNA-52320) into epithelial cells that suppresses cytokine secretion and neutrophil infiltration, resulting in weakened immune responses (Koeppen et al., 2016).

While fungi and other eukaryotic microbes have been well documented to produce EVs, direct evidence for EVs' role in the virulence of such pathogens is still emerging. In the human pathogen *C. albicans*, EVs produced *in vitro* were shown to contain 34 proteins associated with virulence including agglutinins, lysophospholipases, and secreted aspartic proteases (Konecňá et al., 2019). In the gastrointestinal nematode *Heligmosomoides polygyrus*, nematode EVs were shown to carry microRNAs, Y RNAs and an Argonaute protein, and could

suppress innate immune responses in mice (Buck et al., 2014). In the human pathogens *C. gatti* (Bielska et al., 2018) and *Sporothrix brasiliensis* (Ikeda et al., 2018), EVs were shown to carry infection-promoting factors, though the factors were not identified. In the malaria parasite, *Plasmodium falciparum*, EVs derived from parasite-infected red blood cells (IRBCs) could trigger host systemic inflammatory responses and could contribute to the pathology of malaria infection by altering the host barrier properties of endothelial cells (Mantel et al., 2016).

An important role for EVs is also implied by data showing that small RNAs from fungi and oomycetes interact with the RISC machinery of hosts to modulate host immune responses (Figure 6). Examples include the entomopathogenic fungus *Beauveria bassiana* (Cui et al., 2019), the fungal plant pathogens *Botrytis cinerea* (Weiberg et al., 2013), *Verticillium dahliae* (Wang et al., 2016), *Puccinia striiformis* f. sp. *tritici* (Wang M. et al., 2017), and *Ustilago maydis* (Kwon et al., 2021), and the oomycete plant pathogen *Hyaloperonospora arabidopsidis* (Dunker et al., 2020). In these cases, EVs are hypothesized to carry both sRNAs and mRNAs to the host. In malaria, over 20 human miRNAs were identified in EVs that were released from IRBCs. Among them, miR-451a could modify the permeability of host endothelial cells by silencing target genes within those cells (Mantel et al., 2016; Figure 5).

Microbial pathogens of plants and animals produce effector proteins that modify the physiology and morphology of host tissues to promote infection (Torto-Alalibo et al., 2010). Many classes of effector proteins are delivered into the cytoplasm of host cells (Torto-Alalibo et al., 2010). While effector delivery pathways have been well characterized in bacteria (Tseng et al., 2009), the entry of effector proteins from fungal and oomycete pathogens is still poorly understood (Ellis et al., 2006; Khang et al., 2010; Kale and Tyler, 2011; Jiang and Tyler, 2012; Presti and Kahmann, 2017). In principle, EVs offer an attractive mechanism for effector delivery from fungi and oomycetes into the cytoplasm of host cells. Some evidence suggests that effectors targeted to the host cytoplasm are secreted *via* distinct pathways (Giraldo et al., 2013; Wang S. et al., 2017). Furthermore, some oomycete effectors appear to reach the host cytoplasm *via* non-conventional secretion pathways (Liu et al., 2014). However, clear evidence is currently lacking that would support the role of EVs in effector delivery.

Extracellular Vesicle Functions in Host Disease and Immune Responses

Extracellular vesicles have emerged as major players in host responses to microbes. Host EVs can contribute to combatting microbial infection either by targeting pathogen cells directly, or by participating in the regulation of immune responses (Chen et al., 2019; Rybak and Robatzek, 2019; Macia et al., 2020; Cai et al., 2021; Figures 5, 6). At the same time, host responses triggered by microbial EVs may contribute to immunity but also may lead to disease symptoms (Chen et al., 2019; Rybak and Robatzek, 2019; Macia et al., 2020; Cai et al., 2021; Qiao et al., 2021). Common themes that have emerged across animal and plant interactions include recognition of microbe-associated

molecular patterns (MAMPs) carried by microbial EVs by pattern recognition receptors (PRRs), resulting in the activation of innate immune responses *via* mitogen-activated protein kinase (MAPK) pathways (Zhou et al., 2020; Cai et al., 2021; Díaz-Garrido et al., 2021a,b). Some differences include the extensive role of mammalian EVs in modulating immune responses (Díaz-Garrido et al., 2021a,b), especially in the gut, while in plants, an important role of EVs is as carriers of anti-microbial RNA molecules (Cai et al., 2018, 2021). However, these differences may fade as investigations of these systems progress.

Roles in Animal Disease and Immune Responses

During mammalian immune responses, host EVs have been documented to play a wide range of regulatory roles, including antigen presentation, immune homeostasis *via* pro- or anti-inflammatory mediators, and transfer of damage-associated molecular patterns (DAMPs), membrane receptors, enzymes, mRNAs and non-coding RNAs. EVs may also modulate the complement and coagulation systems (Chen et al., 2019; Macia et al., 2020). Mammalian immune systems also respond to EVs carrying pathogen-associated molecular patterns (PAMPs) that are recognized by pattern recognition receptors (PRRs), resulting in inflammasome activation, and activation of cells of the innate immune system. In some cases, host pro-inflammatory responses can become pathological, producing tissue damage and sepsis (Chen et al., 2019; Macia et al., 2020). For example, host microvesicles are greatly elevated in the blood of cerebral malaria patients, and were subsequently shown in a mouse model to be the principal effectors of neurovascular pathology, especially microvesicles derived from *Plasmodium*-infected red blood cells (Combes et al., 2005; El-Assaad et al., 2014; Macia et al., 2020). Immune responses may also be directed against microbial EVs. For example, binding of antibodies could modulate both the composition and the production of EVs by the fungal pathogen *Histoplasma capsulatum*, reducing its virulence in mice (Baltazar et al., 2018).

The roles of host EVs in maintaining healthy mucosal surfaces in the presence of abundant microbial populations have been extensively studied in the mammalian gut (reviewed in Díaz-Garrido et al., 2021b). In the gut, EVs mediate extensive communications among epithelial cells, phagocytic cells, dendritic cells, mast cells and mesenchymal stem cells. The cargos of these EVs include not only cytokines and other regulatory proteins but also a wide spectrum of miRNAs involved in immuno-modulation (Díaz-Garrido et al., 2021b). The functions of those cargoes include support of epithelial barrier integrity, tissue repair and wound healing, intestinal immune responses, pathogen control, and microbiota modulation (Díaz-Garrido et al., 2021b). Furthermore, study of BEVs produced by probiotic bacteria, such as *E. coli* Nissle 1917 (EcN) (Cañas et al., 2016) and kefir *Lactobacillus* strains (Seo et al., 2018) demonstrated that BEVs produced by these strains could reduce the increased expression of pro-inflammatory cytokines and down-regulate enzymes associated with injury and inflammation (Díaz-Garrido et al., 2021b). Associations of host EVs with

airway pathologies present in asthma and COPD have also been extensively documented (reviewed in Fucarino et al., 2021). While EVs may be expected to play similar key roles in the interaction of plants with their microbiomes, this research area is still in its infancy (Motaung and Steenkamp, 2021).

Roles in Plant Disease and Immune Responses

Extracellular vesicles from plants have been demonstrated to directly participate in anti-microbial functions (**Figure 6**). EV production by plants is stimulated by infection as well as by activators of immune signaling such as salicylic acid (Rybak and Robatzek, 2019). One important role of EVs in plant defense is to target the deposition of cell-wall-strengthening callose to sites of attempted pathogen invasion, which is part of the polarized defense response. The polarized defense response has been observed in diverse plant species, including barley, bean and *Arabidopsis*, protecting against diverse fungal pathogens (Rybak and Robatzek, 2019). EVs also target chemical defenses to sites of pathogen attack. In *Arabidopsis*, products of indole glucosinolate metabolism form important anti-microbial defenses against fungi and insects. Glucosinolate transporters and the activating enzyme, myrosinase, are associated with *Arabidopsis* EVs during infection (Rybak and Robatzek, 2019). In sunflower, EVs from seedlings could deliver defense proteins to ascospores of *Sclerotinia sclerotiorum* and could inhibit their germination and growth (Regente et al., 2017).

In plants, EVs have emerged as carriers of host small RNAs that target pathogen genes (**Figure 6**). TET8-positive exosomes from *Arabidopsis* were found to be enriched in a selective set of miRNAs and small interfering RNAs (siRNAs), including phased secondary siRNAs (phasiRNAs). Those exosomes could readily be taken up by the fungal pathogen *Botrytis cinerea*. Furthermore, specific sRNAs such as TAS1c-siR483 and TAS2-siR453 could regulate the expression of fungal pathogenicity genes *Bc-VPS51*, *Bc-DCTN1* and *Bc-SAC1* (Cai et al., 2018). *Arabidopsis* also produced phasi-RNAs capable of silencing genes in the pathogen *P. capsici* (Hou et al., 2019). *Arabidopsis tet8*, *tet9* double knock-out mutants were substantially more susceptible to *B. cinerea*, and *B. cinerea* hyphae from infected *tet8*, *tet9* plants contained substantially fewer plant sRNAs (Cai et al., 2018).

The ability of EVs to deliver sRNAs from plants to pathogens likely underlies the phenomenon of host-induced gene silencing, in which silencing RNAs produced from transgenes or endogenous genes can effectively target pathogen genes to reduce disease (Baulcombe, 2015; Koch and Wassenegger, 2021; Qiao et al., 2021). In a recent study, EVs isolated from *Arabidopsis* leaves contained siRNAs derived from a transgene-encoded dsRNA targeting three *CYP51* genes of the fungal pathogen *Fusarium graminearum*, conferring increased resistance against this pathogen. Disruption of the ESCRT III complex, which is vital to the EV formation in plants, weakened the resistance of these transgenic plants against *F. graminearum* (Koch et al., 2020).

The plant innate immune system also responds to the presence of microbial EVs (Bahar et al., 2016; Rybak and Robatzek, 2019;

McMillan et al., 2021; **Figure 6**). For example, outer membrane-derived EVs (OMVs) from *Xanthomonas campestris* pv. *campestris* could induce a reactive oxygen species (ROS) burst and trigger defense gene expression in *Arabidopsis* (Bahar et al., 2016). The EVs could be perceived by the plant coreceptor brassinosteroid insensitive 1 associated kinase (BAK1) and suppressor of BAK1 interacting receptor-like kinase 1 (SOBIR1) to induce PAMP-triggered immunity (PTI) (Bahar et al., 2016). Similarly, exposure to OMVs from *Pseudomonas syringae* and *Pseudomonas fluorescens* could protect *Arabidopsis* and tomato plants from infection by *P. syringae* or by oomycetes (McMillan et al., 2021).

FUNCTION OF EXTRACELLULAR VESICLES IN CELL-TO-CELL COMMUNICATION

Extracellular vesicles play an important role not only in microbe-host interactions but are also important to intercellular communication among microbes, either of the same or different species, and among cells of higher organisms (Caruana and Walper, 2020; Munhoz da Rocha et al., 2020; Díaz-Garrido et al., 2021b).

For instance, within mammals, EVs shed from stem cells contained mRNA and miRNAs which could alter the phenotype of other stem cells (Ratajczak et al., 2006). As another example, tumor cell-derived EVs contained abundant soluble proteins, signal factors and chemokine receptors, that could promote tumor invasion and influence tumor progression (Turturici et al., 2014). Within the mammalian gut, host EVs play a key role, together with microbial EVs, in maintaining a balanced immunological and mucosal environment that controls microbiota populations at a beneficial level, while avoiding pathological levels of inflammation (Díaz-Garrido et al., 2021b).

The role of EVs in microbe-microbe communication within complex microbial communities (microbiomes) has been examined in detail in the context of the human gut, especially the role of BEVs (Caruana and Walper, 2020). The majority of beneficial community functions mediated by BEVs involve “common good” functions such as provision of BEVs carrying carbohydrate degradation enzymes, iron acquisition proteins, antibiotic or antimicrobial resistance molecules (enzymes such as β -lactamases or catalases, or excess antibiotic target proteins or lipids), and biofilm matrix components such as DNA (Caruana and Walper, 2020; Munhoz da Rocha et al., 2020). For example, in the bacterium, *E. coli*, BEVs released from β -lactam antibiotic-resistant strains contained abundant antibiotic-resistance proteins including Blc, OmpC, OmpF, and OmpW. Cells of antibiotic-susceptible strains could incorporate these resistance proteins from BEVs, protecting themselves from the antibiotic inhibition (Kim et al., 2018). The transmission of developmental signals directly from cell to cell by BEVs and AEVs includes the transfer of DNA molecules and quorum sensing signals. For example, the *P. aeruginosa* quorum sensing molecule PQS (*Pseudomonas* Quinolone Signal) is highly hydrophobic and primarily exported on the surface of BEVs

(Mashburn and Whiteley, 2005). Similarly, the hydrophobic quorum sensing molecules of *Paracoccus denitrificans* and *Vibrio harveyi* are packaged into BEVs (Toyofuku et al., 2017; Brameyer et al., 2018). On the other hand, developmental signaling by transfer of protein or RNA molecules among eubacteria has yet to be demonstrated.

While the mechanisms by which bacteria, especially gram-negatives, could internalize incoming EVs remain speculative, the mechanisms for eukaryotes to internalize EVs have been extensively studied in mammalian cells (Caruana and Walper, 2020). Five different endocytic pathways have been identified by which EVs can be taken into non-phagocytic host cells: macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, lipid raft-mediated endocytosis, and direct membrane fusion (Caruana and Walper, 2020). By extrapolation therefore, eukaryotic microbes are expected to be efficient recipients of EVs, as demonstrated in plant-fungal interactions (Cai et al., 2018, 2021). For example, in the human fungal pathogen *C. gattii*, EVs could induce pathogen proliferation and regulate the virulence phenotype of the recipient pathogen cells over a long distance (Bielska et al., 2018). EVs released from a highly virulent strain of *C. gattii* could be phagocytosed by host macrophages and there stimulate the growth rate of a less virulent strain and enhance its survival. The components transported in EVs were important to induce fungi proliferation, and only EVs from the virulent strain had this capability. However, the exact nature of the stimulatory molecules and the mechanism of stimulation were unknown (Bielska et al., 2018). Likewise, during infection by the malaria parasite *Plasmodium falciparum*, EVs purified from IRBCs could transfer drug-resistance plasmids into drug sensitive strains and increase survival of the parasite under drug pressure (Regev-Rudzki et al., 2013; **Figure 5**). In addition, IRBC-derived EVs could regulate the transmission stage production of parasites *in vivo* (Mantel et al., 2013; **Figure 5**). The EVs could promote gametocyte formation in the parasite population; gametocytes are the form of the parasite transmitted to the mosquito vector. It was proposed that EVs released from IRBCs serve as a sensor for parasite density, signaling a switch to increased gametocyte formation (Mantel et al., 2013).

Complex microbial communities also are common in non-biotic environments such soils, sediments, rocks, and human-built structures, especially where water is abundant. These communities commonly form biofilms that serve to protect and structure the communities, and concentrate nutrients, metabolites and signal molecules. These biofilms likely are rich in microbial EVs. However, this topic is barely explored, except in the case of Archaea, which dominate environments of extreme temperatures, salinity and/or acidity. Two specific functions have been associated with AEVs, namely the dissemination of toxins that target competitors, and the transmission of genomic and plasmid DNA molecules. *Sulfolobus* species produce a family of AEV-associated anti-microbial proteins, called sulfolobocins, that inhibit the growth of related *Sulfolobus* species (Ellen et al., 2011). The encapsulation of DNA by EVs is found in multiple phyla including the Crenarchaeota and the Euryarchaeota. *Thermococcus onnurineus* EVs always contain random ~14 kb fragments of DNA that represent most of the

genome, suggesting that these AEVs mediate genetic exchange within the same or related species (Choi et al., 2015). AEVs capable of transmitting specific plasmids have also been described in *Thermococcus kodakarensis*, *Thermococcus nautili*, *Sulfolobus* species and *Halorubrum* species (Liu et al., 2021a,b). In addition to these specific functions, AEVs have been observed to carry a wide diversity of proteins, including diverse proteases and nucleases (Liu et al., 2021b) and could support heterotrophic growth of *S. islandicus* in minimal medium, implying roles in nutrition (Liu et al., 2021a).

It is likely that EVs play key roles in cell-cell communication relevant to a very wide range of biological activities, beyond the few examples given above. There is growing evidence, for example, that BEVs can transit gut mucosal barriers and reach intestinal immune cells, the blood, and distal organs such as the brain, with measurable physiological effects (Díaz-Garrido et al., 2021a), opening further frontiers in the interaction between microbes (from all kingdoms) and multicellular animals. It is likely also that EVs play similarly important roles in cell-cell communication in plants and in plant-associated microbes, though studies on this topic are currently lacking. The role of EVs in communication among microbes that constitute microbial communities (microbiomes), especially the exchange of developmental signals, remains largely unexplored.

CONCLUSION AND FUTURE DIRECTIONS

Extracellular vesicles have emerged as an exciting and long under-appreciated component of cells. EVs extend the sphere of influence of cells well beyond what can be achieved by conventional secretory mechanisms. The importance of EVs is underlined by the finding that they are produced by all forms of living organisms, including numerous kingdoms of microbes. Because of their ability to transport cell contents, including RNA and DNA, from one organism to another, they have emerged as key players in microbial interactions with plants and animals, as well as in diseases such as cancer. As such they have also emerged as useful biomarkers for diagnosis of some clinical diseases and are being modified for delivery of vaccines and other therapies (Robbins and Morelli, 2014). Compared with the research into mammalian, parasite and bacterial EVs, research into the functions of fungal and oomycete EVs is still in its infancy, especially in the context of plant interactions, and many questions need to be addressed.

REFERENCES

- Ahat, E., Li, J., and Wang, Y. (2019). New insights into the Golgi stacking proteins. *Front. Cell Dev. Biol.* 7:131. doi: 10.3389/fcell.2019.00131
- Bahar, O., Mordukhovich, G., Luu, D. D., Schwessinger, B., Daudi, A., Jehle, A. K., et al. (2016). Bacterial outer membrane vesicles induce plant immune responses. *Mol. Plant Microbe Interact.* 29, 374–384. doi: 10.1094/MPMI-12-15-0270-R
- Baltazar, L. M., Zamith-Miranda, D., Burnet, M. C., Choi, H., Nimrichter, L., Nakayasu, E. S., et al. (2018). Concentration-dependent protein loading of extracellular vesicles released by *Histoplasma capsulatum* after antibody treatment and its modulatory action upon macrophages. *Sci. Rep.* 8:8065. doi: 10.1038/s41598-018-25665-5
- Baulcombe, D. C. (2015). VIGS, HIGS and FIGS: small RNA silencing in the interactions of viruses or filamentous organisms with their plant hosts. *Curr. Opin. Plant Biol.* 26, 141–146. doi: 10.1016/j.pbi.2015.06.007
- Bielaszewska, M., Ruter, C., Bauwens, A., Greune, L., Jarosch, K. A., Steil, D., et al. (2017). Host cell interactions of outer membrane vesicle-associated virulence factors of enterohemorrhagic *Escherichia coli* O157: intracellular delivery, trafficking and mechanisms of cell injury. *PLoS Pathog.* 13:e1006159. doi: 10.1371/journal.ppat.1006159
- A major challenge for EV research is the ability to discern and purify different sub-types of EVs. While progress has been made on separating EVs by size and density, and in some cases immunologically (Rybak and Robatzek, 2019), a much more complete set of biomarkers is needed to accurately determine the origins, contents, functions and targeting of diverse EVs. This information is also needed to fully realize the potential of synthetic EVs as therapeutic agents (Huyan et al., 2020). Key questions remain around the contents of EVs, including whether and how specific cargoes are loaded into EVs. Cargoes of special interest include virulence effector proteins produced by fungal and oomycete pathogens of plants and animals. How these effectors are delivered into host cells remains poorly understood, and EVs stand out as a likely delivery mechanism, both for conventionally and unconventionally secreted effectors. Another cargo of major interest is RNA molecules. Strong evidence has emerged, especially from plant systems, that hosts and pathogens can use EVs to convey specific RNA molecules to each other to modify the physiology, immunity and virulence of their antagonist (Cai et al., 2018). However, the full scope of this mechanism remains to be explored. For example, is EV delivery confined to small RNAs or are full length mRNAs or non-coding RNAs conveyed by this mechanism? Also, what counter-measures might the antagonists have evolved against each other's EVs (Qiao et al., 2013, 2021)? Another interesting question is whether EVs may play a role in horizontal gene transfer between species or even between kingdoms. Finally, the roles of EVs in communication within communities of microbes (microbiomes) remain largely unexplored.

AUTHOR CONTRIBUTIONS

YF and BT wrote the manuscript with contributions from all authors. YF, ZW, XL, and BT conceived the manuscript, read, and agreed to the published version of the manuscript.

FUNDING

YF was supported by an award from the China Scholarship Council. This research was partially supported by the Changjiang Scholars Program of China (T2016106) and Oregon State University. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

- Bielska, E., Sisquella, M. A., Aldeieg, M., Birch, C., O'Donoghue, E. J., and May, R. C. (2018). Pathogen-derived extracellular vesicles mediate virulence in the fatal human pathogen *Cryptococcus gattii*. *Nat. Commun.* 9:1556. doi: 10.1038/s41467-018-03991-6
- Blanc, L., and Vidal, M. (2018). New insights into the function of Rab GTPases in the context of exosomal secretion. *Small GTPases* 9, 95–106. doi: 10.1080/21541248.2016.1264352
- Bomberger, J. M., MacEachran, D. P., Coutermarsh, B. A., Ye, S., O'Toole, G. A., and Stanton, B. A. (2009). Long-distance delivery of bacterial virulence factors by *Pseudomonas aeruginosa* outer membrane vesicles. *PLoS Pathog.* 5:e1000382. doi: 10.1371/journal.ppat.1000382
- Brameyer, S., Plener, L., Müller, A., Klingl, A., Wanner, G., and Jung, K. (2018). Outer membrane vesicles facilitate trafficking of the hydrophobic signaling molecule CAI-1 between *Vibrio harveyi* cells. *J. Bacteriol.* 200:e00740-17. doi: 10.1128/JB.00740-17
- Briaud, P., and Carroll, R. K. (2020). Extracellular vesicle biogenesis and functions in gram-positive bacteria. *Infect. Immun.* 88:e00433-20. doi: 10.1128/IAI.00433-20
- Brown, L., Wolf, J. M., Prados-Rosales, R., and Casadevall, A. (2015). Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. *Nat. Rev. Microbiol.* 13, 620–630. doi: 10.1038/nrmicro3480
- Buck, A. H., Coakley, G., Simbari, F., McSorley, H. J., Quintana, J. F., Le Bihan, T., et al. (2014). Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity. *Nat. Commun.* 5:5488. doi: 10.1038/ncomms6488
- Cai, Q., He, B., Wang, S., Fletcher, S., Niu, D., Mitter, N., et al. (2021). Message in a bubble: shuttling small RNAs and proteins between cells and interacting organisms using extracellular vesicles. *Annu. Rev. Plant Biol.* 72, 497–524. doi: 10.1146/annurev-arplant-081720-010616
- Cai, Q., Qiao, L., Wang, M., He, B., Lin, F.-M., Palmquist, J., et al. (2018). Plants send small RNAs in extracellular vesicles to fungal pathogen to silence virulence genes. *Science* 360, 1126–1129. doi: 10.1126/science.aar4142
- Cai, Y., Zhuang, X., Gao, C., Wang, X., and Jiang, L. (2014). The Arabidopsis endosomal sorting complex required for transport III regulates internal vesicle formation of the prevacuolar compartment and is required for plant development. *Plant Physiol.* 165, 1328–1343. doi: 10.1104/pp.114.238378
- Cañas, M.-A., Giménez, R., Fábrega, M.-J., Toloza, L., Baldomà, L., and Badia, J. (2016). Outer membrane vesicles from the probiotic *Escherichia coli* Nissle 1917 and the commensal ECOR12 enter intestinal epithelial cells via clathrin-dependent endocytosis and elicit differential effects on DNA damage. *PLoS One* 11:e0160374. doi: 10.1371/journal.pone.0160374
- Chatterjee, S. N., and Das, J. (1967). Electron microscopic observations on the excretion of cell-wall material by *Vibrio cholerae*. *J. Gen. Microbiol.* 1, 1–11. doi: 10.1099/00221287-49-1-1
- Caruana, J. C., and Walper, S. A. (2020). Bacterial membrane vesicles as mediators of microbe-microbe and microbe-host community interactions. *Front. Microbiol.* 11:432. doi: 10.3389/fmicb.2020.00432
- Chen, Z., Larregina, A. T., and Morelli, A. E. (2019). Impact of extracellular vesicles on innate immunity. *Curr. Opin. Organ. Transplant.* 24, 670–678. doi: 10.1097/MOT.0000000000000701
- Choi, D. H., Kwon, Y. M., Chiura, H. X., Yang, E. C., Bae, S. S., Kang, S. G., et al. (2015). Extracellular vesicles of the hyperthermophilic archaeon “*Thermococcus onnurineus*” NA1T. *Appl. Environ. Microbiol.* 81, 4591–4599. doi: 10.1128/AEM.00428-15
- Colombo, M., Raposo, G., and Thery, C. (2014). Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu. Rev. Cell Dev. Biol.* 30, 255–289. doi: 10.1146/annurev-cellbio-101512-122326
- Combes, V., Coltel, N., Alibert, M., van Eck, M., Raymond, C., Juhan-Vague, I., et al. (2005). ABCA1 gene deletion protects against cerebral malaria: potential pathogenic role of microparticles in neuropathology. *Am. J. Pathol.* 166, 295–302. doi: 10.1016/S0002-9440(10)62253-5
- Cui, C., Wang, Y., Liu, J., Zhao, J., Sun, P., and Wang, S. (2019). A fungal pathogen deploys a small silencing RNA that attenuates mosquito immunity and facilitates infection. *Nat. Commun.* 10:4298. doi: 10.1038/s41467-019-12323-1
- Díaz-Garrido, N., Badia, J., and Baldomà, L. (2021a). Microbiota-derived extracellular vesicles in interkingdom communication in the gut. *J. Extracell. Vesicles* 10:e12161. doi: 10.1002/jev2.12161
- Díaz-Garrido, N., Cordero, C., Olivo-Martinez, Y., Badia, J., and Baldomà, L. (2021b). Cell-to-cell communication by host-released extracellular vesicles in the gut: implications in health and disease. *Int. J. Mol. Sci.* 22:2213. doi: 10.3390/ijms22042213
- D'Souza-Schorey, C., and Schorey, J. S. (2018). Regulation and mechanisms of extracellular vesicle biogenesis and secretion. *Essays Biochem.* 62, 125–133. doi: 10.1042/EBC20170078
- Dunker, F., Trutzenberg, A., Rothenpieler, J. S., Kuhn, S., Prols, R., Schreiber, T., et al. (2020). Oomycete small RNAs bind to the plant RNA-induced silencing complex for virulence. *Elife* 9:e56096. doi: 10.7554/eLife.56096
- Duran, J. M., Anjard, C., Stefan, C., Loomis, W. F., and Malhotra, V. (2010). Unconventional secretion of Acb1 is mediated by autophagosomes. *J. Cell Biol.* 188, 527–536. doi: 10.1083/jcb.200911154
- El-Assaad, F., Wheway, J., Hunt, N. H., Grau, G. E., and Combes, V. (2014). Production, fate and pathogenicity of plasma microparticles in murine cerebral malaria. *PLoS Pathog.* 10:e1003839. doi: 10.1371/journal.ppat.1003839
- Ellen, A. F., Rohulya, O. V., Fusetti, F., Wagner, M., Albers, S.-V., and Driessen, A. J. (2011). The sulfolobin genes of *Sulfolobus acidocaldarius* encode novel antimicrobial proteins. *J. Bacteriol.* 193, 4380–4387. doi: 10.1128/JB.05028-11
- Ellis, J., Catanzariti, A. M., and Dodds, P. (2006). The problem of how fungal and oomycete avirulence proteins enter plant cells. *Trends Plant Sci.* 11, 61–63. doi: 10.1016/j.tplants.2005.12.008
- Erdmann, S., Tschitschko, B., Zhong, L., Raftery, M. J., and Cavicchioli, R. (2017). A plasmid from an antarctic haloarchaeon uses specialized membrane vesicles to disseminate and infect plasmid-free cells. *Nat. Microbiol.* 2, 1446–1455. doi: 10.1038/s41564-017-0009-2
- Feitosa-Junior, O. R., Stefanello, E., Zaini, P. A., Nascimento, R., Pierry, P. M., Dandekar, A. M., et al. (2019). Proteomic and metabolomic analyses of *Xylella fastidiosa* OMV-enriched fractions reveal association with virulence factors and signaling molecules of the DSF family. *Phytopathology* 109, 1344–1353. doi: 10.1094/PHYTO-03-19-0083-R
- Fucarino, A., Pitruzzella, A., Burgio, S., Zarcone, M. C., Modica, D. M., Cappello, F., et al. (2021). Extracellular vesicles in airway homeostasis and pathophysiology. *Appl. Sci.* 11:933. doi: 10.3390/app11219933
- Gao, C., Luo, M., Zhao, Q., Yang, R., Cui, Y., Zeng, Y., et al. (2014). A unique plant ESCRT component, FREE1, regulates multivesicular body protein sorting and plant growth. *Curr. Biol.* 24, 2556–2563. doi: 10.1016/j.cub.2014.09.014
- Gao, C., Zhuang, X., Shen, J., and Jiang, L. (2017). Plant ESCRT complexes: moving beyond endosomal sorting. *Trends Plant Sci.* 22, 986–998. doi: 10.1016/j.tplants.2017.08.003
- Giraldo, M. C., Dagdas, Y. F., Gupta, Y. K., Mentlak, T. A., Yi, M., Martinez-Rocha, A. L., et al. (2013). Two distinct secretion systems facilitate tissue invasion by the rice blast fungus *Magnaporthe oryzae*. *Nat. Commun.* 4:1996. doi: 10.1038/ncomms2996
- Hansen, L. L., and Nielsen, M. E. (2018). Plant exosomes: using an unconventional exit to prevent pathogen entry? *J. Exp. Bot.* 69, 59–68. doi: 10.1093/jxb/erx319
- Hou, Y., Zhai, Y., Feng, L., Karimi, H. Z., Rutter, B. D., Zeng, L., et al. (2019). A *Phytophthora* effector suppresses trans-kingdom RNAi to promote disease susceptibility. *Cell Host Microbe* 25, 153–165.e5. doi: 10.1016/j.chom.2018.11.007
- Huyan, T., Li, H., Peng, H., Chen, J., Yang, R., Zhang, W., et al. (2020). Extracellular vesicles—advanced nanocarriers in cancer therapy: progress and achievements. *Int. J. Nanomed.* 15, 6485–6502. doi: 10.2147/IJN.S238099
- Ikeda, M. A. K., de Almeida, J. R. F., Jannuzzi, G. P., Cronemberger-Andrade, A., Torrecilhas, A. C. T., Moretti, N. S., et al. (2018). Extracellular vesicles from *Sporothrix brasiliensis* are an important virulence factor that induce an increase in fungal burden in experimental sporotrichosis. *Front. Microbiol.* 9:2286. doi: 10.3389/fmicb.2018.02286
- Jiang, R. H. Y., and Tyler, B. M. (2012). Mechanisms and evolution of virulence in oomycetes. *Ann. Rev. Phytopath.* 50, 295–318. doi: 10.1146/annurev-phyto-081211-172912
- Joffe, L. S., Nimrichter, L., Rodrigues, M. L., and Del Poeta, M. (2016). Potential roles of fungal extracellular vesicles during infection. *mSphere* 1:e00099-16. doi: 10.1128/mSphere.00099-16
- Jones, E. J., Booth, C., Fonseca, S., Parker, A., Cross, K., Miquel-Clopés, A., et al. (2020). The uptake, trafficking, and biodistribution of *Bacteroides thetaiotaomicron* generated outer membrane vesicles. *Front. Microbiol.* 11:57. doi: 10.3389/fmicb.2020.00057

- Kale, S. D., and Tyler, B. M. (2011). Entry of oomycete and fungal effectors into plant and animal cells. *Cell. Microbiol.* 13, 1839–1848. doi: 10.1111/j.1462-5822.2011.01659.x
- Khang, C. H., Berruyer, R., Giraldo, M. C., Kankanala, P., Park, S.-Y., Czymmek, K., et al. (2010). Translocation of *Magnaporthe oryzae* effectors into rice cells and their subsequent cell-to-cell movement. *Plant Cell* 22, 1388–1403. doi: 10.1105/tpc.109.069666
- Kim, S. W., Park, S. B., Im, S. P., Lee, J. S., Jung, J. W., Gong, T. W., et al. (2018). Outer membrane vesicles from beta-lactam-resistant *Escherichia coli* enable the survival of beta-lactam-susceptible *E. coli* in the presence of beta-lactam antibiotics. *Sci. Rep.* 8:5402. doi: 10.1038/s41598-018-23656-0
- Koch, A., and Wassenegger, M. (2021). Host-induced gene silencing—mechanisms and applications. *New Phytol.* 231, 54–59. doi: 10.1111/nph.17364
- Koch, A., Schlemmer, T., Höfle, L., Werner, B. T., Preußner, C., Hardt, M., et al. (2020). Host-induced gene silencing involves transfer of dsRNA-derived siRNA via extracellular vesicles. *bioRxiv* [Preprint]. bioRxiv 02.12: 945154.
- Koeppen, K., Hampton, T. H., Jarek, M., Scharfe, M., Gerber, S. A., Mielcarz, D. W., et al. (2016). A novel mechanism of host-pathogen interaction through sRNA in bacterial outer membrane vesicles. *PLoS Pathog.* 12:e1005672. doi: 10.1371/journal.ppat.1005672
- Konečná, K., Klimentová, J., Benada, O., Němečková, I., Jandourek, O., Julek, P., et al. (2019). A comparative analysis of protein virulence factors released via extracellular vesicles in two *Candida albicans* strains cultivated in a nutrient-limited medium. *Microb. Pathog.* 136:103666. doi: 10.1016/j.micpath.2019.103666
- Kowal, J., Tkach, M., and Thery, C. (2014). Biogenesis and secretion of exosomes. *Curr. Opin. Cell Biol.* 29, 116–125. doi: 10.1016/j.ccb.2014.05.004
- Kuehn, M. J., and Kesty, N. C. (2005). Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes Dev.* 19, 2645–2655. doi: 10.1101/gad.1299905
- Kwon, S., Rupp, O., Brachmann, A., Blum, C. F., Kraege, A., Goesmann, A., et al. (2021). mRNA inventory of extracellular vesicles from *Ustilago maydis*. *J. Fungi* 7:562. doi: 10.3390/jof7070562
- Liu, J., Soler, N., Gorlas, A., Cvirkaitė-Krupovic, V., Krupovic, M., and Forterre, P. (2021a). Extracellular membrane vesicles and nanotubes in Archaea. *microLife* 2:uqab007. doi: 10.1093/femsm/l/2uqab007
- Liu, J., Cvirkaitė-Krupovic, V., Commere, P.-H., Yang, Y., Zhou, F., Forterre, P., et al. (2021b). Archaeal extracellular vesicles are produced in an ESCRT-dependent manner and promote gene transfer and nutrient cycling in extreme environments. *ISME J.* 15, 2892–2905. doi: 10.1038/s41396-021-00984-0
- Liu, T., Song, T., Zhang, X., Yuan, H., Su, L., Li, W., et al. (2014). Unconventionally secreted effectors of two filamentous pathogens target plant salicylate biosynthesis. *Nat. Commun.* 5:4686. doi: 10.1038/ncomms5686
- Macia, L., Nanan, R., Hosseini-Beheshti, E., and Grau, G. E. (2020). Host- and microbiota-derived extracellular vesicles, immune function, and disease development. *Int. J. Mol. Sci.* 21, 107–124. doi: 10.3390/ijms21010107
- Mantel, P. Y., Hjeltnqvist, D., Walch, M., Kharoubi-Hess, S., Nilsson, S., Ravel, D., et al. (2016). Infected erythrocyte-derived extracellular vesicles alter vascular function via regulatory Ago2-miRNA complexes in malaria. *Nat. Commun.* 7:12727. doi: 10.1038/ncomms12727
- Mantel, P. Y., Hoang, A. N., Goldowitz, I., Potashnikova, D., Hamza, B., Vorobjev, I., et al. (2013). Malaria-infected erythrocyte-derived microvesicles mediate cellular communication within the parasite population and with the host immune system. *Cell Host Microbe* 13, 521–534. doi: 10.1016/j.chom.2013.04.009
- Mashburn, L. M., and Whiteley, M. (2005). Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature* 437, 422–425. doi: 10.1038/nature03925
- McMillan, H. M., Zebell, S. G., Ristaino, J. B., Dong, X., and Kuehn, M. J. (2021). Protective plant immune responses are elicited by bacterial outer membrane vesicles. *Cell Rep.* 34:108645. doi: 10.1016/j.celrep.2020.108645
- Motaung, T. E., and Steenkamp, E. T. (2021). Extracellular vesicles in teasing apart complex plant-microbiota links: implications for microbiome-based biotechnology. *mSystems* 6:e0073421. doi: 10.1128/mSystems.00734-21
- Mei, K., and Guo, W. (2018). The exocyst complex. *Curr. Biol.* 28, 922–925. doi: 10.1016/j.cub.2018.06.042
- Munhoz da Rocha, I. F., Amatuzzi, R. F., Lucena, A. C. R., Faoro, H., and Alves, L. R. (2020). Cross-kingdom extracellular vesicles EV-RNA communication as a mechanism for host-pathogen interaction. *Front. Cell Infect. Microbiol.* 10:593160. doi: 10.3389/fcimb.2020.593160
- Ñahui Palomino, R. A., Vanpouille, C., Costantini, P. E., and Margolis, L. (2021). Microbiota–host communications: bacterial extracellular vesicles as a common language. *PLoS Pathog.* 17:e1009508. doi: 10.1371/journal.ppat.1009508
- Oliveira, D. L., Nakayasu, E. S., Joffe, L. S., Guimaraes, A. J., Sobreira, T. J., Nosanchuk, J. D., et al. (2010). Characterization of yeast extracellular vesicles: evidence for the participation of different pathways of cellular traffic in vesicle biogenesis. *PLoS One* 5:e11113. doi: 10.1371/journal.pone.0011113
- Oliveira, D. L., Rizzo, J., Joffe, L. S., Godinho, R. M., and Rodrigues, M. L. (2013). Where do they come from and where do they go: candidates for regulating extracellular vesicle formation in fungi. *Int. J. Mol. Sci.* 14, 9581–9603. doi: 10.3390/ijms14059581
- Park, K. S., Lee, J., Lee, C., Park, H. T., Kim, J. W., Kim, O. Y., et al. (2018). Sepsis-like systemic inflammation induced by nano-sized extracellular vesicles from feces. *Front. Microbiol.* 9:1735. doi: 10.3389/fmicb.2018.01735
- Park, Y. D., Chen, S. H., Camacho, E., Casadevall, A., and Williamson, P. R. (2020). Role of the ESCRT pathway in laccase trafficking and virulence of *Cryptococcus neoformans*. *Infect. Immun.* 88:e00954-19. doi: 10.1128/IAI.00954-19
- Peres da Silva, R., Martins, S. T., Rizzo, J., Dos Reis, F. C. G., Joffe, L. S., Vainstein, M., et al. (2018). Golgi reassembly and stacking protein (GRASP) participates in vesicle-mediated RNA export in *Cryptococcus neoformans*. *Genes* 9:400. doi: 10.3390/genes9080400
- Peres da Silva, R., Puccia, R., Rodrigues, M. L., Oliveira, D. L., Joffe, L. S., Cesar, G. V., et al. (2015). Extracellular vesicle-mediated export of fungal RNA. *Sci. Rep.* 5:7763. doi: 10.1038/srep07763
- Pérez-Cruz, C., Delgado, L., López-Iglesias, C., and Mercade, E. (2015). Outer-inner membrane vesicles naturally secreted by gram-negative pathogenic bacteria. *PLoS One* 10:e0116896. doi: 10.1371/journal.pone.0116896
- Prado, N., de Dios Alché, J., Casado-Vela, J., Mas, S., Villalba, M., Rodríguez, R., et al. (2014). Nanovesicles are secreted during pollen germination and pollen tube growth: a possible role in fertilization. *Mol. Plant* 7, 573–577. doi: 10.1093/mp/sst153
- Presti, L. L., and Kahmann, R. (2017). How filamentous plant pathogen effectors are translocated to host cells. *Curr. Opin. Plant Biol.* 38, 19–24. doi: 10.1016/j.pbi.2017.04.005
- Qiao, Y., Liu, L., Xiong, Q., Flores, C., Wong, J., Shi, J., et al. (2013). Oomycete pathogens encode RNA silencing suppressors. *Nat. Genet.* 45, 330–333. doi: 10.1038/ng.2525
- Qiao, Y., Xia, R., Zhai, J., Hou, Y., Feng, L., Zhai, Y., et al. (2021). Small RNAs in plant immunity and virulence of filamentous pathogens. *Annu. Rev. Phytopathol.* 59, 265–288. doi: 10.1146/annurev-phyto-121520-023514
- Ratajczak, J., Wysoczynski, M., Hayek, F., Janowska-Wieczorek, A., and Ratajczak, M. Z. (2006). Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. *Leukemia* 20, 1487–1495. doi: 10.1038/sj.leu.2404296
- Regente, M., Pinedo, M., San Clemente, H., Balliau, T., Jamet, E., and de la Canal, L. (2017). Plant extracellular vesicles are incorporated by a fungal pathogen and inhibit its growth. *J. Exp. Bot.* 68, 5485–5495. doi: 10.1093/jxb/erx355
- Regev-Rudski, N., Wilson, D. W., Carvalho, T. G., Sisquella, X., Coleman, B. M., Rug, M., et al. (2013). Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles. *Cell* 153, 1120–1133. doi: 10.1016/j.cell.2013.04.029
- Ren, B., Wang, X., Duan, J., and Ma, J. (2019). Rhizobial tRNA-derived small RNAs are signal molecules regulating plant nodulation. *Science* 365, 919–922. doi: 10.1126/science.aav8907
- Rizzo, J., Rodrigues, M. L., and Janbon, G. (2020). Extracellular vesicles in fungi: past, present, and future perspectives. *Front. Cell Infect. Microbiol.* 10:346. doi: 10.3389/fcimb.2020.00346
- Robbins, P. D., and Morelli, A. E. (2014). Regulation of immune responses by extracellular vesicles. *Nat. Rev. Immunol.* 14, 195–208. doi: 10.1038/nri3622
- Rodrigues, M. L., and Casadevall, A. (2018). A two-way road: novel roles for fungal extracellular vesicles. *Mol. Microbiol.* 110, 11–15. doi: 10.1111/mmi.14095
- Rodrigues, M. L., Nimrichter, L., Oliveira, D. L., Frases, S., Miranda, K., Zaragoza, O., et al. (2007). Vesicular polysaccharide export in *Cryptococcus neoformans* is a eukaryotic solution to the problem of fungal trans-cell wall transport. *Eukaryot. Cell* 6, 48–59. doi: 10.1128/EC.00318-06

- Roth, R., Hillmer, S., Funaya, C., Chiappello, M., Schumacher, K., Lo Presti, L., et al. (2019). Arbuscular cell invasion coincides with extracellular vesicles and membrane tubules. *Nat. Plants* 5, 204–211. doi: 10.1038/s41477-019-0365-4
- Rutter, B. D., and Innes, R. W. (2017). Extracellular vesicles isolated from the leaf apoplast carry stress-response proteins. *Plant Physiol.* 173, 728–741. doi: 10.1104/pp.16.01253
- Rybák, K., and Robatzek, S. (2019). Functions of extracellular vesicles in immunity and virulence. *Plant Physiol.* 179, 1236–1247. doi: 10.1104/pp.18.01557
- Sahu, R., Kaushik, S., Clement, C. C., Cannizzo, E. S., Scharf, B., Follenzi, A., et al. (2011). Microautophagy of cytosolic proteins by late endosomes. *Dev. Cell* 20, 131–139. doi: 10.1016/j.devcel.2010.12.003
- Schwechheimer, C., and Kuehn, M. J. (2015). Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nat. Rev. Microbiol.* 13, 605–619. doi: 10.1038/nrmicro3525
- Seo, M., Park, E., Ko, S., Choi, E., and Kim, S. (2018). Therapeutic effects of kefir grain lactobacillus-derived extracellular vesicles in mice with 2, 4, 6-trinitrobenzene sulfonic acid-induced inflammatory bowel disease. *J. Dairy Sci.* 101, 8662–8671. doi: 10.3168/jds.2018-15014
- Shahriari, M., Keshavaiah, C., Scheuring, D., Sabovljevic, A., Pimpl, P., Hausler, R. E., et al. (2010). The AAA-type ATPase AtSKD1 contributes to vacuolar maintenance of *Arabidopsis thaliana*. *Plant J.* 64, 71–85. doi: 10.1111/j.1365-3113.2010.04310.x
- Stenmark, H. (2009). Rab GTPases as coordinators of vesicle traffic. *Nat. Rev. Mol. Cell Biol.* 10, 513–525. doi: 10.1038/nrm2728
- Tao, K., Waletzki, J. R., Wise, H., Arredondo, F., and Tyler, B. M. (2019). Tethering of multi-vesicular bodies and the tonoplast to the plasma membrane in plants. *Front. Plant Sci.* 10:636. doi: 10.3389/fpls.2019.00636
- Torto-Alalibo, T., Collmer, C. W., Gwinn-Giglio, M., Lindeberg, M., Meng, S.-W., Chibucos, M. C., et al. (2010). Unifying themes in microbial associations with animal and plant hosts described using the gene ontology. *Microbiol. Mol. Biol. Rev.* 74, 479–503. doi: 10.1128/MMBR.00017-10
- Toyofuku, M., Cárcamo-Oyarce, G., Yamamoto, T., Eisenstein, F., Hsiao, C.-C., Kurosawa, M., et al. (2017). Prophage-triggered membrane vesicle formation through peptidoglycan damage in *Bacillus subtilis*. *Nat. Commun.* 8, 1–10. doi: 10.1038/s41467-017-00492-w
- Toyofuku, M., Nomura, N., and Eberl, L. (2019). Types and origins of bacterial membrane vesicles. *Nat. Rev. Microbiol.* 17, 13–24. doi: 10.1038/s41579-018-0112-2
- Tseng, T. T., Tyler, B. M., and Setubal, J. C. (2009). Protein secretion systems in bacterial-host associations, and their description in the gene ontology. *BMC Microbiol.* 9(Suppl. 1):S2. doi: 10.1186/1471-2180-9-S1-S2
- Turnbull, L., Toyofuku, M., Hynen, A. L., Kurosawa, M., Pessi, G., Petty, N. K., et al. (2016). Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat. Commun.* 7, 1–13. doi: 10.1038/ncomms11220
- Turturici, G., Tinnirello, R., Sconzo, G., and Geraci, F. (2014). Extracellular membrane vesicles as a mechanism of cell-to-cell communication: advantages and disadvantages. *Am. J. Physiol. Cell Physiol.* 306, 621–633. doi: 10.1152/ajpcell.00228.2013
- Tyler, B. M. (2009). Viewing the microbial world through the lens of the gene ontology. *Trends Microbiol.* 17, 259–261. doi: 10.1016/j.tim.2009.05.002
- Vietri, M., Radulovic, M., and Stenmark, H. (2020). The many functions of ESCRTs. *Nat. Rev. Mol. Cell Biol.* 21, 25–42. doi: 10.1038/s41580-019-0177-4
- Walker, L., Sood, P., Lenardon, M. D., Milne, G., Olson, J., Jensen, G., et al. (2018). The viscoelastic properties of the fungal cell wall allow traffic of ambisome as intact liposome vesicles. *mBio* 9:e02383-17. doi: 10.1128/mBio.02383-17
- Wang, F., Yang, Y., Wang, Z., Zhou, J., Fan, B., and Chen, Z. (2015). A critical role of Lyst-Interacting Protein5, a positive regulator of multivesicular body biogenesis, in plant responses to heat and salt stresses. *Plant Physiol.* 169, 497–511. doi: 10.1104/pp.15.00518
- Wang, H. J., Hsu, Y. W., Guo, C. L., Jane, W. N., Wang, H., Jiang, L., et al. (2017). VPS36-dependent multivesicular bodies are critical for plasmamembrane protein turnover and vacuolar biogenesis. *Plant Physiol.* 173, 566–581. doi: 10.1104/pp.16.01356
- Wang, J., Ding, Y., Wang, J., Hillmer, S., Miao, Y., Lo, S. W., et al. (2010). EXPO, an exocyst-positive organelle distinct from multivesicular endosomes and autophagosomes, mediates cytosol to cell wall exocytosis in *Arabidopsis* and tobacco cells. *Plant Cell* 22, 4009–4030. doi: 10.1105/tpc.110.080697
- Wang, M., Thomas, N., and Jin, H. (2017). Cross-kingdom RNA trafficking and environmental RNAi for powerful innovative pre- and post-harvest plant protection. *Curr. Opin. Plant Biol.* 38, 133–141. doi: 10.1016/j.pbi.2017.05.003
- Wang, M., Weiberg, A., Lin, F. M., Thomma, B. P., Huang, H. D., and Jin, H. (2016). Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nat. Plants* 2:16151. doi: 10.1038/nplants.2016.151
- Wang, S., Boevink, P. C., Welsh, L., Zhang, R., Whisson, S. C., and Birch, P. R. J. (2017). Delivery of cytoplasmic and apoplastic effectors from *Phytophthora infestans* haustoria by distinct secretion pathways. *New Phytol.* 216, 205–215. doi: 10.1111/nph.14696
- Weiberg, A., Wang, M., Lin, F. M., Zhao, H., Zhang, Z., Kaloshian, I., et al. (2013). Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. *Science* 342, 118–123. doi: 10.1126/science.1239705
- Woith, E., Fuhrmann, G., and Melzig, M. F. (2019). Extracellular vesicles-connecting kingdoms. *Int. J. Mol. Sci.* 20:5695. doi: 10.3390/ijms20225695
- Wolf, J. M., Espadas-Moreno, J., Luque-Garcia, J. L., and Casadevall, A. (2014). Interaction of *Cryptococcus neoformans* extracellular vesicles with the cell wall. *Eukaryot. Cell* 13, 1484–1493. doi: 10.1128/EC.00111-14
- Yang, H., Liu, J., Lin, J., Deng, L., Fan, S., Guo, Y., et al. (2016). Overexpression of CHMP7 from rapeseed and *Arabidopsis* causes dwarfism and premature senescence in *Arabidopsis*. *J. Plant Physiol.* 204, 16–26. doi: 10.1016/j.jplph.2016.06.023
- Yoon, Y. J., Kim, O. Y., and Ghoo, Y. S. (2014). Extracellular vesicles as emerging intercellular communication. *BMB Rep.* 47, 531–539. doi: 10.5483/bmbrep.2014.47.10.164
- Zarnowski, R., Sanchez, H., Covelli, A. S., Dominguez, E., Jaromin, A., Bernhardt, J., et al. (2018). *Candida albicans* biofilm-induced vesicles confer drug resistance through matrix biogenesis. *PLoS Biol.* 16:e2006872. doi: 10.1371/journal.pbio.2006872
- Zhao, K., Blackley, M., Chisanga, D., Gangoda, L., Fonseka, P., Liem, M., et al. (2019). Extracellular vesicles secreted by *Saccharomyces cerevisiae* are involved in cell wall remodelling. *Commun. Biol.* 2:305. doi: 10.1038/s42003-019-0538-8
- Zhou, B., Yuan, Y., Zhang, S., Guo, C., Li, X., Li, G., et al. (2020). Intestinal flora and disease mutually shape the regional immune system in the intestinal tract. *Front. Immunol.* 11:575. doi: 10.3389/fimmu.2020.00575

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Fang, Wang, Liu and Tyler. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



The Role of Extracellular Vesicles in Systemic Lupus Erythematosus

Chenghui Zheng[†], Lin Xie^{*†}, Haihong Qin, Xiao Liu, Xi Chen, Fan Lv, Li Wang, Xiaohua Zhu^{*} and Jinhua Xu^{*}

Department of Dermatology, Huashan Hospital, Fudan University, Shanghai, China

OPEN ACCESS

Edited by:

Gal Bitan,
University of California, Los Angeles,
United States

Reviewed by:

Dwijendra K. Gupta,
Jai Prakash Vishwavidyalaya, India
Muhammad Nawaz,
University of Gothenburg, Sweden

*Correspondence:

Lin Xie
xielin126_2007@126.com
Xiaohua Zhu
alphabetist@163.com
Jinhua Xu
jinhuaXu@fudan.edu.cn

[†]These authors have contributed
equally to this work and share first
authorship

Specialty section:

This article was submitted to
Membrane Traffic,
a section of the journal
Frontiers in Cell and Developmental
Biology

Received: 14 December 2021

Accepted: 07 February 2022

Published: 02 March 2022

Citation:

Zheng C, Xie L, Qin H, Liu X, Chen X,
Lv F, Wang L, Zhu X and Xu J (2022)
The Role of Extracellular Vesicles in
Systemic Lupus Erythematosus.
Front. Cell Dev. Biol. 10:835566.
doi: 10.3389/fcell.2022.835566

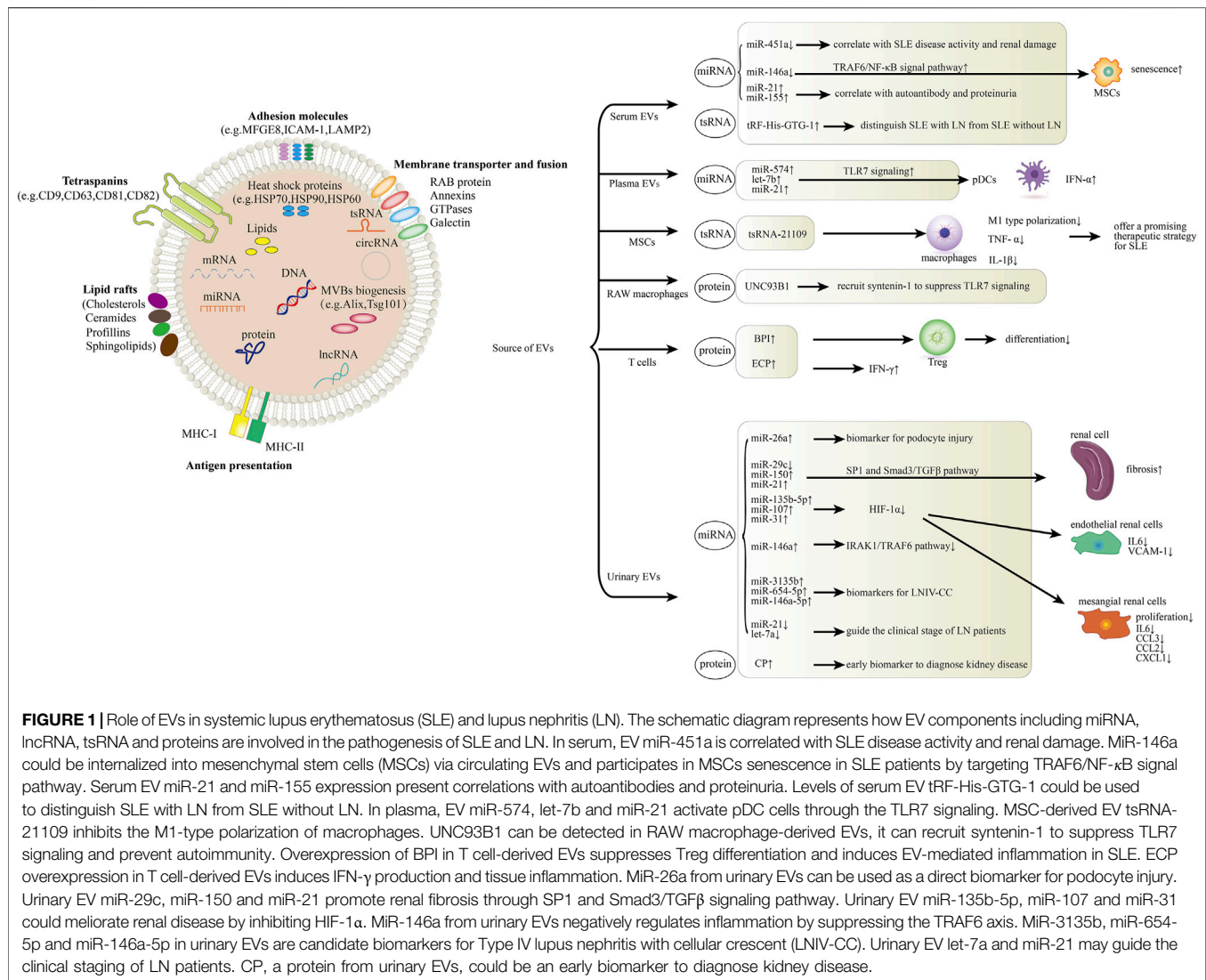
Extracellular Vesicles (EVs) are small vesicles that can be actively secreted by most cell types into the extracellular environment. Evidence indicates that EVs can carry microRNAs (miRNAs), long non-coding RNAs (lncRNAs), tRNA-derived small RNAs (tsRNAs), proteins, and lipids to target cells or tissue organizations. Latest studies show that EVs play a vital role in the immune modulation and may contribute to the pathogenesis of autoimmune diseases. Systemic lupus erythematosus (SLE) is a common autoimmune disease characterized by abnormal T cell activation and sustained production of autoantibodies against self-antigens, resulting in inflammation and damage to multiple systems. Pathogenic mechanisms of SLE, however, are still not well understood. In this review, we summarize the latest research advances on the functions and mechanisms of EVs, and its role in the pathogenesis, diagnosis, and treatment of SLE.

Keywords: exosome, systemic erythematosus lupus, lupus nephritis, intercellular communication, research progress, extracellular vesicle

1 INTRODUCTION

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by loss of tolerance and sustained production of autoantibodies against self-antigens that form immune complex deposits (Crispin et al., 2010). The prevalence of SLE varies from 30/100,000 to 50/100,000, and the disease is more common in women of childbearing age. SLE is hard to diagnose due to its complex pathogenesis and variable clinical symptoms. Most patients are diagnosed based on the 1997 American College of Rheumatology classification criteria, and the disease activity is assessed based on the SLE Disease Activity Index (SLEDAI). Nevertheless, it is not always effective. At present, a majority of scholars believe that it is the interaction of genetic susceptibility, environment, immunology, and hormone factors that lead to SLE, but the exact mechanism is not clear (Tsokos, 2011). Although non-steroidal anti-inflammatory drugs such as glucocorticoid (GCs), immunosuppressants, and biological agents are commonly used in the treatment of SLE, hurdles such as toxic side effects, the lack of target tissue, and non-response to treatment remain to be crossed (Tsokos, 2011).

First described as “platelet dust” by Peter Wolf in 1967, extracellular vesicles (EVs) are a collective term for phospholipids bilayer structures secreted by cells, which contain microRNAs (miRNAs), long non-coding RNAs (lncRNAs), tRNA-derived small RNAs (tsRNAs), proteins, lipids, and other substances (Raposo and Stoorvogel, 2013). The term “extracellular vesicles (EVs)” includes multiple types of vesicles. Specifically, there exist three main classified subtypes based on their biogenesis, size, and release mechanisms, namely microvesicles (100 nm–1 μm), apoptotic bodies (1–5 μm), and exosomes (30–100 nm in diameter) (Yanez-Mo et al., 2015). Microvesicles (MVs, also called microparticles) are larger than exosomes and pinch directly off from the outer cell membrane (Akers et al., 2013). Microvesicles formation is the result of molecular rearrangements of the plasma



membrane regarding phospholipid and cytoskeletal protein composition as well as Ca^{2+} levels (Taylor and Bebawy, 2019). Apoptotic bodies are large structures, which are also produced by direct budding of the membrane and differ from exosomes and MVs as apoptotic bodies are formed only during programmed cell death. They are characterized by the presence of closely packed cellular organelles and fragments of nucleus (Saraste and Pulkki, 2000). Over the past few years, the cutting-edge knowledge about EV research provides insights into new tools for diagnosis, prognosis, and disease activity monitoring, novel therapeutic strategy, and innovative evaluation approaches for treatment effectiveness in SLE (Perez-Hernandez and Cortes, 2015; Perez-Hernandez et al., 2017; Wu et al., 2020; Zhang et al., 2020; Zhao et al., 2020).

As the smallest vesicles and probably the most prominently described class of EV, exosomes are ranging from 30–100 nm in diameter, and are released by almost all cell types, including stem cells, T and B lymphocytes, dendritic cells (DCs), macrophages, endothelial cells, neurons, adipocytes, and epithelial cells

(Obregon et al., 2009; Mashouri et al., 2019; Rayamajhi et al., 2019). They can be found in a wide range of bodily fluids, such as blood, urine, saliva, breast milk, and in the supernatants of cultured cells after being released into the extracellular environment (Record et al., 2011; Matsumura et al., 2015). Exosome has a lipid bilayer membrane structure and contains bio-reactive macromolecules such as cell-specific proteins, lipids, and nucleic acids, which can protect the coating substances, targeting specific tissues and cells to perform their biological functions. Recently, evidence indicates that exosomes play important roles not only in physiological events, such as intracellular communication, immune modulation, and inflammation, but also in pathological conditions, including autoimmune and cardio-metabolic diseases, as well as development and metastasis of tumors (Shah et al., 2018; Stahl and Raposo, 2019). In this review, we summarize the recent progress of the potential role of exosomes in the pathogenesis, diagnosis, and treatment of SLE (Figure 1 and Table 1). However, there is always a heterogeneous population of EVs regardless of the

TABLE 1 | Diagnostic role of EVs in systemic lupus erythematosus (SLE) and lupus nephritis (LN).

Source of EVs	Isolation method	Candidate markers	Function
Serum EVs	—	MiRNA	—
	ExoQuick Kit	miR-451a↓ Tan et al. (2021)	Correlate with SLE disease activity and renal damage, involved in intercellular communication.
	ExoQuick Kit	miR-146a↓ Dong et al. (2019), Li et al. (2020)	miR-146a is negatively correlated with anti-dsDNA antibodies and participates in mesenchymal stem cells (MSCs) senescence in SLE patients by targeting TRAF6/NF- κ B signal pathway
	ExoQuick Kit ExoQuick Kit — N.A.	miR-21↑ Li et al. (2020) miR-155↑ Li et al. (2020) TsRNA tRF-His-GTG-1↑ Yang et al. (2021)	miR-21 is negatively correlated with anti-SSA/Ro antibodies. miR-21 and miR-155 show positive correlations with proteinuria — Can be used to distinguish SLE with LN from SLE without LN.
Plasma EVs	— Ultracentrifugation	MiRNA miR-574↑ Salvi et al. (2018) let-7b↑ Salvi et al. (2018) miR-21↑ Salvi et al. (2018)	— Activate pDC cells through the TLR7 signaling, allowing them to produce IFN- α and proinflammatory cytokines
Mesenchymal stem cells (MSCs)	— Cell culture media exosome purification kit	tsRNA tsRNA-21109 Dou et al. (2021)	— Inhibit the M1-type polarization of macrophages
RAW macrophages	Ultracentrifugation	Protein UNC93B1 Majer et al. (2019)	Recruit syntenin-1 to suppress TLR7 signaling and prevent autoimmunity
T cells	ExoQuick Kit ExoQuick Kit	Protein BPI↑ Chuang et al. (2021) ECP↑ Chuang et al. (2022)	Suppress Treg differentiation and induce EV-mediated inflammation in SLE. Induce IFN- γ production and tissue inflammation
Urinary EVs	—	MiRNA	—
	Ultracentrifugation	miR-26a↑ Ichii et al. (2014)	Can be used as a direct biomarker for podocyte injury
	Ultracentrifugation, miRCURY Exosome Isolation Kit	miR-29c↓ Solé et al. (2015), Solé et al. (2019) miR-150↑ Solé et al. (2019) miR-21↑ Solé et al. (2019)	Correlate with renal chronicity (CI) and promote renal fibrosis in LN through SP1 and Smad3/TGF β signaling pathway
	miRCURY Exosome Isolation Kit	miR-135b-5p↑ Garcia-Vives et al. (2020) miR-107↑ Garcia-Vives et al. (2020) miR-31↑ Garcia-Vives et al. (2020)	Meliorate renal disease by inhibiting HIF-1 α , can be early markers for predicting LN clinical response
	Ultracentrifugation	miR-146a↑ Perez-Hernandez et al. (2015), Perez-Hernandez et al. (2021)	Correlate with lupus activity, proteinuria, and histological features. Negatively regulate inflammation by suppressing the TRAF6 axis
	Ultracentrifugation	miR-3135b↑ Li et al. (2018) miR-654-5p↑ Li et al. (2018) miR-146a-5p↑ Li et al. (2018)	Candidate biomarkers for Type IV lupus nephritis with cellular crescent (LNIV-CC)
	Ultracentrifugation	miR-21↓ Tangtanatakul et al. (2019) let-7a↓ Tangtanatakul et al. (2019)	Guide the clinical stage of LN patients
Urinary EVs	Ultracentrifugation	Protein CP↑ Gudehithlu et al. (2019)	Early biomarker to diagnose kidney disease

isolation method used. Additionally, none of the involved studies published to date can prove that the isolated fractions are exosomes only. In this context, we utilized the generic term “extracellular vesicles (EVs)” instead of “exosomes” throughout the rest of this survey. It is also in line with the recommended terminology from the international society for extracellular vesicles (ISEV) (Thery et al., 2018).

2 BIOLOGICAL CHARACTERISTICS OF EVS

2.1 The Biogenesis of EVs

The formation of EVs involves a variety of proteins and transport complexes, and the fusion of primary endocytic vesicles should be the first step of the early endosomes (EEs) formation. Then, two pathways are shown by the EEs. One is that EEs become recycling endosomes, returning to plasma membrane, and the other way is converting into “late endosomes” (LEs)/multivesicular bodies (MVBs) via inward budding of the membrane under the endosomal sorting complex required for transport (ESCRT)-dependent or ESCRT-independent mechanism. Afterwards, LEs are fused with cell membranes, released into the extracellular space under the control of Ras-related proteins in barin (Rab) GTPases and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), which are called EVs (Thery et al., 2002b; Simons and Raposo, 2009; Hessvik and Llorente, 2018).

2.2 Isolation and Extraction of EVs

EVs play an essential role in mediating cell communications and participate in the pathological process of multiple diseases. How to extract EVs efficiently with high purity, high recovery, and low cost has become the key to further downstream cell function research. EV samples contain a large number of vesicles or proteins that have similar volume, density or surface charge to EVs, which can interfere with the result of the experiment. A variety of methods have been developed in this regard (Ramirez et al., 2018). Among them, ultracentrifugation is the most widely used protocol and has also evolved as the gold standard for EV separation. There are, however, still some inevitable downsides such as high instrument cost and long extraction time. Moreover, factors (e.g., multiple cleaning, high sample viscosity, etc.) are likely to shape the downstream analyses negatively in an uncertain manner (Momen-Heravi et al., 2012a; Momen-Heravi et al., 2012b). Size exclusion chromatography is a scheme using EV purification columns to separate EVs. It does not require the use of expensive centrifuges, and the obtained EVs have high purity. However, compared to ultracentrifugation, it is more challenging to handle large samples due to the limitations of the purification columns (Koh et al., 2018; Monguio-Tortajada et al., 2019). Faced with bodily fluids and other large-volume samples, ultrafiltration can be perceived as a solution. The principle of ultrafiltration is the same as membrane separation, and it takes less time, but impurities such as other vesicles and proteins tend to block the pores and reduce the extraction efficiency (Cheruvanky et al., 2007; Konoshenko et al., 2018). The above-mentioned

traditional extraction methods have multiple drawbacks including low purity, low recovery rate, and low efficiency. Nowadays, a mounting number of new extraction methods are discovered. Lewis et al. (2018) utilized static electricity to adsorb EVs around the positive electrode, which largely improves the purity and specificity of the EVs (Lewis et al., 2018). Wu et al. developed a sonic-based separation method that can directly isolate EVs from whole blood, greatly reducing the time required to extract EVs (Wu M. et al., 2017). These new technologies bring new opportunities for the diagnosis and treatment of diseases in the future.

2.3 Function of EVs

EVs were originally considered to be vesicles employed to expel excess transferrin receptor vesicles (Pan and Johnstone, 1983). With the development of the research, various functions of EVs were gradually revealed to the public. The vesicle structure of EVs can protect its internal transported substances from the interference of soluble substances such as proteases *in vivo*. At present, it is generally believed that EVs serve as carriers and play a big part in mediating information exchange between cells by transporting microRNAs (miRNAs), long non-coding RNAs (lncRNAs), tRNA-derived small RNAs (tsRNAs), proteins, lipids, and other substances (Mathivanan et al., 2012; Barile and Vassalli, 2017; Mathieu et al., 2019). These substances may be involved in the pathogenesis of different diseases. EVs of nasal epithelial cells in patients with chronic rhinosinusitis with nasal polyps contain differentially expressed proteins, which are mainly involved in epithelial remodeling through p53 and other pathways, leading to sinus mucosal remodeling (Zhou et al., 2020). EVs can carry β -Amyloid, prion, and α -synuclein, thus spread disease-causing proteins in the brain, which may be involved in Alzheimer's disease progression (Nath et al., 2012; Arellano-Anaya et al., 2015; Lööv et al., 2016). Evidence shows that EV-associated miRNAs and lncRNAs play essential roles in the pathogenesis of osteoarthritis (OA), including OA diagnosis, pathogenesis, and treatment (Maehara et al., 2021; Miao et al., 2021). In addition, EVs are involved in many physiological processes, such as intracellular communication, signal transduction, transport of genetic materials, and modulation of immune response (Natasha et al., 2014). Evidence in previous studies indicates that EVs are also involved in the progression of diseases, including cancers, neurodegenerative diseases, and autoimmune diseases (Anderson et al., 2010), such as rheumatoid arthritis (RA), Sjogren's syndrome (SS), and SLE. In this review, we summarize the latest progress and recent advances in EV research, therapeutic potential, and mechanism of EVs in the pathogenesis of SLE, as well as their clinical implications.

3 ROLE OF EVS IN IMMUNE FUNCTION

It was not until 1996 that B cells were found to induce T cell responses by secreting EVs with major histocompatibility complex (MHC) class II, which indicated the relationship between EVs and immune regulation (Raposo et al., 1996).

After that, EVs from other immunocytes, such as T cells, natural killer (NK) cells, and dendritic cells (DCs), have also been proven to mediate either immune stimulation or immune modulation (Gutierrez-Vazquez et al., 2013; Zhang et al., 2014; Jong et al., 2017; Reis et al., 2018).

3.1 EVs and Innate Immune Cells

Immune cell-derived EVs are involved in the regulation of the innate immune responses. EVs released by neutrophils, macrophages, NK cells, and DCs act on the innate immune system as pro-inflammatory mediators via paracrine messengers (Yanez-Mo et al., 2015).

3.1.1 Neutrophils

Neutrophils are the most abundant leukocyte population in peripheral blood and are the first line of host defense against a wide range of infectious pathogens (Mayadas et al., 2014). In addition to regulating macrophage activation (Gasser and Schifferli, 2004), neutrophil-derived EVs have inhibitory effects on monocyte-derived DCs (Eken et al., 2008). These EVs modify the morphology of monocyte derived DCs (MoDCs) by inhibiting the formation of dendrites, downregulate their phagocytic activity and maturation, and inhibit the cytokine release of MoDCs, resulting in an attenuated capacity to stimulate T cell proliferation (Eken et al., 2008). Other studies identified several neutrophil-derived EV associated molecules which can influence DC and T cell function potentially, such as annexin A1 (Dalli et al., 2008; Gavins and Hickey, 2012) and arginase-1 (Leliefeld et al., 2015; Shen et al., 2017). What's more, it was found that several proteases in neutrophil-derived EVs such as myeloperoxidase (MPO), elastase, cathepsin G and proteinase 3 may influence adaptive immunity (Hess et al., 1999; Gasser et al., 2003; Dalli et al., 2013; Timar et al., 2013; Slater et al., 2017).

3.1.2 Macrophages

Another type of innate immune cells which is a rich source of EVs are macrophages (Wang et al., 2020). Macrophages are important phagocytic cells distributed in essentially all tissues, where they respond to a complex variety of regulatory signals to coordinate immune functions involved in tissue development, homeostasis, metabolism, and repair (Wynn and Vannella, 2016). EVs secreted by bacterially infected macrophages have a pro-inflammatory effect, which can induce the maturation of DCs and activate CD4⁺ and CD8⁺ T cells (Giri and Schorey, 2008; Ramachandra et al., 2010). Besides, these macrophage-derived EVs promote the release of multiple pro-inflammatory cytokines and chemokines (Singh et al., 2012). Furthermore, several studies characterized EVs content and their effects on uninfected macrophages which revealed that EVs released from infected macrophages holds a vital role in immune surveillance (Bhatnagar et al., 2007; Bhatnagar and Schorey, 2007).

3.1.3 Natural Killer Cells

Natural killer (NK) cells are innate lymphoid cells with potent cytolytic function toward viral invasion and prevent survival or

spread of tumor cells (Morvan and Lanier, 2016). NK cells have multiple activating receptors (e.g., NKG2D) and inhibitory receptors (e.g., killer-cell immunoglobulin-like receptors, KIRs), and the balance between these signals determines whether or not NK cells are activated (Fernandez-Messina et al., 2012; Sivori et al., 2019). NK cells are found to secrete EVs in a constitutive way and independent of their activation status (Lugini et al., 2012). Several studies reported that NK cell-derived EVs show cytotoxic activity against tumor cells (Fais, 2013; Zhu et al., 2017) and activate immune cells (Lugini et al., 2012).

3.1.4 Dendritic Cells

As the sentinel antigen-presenting cells (APCs) of the immune system, dendritic cells (DCs) function as the link between innate and adaptive immunity, leading to either antigen-specific immunity initiation or tolerance (Steinman, 2012). Like DCs, EVs secreted by DCs were found to possess functional MHC-peptide complexes, costimulatory molecules, and other components that interact with immune cells (Thery et al., 1999; Thery et al., 2001; Thery et al., 2002a). EVs secreted by mature DCs contain class II MHC complexes and costimulatory molecules, which can directly interact with T cells to activate the immune system (Segura et al., 2005). On the other hand, EVs secreted by immature DCs can regulate the immune response, but do not function in direct T cell activation (Quah and O'Neill, 2005). In addition, studies have shown that DC-derived EVs can be absorbed by epithelial cells and promote the release of inflammatory mediators (MCP-1, IL-8, TNF α , RANTES) secreted by epithelial cells, suggesting that EVs promote immune-inflammatory response (Obregon et al., 2009).

3.2 EVs and Adaptive Immune Cells

The adaptive immune cells mainly include T and B lymphocytes.

3.2.1 T Cells

DC-T cell interaction results in T cell activation. The interaction is transmitted from T cells to DCs via the transfer of EV-DNA, making DCs more resistant to infections (Torralba et al., 2018). EVs derived from activated CD3⁺ T cell together with IL-2 can modulate the activity of immune cells, including other T cells (Wahlgren et al., 2012). In addition, depending on their activation status, CD4⁺ T cells regulate the release of distinct vesicle subpopulations with various abilities to activate other untouched T cells (van der Vlist et al., 2012). T cell tolerance is shown due to EVs secreted by CD8⁺ suppressor T cells (Bryniarski et al., 2013). The protein expression profile of T cell EVs change substantially after different stimuli (activation vs apoptosis induction). Induction of apoptosis causes T cells to release more apoptotic bodies than exosomes, while activated T cells release exosomes and microvesicles both in lower amounts (Tucher et al., 2018). Studies have shown that Treg-derived EVs express immunomodulatory molecules (CD25, CD73, CTLA4), which have immunosuppressive effects and can regulate effector T cell proliferation and cytokine secretion to regulate immune response (Agarwal et al., 2014).

3.2.2 B Cells

EVs derived from B cells exert a predominant role in antigen presentation and immunoregulation. B cell-derived EVs can induce antigen-specific MHC II-restricted T cell responses, suggesting antigen presentation capacities just like B cells (Raposo et al., 1996). Different types of antigens, carried by B cell-derived EVs, may dictate different types of immune responses (Hood, 2017). Recently, it has been suggested that B cell-derived EVs may have immunoregulatory functions which are independent of their ability to present antigen (Zhang et al., 2019). In addition, the role of different lymphocytes subsets ($CD4^+$ T cells, $CD8^+$ T cells, and NK cells) and DCs in CTL immune response to antigen presented on B-cell derived EVs has been described, demonstrating a complex interplay of cooperating lymphocytes for EV immunogenicity (Saunderson and McLellan, 2017).

4 ROLE OF EVS IN SLE AND LN

4.1 EVs, SLE

EVs were found to be increased (Pereira et al., 2006; Sellam et al., 2009; Lee et al., 2016; Lopez et al., 2020) or decreased (Nielsen et al., 2011) in SLE patients compared to healthy controls. Proteins, mRNAs, miRNAs, lncRNAs, tsRNAs and other noncoding RNAs have been shown to be associated with EVs (Mathivanan et al., 2012; Barile and Vassalli, 2017; Mathieu et al., 2019). Recent studies have revealed that the ncRNAs play dominant roles in the pathogenesis of SLE (Long et al., 2018; Xie and Xu, 2018; Zhao et al., 2018; Chen et al., 2019; Liu et al., 2021). In this sense, miRNAs, lncRNAs, tsRNAs and proteins in SLE EVs might serve as biomarkers for disease diagnosis and therapeutic targets (Tan et al., 2016; Ortega et al., 2020).

4.1.1 EVs, miRNA, SLE

MiRNA is a type of single-stranded non-coding RNA with a length of about 19–24 nucleotides, which can regulate the expression of many genes *in vivo* and participate in the pathogenesis of many diseases. Abnormal expression of circulating miRNAs in SLE patients have been found, and some of these miRNAs are related to clinical parameters (Carlsen et al., 2013; Ishibe et al., 2018). Circulating miRNAs are extracellularly secreted miRNAs circulating in the peripheral blood, which are either encapsulated by extracellular vesicles such as exosomes and microvesicles or bound to molecules such as the Argonaute protein or HDL cholesterol (Arroyo et al., 2011; Vickers et al., 2011). Tan et al. (2021) have reported that compared with healthy controls, serum EV miR-451a was decreased in SLE patients, which correlated with SLE disease activity and renal damage (Tan et al., 2021). Moreover, they found that EV shuttled miR-451a was involved in intercellular communication (Tan et al., 2021). Li et al. (2020) demonstrated that compared with healthy controls, serum EV miR-21 and miR-155 of SLE patients were up-regulated, whereas the expression of miR-146a was down-regulated (Li et al., 2020). Additionally, the expression of miR-21 and miR-146a were negatively correlated with anti-SSA/Ro antibodies and anti-dsDNA antibodies,

respectively (Li et al., 2020). What's more, both EV miR-21 and miR-155 expression presented positive correlations with proteinuria. These findings indicated that the expression levels of EV miR-21 and miR-155 might serve as potential biomarkers for the diagnosis of SLE. The aforementioned studies, however, have some limitations yet to be addressed. The mechanism underlying the reported dysregulation of the EV-associated miRNAs expression and the cell origin of the EVs remain unclear in the studies, which are performed based on relatively limited samples.

With the continual advances in this thread, the mechanism of EV-associated miRNAs in SLE pathogenesis has been revealed gradually. It is shown that miR-574, let-7b and miR-21 in plasma EVs can activate plasmacytoid DCs (pDCs) through the TLR7 pathway, enabling them to continuously produce IFN- α and proinflammatory cytokines, which may contribute to the pathogenesis of SLE (Salvi et al., 2018). Another study suggests that miR-146a could be internalized into mesenchymal stem cells (MSCs) via circulating EVs and participates in MSCs senescence in SLE patients by targeting TRAF6/NF- κ B signal pathway (Dong et al., 2019).

4.1.2 EVs, lncRNA, SLE

lncRNA is another regulatory noncoding RNA longer than 200 nucleotides, capable of modulating many biological functions more specifically than miRNA. Aberrant circulating lncRNA expressions are found in SLE patients as well. Wu et al. (2017), Wu et al. (2019) found that plasma levels of GAS5, lnc7074 and lnc-DC were significantly reduced, whereas levels of linc0597, linc0640 and lnc5150 were elevated in SLE patients compared with those of healthy controls (Wu G.-C. et al., 2017; Wu et al., 2019). However, due to the complexity of its role, there is no literature regarding EV-associated lncRNA's role in the pathogenesis of SLE. Thus, this promising research line is worthwhile to be investigated.

4.1.3 EVs, tsRNA, SLE

Transfer RNAs (tRNAs) are a group of classic ncRNAs with a well-defined role in protein translation (Schulman and Abelson, 1988). tRNA-derived small RNAs (tsRNAs) are cleaved from precursor or mature tRNAs with a length of 18–40 nt and can be broadly classified into two main groups: tRNA halves and tRNA-derived fragments (tRFs) (Anderson and Ivanov, 2014). tRFs have been implicated to participate in diverse physiological processes and involved in many diseases through the protein synthesis by regulating mRNA expression (Zhu et al., 2018; Kim et al., 2020). In a recent study, Xu et al. (2020) found that tRNAs and tsRNAs were significantly differentially expressed in the PBMCs of SLE patients, compared with those of healthy donors, and the targeted genes of the differentially expressed tsRNAs were enriched in the signaling pathway involved in primary immunodeficiency, T cell receptor and Th cell differentiation, suggesting that tRNAs and tsRNAs play important roles in the pathogenesis of SLE (Xu et al., 2020). More precisely, Geng et al. (2021) showed that tRF-3009 was substantially over-expressed in $CD4^+$ T cells of SLE patients than those of healthy donors. What's more, tRF-3009 may be involved in SLE pathogenesis by modulation of IFN- α -induced

CD4⁺ T cell oxidative phosphorylation (Geng et al., 2021). As for the EV, Dou et al. (2021) revealed that mesenchymal stem cell (MSC)-derived EV tsRNA-21109 inhibited the M1-type polarization of macrophages, offering a promising therapeutic strategy for SLE (Dou et al., 2021). Nowadays, Yang et al. (2021) found that tRF-His-GTG-1 was significantly upregulated both in serum of SLE without LN, and in serum EVs of SLE with LN compared with healthy controls, suggesting that it could be employed as a noninvasive biomarker for diagnosis and prediction of nephritis in SLE (Yang et al., 2021). Nonetheless, the exact mechanism underlying tsRNA-21109 and tRF-His-GTG-1 mediated SLE development remains yet to be elucidated.

4.1.4 EVs, Protein, SLE

Proteins in EVs are also involved in the pathogenesis of SLE. Majer et al. (2019) found that UNC93B1 can be detected in RAW macrophage-derived EVs, it can limit TLR7 signaling and prevent TLR7-dependent autoimmunity in mice (Majer et al., 2019). Moreover, UNC93B1 mutation can enhance the TLR7 signaling pathway, leading to the development of autoimmune diseases (Majer et al., 2019). More recently, Chuang et al. (2021) proved that bactericidal/permeability-increasing protein (BPI) is a negative regulator of Treg differentiation (Chuang et al., 2021). They identified the overexpression of BPI in T cells and T cell-derived EVs contributed to autoimmune responses through both intrinsic (inhibition of Treg population) and extrinsic (induction of inflammatory EVs) pathways, which might be a biomarker and a pathogenic factor for SLE (Chuang et al., 2021). And just this month, they reported another EV-associated protein, Eosinophil Cationic Protein (ECP, also named RNase 3), which was overexpressed in SLE T cell-derived EVs (Chuang et al., 2022). What's more, ECP overexpression in T cells resulted in an increase of inflammatory responses and T-cell activation. Notably, ECP-containing EVs from T cells led to tissue inflammation of the recipient mice. These results suggest that ECP-overexpressing T cells or ECP-containing EVs may play an important role in SLE pathogenesis (Chuang et al., 2022). Nevertheless, except for the small sample size, it would be challenging yet essential to dig into the fundamental mechanisms of BPI/ECP-induced inflammation via EVs in the future.

4.2 EVs, LN

Lupus nephritis (LN) is one of the most devastating manifestations of SLE, and a primary cause of morbidity and mortality of SLE (Hahn et al., 2012). At present, renal biopsy is still the gold standard for diagnosis and evaluation of residual nephron function. Renal puncture, however, presents many perilous complications and can only reflect the state of a small part of the renal tissue. Therefore, pursuing a non-invasive and sensitive diagnostic method is urgently needed (Ortega et al., 2010; Morell et al., 2021). Over the past few years, changes in urinary miRNAs have been reported in LN patients, and its expression may be relevant to disease activity (So et al., 2021).

4.2.1 EVs, miRNA, LN

Urinary EV-associated miRNAs are promising novel markers for the diagnosis and prognosis of disease, and also clinical outcomes. Lv et al. (2013) showed that high levels of miRNA

were confined to urinary EVs in patients with a diversity of chronic diseases (Lv et al., 2013). Urinary EV in lupus nephritis was first described in 2014, Ichii et al. (2014) first found that in patients with lupus nephritis, the expression level of miR-26a in urinary EVs was significantly higher than that in the control group, and miR-26a expression was related to podocyte injury, suggesting that miR-26a can be used as a direct biomarker for podocyte injury in autoimmune glomerulonephritis (Ichii et al., 2014). Solé et al. (2015) showed reduced expression level of miR-29c in LN patients compared with healthy controls, and its level in urinary EVs was negatively correlated with the histological chronicity index and glomerular sclerosis, indicating that miR-29c level could be used as a novel non-invasive marker for predicting histological fibrosis of LN (Solé et al., 2015). As the research proceeds, the mechanism of urinary EV-associated miRNAs in LN pathogenesis has been gradually revealed. Recently, their team revealed that miR-21 and miR-150 were substantially up-regulated while miR-29c was down-regulated in the urinary EVs of LN patients, and their expression was strongly correlated with renal chronicity. They also demonstrated that these miRNAs promoted renal fibrosis through SP1 and Smad3/TGF β signaling pathway (Solé et al., 2019). And a more recent study by their team found that the overexpression of urinary EV miR-135b-5p, miR-107, and miR-31 could meliorate renal disease by inhibiting HIF-1 α , suggesting their potential to become early markers for predicting clinical response in LN (Garcia-Vives et al., 2020). Perez-Hernandez et al. (2015) confirmed that urinary miRNAs were contained mainly in EVs, and they reported an impressive increase in miRNA-146a in urinary EVs in patients with active lupus nephropathy, implying that miRNA-146a in EVs may be able to distinguish SLE patients with active LN from control group or SLE patients in absence of LN (Perez-Hernandez et al., 2015). Recently, their group identified a protective role that urinary EV miR-146a played in LN progression through negative regulation of inflammation by suppressing the TRAF6 axis (Perez-Hernandez et al., 2021). Furthermore, it has been evidenced that Type IV lupus nephritis with cellular crescent (LNIV-CC) has a unique urinary EV-associated miRNA expression profile, and urinary EV miR-3135b, miR-654-5p and miR-146a-5p are candidate biomarkers for LNIV-CC (Li et al., 2018). In addition, another study discovered that compared with inactive disease, let-7a and miR-21 in urine EVs were significantly down-regulated in LN patients with active disease. Interestingly, their expression increased after the entire course of treatment, indicating that urinary EV-related miRNA, let-7a and miR-21, may be leveraged to guide the clinical stage of LN patients (Tangtanatakul et al., 2019). The above findings indicate that miRNAs in urinary EVs may have great potential to serve as biomarkers in LN diagnosis and monitoring.

4.2.2 EVs, Protein, LN

Proteins in urine EVs are also involved in the pathogenesis of LN. Gudehithlu et al. (2019) found that urine EV ceruloplasmin (CP) was increased in LN patients. What's more, in biopsied cases, CP

was strongly localized to kidney tubules, suggesting that the CP found in urine EVs came from the kidney. Moreover, in mouse models, urine EV CP were observed to increase prior to proteinuria, indicating it could be an early biomarker to diagnose kidney disease (Gudehithlu et al., 2019).

These findings strongly suggest the potential role of urinary EVs as a non-invasive biomarker in the diagnosis and treatment of LN. Multiple limitations of the present studies, however, need to be acknowledged and are summarized as follows. Firstly, studies involving a reasonably larger patient cohort are necessary for further analyzing and validating these findings, especially for diagnosis. Secondly, most of the research works described above are cross-sectional studies. The longitudinal studies such as during disease flare or before and after treatment are also needed to further extend these findings. Thirdly, the thorough comparative analysis between the reported urinary EV-associated miRNAs/protein and the existing inflammatory and clinical markers of disease is missing and thus worthwhile to be performed in the further. Fourthly, the differentially expressed urinary EV-associated miRNAs/protein is delivered from various kidney cell types. Further study to characterize specific cell types that contribute to the dysregulation of miRNAs/protein in urine EVs is thus demanded. What's more, the present studies lack functional experiments at molecular or cellular level to verify the association between miRNAs/protein and LN. All these imperfections are likely due to the low recovery ration, low yield and purity of EV extraction as well as the immature technology of EV transfection and infection.

5 CONCLUSION

In recent years, EVs have emerged as an important endogenous “nanovehicles” for carrying and transferring molecular mediators such as nucleic acids, proteins, and bioactive lipids for intercellular communications and signal transduction. Besides,

it is well established that EVs are involved in a multitude of physiological and pathological processes, such as immune response, antigen presentation, cell differentiation, cell migration, and tumor invasion. A rapidly expanding body of evidence indicates that the presence of EV-specific patterns and their cargo play crucial physiological and pathological roles in SLE. In this context, miRNAs, tsRNAs, and proteins transported into serum/plasma/urinary EVs are correlated with glomerular damage, SLE disease activity, clinical stage and response, proteinuria as well as the severity of renal fibrosis in lupus nephritis. In this article, we provide the up-to-date survey of relevant literature evidencing that EVs have great potential in SLE disease diagnosis, prediction, prognosis, and targeted treatment (Figure 1 and Table 1). However, the underlying pathophysiological mechanisms of EVs in SLE pathogenesis and their functionality as therapeutic agents or targets are not fully understood. Future investigations into the exact mechanisms of EVs in SLE will undoubtedly bring new breakthroughs for SLE disease diagnosis and therapies.

AUTHOR CONTRIBUTIONS

LX and CZ analyzed the literature and wrote the manuscript. HQ, XL, XC, FL and LW assisted with constructing figures and polishing the language. LX, JX and XZ revised the manuscript. All authors listed have made substantial, direct, and intellectual contributions to the work, and approved the article for publication.

FUNDING

This work was supported by grants from the National Natural Science Foundation of China (81903216 and 81872526) and from Shanghai Science and Technology Committee (21YF1404500).

REFERENCES

- Agarwal, A., Fanelli, G., Letizia, M., Tung, S. L., Boardman, D., Lechler, R., et al. (2014). Regulatory T Cell-Derived Exosomes: Possible Therapeutic and Diagnostic Tools in Transplantation. *Front. Immunol.* 5, 555. doi:10.3389/fimmu.2014.00555
- Akers, J. C., Gonda, D., Kim, R., Carter, B. S., and Chen, C. C. (2013). Biogenesis of Extracellular Vesicles (EV): Exosomes, Microvesicles, Retrovirus-Like Vesicles, and Apoptotic Bodies. *J. Neurooncol.* 113 (1), 1–11. doi:10.1007/s11060-013-1084-8
- Anderson, H. C., Mulhall, D., and Garimella, R. (2010). Role of Extracellular Membrane Vesicles in the Pathogenesis of Various Diseases, Including Cancer, Renal Diseases, Atherosclerosis, and Arthritis. *Lab. Invest.* 90 (11), 1549–1557. doi:10.1038/labinvest.2010.152
- Anderson, P., and Ivanov, P. (2014). tRNA Fragments in Human Health and Disease. *FEBS Lett.* 588 (23), 4297–4304. doi:10.1016/j.febslet.2014.09.001
- Arellano-Anaya, Z. E., Huor, A., Leblanc, P., Lehmann, S., Provansal, M., Raposo, G., et al. (2015). Prion Strains are Differentially Released through the Exosomal Pathway. *Cell. Mol. Life Sci.* 72 (6), 1185–1196. doi:10.1007/s00018-014-1735-8
- Arroyo, J. D., Chevillet, J. R., Kroh, E. M., Ruf, I. K., Pritchard, C. C., Gibson, D. F., et al. (2011). Argonaute2 Complexes Carry a Population of Circulating microRNAs Independent of Vesicles in Human Plasma. *Proc. Natl. Acad. Sci.* 108 (12), 5003–5008. doi:10.1073/pnas.1019055108
- Barile, L., and Vassalli, G. (2017). Exosomes: Therapy Delivery Tools and Biomarkers of Diseases. *Pharmacol. Ther.* 174, 63–78. doi:10.1016/j.pharmthera.2017.02.020
- Bhatnagar, S., and Schorey, J. S. (2007). Exosomes Released from Infected Macrophages Contain *Mycobacterium A* Glycopeptidolipids and Are Proinflammatory. *J. Biol. Chem.* 282 (35), 25779–25789. doi:10.1074/jbc.M702277200
- Bhatnagar, S., Shinagawa, K., Castellino, F. J., and Schorey, J. S. (2007). Exosomes Released from Macrophages Infected with Intracellular Pathogens Stimulate a Proinflammatory Response *In Vitro* and *In Vivo*. *Blood* 110 (9), 3234–3244. doi:10.1182/blood-2007-03-079152
- Bryniarski, K., Ptak, W., Jayakumar, A., Püllmann, K., Caplan, M. J., Chairoungdua, A., et al. (2013). Antigen-Specific, Antibody-Coated, Exosome-Like Nanovesicles Deliver Suppressor T-Cell microRNA-150 to Effector T Cells to Inhibit Contact Sensitivity. *J. Allergy Clin. Immunol.* 132 (1), 170–181. doi:10.1016/j.jaci.2013.04.048
- Carlsen, A. L., Schetter, A. J., Nielsen, C. T., Lood, C., Knudsen, S., Voss, A., et al. (2013). Circulating microRNA Expression Profiles Associated with Systemic Lupus Erythematosus. *Arthritis Rheum.* 65 (5), 1324–1334. doi:10.1002/art.37890

- Chen, S., Wang, Y., Qin, H., Lin, J., Xie, L., Chen, S., et al. (2019). Downregulation of miR-633 Activated AKT/mTOR Pathway by Targeting AKT1 in Lupus CD4+ T Cells. *Lupus* 28 (4), 510–519. doi:10.1177/0961203319829853
- Cheruvanky, A., Zhou, H., Pisitkun, T., Kopp, J. B., Knepper, M. A., Yuen, P. S. T., et al. (2007). Rapid Isolation of Urinary Exosomal Biomarkers Using a Nanomembrane Ultrafiltration Concentrator. *Am. J. Physiology-Renal Physiol.* 292 (5), F1657–F1661. doi:10.1152/ajprenal.00434.2006
- Chuang, H.-C., Chen, M.-H., Chen, Y.-M., Yang, H.-Y., Ciou, Y.-R., Hsueh, C.-H., et al. (2021). BPI Overexpression Suppresses Treg Differentiation and Induces Exosome-Mediated Inflammation in Systemic Lupus Erythematosus. *Theranostics* 11 (20), 9953–9966. doi:10.7150/thno.63743
- Chuang, H. C., Chen, M. H., Chen, Y. M., Ciou, Y. R., Hsueh, C. H., Tsai, C. Y., et al. (2022). Induction of Interferon- γ and Tissue Inflammation by Overexpression of Eosinophil Cationic Protein in T Cells and Exosomes. *Arthritis Rheumatol.* 74 (1), 92–104. doi:10.1002/art.41920
- Crispin, J. C., Liossis, S.-N. C., Kis-Toth, K., Lieberman, L. A., Kyttaris, V. C., Juang, Y.-T., et al. (2010). Pathogenesis of Human Systemic Lupus Erythematosus: Recent Advances. *Trends Mol. Med.* 16 (2), 47–57. doi:10.1016/j.molmed.2009.12.005
- Dalli, J., Montero-Melendez, T., Norling, L. V., Yin, X., Hinds, C., Haskard, D., et al. (2013). Heterogeneity in Neutrophil Microparticles Reveals Distinct Proteome and Functional Properties. *Mol. Cell Proteomics* 12 (8), 2205–2219. doi:10.1074/mcp.M113.028589
- Dalli, J., Norling, L. V., Renshaw, D., Cooper, D., Leung, K.-Y., and Perretti, M. (2008). Annexin 1 Mediates the Rapid Anti-Inflammatory Effects of Neutrophil-Derived Microparticles. *Blood* 112 (6), 2512–2519. doi:10.1182/blood-2008-02-140533
- Dong, C., Zhou, Q., Fu, T., Zhao, R., Yang, J., Kong, X., et al. (2019). Circulating Exosomes Derived-miR-146a from Systemic Lupus Erythematosus Patients Regulates Senescence of Mesenchymal Stem Cells. *Biomed. Res. Int.* 2019, 6071308. doi:10.1155/2019/6071308
- Dou, R., Zhang, X., Xu, X., Wang, P., and Yan, B. (2021). Mesenchymal Stem Cell Exosomal tsRNA-21109 Alleviate Systemic Lupus Erythematosus by Inhibiting Macrophage M1 Polarization. *Mol. Immunol.* 139, 106–114. doi:10.1016/j.molimm.2021.08.015
- Eken, C., Gasser, O., Zenhausem, G., Oehri, I., Hess, C., and Schifferli, J. A. (2008). Polymorphonuclear Neutrophil-Derived Ectosomes Interfere with the Maturation of Monocyte-Derived Dendritic Cells. *J. Immunol.* 180 (2), 817–824. doi:10.4049/jimmunol.180.2.817
- Fais, S. (2013). NK Cell-Released Exosomes: Natural Nanobullets against Tumors. *Oncoimmunology* 2 (1), e22337. doi:10.4161/onci.22337
- Fernández-Messina, L., Reyburn, H. T., and Valés-Gómez, M. (2012). Human NKG2D-Ligands: Cell Biology Strategies to Ensure Immune Recognition. *Front. Immun.* 3, 299. doi:10.3389/fimmu.2012.00299
- García-Vives, E., Solé, C., Moliné, T., Vidal, M., Agraz, I., Ordi-Ros, J., et al. (2020). The Urinary Exosomal miRNA Expression Profile Is Predictive of Clinical Response in Lupus Nephritis. *Int. J. Mol. Sci.* 21 (4), 1372. doi:10.3390/ijms21041372
- Gasser, O., Hess, C., Miot, S., Deon, C., Sanchez, J.-C., and Schifferli, J. U. A. (2003). Characterisation and Properties of Ectosomes Released by Human Polymorphonuclear Neutrophils. *Exp. Cell Res.* 285 (2), 243–257. doi:10.1016/s0014-4827(03)00055-7
- Gasser, O., and Schifferli, J. A. (2004). Activated Polymorphonuclear Neutrophils Disseminate Anti-Inflammatory Microparticles by Ectocytosis. *Blood* 104 (8), 2543–2548. doi:10.1182/blood-2004-01-0361
- Gavins, F. N. E., and Hickey, M. J. (2012). Annexin A1 and the Regulation of Innate and Adaptive Immunity. *Front. Immun.* 3, 354. doi:10.3389/fimmu.2012.00354
- Geng, G., Wang, H., Xin, W., Liu, Z., Chen, J., Danting, Z., et al. (2021). tRNA Derived Fragment (tRF)-3009 Participates in Modulation of IFN- α -Induced CD4+ T Cell Oxidative Phosphorylation in Lupus Patients. *J. Transl. Med.* 19 (1), 305. doi:10.1186/s12967-021-02967-3
- Giri, P. K., and Schorey, J. S. (2008). Exosomes Derived from M. Bovis BCG Infected Macrophages Activate Antigen-Specific CD4+ and CD8+ T Cells *In Vitro* and *In Vivo*. *PLoS One* 3 (6), e2461. doi:10.1371/journal.pone.0002461
- Gudeithlu, K. P., Hart, P., Joshi, A., Garcia-Gomez, I., Cimbalku, D. J., Dunea, G., et al. (2019). Urine Exosomal Ceruloplasmin: A Potential Early Biomarker of Underlying Kidney Disease. *Clin. Exp. Nephrol.* 23 (8), 1013–1021. doi:10.1007/s10157-019-01734-5
- Gutiérrez-Vázquez, C., Villarroja-Beltri, C., Mittelbrunn, M., and Sánchez-Madrid, F. (2013). Transfer of Extracellular Vesicles during Immune Cell-Cell Interactions. *Immunol. Rev.* 251 (1), 125–142. doi:10.1111/imr.12013
- Hahn, B. H., McMahon, M. A., Wilkinson, A., Wallace, W. D., Daikh, D. I., Fitzgerald, J. D., et al. (2012). American College of Rheumatology Guidelines for Screening, Treatment, and Management of Lupus Nephritis. *Arthritis Care Res.* 64 (6), 797–808. doi:10.1002/acr.21664
- Hess, C., Sadallah, S., Hefti, A., Landmann, R., and Schifferli, J. A. (1999). Ectosomes Released by Human Neutrophils Are Specialized Functional Units. *J. Immunol.* 163 (8), 4564–4573.
- Hessvik, N. P., and Llorente, A. (2018). Current Knowledge on Exosome Biogenesis and Release. *Cel. Mol. Life Sci.* 75 (2), 193–208. doi:10.1007/s00018-017-2595-9
- Hood, J. L. (2017). The Association of Exosomes with Lymph Nodes. *Semin. Cel. Develop. Biol.* 67, 29–38. doi:10.1016/j.semcdb.2016.12.002
- Ichii, O., Otsuka-Kanazawa, S., Horino, T., Kimura, J., Nakamura, T., Matsumoto, M., et al. (2014). Decreased miR-26a Expression Correlates with the Progression of Podocyte Injury in Autoimmune Glomerulonephritis. *PLoS One* 9 (10), e110383. doi:10.1371/journal.pone.0110383
- Ishibe, Y., Kusaoi, M., Murayama, G., Nemoto, T., Kon, T., Ogasawara, M., et al. (2018). Changes in the Expression of Circulating microRNAs in Systemic Lupus Erythematosus Patient Blood Plasma after Passing through a Plasma Adsorption Membrane. *Ther. Apher. Dial.* 22 (3), 278–289. doi:10.1111/1744-9987.12695
- Jong, A. Y., Wu, C.-H., Li, J., Sun, J., Fabbri, M., Wayne, A. S., et al. (2017). Large-Scale Isolation and Cytotoxicity of Extracellular Vesicles Derived from Activated Human Natural Killer Cells. *J. Extracellular Vesicles* 6 (1), 1294368. doi:10.1080/20013078.2017.1294368
- Kim, H. K., Yeom, J.-H., and Kay, M. A. (2020). Transfer RNA-Derived Small RNAs: Another Layer of Gene Regulation and Novel Targets for Disease Therapeutics. *Mol. Ther.* 28 (11), 2340–2357. doi:10.1016/j.jymthe.2020.09.013
- Koh, Y. Q., Almughliq, F. B., Vaswani, K., Peiris, H. N., and Mitchell, M. D. (2018). Exosome Enrichment by Ultracentrifugation and Size Exclusion Chromatography. *Front. Biosci.* 23, 865–874. doi:10.2741/4621
- Konoshenko, M. Y., Lekchnov, E. A., Vlassov, A. V., and Laktionov, P. P. (2018). Isolation of Extracellular Vesicles: General Methodologies and Latest Trends. *Biomed. Res. Int.* 2018, 8545347. doi:10.1155/2018/8545347
- Lee, J. Y., Park, J. K., Lee, E. Y., Lee, E. B., and Song, Y. W. (2016). Circulating Exosomes from Patients with Systemic Lupus Erythematosus Induce a Proinflammatory Immune Response. *Arthritis Res. Ther.* 18 (1), 264. doi:10.1186/s13075-016-1159-y
- Lelifeld, P. H. C., Koenderman, L., and Pillay, J. (2015). How Neutrophils Shape Adaptive Immune Responses. *Front. Immunol.* 6, 471. doi:10.3389/fimmu.2015.00471
- Lewis, J. M., Vyas, A. D., Qiu, Y., Messer, K. S., White, R., and Heller, M. J. (2018). Integrated Analysis of Exosomal Protein Biomarkers on Alternating Current Electrokinetic Chips Enables Rapid Detection of Pancreatic Cancer in Patient Blood. *ACS Nano* 12 (4), 3311–3320. doi:10.1021/acsnano.7b08199
- Li, W., Liu, S., Chen, Y., Weng, R., Zhang, K., He, X., et al. (2020). Circulating Exosomal microRNAs as Biomarkers of Systemic Lupus Erythematosus. *Clinics (Sao Paulo)* 75, e1528. doi:10.6061/clinics/2020/e1528
- Li, Y., Xu, X., Tang, X., Bian, X., Shen, B., Zhao, H., et al. (2018). MicroRNA Expression Profile of Urinary Exosomes in Type IV Lupus Nephritis Complicated by Cellular Crescent. *J. Biol. Res-thessaloniki* 25, 16. doi:10.1186/s40709-018-0088-0
- Liu, X., Lin, J., Wu, H., Wang, Y., Xie, L., Wu, J., et al. (2021). A Novel Long Noncoding RNA lincRNA00892 Activates CD4(+) T Cells in Systemic Lupus Erythematosus by Regulating CD40L. *Front. Pharmacol.* 12, 733902. doi:10.3389/fphar.2021.733902
- Long, H., Wang, X., Chen, Y., Wang, L., Zhao, M., and Lu, Q. (2018). Dysregulation of microRNAs in Autoimmune Diseases: Pathogenesis, Biomarkers and Potential Therapeutic Targets. *Cancer Lett.* 428, 90–103. doi:10.1016/j.canlet.2018.04.016
- Lööv, C., Scherzer, C. R., Hyman, B. T., Breakefield, X. O., and Ingelsson, M. (2016). α -Synuclein in Extracellular Vesicles: Functional Implications and Diagnostic Opportunities. *Cell Mol Neurobiol* 36 (3), 437–448. doi:10.1007/s10571-015-0317-0
- López, P., Rodríguez-Carrio, J., Caminal-Montero, L., and Suárez, A. (2020). Relationship between T-Cell Exosomes and Cellular Subsets in SLE According to Type I IFN-Signaling. *Front. Med.* 7, 604098. doi:10.3389/fmed.2020.604098

- Lugini, L., Cecchetti, S., Huber, V., Luciani, F., Macchia, G., Spadaro, F., et al. (2012). Immune Surveillance Properties of Human NK Cell-Derived Exosomes. *J. Immunol.* 189 (6), 2833–2842. doi:10.4049/jimmunol.1101988
- Lv, L.-L., Cao, Y., Liu, D., Xu, M., Liu, H., Tang, R.-N., et al. (2013). Isolation and Quantification of microRNAs from Urinary Exosomes/microvesicles for Biomarker Discovery. *Int. J. Biol. Sci.* 9 (10), 1021–1031. doi:10.7150/ijbs.6100
- Maehara, M., Toyoda, E., Takahashi, T., Watanabe, M., and Sato, M. (2021). Potential of Exosomes for Diagnosis and Treatment of Joint Disease: Towards a Point-of-Care Therapy for Osteoarthritis of the Knee. *Int. J. Mol. Sci.* 22 (5), 2666. doi:10.3390/ijms22052666
- Majer, O., Liu, B., Kreuk, L. S. M., Krogan, N., and Barton, G. M. (2019). UNC93B1 Recruits Syntenin-1 to Dampen TLR7 Signalling and Prevent Autoimmunity. *Nature* 575 (7782), 366–370. doi:10.1038/s41586-019-1612-6
- Mashouri, L., Yousefi, H., Aref, A. R., Ahadi, A. M., Molaei, F., and Alahari, S. K. (2019). Exosomes: Composition, Biogenesis, and Mechanisms in Cancer Metastasis and Drug Resistance. *Mol. Cancer* 18 (1), 75. doi:10.1186/s12943-019-0991-5
- Mathieu, M., Martin-Jaular, L., Lavieu, G., and Théry, C. (2019). Specificities of Secretion and Uptake of Exosomes and Other Extracellular Vesicles for Cell-To-Cell Communication. *Nat. Cell Biol.* 21 (1), 9–17. doi:10.1038/s41556-018-0250-9
- Mathivanan, S., Fahner, C. J., Reid, G. E., and Simpson, R. J. (2012). ExoCarta 2012: Database of Exosomal Proteins, RNA and Lipids. *Nucleic Acids Res.* 40 (Database issue), D1241–D1244. doi:10.1093/nar/gkr828
- Matsumura, T., Sugimachi, K., Iinuma, H., Takahashi, Y., Kurashige, J., Sawada, G., et al. (2015). Exosomal microRNA in Serum Is a Novel Biomarker of Recurrence in Human Colorectal Cancer. *Br. J. Cancer* 113 (2), 275–281. doi:10.1038/bjc.2015.201
- Mayadas, T. N., Cullere, X., and Lowell, C. A. (2014). The Multifaceted Functions of Neutrophils. *Annu. Rev. Pathol. Mech. Dis.* 9, 181–218. doi:10.1146/annurev-pathol-020712-164023
- Miao, C., Zhou, W., Wang, X., and Fang, J. (2021). The Research Progress of Exosomes in Osteoarthritis, with Particular Emphasis on the Mediating Roles of miRNAs and lncRNAs. *Front. Pharmacol.* 12, 685623. doi:10.3389/fphar.2021.685623
- Momen-Heravi, F., Balaj, L., Alian, S., Tigges, J., Toxavidis, V., Ericsson, M., et al. (2012a). Alternative Methods for Characterization of Extracellular Vesicles. *Front. Physio.* 3, 354. doi:10.3389/fphys.2012.00354
- Momen-Heravi, F., Balaj, L., Alian, S., Trachtenberg, A. J., Hochberg, F. H., Skog, J., et al. (2012b). Impact of Biofluid Viscosity on Size and Sedimentation Efficiency of the Isolated Microvesicles. *Front. Physio.* 3, 162. doi:10.3389/fphys.2012.00162
- Monguió-Tortajada, M., Gálvez-Montón, C., Bayes-Genis, A., Roura, S., and Borrás, F. E. (2019). Extracellular Vesicle Isolation Methods: Rising Impact of Size-Exclusion Chromatography. *Cel. Mol. Life Sci.* 76 (12), 2369–2382. doi:10.1007/s00018-019-03071-y
- Morell, M., Pérez-Cózar, F., and Marañón, C. (2021). Immune-Related Urine Biomarkers for the Diagnosis of Lupus Nephritis. *Int. J. Mol. Sci.* 22 (13), 7143. doi:10.3390/ijms22137143
- Morvan, M. G., and Lanier, L. L. (2016). NK Cells and Cancer: You Can Teach Innate Cells New Tricks. *Nat. Rev. Cancer* 16 (1), 7–19. doi:10.1038/nrc.2015.5
- Natasha, G., Gundogan, B., Tan, A., Farhatnia, Y., Wu, W., Rajadas, J., et al. (2014). Exosomes as Immunotherapeutic Nanoparticles. *Clin. Ther.* 36 (6), 820–829. doi:10.1016/j.clinthera.2014.04.019
- Nath, S., Agholme, L., Kurudenkandy, F. R., Granseth, B., Marcusson, J., and Hallbeck, M. (2012). Spreading of Neurodegenerative Pathology via Neuron-To-Neuron Transmission of α -Amyloid. *J. Neurosci.* 32 (26), 8767–8777. doi:10.1523/jneurosci.0615-12.2012
- Nielsen, C. T., Østergaard, O., Johnsen, C., Jacobsen, S., and Heegaard, N. H. H. (2011). Distinct Features of Circulating Microparticles and Their Relationship to Clinical Manifestations in Systemic Lupus Erythematosus. *Arthritis Rheum.* 63 (10), 3067–3077. doi:10.1002/art.30499
- Obregon, C., Rothen-Rutishauser, B., Gerber, P., Gehr, P., and Nicod, L. P. (2009). Active Uptake of Dendritic Cell-Derived Exovesicles by Epithelial Cells Induces the Release of Inflammatory Mediators through a TNF- α -Mediated Pathway. *Am. J. Pathol.* 175 (2), 696–705. doi:10.2353/ajpath.2009.080716
- Ortega, A., Martínez-Arroyo, O., Forner, M. J., and Cortes, R. (2020). Exosomes as Drug Delivery Systems: Endogenous Nanovehicles for Treatment of Systemic Lupus Erythematosus. *Pharmaceutics* 13 (1), 3. doi:10.3390/pharmaceutics13010003
- Ortega, L., Schultz, D., Lenz, O., Pardo, V., and Contreras, G. (2010). Review: Lupus Nephritis: Pathologic Features, Epidemiology and a Guide to Therapeutic Decisions. *Lupus* 19 (5), 557–574. doi:10.1177/0961203309358187
- Pan, B.-T., and Johnstone, R. M. (1983). Fate of the Transferrin Receptor during Maturation of Sheep Reticulocytes *In Vitro*: Selective Externalization of the Receptor. *Cell* 33 (3), 967–978. doi:10.1016/0092-8674(83)90040-5
- Pereira, J., Alfaro, G., Goycoolea, M., Quiroga, T., Ocqueteau, M., Massardo, L., et al. (2006). Circulating Platelet-Derived Microparticles in Systemic Lupus Erythematosus. Association with Increased Thrombin Generation and Procoagulant State. *Thromb. Haemost.* 95 (1), 94–99. doi:10.1160/TH05-05-0310
- Perez-Hernandez, J., and Cortes, R. (2015). Extracellular Vesicles as Biomarkers of Systemic Lupus Erythematosus. *Dis. Markers* 2015, 613536. doi:10.1155/2015/613536
- Perez-Hernandez, J., Forner, M. J., Pinto, C., Chaves, F. J., Cortes, R., and Redon, J. (2015). Increased Urinary Exosomal MicroRNAs in Patients with Systemic Lupus Erythematosus. *PLoS One* 10 (9), e0138618. doi:10.1371/journal.pone.0138618
- Perez-Hernandez, J., Martínez-Arroyo, O., Ortega, A., Galera, M., Solis-Salguero, M. A., Chaves, F. J., et al. (2021). Urinary Exosomal miR-146a as a Marker of Albuminuria, Activity Changes and Disease Flares in Lupus Nephritis. *J. Nephrol.* 34 (4), 1157–1167. doi:10.1007/s40620-020-00832-y
- Perez-Hernandez, J., Redon, J., and Cortes, R. (2017). Extracellular Vesicles as Therapeutic Agents in Systemic Lupus Erythematosus. *Int. J. Mol. Sci.* 18 (4), 717. doi:10.3390/ijms18040717
- Quah, B. J. C., and O'Neill, H. C. (2005). The Immunogenicity of Dendritic Cell-Derived Exosomes. *Blood Cell Mol. Dis.* 35 (2), 94–110. doi:10.1016/j.bcmd.2005.05.002
- Ramachandra, L., Qu, Y., Wang, Y., Lewis, C. J., Cobb, B. A., Takatsu, K., et al. (2010). *Mycobacterium T* Synergizes with ATP to Induce Release of Microvesicles and Exosomes Containing Major Histocompatibility Complex Class II Molecules Capable of Antigen Presentation. *Infect. Immun.* 78 (12), 5116–5125. doi:10.1128/IAI.01089-09
- Ramirez, M. I., Amorim, M. G., Gadelha, C., Milic, I., Welsh, J. A., Freitas, V. M., et al. (2018). Technical Challenges of Working with Extracellular Vesicles. *Nanoscale* 10 (3), 881–906. doi:10.1039/c7nr08360b
- Raposo, G., Nijman, H. W., Stoorvogel, W., Liejendekker, R., Harding, C. V., Melief, C. J., et al. (1996). B Lymphocytes Secrete Antigen-Presenting Vesicles. *J. Exp. Med.* 183 (3), 1161–1172. doi:10.1084/jem.183.3.1161
- Raposo, G., and Stoorvogel, W. (2013). Extracellular Vesicles: Exosomes, Microvesicles, and Friends. *J. Cell Biol.* 200 (4), 373–383. doi:10.1083/jcb.201211138
- Rayamajhi, S., Nguyen, T. D. T., Marasini, R., and Aryal, S. (2019). Macrophage-derived Exosome-Mimetic Hybrid Vesicles for Tumor Targeted Drug Delivery. *Acta Biomater.* 94, 482–494. doi:10.1016/j.actbio.2019.05.054
- Record, M., Subra, C., Silvente-Poirot, S., and Poirot, M. (2011). Exosomes as Intercellular Signalosomes and Pharmacological Effectors. *Biochem. Pharmacol.* 81 (10), 1171–1182. doi:10.1016/j.bcp.2011.02.011
- Reis, M., Mavin, E., Nicholson, L., Green, K., Dickinson, A. M., and Wang, X.-N. (2018). Mesenchymal Stromal Cell-Derived Extracellular Vesicles Attenuate Dendritic Cell Maturation and Function. *Front. Immunol.* 9, 2538. doi:10.3389/fimmu.2018.02538
- Salvi, V., Gianello, V., Busatto, S., Bergese, P., Andreoli, L., D'Oro, U., et al. (2018). Exosome-Delivered microRNAs Promote IFN- α Secretion by Human Plasmacytoid DCs via TLR7. *JCI Insight* 3 (10), e98204. doi:10.1172/jci.insight.98204
- Saraste, A., and Pulkki, K. (2000). Morphologic and Biochemical Hallmarks of Apoptosis. *Cardiovasc. Res.* 45 (3), 528–537. doi:10.1016/s0008-6363(99)00384-3
- Saunderson, S. C., and McLellan, A. D. (2017). Role of Lymphocyte Subsets in the Immune Response to Primary B Cell-Derived Exosomes. *J. Immunol.* 199 (7), 2225–2235. doi:10.4049/jimmunol.1601537
- Schulman, L. H., and Abelson, J. (1988). Recent Excitement in Understanding Transfer RNA Identity. *Science* 240 (4859), 1591–1592. doi:10.1126/science.2454505
- Segura, E., Amigorena, S., and Théry, C. (2005). Mature Dendritic Cells Secrete Exosomes with strong Ability to Induce Antigen-specific Effector Immune Responses. *Blood Cell Mol. Dis.* 35 (2), 89–93. doi:10.1016/j.bcmd.2005.05.003

- Sellam, J., Proulle, V., Jüngel, A., Ittah, M., Miceli Richard, C., Gottenberg, J.-E., et al. (2009). Increased Levels of Circulating Microparticles in Primary Sjögren's Syndrome, Systemic Lupus Erythematosus and Rheumatoid Arthritis and Relation with Disease Activity. *Arthritis Res. Ther.* 11 (5), R156. doi:10.1186/ar2833
- Shah, R., Patel, T., and Freedman, J. E. (2018). Circulating Extracellular Vesicles in Human Disease. *N. Engl. J. Med.* 379 (10), 958–966. doi:10.1056/NEJMra1704286
- Shen, G., Krienke, S., Schiller, P., Niessen, A., Neu, S., Eckstein, V., et al. (2017). Microvesicles Released by Apoptotic Human Neutrophils Suppress Proliferation and IL-2/IL-2 Receptor Expression of Resting T Helper Cells. *Eur. J. Immunol.* 47 (5), 900–910. doi:10.1002/eji.201546203
- Simons, M., and Raposo, G. (2009). Exosomes - Vesicular Carriers for Intercellular Communication. *Curr. Opin. Cell Biol.* 21 (4), 575–581. doi:10.1016/j.ccb.2009.03.007
- Singh, P. P., Smith, V. L., Karakousis, P. C., and Schorey, J. S. (2012). Exosomes Isolated from Mycobacteria-Infected Mice or Cultured Macrophages Can Recruit and Activate Immune Cells *In Vitro* and *In Vivo*. *J. Immunol.* 189 (2), 777–785. doi:10.4049/jimmunol.1103638
- Sivori, S., Vacca, P., Del Zotto, G., Munari, E., Mingari, M. C., and Moretta, L. (2019). Human NK Cells: Surface Receptors, Inhibitory Checkpoints, and Translational Applications. *Cell Mol Immunol* 16 (5), 430–441. doi:10.1038/s41423-019-0206-4
- Slater, T. W., Finkelsztain, A., Mascarenhas, L. A., Mehl, L. C., Butin-Israeli, V., and Sumagin, R. (2017). Neutrophil Microparticles Deliver Active Myeloperoxidase to Injured Mucosa to Inhibit Epithelial Wound Healing. *J. Immunol.* 198 (7), 2886–2897. doi:10.4049/jimmunol.1601810
- So, B. Y. F., Yap, D. Y. H., and Chan, T. M. (2021). MicroRNAs in Lupus Nephritis-Role in Disease Pathogenesis and Clinical Applications. *Int. J. Mol. Sci.* 22 (19), 10737. doi:10.3390/ijms221910737
- Solé, C., Cortés-Hernández, J., Felip, M. L., Vidal, M., and Ordi-Ros, J. (2015). miR-29c in Urinary Exosomes as Predictor of Early Renal Fibrosis in Lupus Nephritis. *Nephrol. Dial. Transpl.* 30 (9), 1488–1496. doi:10.1093/ndt/gfv128
- Solé, C., Moliné, T., Vidal, M., Ordi-Ros, J., and Cortés-Hernández, J. (2019). An Exosomal Urinary miRNA Signature for Early Diagnosis of Renal Fibrosis in Lupus Nephritis. *Cells* 8 (8), 773. doi:10.3390/cells8080773
- Stahl, P. D., and Raposo, G. (2019). Extracellular Vesicles: Exosomes and Microvesicles, Integrators of Homeostasis. *Physiology* 34 (3), 169–177. doi:10.1152/physiol.00045.2018
- Steinman, R. M. (2012). Decisions about Dendritic Cells: Past, Present, and Future. *Annu. Rev. Immunol.* 30, 1–22. doi:10.1146/annurev-immunol-100311-102839
- Tan, L., Wu, H., Liu, Y., Zhao, M., Li, D., and Lu, Q. (2016). Recent Advances of Exosomes in Immune Modulation and Autoimmune Diseases. *Autoimmunity* 49 (6), 357–365. doi:10.1080/08916934.2016.1191477
- Tan, L., Zhao, M., Wu, H., Zhang, Y., Tong, X., Gao, L., et al. (2021). Downregulated Serum Exosomal miR-451a Expression Correlates with Renal Damage and its Intercellular Communication Role in Systemic Lupus Erythematosus. *Front. Immunol.* 12, 630112. doi:10.3389/fimmu.2021.630112
- Tangtanatakul, P., Klinchanhom, S., Sodsai, P., Sutichet, T., Promjeen, C., Avihingsanon, Y., et al. (2019). Down-regulation of Let-7a and miR-21 in Urine Exosomes from Lupus Nephritis Patients during Disease Flare. *Asian Pac. J. Allergy Immunol.* 37 (4), 189–197. doi:10.12932/ap-130318-0280
- Taylor, J., and Bebaawy, M. (2019). Proteins Regulating Microvesicle Biogenesis and Multidrug Resistance in Cancer. *Proteomics* 19 (1–2), e1800165. doi:10.1002/pmic.201800165
- Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., et al. (2018). Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): A Position Statement of the International Society for Extracellular Vesicles and Update of the MISEV2014 Guidelines. *J. Extracell. Vesicles* 7 (1), 1535750. doi:10.1080/20013078.2018.1535750
- Théry, C., Boussac, M., Véron, P., Ricciardi-Castagnoli, P., Raposo, G., Garin, J., et al. (2001). Proteomic Analysis of Dendritic Cell-Derived Exosomes: A Secreted Subcellular Compartment Distinct from Apoptotic Vesicles. *J. Immunol.* 166 (12), 7309–7318. doi:10.4049/jimmunol.166.12.7309
- Théry, C., Duban, L., Segura, E., Véron, P., Lantz, O., and Amigorena, S. (2002a). Indirect Activation of Naïve CD4⁺ T Cells by Dendritic Cell-Derived Exosomes. *Nat. Immunol.* 3 (12), 1156–1162. doi:10.1038/nri854
- Théry, C., Regnault, A., Garin, J., Wolfers, J., Zitvogel, L., Ricciardi-Castagnoli, P., et al. (1999). Molecular Characterization of Dendritic Cell-Derived Exosomes. Selective Accumulation of the Heat Shock Protein Hsc73. *J. Cell Biol.* 147 (3), 599–610. doi:10.1083/jcb.147.3.599
- Théry, C., Zitvogel, L., and Amigorena, S. (2002b). Exosomes: Composition, Biogenesis and Function. *Nat. Rev. Immunol.* 2 (8), 569–579. doi:10.1038/nri855
- Timár, C. I., Lőrincz, Á. M., Csépanyi-Kömi, R., Vályi-Nagy, A., Nagy, G., Buzás, E. I., et al. (2013). Antibacterial Effect of Microvesicles Released from Human Neutrophilic Granulocytes. *Blood* 121 (3), 510–518. doi:10.1182/blood-2012-05-431114
- Torralba, D., Baixauli, F., Villarroya-Beltri, C., Fernández-Delgado, I., Latorre-Pellicer, A., Acín-Pérez, R., et al. (2018). Priming of Dendritic Cells by DNA-Containing Extracellular Vesicles from Activated T Cells through Antigen-Driven Contacts. *Nat. Commun.* 9 (1), 2658. doi:10.1038/s41467-018-05077-9
- Tsokos, G. C. (2011). Systemic Lupus Erythematosus. *N. Engl. J. Med.* 365 (22), 2110–2121. doi:10.1056/NEJMra1100359
- Tucher, C., Bode, K., Schiller, P., Classen, L., Birr, C., Souto-Carneiro, M. M., et al. (2018). Extracellular Vesicle Subtypes Released from Activated or Apoptotic T-Lymphocytes Carry a Specific and Stimulus-Dependent Protein Cargo. *Front. Immunol.* 9, 534. doi:10.3389/fimmu.2018.00534
- van der Vlist, E. J., Arksteijn, G. J. A., van de Lest, C. H. A., Stoorvogel, W., Nolte-’t Hoen, E. N. M., and Wauben, M. H. M. (2012). CD4(+) T Cell Activation Promotes the Differential Release of Distinct Populations of Nanosized Vesicles. *J. Extracellular Vesicles* 1, 18364. doi:10.3402/jev.v1i0.18364
- Vickers, K. C., Palmisano, B. T., Shoucri, B. M., Shamburek, R. D., and Remaley, A. T. (2011). MicroRNAs Are Transported in Plasma and Delivered to Recipient Cells by High-Density Lipoproteins. *Nat. Cell Biol.* 13 (4), 423–433. doi:10.1038/ncb2210
- Wahlgen, J., Karlson, T. D. L., Glader, P., Telemo, E., and Valadi, H. (2012). Activated Human T Cells Secrete Exosomes that Participate in IL-2 Mediated Immune Response Signaling. *PLoS One* 7 (11), e49723. doi:10.1371/journal.pone.0049723
- Wang, Y., Zhao, M., Liu, S., Guo, J., Lu, Y., Cheng, J., et al. (2020). Macrophage-Derived Extracellular Vesicles: Diverse Mediators of Pathology and Therapeutics in Multiple Diseases. *Cell Death Dis* 11 (10), 924. doi:10.1038/s41419-020-03127-z
- Wu, G.-C., Hu, Y., Guan, S.-Y., Ye, D.-Q., and Pan, H.-F. (2019). Differential Plasma Expression Profiles of Long Non-Coding RNAs Reveal Potential Biomarkers for Systemic Lupus Erythematosus. *Biomolecules* 9 (6), 206. doi:10.3390/biom9060206
- Wu, G.-C., Li, J., Leng, R.-X., Li, X.-P., Li, X.-M., Wang, D.-G., et al. (2017a). Identification of Long Non-coding RNAs GAS5, Linc0597 and Lnc-DC in Plasma as Novel Biomarkers for Systemic Lupus Erythematosus. *Oncotarget* 8 (14), 23650–23663. doi:10.18632/oncotarget.15569
- Wu, M., Ouyang, Y., Wang, Z., Zhang, R., Huang, P.-H., Chen, C., et al. (2017b). Isolation of Exosomes from Whole Blood by Integrating Acoustics and Microfluidics. *Proc. Natl. Acad. Sci. USA* 114 (40), 10584–10589. doi:10.1073/pnas.1709210114
- Wu, W.-C., Song, S.-J., Zhang, Y., and Li, X. (2020). Role of Extracellular Vesicles in Autoimmune Pathogenesis. *Front. Immunol.* 11, 579043. doi:10.3389/fimmu.2020.579043
- Wynn, T. A., and Vannella, K. M. (2016). Macrophages in Tissue Repair, Regeneration, and Fibrosis. *Immunity* 44 (3), 450–462. doi:10.1016/j.immuni.2016.02.015
- Xie, L., and Xu, J. (2018). Role of MiR-98 and its Underlying Mechanisms in Systemic Lupus Erythematosus. *J. Rheumatol.* 45 (10), 1397–1405. doi:10.3899/jrheum.171290
- Xu, H., Chen, W., Zheng, F., Tang, D., Dai, W., Huang, S., et al. (2020). The Potential Role of tRNAs and Small RNAs Derived from tRNAs in the Occurrence and Development of Systemic Lupus Erythematosus. *Biochem. Biophysical Res. Commun.* 527 (2), 561–567. doi:10.1016/j.bbrc.2020.04.114
- Yáñez-Mó, M., Siljander, P. R.-M., Andreu, Z., Bedina Zavec, A., Borrás, F. E., Buzas, E. I., et al. (2015). Biological Properties of Extracellular Vesicles and Their Physiological Functions. *J. Extracellular Vesicles* 4, 27066. doi:10.3402/jev.v4.27066
- Yang, P., Zhang, X., Chen, S., Tao, Y., Ning, M., Zhu, Y., et al. (2021). A Novel Serum tsRNA for Diagnosis and Prediction of Nephritis in SLE. *Front. Immunol.* 12, 735105. doi:10.3389/fimmu.2021.735105
- Zhang, B., Yin, Y., Lai, R. C., and Lim, S. K. (2014). Immunotherapeutic Potential of Extracellular Vesicles. *Front. Immunol.* 5, 518. doi:10.3389/fimmu.2014.00518

- Zhang, B., Zhao, M., and Lu, Q. (2020). Extracellular Vesicles in Rheumatoid Arthritis and Systemic Lupus Erythematosus: Functions and Applications. *Front. Immunol.* 11, 575712. doi:10.3389/fimmu.2020.575712
- Zhang, F., Li, R., Yang, Y., Shi, C., Shen, Y., Lu, C., et al. (2019). Specific Decrease in B-Cell-Derived Extracellular Vesicles Enhances Post-Chemotherapeutic CD8(+) T Cell Responses. *Immunity* 50 (3), 738–750. doi:10.1016/j.immuni.2019.01.010
- Zhao, C.-N., Mao, Y.-M., Liu, L.-N., Li, X.-M., Wang, D.-G., and Pan, H.-F. (2018). Emerging Role of lncRNAs in Systemic Lupus Erythematosus. *Biomed. Pharmacother.* 106, 584–592. doi:10.1016/j.biopha.2018.06.175
- Zhao, Y., Wei, W., and Liu, M.-L. (2020). Extracellular Vesicles and Lupus Nephritis - New Insights into Pathophysiology and Clinical Implications. *J. Autoimmun.* 115, 102540. doi:10.1016/j.jaut.2020.102540
- Zhou, M., Tan, K. S., Guan, W.-J., Jiang, L.-J., Deng, J., Gao, W.-X., et al. (2020). Proteomics Profiling of Epithelium-Derived Exosomes from Nasal Polyps Revealed Signaling Functions Affecting Cellular Proliferation. *Respir. Med.* 162, 105871. doi:10.1016/j.rmed.2020.105871
- Zhu, L., Kalimuthu, S., Gangadaran, P., Oh, J. M., Lee, H. W., Baek, S. H., et al. (2017). Exosomes Derived from Natural Killer Cells Exert Therapeutic Effect in Melanoma. *Theranostics* 7 (10), 2732–2745. doi:10.7150/thno.18752
- Zhu, L., Liu, X., Pu, W., and Peng, Y. (2018). tRNA-Derived Small Non-Coding RNAs in Human Disease. *Cancer Lett.* 419, 1–7. doi:10.1016/j.canlet.2018.01.015
- Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
- Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.
- Copyright © 2022 Zheng, Xie, Qin, Liu, Chen, Lv, Wang, Zhu and Xu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



A Novel Tissue Atlas and Online Tool for the Interrogation of Small RNA Expression in Human Tissues and Biofluids

OPEN ACCESS

Edited by:

Georg Haase,
INSERM U1106 Institut de
Neurosciences des Systèmes, France

Reviewed by:

Axel Freischmidt,
University of Ulm, Germany
Silvia Fischer,
University of Giessen, Germany

*Correspondence:

Kendall Van Keuren-Jensen
kjensen@tgen.org

[†]These authors have contributed
equally to this work and share first
authorship

[‡]These authors share senior
authorship

Specialty section:

This article was submitted to
Membrane Traffic,
a section of the journal
Frontiers in Cell and Developmental
Biology

Received: 28 October 2021

Accepted: 28 January 2022

Published: 04 March 2022

Citation:

Alsop E, Meechoovet B, Kitchen R,
Sweeney T, Beach TG, Serrano GE,
Hutchins E, Ghiran I, Reiman R,
Syring M, Hsieh M, Courtright-Lim A,
Valkov N, Whitsett TG, Rakela J,
Pockros P, Rozowsky J, Gallego J,
Huentelman MJ, Shah R, Nakaji P,
Kalani MYS, Laurent L, Das S and
Van Keuren-Jensen K (2022) A Novel
Tissue Atlas and Online Tool for the
Interrogation of Small RNA Expression
in Human Tissues and Biofluids.
Front. Cell Dev. Biol. 10:804164.
doi: 10.3389/fcell.2022.804164

Eric Alsop^{1†}, Bessie Meechoovet^{1†}, Robert Kitchen², Thadryan Sweeney²,
Thomas G. Beach³, Geidy E. Serrano³, Elizabeth Hutchins¹, Ionita Ghiran⁴,
Rebecca Reiman¹, Michael Syring¹, Michael Hsieh¹, Amanda Courtright-Lim¹,
Nedyalka Valkov², Timothy G. Whitsett¹, Jorge Rakela⁵, Paul Pockros⁶, Joel Rozowsky⁷,
Juan Gallego^{8,9,10}, Matthew J. Huentelman¹, Ravi Shah², Peter Nakaji¹¹,
M. Yashar S. Kalani¹², Louise Laurent¹³, Saumya Das^{2‡} and Kendall Van Keuren-Jensen^{1*‡}

¹Neurogenomics Division, The Translational Genomics Research Institute, Phoenix, AZ, United States, ²Cardiovascular Research Center, Massachusetts General Hospital and Harvard Medical School, Boston, MA, United States, ³Banner Sun Health Research Institute, Sun City, AZ, United States, ⁴Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, United States, ⁵Mayo Clinic, Scottsdale, AZ, United States, ⁶Division of Gastroenterology/Hepatology, Scripps Clinic, La Jolla, CA, United States, ⁷Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, United States, ⁸Institute for Behavioral Science, The Feinstein Institute for Medical Research, Manhasset, NY, United States, ⁹Division of Psychiatry Research, The Zucker Hillside Hospital, Glen Oaks, NY, United States, ¹⁰Department of Psychiatry, Zucker School of Medicine at Hofstra/Northwell, Hempstead, NY, United States, ¹¹Department of Neurosurgery, Banner Health, Phoenix, AZ, United States, ¹²Department of Neurosurgery, St. John Medical Center, Tulsa, OK, United States, ¹³Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Diego, San Diego, CA, United States

One promising goal for utilizing the molecular information circulating in biofluids is the discovery of clinically useful biomarkers. Extracellular RNAs (exRNAs) are one of the most diverse classes of molecular cargo, easily assayed by sequencing and with expressions that rapidly change in response to subject status. Despite diverse exRNA cargo, most evaluations from biofluids have focused on small RNA sequencing and analysis, specifically on microRNAs (miRNAs). Another goal of characterizing circulating molecular information, is to correlate expression to injuries associated with specific tissues of origin. Biomarker candidates are often described as being specific, enriched in a particular tissue or associated with a disease process. Likewise, miRNA data is often reported to be specific, enriched for a tissue, without rigorous testing to support the claim. Here we provide a tissue atlas of small RNAs from 30 different tissues and three different blood cell types. We analyzed the tissues for enrichment of small RNA sequences and assessed their expression in biofluids: plasma, cerebrospinal fluid, urine, and saliva. We employed published data sets representing physiological (resting vs. acute exercise) and pathologic states (early- vs. late-stage liver fibrosis, and differential subtypes of stroke) to determine differential tissue-enriched small RNAs. We also developed an online tool that provides information about exRNA sequences found in different biofluids and tissues. The data can be used to better understand the various types of small RNA sequences in different tissues as well as their potential release into biofluids, which should help in the validation or design of biomarker studies.

Keywords: small RNA, plasma, urine, saliva, cerebrospinal fluid, extracellular vesicle, tissue atlas, extracellular RNA

INTRODUCTION

Extracellular RNAs (exRNAs) have been identified in every biofluid tested to-date (Yáñez-Mó et al., 2015; Yeri et al., 2017; Murillo et al., 2019; Srinivasan et al., 2019; Hulstaert et al., 2020) and are found within extracellular vesicles (EVs) and associated with RNA binding proteins or lipoprotein particles (Valadi et al., 2007; Skog et al., 2008; Arroyo et al., 2011; Turchinovich et al., 2011; Tabet et al., 2014). While longer RNAs (>200 nucleotides) can be detected in biofluids (Wei et al., 2017; Everaert et al., 2019; Hulstaert et al., 2020; Rodosthenous et al., 2020), most studies have focused on small RNA biotypes (<50 nucleotides) (Weiland et al., 2012; Max et al., 2018) for their potential as biomarkers (Witwer, 2015; Nik Mohamed Kamal and Shahidan, 2019). Each biofluid has a distinct small RNA profile. For example, plasma has large amounts of miRNAs, but also high levels of YRNA fragments (Dhahbi et al., 2013; Max et al., 2018; Driedonks et al., 2020). Urine and cerebrospinal fluid (CSF) samples, by comparison, have large numbers of tRNA fragments (tRFs), and smaller numbers of miRNAs (Ben-Dov et al., 2016; Yeri et al., 2017; Godoy et al., 2018; Srinivasan et al., 2019; Tosar and Cayota, 2020). Biofluids also contain measurable piRNA, snoRNA, snRNA, rRNA, fragments of protein-coding genes and lncRNAs found in biofluids (Ishikawa et al., 2017; Yeri et al., 2017; Ferrero et al., 2018; Umu et al., 2018; Akat et al., 2019; Dhahbi et al., 2019; Srinivasan et al., 2019).

These distinct RNA profiles in biofluids suggest coordination of biological processes, such as specific cargo loading into EVs [reviewed in (O'Brien et al., 2020)] and enhanced EV contributions from organs that produce or are more proximally perfused by the biofluid interrogated. While blood cells certainly contribute to RNA patterns found in plasma/serum (Savelyeva et al., 2017; Max et al., 2018), the contributions of specific tissues to acellular biofluids such as urine and CSF are poorly investigated (Cui and Cui, 2020). These are important considerations as many biomarker studies place greater importance on “tissue-specific” miRNAs even though the data used to support these designations are generally poorly defined. Differences in less-studied circulating exRNA biotypes (such as tRNAs/tRFs) have exhibited increased tissue and disease specificity (Magee et al., 2019; Qu et al., 2019; Maleki Dana et al., 2020). For example, the abundance of tRNAs varies temporally and between tissues, and are demonstrated to play a role in a variety of diseases (Dittmar et al., 2006; Sagi et al., 2016; Pan, 2018). piRNAs have also been examined for somatic expression and tissue-enrichment (Martinez et al., 2015; Perera et al., 2019; Chen et al., 2020). While substantial, long RNA tissue atlases have been developed (GTEx, FANTOM, Human Body Atlas), there are fewer, less complete tissue atlases for small RNAs available (Landgraf et al., 2007; Ludwig et al., 2016; Rahman et al., 2020). These tissue atlases for small RNAs have one or more limitations: lack of consistency in analytical technique, insufficient number of replicates per sample type, or samples from disease cases

without sufficient numbers of normal controls. Here we describe a tissue atlas of small RNA-seq from postmortem samples from 90 human tissue samples (30 tissues with three biological replicates each) and four biological replicates each of red blood cells (RBCs), monocytes, and peripheral blood mononuclear cells (PBMCs). To make this data uniform, easily accessible and usable to other investigators, we have also processed all of the data with a consistent bioinformatic pipeline, *exceRpt* (Rozowsky et al., 2019), which assesses all RNA species and fragments that align to the human genome.

Many bioinformatic tools and small RNA databases, such as miRbase, rely on alignment with the canonical sequence for each miRNA or small RNA, which is typically the first discovered sequence and/or the sequence thought to be most abundantly expressed. While consensus sequences are relied upon for naming and organizing RNA biotypes, it is well known that miRNA sequence variation exists, giving rise to isomiRs and diverse RNA fragments (Luciano et al., 2004; Ebhardt et al., 2009; Lee et al., 2010; Martí et al., 2010). IsomiRs are sequences that exhibit slight nucleotide differences (isoforms) from the canonical sequence in miRBase (Guo and Lu, 2010). miRNAs are the most diverse class of small RNA with 1000s of isomiRs that are often not included in downstream analysis, because many analyses pipelines collapse/sum isomiR counts and attribute them to the corresponding canonical mature sequence. Not only are these other isomiRs and RNA fragment sequences measurable, but some are found at higher levels in certain tissues and biofluids than the canonical sequence (Guo and Chen, 2014; Haseeb et al., 2017; Wu et al., 2018). Tissue-dependent expression of isomiRs were found to be biologically active and to target mRNAs in tissues (Londin et al., 2015). Thus, a more comprehensive view of unique RNA sequences is likely to expand the repertoire of disease- and tissue-representative biomarkers (Guo and Chen, 2014; Haseeb et al., 2017; Wu et al., 2018), which in turn may have utility as biofluid-based biomarkers to enable liquid tissue biopsy. For example, in a 2017 paper, Telonis *et al.* described the ability to discriminate between 32 different cancers in TCGA, based solely on the presence and absence of isomiR sequences (Telonis et al., 2017). They found that some isomiR sequences were specific to some tissues and cancers. Telonis went on to apply this approach in breast cancer where they used isomiRs to identify breast cancer subtypes (Telonis et al., 2015). The shifting of a single base can change the targeting of isomiRs for their downstream mRNA target (Karali et al., 2016; Yu et al., 2017).

RNA-sequencing (RNA-Seq) has had a substantial impact on identifying and measuring EV exRNA cargo and has been gaining momentum as a technique for diagnosing and monitoring disease (Van Keuren-Jensen et al., 2014; Byron et al., 2016; Marco-Puche et al., 2019). Many assays require targeting of specific RNA sequences, and thus limit the scope of detected RNAs. Thus, RNA-seq offers not only the ability to monitor a larger number of canonical small RNAs, but also enables monitoring of non-canonical

sequences, such as isomiRs, which may be superior discriminators for disease and tissue-enrichment, identifying functional changes, differentiating between disease and health or distinguishing between source tissues. Changes at the 5' end of isomiRs can change the position of the seed sequence [nucleotides 2-7; (Bartel, 2009)] thereby altering the binding to the target mRNA (Bartel, 2009; Ellwanger et al., 2011), while nucleotides at the 3' end of the isomiR can also change the stability and targeting of the RNA (Grimson et al., 2007; Agarwal et al., 2015). Although the functions associated with most isomiRs are not known, given the lack of studies in this area, the inclusion of these reproducible sequences increases the number of tissue-enriched targets. It is currently unclear how accurately these slight differences in nucleotides can be measured by qRT-PCR or other methods (Witwer, 2015; Avendaño-Vázquez and Flores-Jasso, 2020).

Here we focused on the use of RNA-seq data to identify individual RNA sequences found in tissues and biofluids. We are aware that there are several advanced adaptations to small RNA sequencing that offer enhanced detection of tRNA fragments with base modifications, mRNA fragments, or sequences with 5' or 3' end modifications more comprehensively (Abdelhamid et al., 2014; Cozen et al., 2015; Gogakos et al., 2017; Giraldez et al., 2019; Xu et al., 2019). However, for these experiments, we chose commonly used sequencing kits for their reproducibility, uniformity (Giraldez et al., 2018; Yeri et al., 2018) and their ability to be directly compared across biofluid samples that are already sequenced and readily obtained from several diverse cohorts. We believe that our findings, and the creation of a searchable, online tool, will aid researchers validating their own small RNA studies across tissue types and biofluids.

MATERIALS AND METHODS

Tissue Samples

RNA Isolation

Fresh frozen tissue was sliced into pieces that were no greater than 0.5 mm in thickness on dry ice to maintain integrity and placed in 1 ml of RNA Later ICE. The RNA later-Ice immersed tissue incubated for at least 16 h at -20°C to allow for absorption. The tissue was then immediately placed in a glass 16 mm $\times$ 100 mm Covaris tube and mixed with 400 μL of cold lysis/binding buffer from the mirVana miRNA Isolation Kit. The Covaris was set to treat each sample at a peak power of 452, a duty factor of 17.4, and cycles/burst of 280. Each sample was then treated for 15–30 s in a Covaris S220 sonicator. Lysate was transferred to a new pre-chilled 2 ml Eppendorf tube with 40 μL of miRNA homogenate solution, and incubated on ice for 3–5 min. Total RNA was extracted according to manufacturer's protocol with the Total Exosome RNA and Protein isolation kit (cat# 4478545, ThermoFisher Scientific, Waltham, MA). RNA was eluted using 100 μL of 95°C ultrapure water. Total

RNA was treated with DNase, Turbo DNA-free kit (AM 1907, ThermoFisher Scientific, Waltham, MA). DNA-free Total RNA was cleaned and concentrated using Zymo clean and concentrator kit (R1016) and eluted in 28 μL of ultrapure water. RIN values for tissues can be found in **Supplementary Table S1**.

Perkin Elmer Small RNA Library Preparation

Small RNA libraries were generated using NEXTflex Small RNA Library Prep Kit v3 following the manufacturer's instructions with 250 ng of RNA using 100% adapter and 16 cycles of PCR amplification. Following PCR amplification, libraries between 140 and 160 bp in size were gel purified using 6% TBE gels followed by ethanol precipitation and resuspension in 11 μL of ultra-pure water.

Small RNA Library QC and Pooling

Gel purified libraries were quantified using Agilent 2100 Bioanalyzer with High-Sense DNA chips. Equimolar amounts of libraries were pooled and quantified by Bioanalyzer with High-Sense DNA chips. Pooled libraries were normalized and denatured at a working concentration range of 6–8 pM with 5% PhiX spike-in for flow cell cluster generation. Samples were sequenced until new miRNA detection plateaued for each tissue (**Supplementary Figure S1**).

Blood Cells

Peripheral Blood Mononuclear Cells and Washed Red Blood Cells

Human blood samples were collected with written consent from donors ≥ 18 years of age under an IRB protocol approved by the Human Research Protections Programs at UCSD. Biofluid samples were labeled with study identifiers.

Whole blood was collected from two male and two female healthy, non-pregnant adult donors, 22–50 years of age. For each donor, blood was collected in the following order: ~8 ml into a serum BD Vacutainer collection tube (Becton Dickinson, PN 368045) followed by 3 ml $\times$ 4.5 ml into CTAD (0.11 M buffered trisodium citrate, 15 M theophylline, 3.7 M adenosine, 0.198 M dipyrindamole) collection tubes (Becton Dickinson, PN 367947). The serum tubes were held at room temperature for 20 min prior to centrifugation at 2000 g for 5 min with no brake. 500 μL aliquots were transferred from the clear upper serum layer into screw cap 2 ml centrifuge tubes and frozen at -80°C until they were processed. PBMC, platelets, and washed red blood cells (RBC) were purified from the CTAD tubes. Wide-bore pipette tips were used at all relevant steps to reduce cell shearing and lysis.

For PBMCs, the CTAD tubes were centrifuged at 100 $\times$ g for 20 min with no brake and all but ~100 μL of the supernatant was removed and discarded. For the PBMCs, the remaining supernatant, buffy coat, and a small portion of the RBCs were transferred from the CTAD tubes into a fresh 15 ml tube. Freshly prepared Prostaglandin I₂ (PGI₂) (Abcam, ab120912-1 mg) was added to ~2 mM final concentration. The material was gently inverted several times to mix and

centrifuged at 100 x g for 20 min with no brake. The supernatant material was removed to near completion and the pellet was mixed in 10 ml of RBC lysis buffer (150 mM NH₄Cl, 10 mM NaHCO₃, 1.27 mM EDTA) placed at room temperature for 20 min. The material was centrifuged at 500 x g for 5 min and the supernatant was discarded. The pellet was washed twice with 10 ml of Dulbecco's phosphate-buffered saline (DPBS) each time and centrifuged as before. The pellet was gently resuspended in 2 ml of DPBS and the material was transferred to a 2 ml centrifuge tube and centrifuged as before. The supernatant was carefully removed and the pellet material in the tube was placed at -80°C until processed.

The remaining RBCs within the CTAD tubes were transferred into a 50 ml conical tube and DPBS was added to 50 ml. The cells were centrifuged at 500 x g for 5 min with no brake and the supernatant was decanted. This washing process was repeated two more times. 200 µL aliquots of the remaining washed RBC pellet were transferred into 2 ml screw cap tubes and stored at -80°C until processed.

Monocytes

Human peripheral blood was obtained from health adult volunteers in accordance with the guidelines of the IRB of Beth Israel Deaconess Medical Center after informed consent was obtained in accordance with the Declaration of Helsinki. Ten ml of blood from healthy donors were collected via cubital venipuncture into a syringe prefilled with 2.3 ml of 6% Dextran 500 (Sigma-Aldrich, St. Louis, MO) and 1 ml of 3.2% Sodium Citrate (Sigma-Aldrich). After gentle mixing the blood was sedimented for 45 min with the syringe's nozzle up. The RBC-free fraction was washed once by centrifugation at 2000 g for 10 min. The resulting pellet was resuspended in 0.5 ml of HBSS⁺⁺.

The cells were sorted using a Becton Dickinson FACSaria IIu cell sorter equipped with five lasers (350, 405, 488, 561, and 640 nm). The cell populations were sorted through a 70 µm nozzle tip at a sheath pressure of 70 psi and a drop drive frequency of 90–95 kHz. A highly pure sorting modality (4-way purity sorting for FACS Aria, Masks at 0-32-0) was chosen for cell sorting. The flow rate was maintained at an approximate speed of 10,000 events/second. Monocytes were gated based on FSC/SSC properties. The FSC values are proportional to the diameter of the interrogated cells, whereas the SSC values provide information about the internal complexity of the interrogated cell or its granularity. Sorted cells were collected in 5 ml polypropylene tubes containing 1 ml collection medium (RPMI supplemented with 50% FBS, 100 µg/ml gentamicin, 4 mM L-glutamine, 20 mM HEPES) and stored at -80°C until processed.

Biofluid Samples

Collection

The cell-free plasma, urine and saliva samples were from a previously published study (Yeri et al., 2017). Cell-free RNA isolations methods were optimized and compared to identify the best return of small RNAs from biofluid samples (Burgos et al., 2013; Burgos and Van Keuren-Jensen, 2014). Samples were

collected from male college athletes ages 18–25. All human subjects provided written consent form prior to enrollment. All samples were collected with consent and approval from the Western Institutional Review Board (WIRB) study ID# 1307009395, dbGaP accession phs001258. Blood samples were collected in EDTA tubes and placed with ice packs until they were processed within 2–3 h of blood draw. Samples were spun for 10 min at 700 x g at 4°C in an Eppendorf 5804 R centrifuge using an A-4-44 rotor to remove cells and debris. The supernatant (plasma) was collected, and stored in 1 ml aliquots at -80°C for RNA isolation and sequencing. Urine was collected in sterile cups and placed in a cooler with ice packs and processed within 2–3 h of collection. Samples were spun at 1900 x g for 10 min at 4°C and 15 ml were pipetted into a 50 ml conical tube for storage at -80°C. Saliva samples were collected by passive drool to collect into a 50 ml conical tube. The sample was spun at 1900 x g for 10 min at 4°C, and 1 ml volumes were placed into 2 ml microcentrifuge tubes and stored at -80°C.

The CSF-EV and plasma-EV samples were collected under the approval of the Institutional Review Board of the North Shore-Long Island Jewish Health System. Lumbar puncture, performed using a standard technique with a 25-gauge, Whitacre point spinal needle after subcutaneous lidocaine was applied. The procedure was conducted with patient sitting up and all procedures took place at 2 p.m. local time. 15–25 ml of CSF was obtained from each subject. The first 2–3 ml obtained after collecting the fluid were sent for routine testing (cell count, proteins, glucose, and VDRL). CSF samples with macroscopic blood as consequence of traumatic procedures were not included in these analyses. A blood draw to obtain 10 ml of peripheral blood into an EDTA tube was also obtained prior to the lumbar puncture procedure. Both CSF and blood samples were centrifuged at 2,000 x g for 10 min at 4°C. Both sample types were flash frozen with liquid nitrogen and stored at -80°C at the biorepository of the Feinstein Institute for Medical Research.

RNA Isolation

CSF-EV and Plasma-EV Samples RNA Extraction

We followed the protocol in the Qiagen exoRNeasy Serum/Plasma kit (Cat No./ID: 77044) to capture extracellular vesicles on an affinity membrane, and then to lyse the vesicles and isolate the extracellular RNA. Briefly, 1 ml plasma samples were thawed at room temperature and centrifuged at 3,000 x g for 5 min at 4°C to remove debris (in an Eppendorf 5804 R centrifuge using an A-4-44). Samples were then applied to the column following the manufacturer instructions. This kit and the EVs isolated have been previously characterized (Enderle et al., 2015; Tang et al., 2017; Srinivasan et al., 2019; Kalani et al., 2020).

Cell-Free Plasma, Saliva, Urine RNA Isolation

The samples used were from a previously published study (Yeri et al., 2017). Plasma and saliva samples were isolated (1 ml) using the mirVana miRNA Isolation Kit (ThermoFisher Scientific, Waltham, MA, AM1560) according to (Burgos et al., 2013). Samples were DNase treated using TURBO DNA-free Kit

(ThermoFisher Scientific, Waltham, MA, AM 1907). Samples were then cleaned and concentrated using Zymo RNA Clean and Concentrator (Zymo Research, Irvine, CA, R1016) using Protocol: Purification of small and large RNAs into separate fractions and combining the fractions at the end. Urine samples (15 ml) were isolated with Urine Total RNA Purification Maxi Kit, Slurry Format (Norgen Biotek Corp., Thorold, ON, Canada, Cat#29600). Samples were DNase treated on column using RNase-Free DNase Set (Qiagen, Germantown, MD, cat# 79254). Samples were concentrated by Speed Vacuum.

Sample Preparation for Sequencing CSF-EV and Plasma-EV Samples Small RNA Library Preparation

Small RNA libraries were generated using NEXTflex Small RNA Library Prep Kit v2 (BIOO) with 16 cycles of PCR amplification. Amplified libraries were resolved on a 6% TBE gel for size selection. The 140- to 160-nucleotide bands corresponding to adapter-ligated libraries were excised and recovered in a DNA elution buffer followed by ethanol precipitation and resuspension in 11 µL of ultra-pure water. Gel purified libraries were quantified using Agilent 2100 Bioanalyzer with High-Sense DNA chips. Equimolar amounts of libraries were pooled and quantified by Bioanalyzer with High-Sense DNA chips. Pooled libraries were normalized and denatured at a working concentration range of 6–8 pM with 5% PhiX spike-in for flow cell cluster generation.

Cell-Free Plasma, Saliva, Urine Sequencing

The samples employed were previously published (Yeri et al., 2017). Plasma, saliva and urine RNAs were quantified in triplicate using Quant-iT Ribogreen RNA Assay kit, Low-Range protocol (R11490; ThermoFisher). The Illumina small RNA TruSeq kit (RS-200-0048; Illumina) was used for sequencing all samples. RNA input for plasma and saliva was 10–20 ng for all samples and the RNA input for urine was 30 ng for all samples. The reagents from the Illumina TruSeq kit were halved (Burgos et al., 2013). Samples were assigned and unique index. 16 PCR cycles were used for all samples. Samples were pooled and placed on Illumina V3 single read flowcells (GD-401-3001; Illumina).

Liver Fibrosis Samples

The samples employed were previously published (Van Keuren-Jensen et al., 2016). All patients were consented at the Mayo and Scripps Clinics and the Institutional Review Boards of both sites approved the collection of the samples. For liver and serum samples, the miRvana kit (AM1560; Life Technologies) was used according to the manufacturer's protocol with a slight modification for two phenol chloroform extractions (Burgos et al., 2013). TruSeq small RNA Library preparation Kit (Illumina RS-200-0048) was used. Kit reagents were used in a half reaction. Each sample was assigned one of 48 indices. 200 ng of RNA were used from tissue samples, with 15 PCR cycles. The total RNA isolated from each serum sample volume (~1 ml) was used for small RNA sequencing. Samples were pooled and placed on a single read Illumina V3 flowcell (GD-401-3001). One lane of

the flowcell was loaded with PhiX as a reference lane to help with low nucleotide diversity in microRNA.

Exercise Samples

The samples employed were previously published (Shah et al., 2017). All subjects provided written informed consent under approved Institutional Review Board protocols at the Beth Israel Deaconess Medical Center and Massachusetts General Hospital. Plasma samples (1 ml) were isolated using a modified mirVana PARIS protocol (AM1556, Life Technologies) with sequential phenol-chloroform extractions (Burgos et al., 2013). RNA was cleaned and concentrated (R1016, Zymo Research, Irvine, CA). Sample preparation was done using the Illumina small RNA TruSeq kit (RS-200-0048, Illumina). The kit reagents were halved at all steps with 16 rounds of PCR amplification. Libraries were quantified with the Agilent High Sensitivity DNA Kit (5067-4626, Agilent) and sequenced on an Illumina flowcell.

Stroke Samples

The samples employed were previously published (Kalani et al., 2020). This study was approved by the institutional review boards of St. Joseph's Hospital and Medical Centre. Blood was collected in EDTA tubes. Extracellular vesicles were isolated from the plasma samples using the exoRNeasy Serum/Plasma kit from Qiagen. We followed the protocol in the Qiagen exoRNeasy Serum/Plasma kit (Cat No./ID: 77044) including a centrifugation at 3000 g for 5 min at 4°C to remove debris, after thawing and before RNA isolation. A total of 10 ng of isolated RNA was used in sample preparation (Quant-iT Ribogreen RNA Assay kit, Low-Range protocol (R11490; ThermoFisher). Samples were sequenced as described above for the liver fibrosis and exercise samples, with 16 PCR cycles.

Data Analysis Processing

All samples were processed using the exceRpt small RNA pipeline (Rozowsky et al., 2019). The gene count tables for miRNAs, tRFs, piRNAs, protein coding genes and yRNAs were taken directly from the summary tables generated by exceRpt. To generate the count tables of sequences in each sample, the adapter trimmed fastq files were first compared against the human genome mapped BAM files from exceRpt to find reads which mapped to the human genome. The genome mapped reads were then identified in the transcriptome mapped BAM file to identify the biotypes of the sequence. Since multi-mappers are common, biotype was assigned in the same priority order as the exceRpt pipeline: miRNA, YRNA, tRNA, piRNA, protein coding, followed by a bin of any other biotype in the annotation. Reads with the same sequence (after adapter trimming) were then collapsed into a counts table with biotype and gene annotations. All counts tables (both genes and sequences) were then loaded into R (v. 4.1.0) for further analysis. Rarefaction curves were generated in R using the vegan package (<https://CRAN.R-project.org/package=vegan>) to assess sequencing saturation.

Tissue Atlas

The count tables from the tissue atlas samples were filtered in R to only genes and sequences present at >25 read counts in >2 of the 3 biological replicates. This allowed us to both include sequences which were well expressed while not removing sequences which were specific to a single tissue type. After filtering, read counts were normalized using median of ratios in DESeq2 (Love et al., 2014). Normalized counts were used to generate diversity and counts boxplots for each tissue type and a UMAP of all sequences was generated using the uwot package in R (<https://CRAN.R-project.org/package=uwot>). Tissue-enriched genes and sequences were determined using the TissueEnrich package in R with the foldChangeThreshold set to 7 (Jain and Tuteja, 2019). Sequences with specificity of “All” were removed, leaving a table of tissue-enriched, group-enriched and tissue-enhanced sequences and genes. Genes and sequences are assigned using the algorithm from the Human Protein Atlas (Uhlén et al., 2015) and can be grouped as follows: 1) Tissue-Enriched: Genes with an expression level greater than 1 normalized count that also have at least five-fold higher expression levels in a single tissue compared to all other tissues; 2) Group-Enriched: Genes with an expression level greater than 1 normalized count that also have at least five-fold higher expression levels in a group of four tissues compared to all other tissues; 3) Tissue-Enhanced: Genes with an expression level greater than 1 normalized count that also have at least five-fold higher expression levels in a single tissue compared to the average levels in all other tissues, and that are not considered Tissue-Enriched or Group-Enriched. Throughout the paper, these three categories were combined and termed; tissue-elevated.

Biofluids

The count tables from the various projects above were processed in a consistent manner. All were loaded into R as counts matrices of genes or sequences. Stacked bar plots of biofluid genome mapping and biotype diversity was generated from the raw count matrices. For further analysis, each biofluid type was filtering independently with a gene or sequence requiring >10 read counts in >50% of the samples for inclusion. As with the tissue atlas samples, filtered count matrices were normalized using median of ratios in DESeq2. Tissue-elevated sequences in biofluids were found based on the overlap of filtered sequences in biofluids samples with tissue-elevated sequences identified above.

Diversity Analysis

We use the term gene diversity in tissues to refer to the traditional analysis of collapsing all detected isoforms of a gene to a single parent gene and counting only the parent. We use the term “sequence diversity” as the total number of unique sequences with a normalized expression >10 in any tissue, without collapsing to the canonical parent gene. Similarly, in biofluids, gene diversity was determined by collapsing all isoforms of a gene to the canonical mature sequence and allowing them to count for that gene, sequence diversity in biofluids were determined by summing the number of detected sequences with normalized counts >10 in a biofluid and allowing them to be counted independently. Sequences detected in the biofluid samples with >10 read counts in 50% of the samples from that biofluid, and were listed

as tissue-enriched, tissue-enhanced or group-enriched – combined they are designated tissue-elevated. Sequences that were elevated in multiple tissues were counted for each tissue.

Differential Expression Analysis

For analyses of liver fibrosis, exercise and stroke, all biofluid samples were loaded into R, filtered as above, and analyzed for differentially expressed sequences using DESeq2. Volcano plots of differentially expressed sequences were generated and colored based on the RNA biotype of the sequences. Differentially expressed sequences (Benjamini-Hochberg p-adjusted values <0.05) were compared to tissue elevated sequences and one sequence was highlighted in a bar graph displaying expression levels.

RESULTS

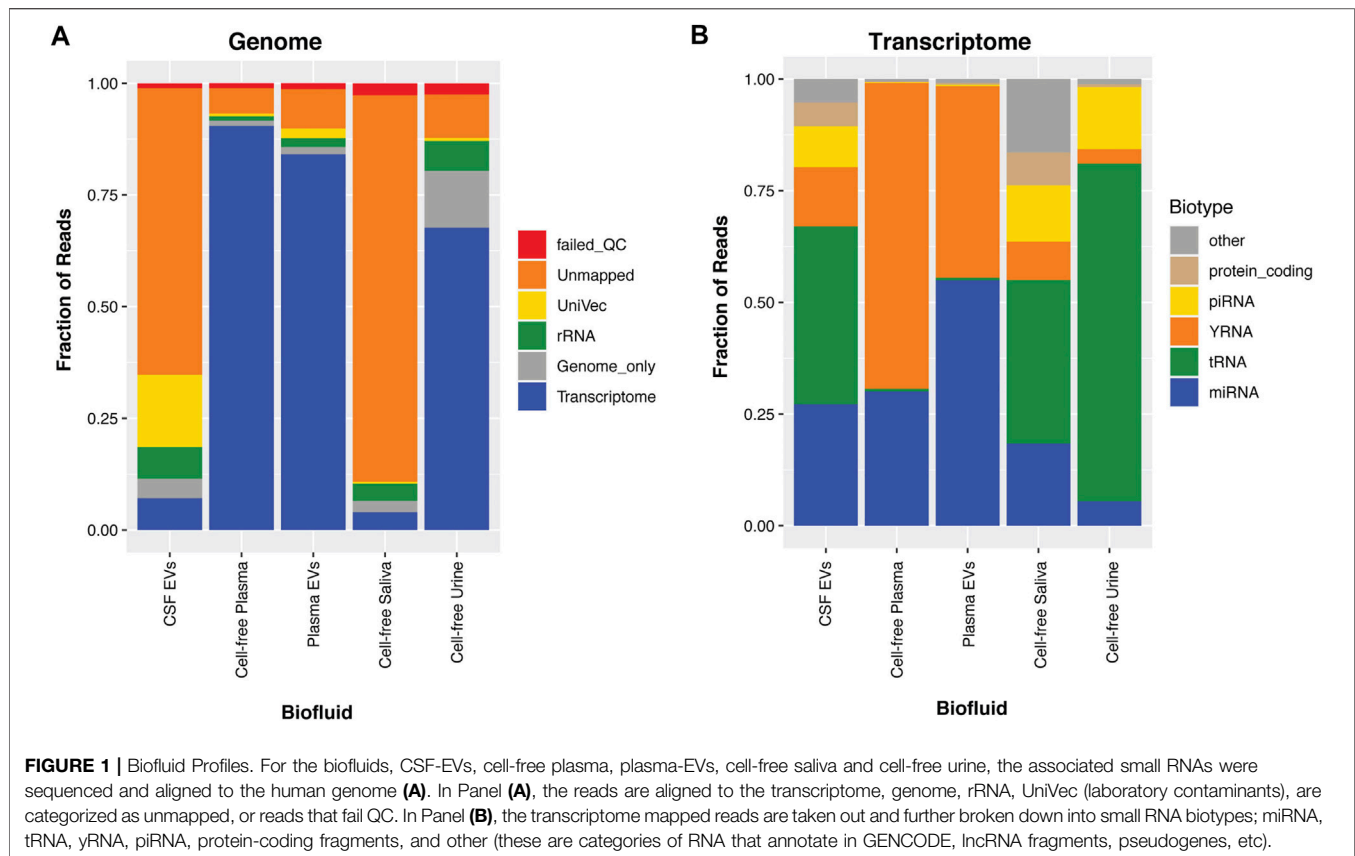
Small RNA Profiles Differ Across Biofluids

We, and others, have found that each biofluid contains a distinct small RNA profile (ref 20; 1, 3), as can be observed in the percentage of reads mapped to the genome (**Figure 1A**) or transcriptome (**Figure 1B**). For this paper, we compare data from a previously published cohort: 179 cell-free plasma samples, 44 cell-free saliva samples, and 203 cell-free urine samples (Yeri et al., 2017). We also included 37 CSF and 35 matched plasma samples isolated with exoRNeasy to enrich for exRNAs contained in EVs.

We show that plasma and urine samples show the highest mapping rates to the human genome, while saliva and CSF samples have lower mapping rates (**Figure 1A**). CSF typically has very little measurable exRNA (Hulstaert et al., 2020; Burgos et al., 2013; Kopkova et al., 2018; Saugstad et al., 2017; Waller et al., 2017). These low-level RNA inputs make these samples more susceptible to amplification of synthetic (adapter) oligos and common laboratory contaminants (UniVec). Taking the portion of the CSF that maps to the transcriptome (**Figure 1B**), many of the exRNAs found in CSF are miRNA and tRNA, with >25% going to yRNA, piRNA and protein-coding fragments (Hulstaert et al., 2020; Godoy et al., 2018). Saliva contains high RNA concentrations, but a large fraction of this RNA is not human, with significant contributions from bacterial sequences (Li et al., 2018; Takeshita et al., 2016). Of the reads that map to the human transcriptome (**Figure 1B**), cell-free plasma is dominated by miRNA and yRNA fragments (yRFs), and EVs enriched from plasma samples have slightly higher levels of mapped miRNA. Finally, urine has low miRNA content, with high levels of tRFs as previously reported (Yeri et al., 2017; Godoy et al., 2018; Srinivasan et al., 2019).

Generation of a Tissue Atlas of Small RNAs

To examine whether tissue-elevated, small RNA sequences (comprised of miRNA, piRNA, snoRNA, snRNA, tRNA and YRNA fragments, etc) were found in specific biofluids and correlated with tissue abundances, we produced a high-



quality tissue atlas of small RNAs across 30 human tissues. Tissue samples were received from the Brain and Body Donation Program (BBDP) at Banner Sun Health Research Institute (<http://www.brainandbodydonationprogram.org>); samples were rapidly collected with a median postmortem interval of 3 h and verified to be pathologically normal (see Methods; donor demographic information is available in **Supplementary Table S2**). Samples were sequenced deeply with at least 9.8 million reads mapping to miRNA. **Supplementary Figure S1** displays rarefaction curves that have plateaued for new miRNA detection in each tissue. The three biological replicates were averaged together for each tissue, the human genome mapping rate was high for all tissues (**Figure 2A**). The RNA biotypes that make up the transcriptome alignments for each tissue are shown in **Figure 2B**. Gallbladder has high levels of tRFs and piRNA sequence alignments; whereas, monocytes and PBMCs display higher levels of yRFs, potentially contributing to the high abundance of yRFs in plasma samples.

Detection and Classification of Tissue-Elevated Sequences

There is overlap in the expression of canonical miRNAs between different tissues, as well as some unique tissue-elevated miRNAs. We sought to expand the number of tissue-elevated sequences by identifying abundant sequences that do not

conform to canonical small RNA annotations. We performed a strict alignment of small RNA sequences from the biofluids and tissues using the *exceRpt* pipeline (see Materials and Methods). We used miR-451a and miR-30a-5p as examples to demonstrate the presence of the mature canonical sequences and the number of isomiRs present in plasma samples. There are 73 isomiRs of miR-451a with >1 read in >25% of plasma samples (**Supplementary Table S3**). The mature canonical sequence for miR-451a, highlighted in **Figure 3A**, is not the most abundant sequence (Guo and Chen, 2014; Liang et al., 2017). MiRNA miR-451a (Griffiths-Jones, 2004; Kozomara et al., 2019) is highly abundant in RBCs (Sun et al., 2020) and we used a heatmap to display expression (**Figure 3B**) of all miR-451a isomiRs in RBC, PBMCs and monocytes. miR-451a is a dicer-independent miRNA (Liang et al., 2017) while the second example in **Figure 3A** is from a dicer-dependent miRNA, miR-30a-5p. miR-30a-5p has 18 detectable isomiRs with >1 read in >25% of cell-free plasma samples with many of them more abundantly detected than the highlighted mature sequence found in miRBase.

The most abundant sequence in urine is GCATTGGTGGTT CAGTGGTAGAATTCTCGC (Yeri et al., 2017), which matches a piRNA entry (hsa_piR_020326) with one less 5' G, as well as being an exact match for a portion of a tRF (**Figure 3C**). Despite being an exact match, typical tRFs and those used by the tRF database in our alignment pipeline, *exceRpt* (Rozowsky et al., 2019), are longer than the sequence we

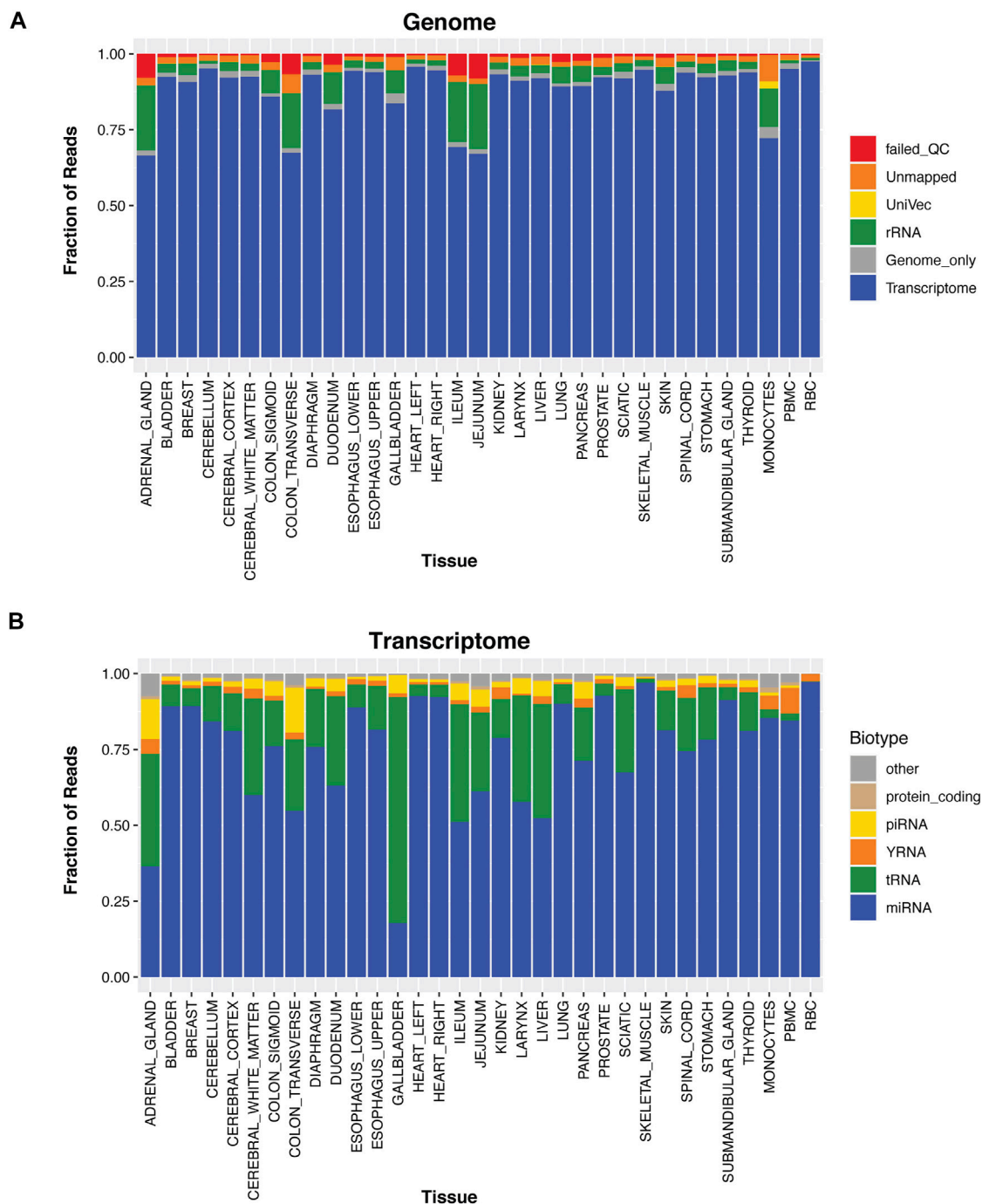


FIGURE 2 | Tissue Profiles. For thirty different tissues, PBMCs, monocytes, and red blood cells we isolated the RNA and sequenced their small RNA contents. In Panel (A), the reads are aligned to the transcriptome, genome, rRNA, UniVec (contaminants), are unmapped, or reads that fail QC. In Panel (B), the transcriptome mapped reads are removed and further broken down into small RNA biotypes; miRNA, tRNA, yRNA, piRNA, protein-coding fragments, and other (these are categories of RNA that annotate in GENCODE, lncRNA fragments, pseudogenes, etc).

detect (see red shading to indicate the detected fragment compared with the full-length fragment in the database). A recent paper introduced a tRF database called MINTbase

(Wang et al., 2019), which describes and counts the sequence abundantly detected here in urine, as an exact match to a tRF (Figure 3C). It is important to keep these

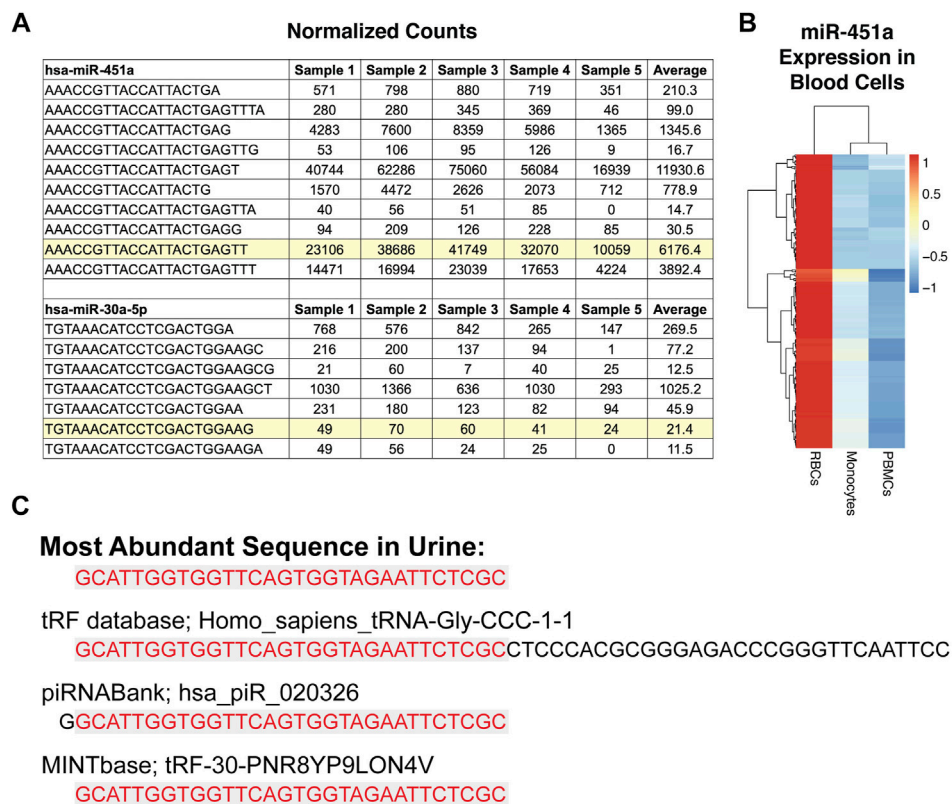


FIGURE 3 | Sequence Variation and Categorization. Panel (A) highlights the canonical miRNA sequence that is entered in miRbase for two miRNAs, miR-451a and miR-30a-5p. The other sequences are isomiRs of the mature sequence (highlighted) listed in miRbase. The table shows the normalized expression for the sequences in plasma from five different subjects and the average across the 179 cell-free plasma samples. In some cases, an isomiR has higher expression in plasma than the highlighted canonical sequence. miR-451a is highly expressed in red blood cells, as can be seen in the heat map in Panel (B). Panel (B) displays the canonical and isomiR sequences for miR-451a found in red blood cells, PBMCs, and monocytes. The most abundant sequence detected in urine samples is displayed in Figure 3c and is = similar to both a piRNA and a tRNA sequence.

alignment and naming challenges in mind, as these sequences are short, and in many cases, cannot be classified with complete certainty back to the original parent RNA.

Using the tissue atlas of small RNAs that we generated, we next explored sequences for tissue-elevation. We generated a list of sequences at least seven-fold enriched in a maximum of four tissues using the R package, TissueEnrich (Jain and Tuteja, 2019). The TissueEnrich package contains three categories of “enrichment.” We employed tissue-elevated as the combination of the three categories (“tissue-enriched,” “group-enriched” and “tissue-enhanced”). The reason for four tissues was due in part to having multiple regions for a particular tissue (e.g., central nervous system - cerebellum, cerebral cortex, cerebral cortex-white matter, spinal cord). Tissue-elevated sequences and their fold-enrichments are available in **Supplementary Table S4**. **Figure 4** describes the number of genes (4a) or individual sequences (4b) that were identified to be elevated in each tissue. **Figure 4A** represents the number of genes detected when all isomiRs and sequence fragments have been collapsed down to the

parent/canonical gene. The sequences in **Figure 4B** represent individual isomiRs, and unique RNA fragments of genes that are expressed at a minimum of 25 counts in at least 2/3 biological replicate samples. The numbers of these tissue-elevated sequences that go to miRNA and piRNA for each tissue and biofluid are displayed in **Table 1** and the full table with all RNA biotypes can be found in **Supplementary Table S5**. The graphs and tables can be used to determine that RBCs and monocytes, and tissues such as bladder and gall bladder, have fewer distinct tissue-defining sequences, while interestingly – jejunum – has many tissue-elevated sequences. **Table 1** and **Supplementary Table S5** describe the sequences detected in each tissue broken out by small RNA biotype. First column for each RNA biotype is the number of detected genes identified for each tissue (all isomiRs and similar fragments are collapsed and counted with the canonical sequence), followed by the full number of isomiRs or unique fragments (not collapsed; column two) for that RNA biotype, for each tissue. We also include the number of tissue-elevated genes (column three) and fragments/isoforms

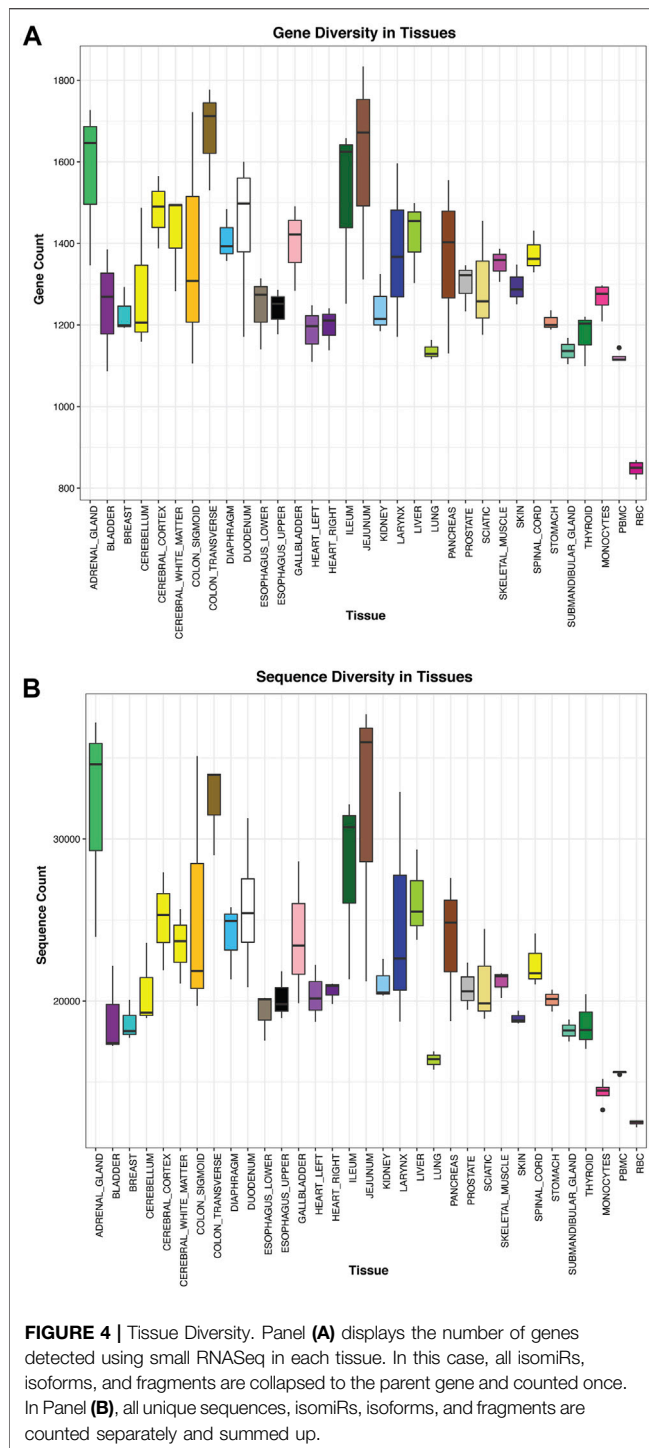


FIGURE 4 | Tissue Diversity. Panel (A) displays the number of genes detected using small RNASeq in each tissue. In this case, all isomiRs, isoforms, and fragments are collapsed to the parent gene and counted once. In Panel (B), all unique sequences, isomiRs, isoforms, and fragments are counted separately and summed up.

(column four) for each RNA biotype in each tissue. While the pattern of tissue-elevated sequences is similar in **Figures 4A,B**, the scales are very different. For example, adrenal gland has 1,572 tissue-elevated genes when collapsed (**Figure 4A**) and has 31,917 tissue-elevated unique sequences with >10 reads in at least 2 of the three replicate tissue samples (**Figure 4B**). We also observe low small RNA diversity in monocytes and RBCs.

Figure 5 displays the normalized counts for a pancreas-elevated miRNA, miR-216-5p, as well as the normalized counts for miR-216-5p and its isomiRs in all tissues. The canonical sequence has 50% of the reads and the other 50% of miR-216-5p reads are distributed to its 6 isomiRs. All of these sequences are elevated in pancreas, compared to other tissues.

Tissue-Enriched miRNAs Cluster in Tissues and Blood Cell Types

We wanted to determine how well tissues clustered based on their related sequences. **Figure 6A** is a UMAP using all sequences to cluster the tissues and blood cells and **Figure 6B** UMAP is subset to using tissue-elevated miRNA and isomiR sequences from each tissue for clustering. Most tissue replicates show consistency and cluster closely, regardless of the sequences used to generate them. Using only the tissue-elevated sequences improved clustering in the CNS tissues. **Supplementary Figure S2** displays tissue clustering using the tissue-enriched tRFs, yRFs and piRNAs, each of which display less tissue specificity than tissue-elevated miRNAs/isomiRs and greater variability among tissue replicates.

Tissue-Elevated Sequences are Detected in Biofluids

We sequenced 30 tissues, representing most major organ systems in the body and classified tissue-elevated small RNA sequences. We next wanted to measure the number of tissue-elevated genes and sequences that were detectable in biofluids: CSF-EVs, plasma-EVs, cell-free plasma, cell-free saliva, and cell-free urine. **Figure 7** displays the diversity of tissue-elevated genes (**Figure 7A**) and sequences (**Figure 7B**, including isomiRs and RNA fragments) detected in each biofluid. **Table 1** provides the number of tissue-enriched sequences, taken from the tissue atlas portion of the paper, and present in each biofluid (**Supplementary Table S5** provides the data for all RNA biotypes). Plasma samples contain the largest number of tissue-elevated sequences compared to the other biofluids.

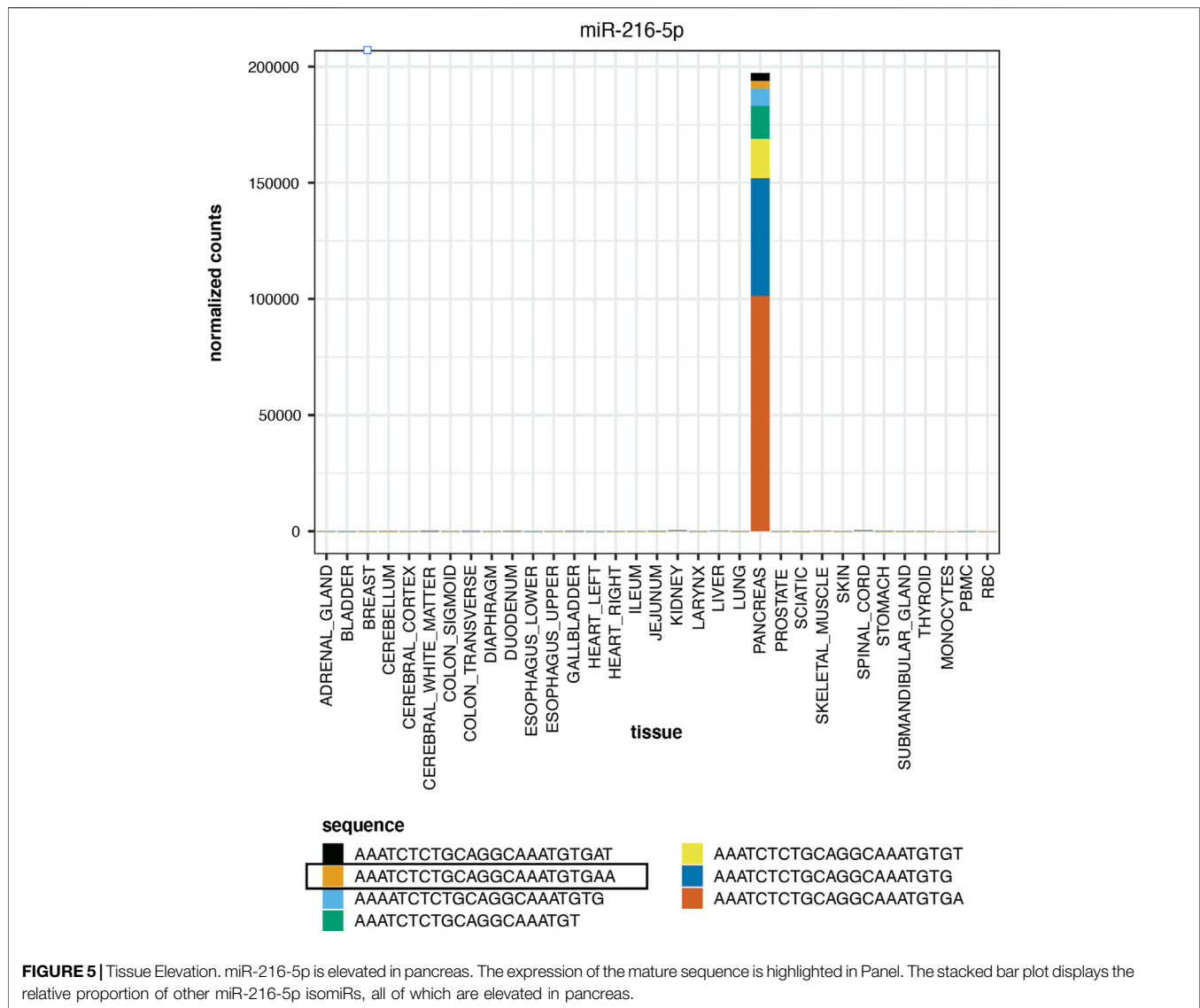
We looked more closely at the number of detectable tissue-elevated sequences in cell-free plasma, plasma-EVs, cell-free saliva, cell-free urine, and CSF-EVs (**Figure 8**). We examined tissue-elevated miRNAs and isomiRs, since they are the most diverse sequences and clustered tissues well in **Figure 6B**. In each panel, **Figures 8A–E**, the first stacked bar plot represents the number of tissue-enriched miRNA and isomiR sequences detected in that biofluid with >10 counts in >50% of samples, and the second stacked bar plot is the sum of all tissue-elevated counts for each tissue. If two tissues share a tissue-elevated sequence, the sequences are counted for both tissues. For **Figure 8**, the number of tissue-elevated sequences detected in each biofluid are provided in **Supplementary Table S6**, the number of tissue-elevated sequences and the fraction of the reads they account for in each biofluid are provided. Cell-free plasma contained tissue-elevated sequences from the largest number of tissues and CSF from the least number of tissues.

TABLE 1 | Tissue-Enriched Sequences. Displays the # of select RNA biotypes detected, the number of isoforms detected, and the number of tissue-enriched sequences observed for each tissue.

Sample	miRNA	isomiRs	Tissue enriched miRNA	Tissue Enriched isomiRs	piRNA	piRNA Isoforms	Tissue Enriched piRNAs	Tissue Enriched piRNA isoforms
Adrenal gland	474	11737	71	554	149	3644	85	1086
Bladder	485	12171	36	65	131	1550	1	1
Breast	465	11676	9	88	132	1234	5	16
Cerebellum	523	14077	137	2141	131	1087	4	4
Cerebral cortex	551	14224	183	2926	142	1571	19	38
Cerebral - white matter	543	13073	111	1698	142	1734	9	13
Colon – sigmoid	464	12638	28	85	142	2998	26	81
Colon - transverse	474	12239	32	419	142	3344	74	1183
Diaphragm	542	13319	57	532	140	1997	6	11
Duodenum	475	12578	19	112	141	2596	3	4
Esophagus - lower	467	11962	6	306	134	1261	3	4
Esophagus - upper	465	11724	11	274	135	1651	1	1
Gallbladder	481	12170	67	173	138	2724	58	819
Heart – left	486	12817	34	320	131	1117	2	6
Heart – right	496	12965	44	390	130	1080	5	17
Ileum	457	11570	10	128	145	3415	41	168
Jejunum	475	11237	13	413	142	3269	44	122
Kidney	455	11255	34	244	135	1371	1	2
Larynx	496	13973	39	446	142	2639	31	172
Liver	436	10343	21	575	142	2585	21	63
Lung	461	11292	12	15	126	893	6	8
Pancreas	470	12084	29	404	140	2543	18	38
Prostate	473	13103	85	493	136	1544	12	47
Sciatic	487	11773	10	22	136	1923	2	3
Skeletal muscle	524	14110	97	1940	134	1107	6	10
Skin	466	10860	16	95	135	1270	4	6
Spinal cord	531	12213	59	928	137	1443	9	18
Stomach	448	12597	12	90	132	1596	2	4
Submandibular gland	451	11663	23	503	130	981	0	0
Thyroid	448	11235	12	114	133	1415	0	0
Monocytes	455	9496	188	1519	119	984	35	79
PBMC	443	9867	79	1323	112	659	14	24
RBC	403	9967	225	3656	73	274	8	11
CSF	122	610	49	136	24	259	14	116
PLASMA	357	5863	152	913	52	281	13	38
SERUM	180	1714	40	177	71	1030	33	129
SALIVA	240	3200	80	503	46	729	26	222
URINE	155	1684	46	131	53	2594	31	377
PLASMA_EVs	381	8069	202	1374	43	297	15	40

There are 474 tissue-enriched RBC miRNA/isomiR sequences found in cell-free plasma, accounting for an average of 82% (874,975 counts) of the tissue-enriched sequences detected in cell-free plasma. Saliva samples display high levels of RBC sequences as well as sequences from esophagus and submandibular gland (**Figure 8C**). Interestingly, pancreas-enriched sequences are also found in high numbers. Kidney and brain tissue-enriched sequences tended to overlap, and both urine and CSF display high levels of kidney and CNS tissue-elevated sequences. The two sequences most abundantly detected in CSF are tissue-elevated for both cerebellum and

kidney, the canonical sequence for miR-204 and an isomiR-204, and are counted for both tissues. Tissue-elevated sequence abundance from RBCs make up the majority of the overall number of counts in plasma-EVs. 3% of the tissue-enriched sequences in plasma-EVs were from skeletal muscle and other tissues that cluster with skeletal muscle in the UMAP in **Figure 6B**. We would like to point out caveats to our approach: 1) tissue-elevation does not equal tissue-specificity, sequences can be associated with and expressed in more than one tissue, 2) we did not sequence every tissue or cell type in the body, 3) each tissue has a different number of elevated sequences, tissues with fewer



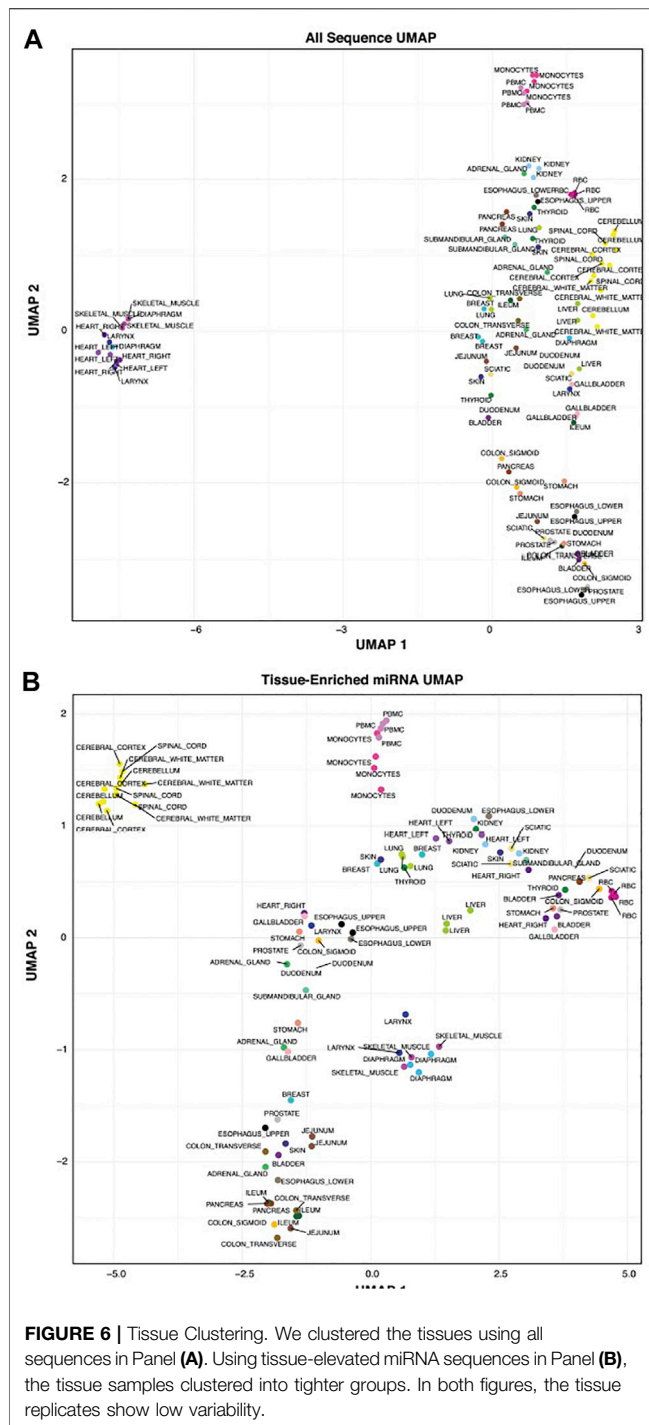
sequences were not weighted differently from tissues with a large number of sequences, and 4) tissue vascularity and size were not accounted for.

tRFs illustrate one area of caution when examining these data. Forty-six tissue-elevated tRF sequences were detected in cell-free plasma, 14 of which were tissue enriched in gallbladder. Gallbladder had a high level of tRFs compared to other tissues (Figure 2). The tRF with the sequence “TCCCTGGTGGTCTAG TGGTTAGGATTCGGCGC” comprises 10.2% of gallbladder tissue small RNASeq reads. Because it is so highly expressed in the gallbladder, it is counted as ‘elevated’ in gallbladder by the criteria we used in this paper. It is nearly 10-fold more highly expressed in gallbladder than in the next most abundant tissue, Ileum. However, as can be seen in Table 2, this tRF is highly expressed in most tissues, but low in blood cell types. This illustrates that while this gallbladder-elevated tRF is detectable

in every biofluid we examined, it is high expression in all tissues makes it a poor marker of tissue specificity.

Circulating, Tissue-Elevated Small RNAs are Differentially Expressed in Physiological (Exercise) and Pathologic States (Liver Fibrosis or Ischemic Stroke)

To assess the potential for tissue-elevated isomiRs to be differentially expressed in physiological or pathological states, we re-examined previously published data from three different studies. The first study compared blood samples from subjects with Hepatitis C and liver fibrosis stage 1 (early) and liver fibrosis stage 4 (advanced) (Van Keuren-Jensen et al., 2016). Figure 9A shows the number of differentially expressed and tissue-elevated sequences and their RNA biotypes identified in



blood samples. miRNAs or isomiRs that were tissue-elevated, in any tissue, are blue dots in the volcano plot, tissue-elevated piRNAs or sequence isoforms are yellow dots, tissue-elevated tRNA sequences are green, tissue-elevated YRNA sequences are orange, and sequences that were differentially expressed but not tissue-elevated are gray. Sequences that do not make the cutoff for p -value or fold-change are black. These RNA biotypes represented the largest number of differentially expressed

sequences. We focused on a tissue-elevated isomiR in **Figure 9A** (miR-122 isomiR, TGGAGTGTGACAATGGTGT), indicated by the red arrow. The expression level of this isomiR in tissues is displayed in **Figure 9B**. This miR-122 isomiR, like the canonical miRNA, is liver elevated and expressed very highly.

Towards employing the tool to distinguish sequences differences in a physiological example, we reanalyzed changes in miRNA detection in plasma samples pre- and post-exercise. Samples were taken from 26 participants at baseline and immediately after peak exercise using the Bruce Treadmill test. Using all unique sequences and isomiRs, we compared baseline expression to expression immediately after exercise (Shah et al., 2017). **Figure 9C** displays the number of tissue-elevated sequences following exercise. An isomiR of miR-10b, which is tissue-elevated in skeletal muscle was significantly down-regulated following exercise, **Figure 9D**.

In the final dataset, we examined plasma samples from patients with two types of stroke, either ischaemic stroke (cerebral vessel occlusion) or a subarachnoid hemorrhage. We examined the differentially expressed sequences that may be able to differentiate these two types of stroke and did differential expression analysis of plasma samples. From our differentially expressed sequences, we focused on a CNS tissue-elevated sequence, an isomiR of miR-181-5p, that was downregulated in stroke and upregulated in subarachnoid hemorrhage, **Figure 9E**. In **Figure 9F**, you can see the elevation of this sequence across the CNS tissues.

The exRNA Expression Atlas—an Online Tool to Display Tissue-Elevated Sequences in Biofluids

To facilitate the widest utility of this data, we created a searchable, online tool with tissue-elevated sequences, and the biofluids in which they are present. **Figure 10** displays output capabilities of the tool, the exRNA Expression Atlas (<https://data.omics.kitchen/miRNAAtlas/>). To illustrate how this tool works, we chose miR-1298 as an example of a miRNA that is highly enriched for tissues of the central nervous system [**Figure 10A**; (Godoy et al., 2018)]. **Figure 10B** displays the biofluids in which this miRNA can be detected. miR-1298 is most highly detected in CSF with minimal counts in other biofluids for most subjects. Knowing the expression of a miRNA such as this one in tissues, and its detection in CSF, may help prioritize biomarker candidates. As such, this searchable, online tool may assist in the validation of studies aiming to demonstrate tissue-specificity for RNA species.

DISCUSSION

Small RNA databases and resources currently exist, but are subject to confounders, such as a lack of overlapping tissues in each dataset, differential RNA isolations that can lead to

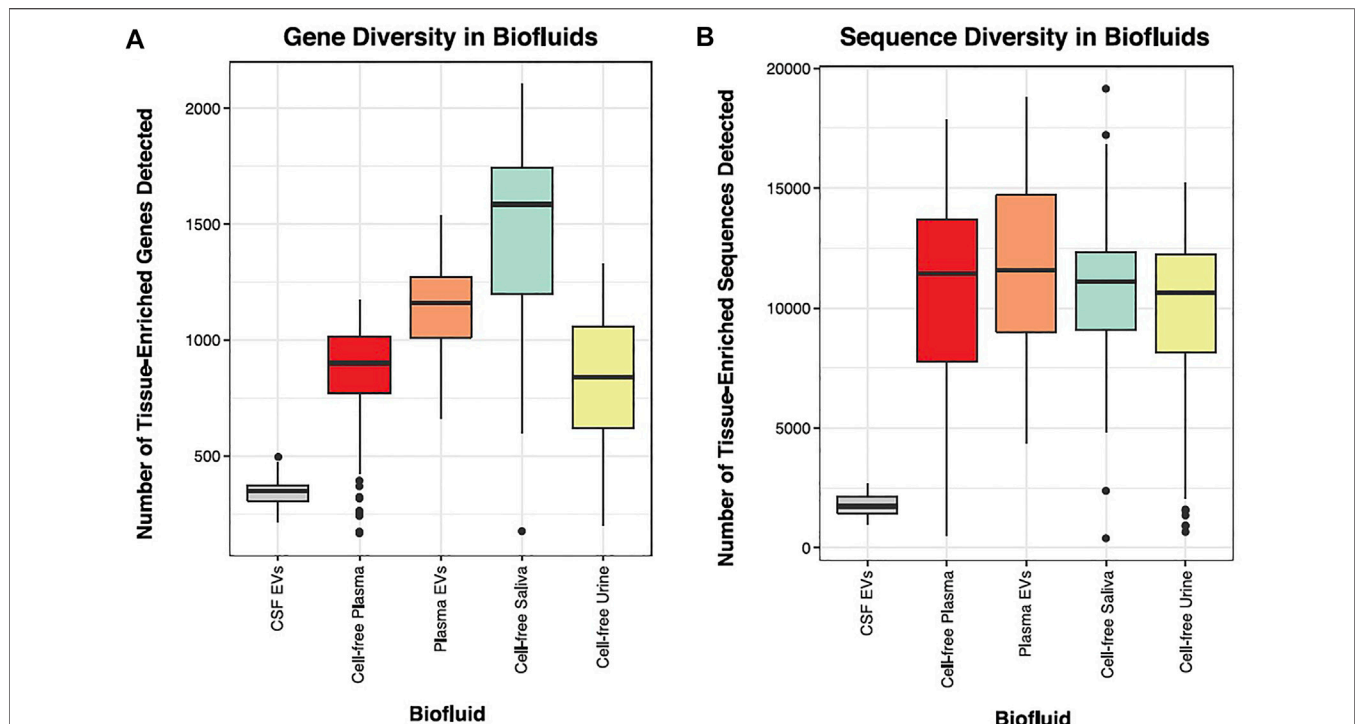


FIGURE 7 | Biofluid Diversity. Panels (A, B) display the number of tissue-enriched genes identified in the small RNA sequenced data. The parent tissue-elevated genes that were detected in each biofluid are displayed in Panel (A). CSF had the lowest number of tissue-elevated genes and saliva had the highest. Panel (B) displays the number of tissue-elevated sequences that were detected in each biofluid.

potential batch effects, and inconsistent sequencing/alignment of the reads across studies. To address these unmet needs, we created a tissue atlas of small RNAs in 30 human tissues with three replicates and multiple blood cell types. Strengths of our study are the inclusion of pathologically normal tissue with low postmortem interval and uniformity in RNA isolation and sequencing and bioinformatic handling. We found that tissues had varying percentages of RNA biotypes and diversity of tissue-elevated sequences. One recent paper that has similarly brought together these disparate resources is by Rahman *et al.* (Rahman *et al.*, 2020), where the investigators examined small RNA biotypes from different tissues to come up with a set that they found to be tissue-enriched.

Using tissue-elevated sequences, especially miRNAs, we clustered the tissue samples with low variability among biological tissue replicates. Tissue-elevated tRFs and yRFs successfully clustered the samples, despite greater variability among replicates. Surprisingly, as a category, the number of tissue-elevated tRF sequences were high for several tissues: gallbladder has 2283 tissue-enriched tRFs, adrenal gland has 3805 tissue-enriched tRFs, and transverse colon has 3782 tissue-enriched tRFs. The use of all unique sequences provided us with interesting observations about some of the tissue-elevated sequences. For example, by expanding the miRNA biotype to include all isomiRs, we found a heart enriched miRNA that was more highly expressed in right heart than the left heart. These data provided us with an

opportunity to use these tissue-elevated sequences to explore their detection in biofluids. One qualification for this dataset is that the RNA from the tissue samples was isolated and prepped uniformly, however different RNA isolation and sample preparation protocols, optimized for low input RNA, were used for the cell-free biofluids and EVs. A second qualification for this dataset is the use of postmortem tissues from older individuals, while most of the biofluid samples are from much younger subjects. It is known that small RNA profiles can change with age (Fehlmann *et al.*, 2020).

There are several previously published approaches to deconvolute the origin of specific RNAs from a varied set of tissue types, but this has not been widely extended to biofluids or EVs. In this case, we decided not to do deconvolution for examining the tissue contributions in biofluids, given the lack of known correspondence between enrichment of sequences in a given tissue with the release of EVs bearing that RNA cargo from that tissue into biofluids. Instead, we relied on a more “direct” approach to measure the diversity and abundance of tissue-elevated sequences detected in each biofluid based on the total frequency of tissue-elevated sequences detected in each biofluid. A unique finding of this approach is the presence of similar transcripts across biologically divergent tissue types. For example, renal and brain RNAs were abundant in samples from the CSF. The biological etiology of this is elusive, but is certainly plausible given the similar physiologic function of filtration and electrolyte regulation in both urine and CSF.

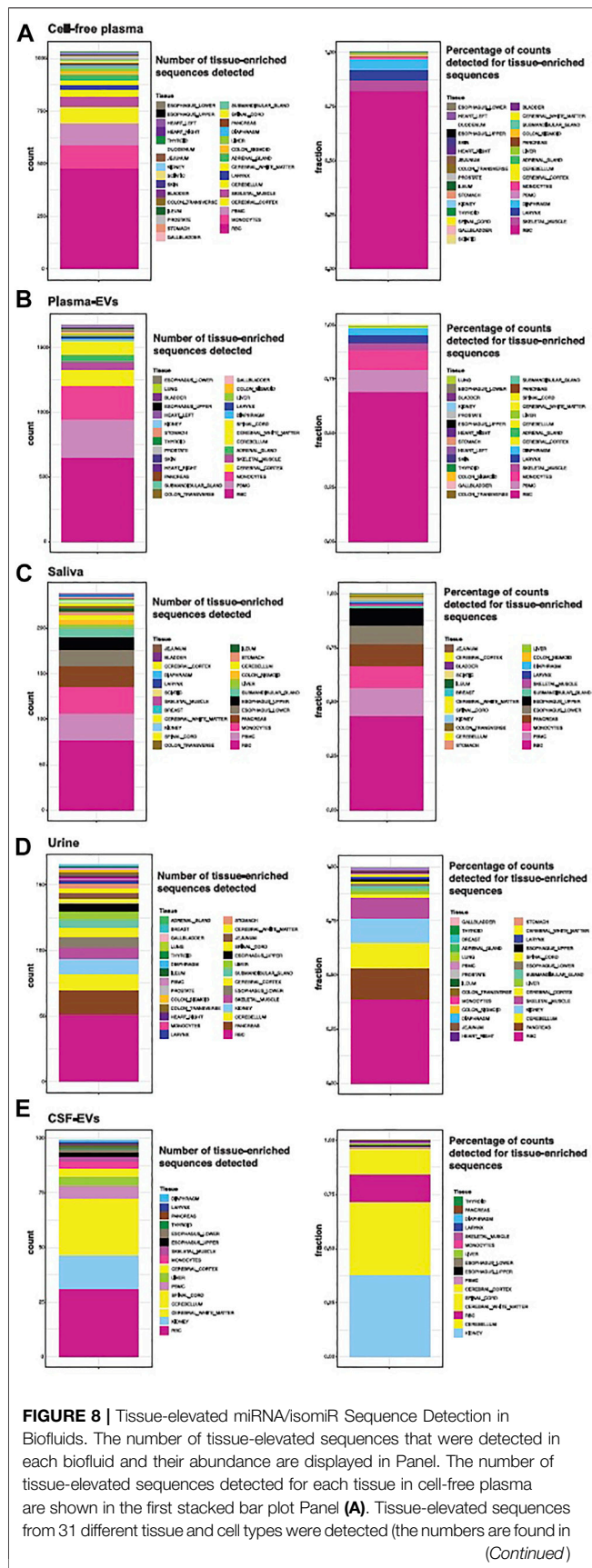


FIGURE 8 | Supplementary Table S5). The second stacked bar plot in Panel (A) displays the fraction of tissue-elevated reads going to each sample type. 69% of the tissue-elevated reads in cell-free plasma go to red blood cells. The number of tissue-elevated sequences detected and the fraction of tissue-elevated reads going to each tissue are displayed for plasma EVs [Panel (B)], saliva [Panel (C)], Urine [Panel (D)], and CSF [Panel (E)].

TABLE 2 | Tissue-enriched gallbladder tRF. A tRF sequence that is enriched in gallbladder is also highly expressed in many tissues.

Tissue	Normalized counts
ADRENAL_GLAND	120,004
BLADDER	127,655
BREAST	44,534
CEREBELLUM	124,302
CEREBRAL_CORTX	137,064
CEREBRAL_WHITE_MATTER	424,818
COLON_TRANSVERSE	75,749
DIAPHRAGM	381,206
DUODENUM	619,992
GALLBLADDER	15,953,357
ILEUM	1,615,212
JEJUNUM	284,800
KIDNEY	139,955
LARYNX	958,023
LEFT_HEART	22,264
LIVER	530,800
LOWER_ESOPHAGUS	58,251
LUNG	66,785
MONO	11,612
PANCREAS	187,907
PBMC	6,166
PROSTATE	64,484
RBC	490
RIGHT_HEART	22,535
SCIATIC	393,225
SIGMOID_COLON	114,936
SKELETAL_MUSCLE	34,388
SKIN	136,642
SPINAL_CORD	164,628
STOMACH	164,674
SUBMANDIBULAR_GLAND	37,790
THYROID	146,499
UPPER_ESOPHAGUS	121,985

The two sequences with the highest detection in CSF are tissue-enriched for both kidney and cerebellum and are detected with >1000 counts each in CSF, miR-204-5p and an isomiR of that miRNA.

In the examination of differentially expressed tissue-elevated sequences found in blood, it was interesting to note that we were able to identify tissue sequences related to the disease or event. Because we do not have corresponding tissue samples from these individuals, it is hard to know if these changes directly correlate with the tissue, or if there is different usage or packaging of these sequences into the extracellular biofluid.

Clinically, this simplistic “deconvolution” within biofluids may facilitate targeting those RNAs that may be more specific for disease monitoring. Certainly, this tissue-enrichment

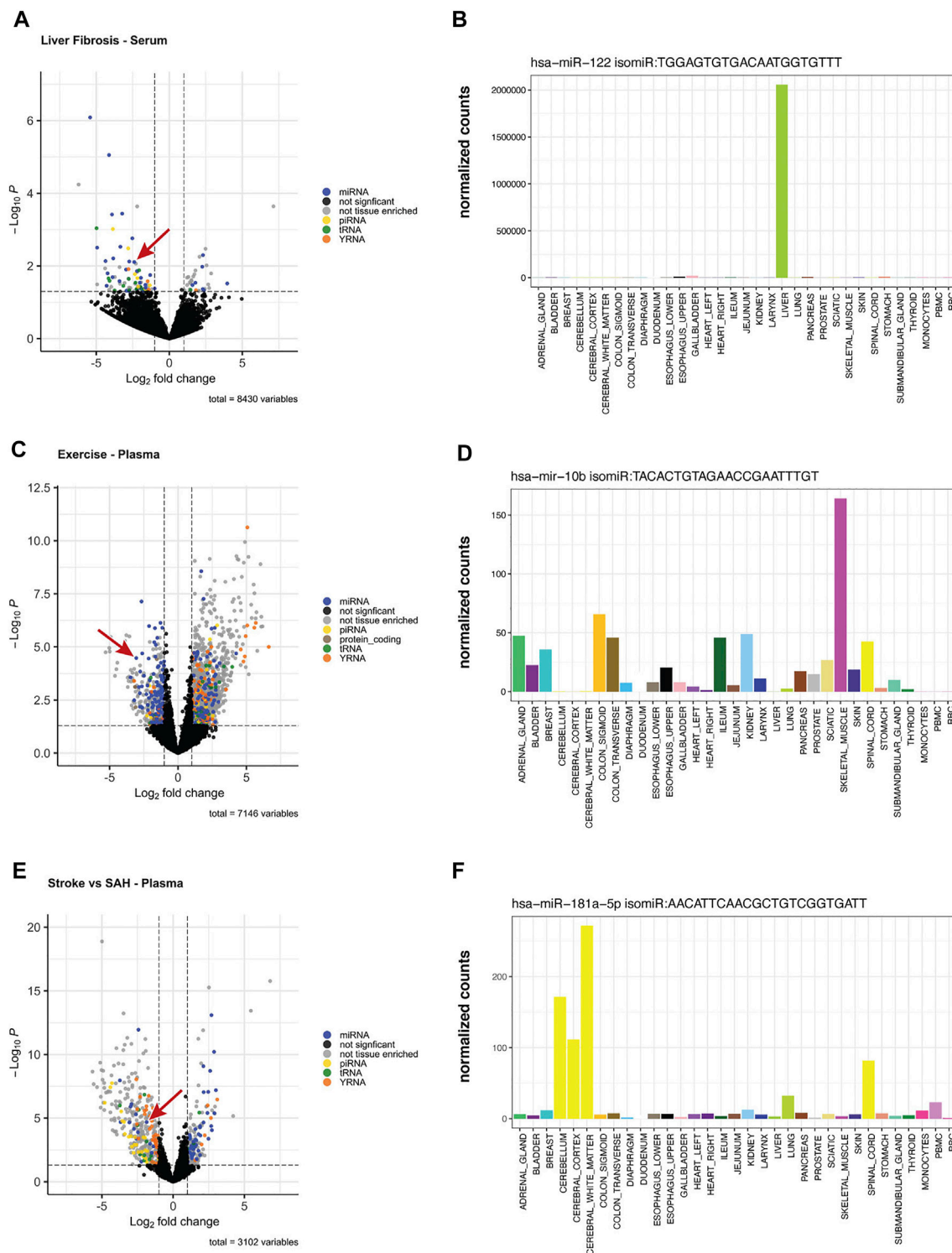


FIGURE 9 | Differentially Expressed Tissue-Elevated Sequences. We examined three different conditions for differentially expressed sequences: **(A)** displays the differentially expressed tissue-elevated sequences found in blood samples from Hepatitis C patients with liver fibrosis stage 1 or stage 4. Panel **(B)** is a tissue-elevated isomiR that is highly expressed in liver. **(C)** are samples taken from participants before and after exercise, and the expression level of a muscle-elevated isomiR across tissues **(D)**. The final example is from a comparison of plasma samples taken from individuals that had either an ischaemic stroke or a subarachnoid hemorrhage, Panel **(E)**. In Panel **(F)**, we selected a CNS-elevated isomiR of miR-181-5p to illustrate tissue-elevation.

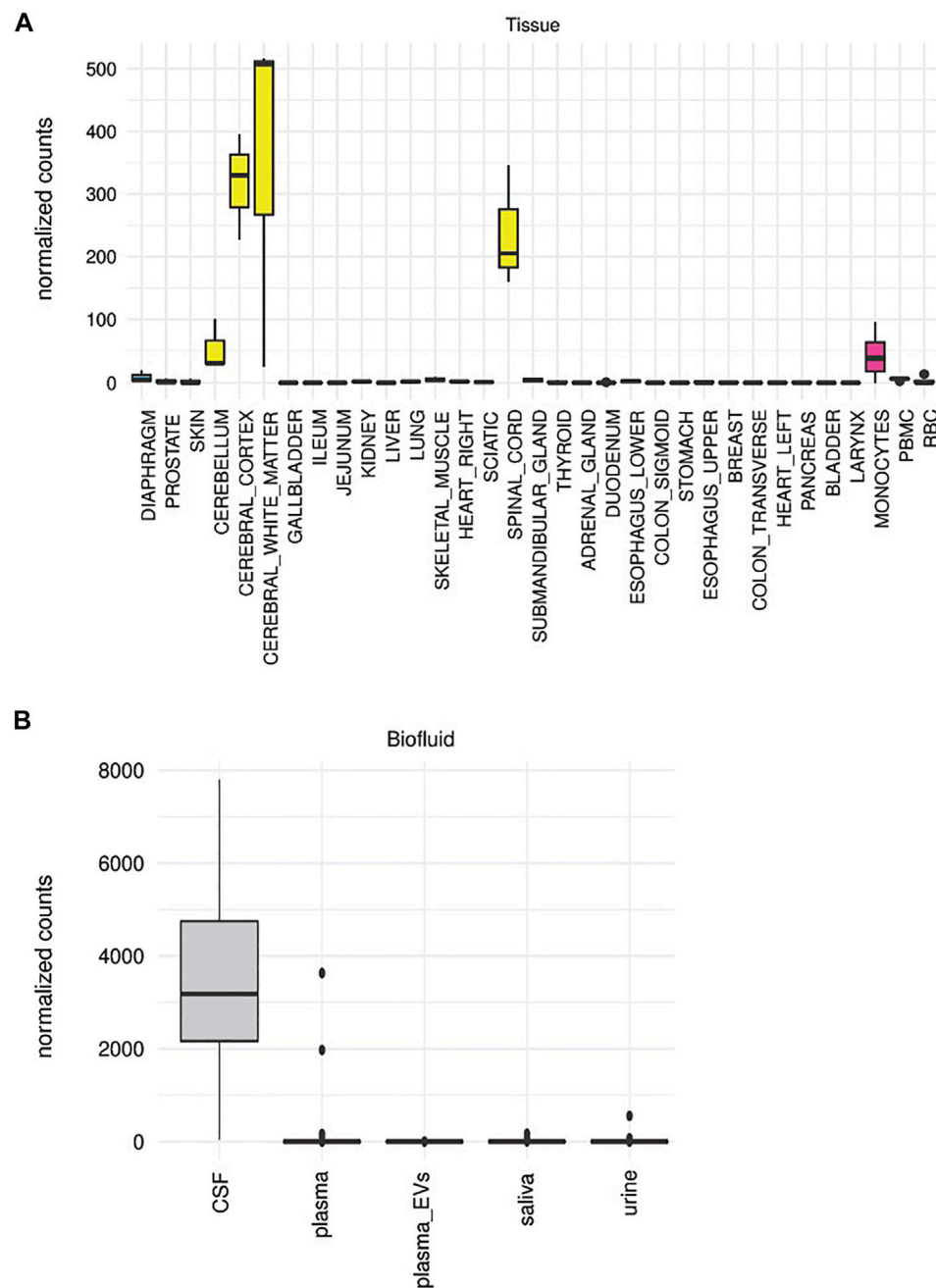


FIGURE 10 | exRNA Expression Atlas. In **(A)** we display the expression of a tissue-elevated miRNA for the CNS (miR-1298) across all tissues. Panel **(B)** displays the detection of tissue-elevated miR-1298 in different biofluids. As can be seen in this figure, this enriched CNS miRNA is detectable in CSF, but poorly detected in the other biofluids. These data will provide insight regarding which biofluids hold which tissue-elevated sequences.

estimation is imperfect. First, we do not have many replicate tissues and samples from every tissue or cell type within that tissue. Nevertheless, we believe that the current study provides a framework to build shared resources to assist in studies of tissue- and cell-specific RNAs and their presence in biofluids (exRNAs). Some obvious confounding factors to target in future experimental designs include: 1) some tissues may

actively release more EVs or EVs with more/specific RNA cargo, 2) vascularization of the tissue may lead to greater abundance in plasma, and 3) selective RNA loading that does not contain tissue enriched sequences for some of the tissues will lead to the appearance of low abundance in plasma, and 4) understanding expression level changes with disease, injury, stress, development or aging. In

addition, care to avoid overinterpretation of “concentration” of a given exRNA species is needed: for example, the abundant gallbladder-enriched tRF is also abundant across many other tissues. Ultimately, with the incorporation of larger numbers of matched tissue and biofluid samples, these data may expand the repertoire of disease-relevant exRNA biomarkers. We have created a tool (the exRNA Expression Atlas) to display tissue-elevated sequences and their abundance in biofluids that is freely accessible to the scientific community.

DATA AVAILABILITY STATEMENT

All sequencing data is available through the ERCC exRNA Atlas: <https://exrna-atlas.org/exat/datasets> (EXR-KJENS1WBaSro-AN), <https://exrna-atlas.org/exat/datasets> (EXRKJENS1sPlvS2AN), <https://exrna-atlas.org/exat/datasets> (EXR-KJENS1sPlvS2-AN), <https://exrna-atlas.org/exat/datasets> (EXR-KJENS1TISSUEDATA-AN); and through accession number phs001258.v1.p1 in dbGaP: <https://www.ncbi.nlm.nih.gov/gap/>.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the appropriate institutional review board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Study Design: EA, BM, RK, LL, SD, and KV. Data Collection and Interpretation: EA, BM, RK, TS, TB, GES, EH, IG, RR, MS, MH, AC-L, NV, TW, JRa, PP, JRo, JG, MJH, RS, LL, SD, and KV. Writing: EA, RK, TW, RS, LL, SD, and KV. Figures: EA, RK, TW, SD, and KV.

FUNDING

This work was funded by a grant from the National Institutes of Health-National Center for Translational Sciences (UH3 TR000891-03S1). The Brain and Body Donation Program is supported by the National Institute of Neurological Disorders and Stroke (U24 NS072026 National Brain and Tissue Resource for Parkinson’s Disease and Related Disorders), the National Institute on Aging (P30 AG19610 Arizona Alzheimer’s Disease Core Center), the Arizona Department of Health Services

(contract 211002, Arizona Alzheimer’s Research Center), the Arizona Biomedical Research Commission (contracts 4001, 0011,05-90 and 1001 to the Arizona Parkinson’s Disease Consortium). The work was also funded by the Flinn Foundation (Award #s 1994 and 2037).

ACKNOWLEDGMENTS

We thank NCATS Extracellular RNA Communication Consortium (UH3 TR000891-03S1) for funding and to the directors that provided encouragement and advice, John Satterlee and Dan Tagle. We thank Aleksander Milosavljevic and his group at Baylor for their help in the ERCC DMRR. We are grateful to the Banner Sun Health Research Institute Brain and Body Donation Program of Sun City, Arizona for the provision of human tissue. We also thank the Michael J. Fox Foundation for Parkinson’s Research for their continued support. We thank Jeff Watkins from TGen for his help with figures and beautification of the manuscript. We thank the Center for Collaborative Sequencing at TGen for maintaining the sequencing machines and related equipment. We acknowledge the Dorrance Family Research Fund for supporting studies of extracellular vesicles at TGen.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2022.804164/full#supplementary-material>

Supplementary Figure S1 | This graph shows the rarefaction curves of each tissue. As can be seen here, the detection rate of new miRNAs rises steeply and then begins to plateau at 1 million reads and 3 million reads. These data indicate that using the most diverse class of small RNA, we are capturing most of the diversity for each tissue with our read depth.

Supplementary Figure S2 | Supplemental figure 1 displays the UMAP clusters when using either tissue-elevated tRFs, piRNAs, or yRFs. tRFs do a better job of clustering tissues than do the other RNA biotypes displayed here. These sequences are not as tissue-elevated and the replicates are not as close as they are with tissue-elevated miRNA and isomiRs.

Supplementary Table S1 | RIN values for tissue samples.

Supplementary Table S2 | Demographic information for tissue samples.

Supplementary Table S3 | IsomiR sequences for miR-451a in monocytes, PBMCs and RBCs.

Supplementary Table S4 | Tissue-elevated sequences and their fold-enrichments.

Supplementary Table S5 | The number of tissue-elevated sequences that go to each RNA biotype for each tissue and biofluid.

Supplementary Table S6 | The number of tissue-elevated sequences detected in each biofluid.

REFERENCES

- Abdelhamid, R. F., Plessy, C., Yamauchi, Y., Taoka, M., de Hoon, M., Gingeras, T. R., et al. (2014). Multiplicity of 5' Cap Structures Present on Short RNAs. *PLoS One* 9 (7), e102895. doi:10.1371/journal.pone.0102895
- Agarwal, V., Bell, G. W., Nam, J.-W., and Bartel, D. P. (2015). Predicting Effective microRNA Target Sites in Mammalian mRNAs. *Elife* 4. doi:10.7554/eLife.05005
- Akat, K. M., Lee, Y. A., Hurley, A., Morozov, P., Max, K. E. A., Brown, M., et al. (2019). Detection of Circulating Extracellular mRNAs by Modified Small-RNA-Sequencing Analysis. *JCI Insight* 4. doi:10.1172/jci.insight.127317
- Arroyo, J. D., Chevillet, J. R., Kroh, E. M., Ruf, I. K., Pritchard, C. C., Gibson, D. F., et al. (2011). Argonaute2 Complexes Carry a Population of Circulating microRNAs Independent of Vesicles in Human Plasma. *Proc. Natl. Acad. Sci.* 108 (12), 5003–5008. doi:10.1073/pnas.1019055108
- Avendaño-Vázquez, S. E., and Flores-Jasso, C. F. (2020). Stumbling on Elusive Cargo: How isomiRs challenge microRNA Detection and Quantification, the Case of Extracellular Vesicles. *J. Extracellular Vesicles* 9 (1), 1784617. doi:10.1080/20013078.2020.1784617
- Bartel, D. P. (2009). MicroRNAs: Target Recognition and Regulatory Functions. *Cell* 136 (2), 215–233. doi:10.1016/j.cell.2009.01.002
- Ben-Dov, I. Z., Whalen, V. M., Goilav, B., Max, K. E. A., and Tuschl, T. (2016). Cell and Microvesicle Urine microRNA Deep Sequencing Profiles from Healthy Individuals: Observations with Potential Impact on Biomarker Studies. *PLoS One* 11 (1), e0147249. doi:10.1371/journal.pone.0147249
- Burgos, K. L., Javaherian, A., Bomprezzi, R., Ghaffari, L., Rhodes, S., Courtright, A., et al. (2013). Identification of Extracellular miRNA in Human Cerebrospinal Fluid by Next-Generation Sequencing. *RNA* 19 (5), 712–722. doi:10.1261/rna.036863.112
- Burgos, K. L., and Van Keuren-Jensen, K. (2014). RNA Isolation for Small RNA Next-Generation Sequencing from Acellular Biofluids. *Methods Mol. Biol.* 1182, 83–92. doi:10.1007/978-1-4939-1062-5_8
- Byron, S. A., Van Keuren-Jensen, K. R., Engelthaler, D. M., Carpten, J. D., and Craig, D. W. (2016). Translating RNA Sequencing into Clinical Diagnostics: Opportunities and Challenges. *Nat. Rev. Genet.* 17 (5), 257–271. doi:10.1038/nrg.2016.10
- Chen, L., Chen, Z., Simões, A., Wu, X., Dai, Y., DiPietro, L. A., et al. (2020). Site-Specific Expression Pattern of PIWI-Interacting RNA in Skin and Oral Mucosal Wound Healing. *Ijms* 21 (2), 521. doi:10.3390/ijms21020521
- Cozen, A. E., Quartley, E., Holmes, A. D., Hrabeta-Robinson, E., Phizicky, E. M., and Lowe, T. M. (2015). ARM-seq: AlkB-Facilitated RNA Methylation Sequencing Reveals a Complex Landscape of Modified tRNA Fragments. *Nat. Methods* 12 (9), 879–884. doi:10.1038/nmeth.3508
- Cui, C., and Cui, Q. (2020). The Relationship of Human Tissue microRNAs with Those from Body Fluids. *Sci. Rep.* 10 (1), 5644. doi:10.1038/s41598-020-62534-6
- Dhabhi, J. M., Spindler, S. R., Atamna, H., Boffelli, D., Mote, P., and Martin, D. I. K. (2013). 5'-YRNA Fragments Derived by Processing of Transcripts from Specific YRNA Genes and Pseudogenes Are Abundant in Human Serum and Plasma. *Physiol. Genomics* 45 (21), 990–998. doi:10.1152/physiolgenomics.00129.2013
- Dhabhi, J., Nunez Lopez, Y. O., Schneider, A., Victoria, B., Saccon, T., Bharat, K., et al. (2019). Profiling of tRNA Halves and YRNA Fragments in Serum and Tissue from Oral Squamous Cell Carcinoma Patients Identify Key Role of 5' tRNA-Val-CAC-2-1 Half. *Front. Oncol.* 9, 959. doi:10.3389/fonc.2019.00959
- Dittmar, K. A., Goodenbour, J. M., and Pan, T. (2006). Tissue-specific Differences in Human Transfer RNA Expression. *Plos Genet.* 2 (12), e221. doi:10.1371/journal.pgen.0020221
- Driedonks, T. A. P., Mol, S., Bruin, S., Peters, A. L., Zhang, X., Lindenbergh, M. F. S., et al. (2020). Y-RNA Subtype Ratios in Plasma Extracellular Vesicles Are Cell Type-Specific and Are Candidate Biomarkers for Inflammatory Diseases. *J. Extracellular Vesicles* 9 (1), 1764213. doi:10.1080/20013078.2020.1764213
- Ebhardt, H. A., Tsang, H. H., Dai, D. C., Liu, Y., Bostan, B., and Fahlman, R. P. (2009). Meta-analysis of Small RNA-Sequencing Errors Reveals Ubiquitous post-transcriptional RNA Modifications. *Nucleic Acids Res.* 37 (8), 2461–2470. doi:10.1093/nar/gkp093
- Ellwanger, D. C., Büttner, F. A., Mewes, H.-W., and Stümpflen, V. (2011). The Sufficient Minimal Set of miRNA Seed Types. *Bioinformatics* 27 (10), 1346–1350. doi:10.1093/bioinformatics/btr149
- Enderle, D., Spiel, A., Coticchia, C. M., Berghoff, E., Mueller, R., Schlumpberger, M., et al. (2015). Characterization of RNA from Exosomes and Other Extracellular Vesicles Isolated by a Novel Spin Column-Based Method. *PLoS One* 10 (8), e0136133. doi:10.1371/journal.pone.0136133
- Everaert, C., Helmsmoortel, H., Decock, A., Hulstaert, E., Van Paemel, R., Verniers, K., et al. (2019). Performance Assessment of Total RNA Sequencing of Human Biofluids and Extracellular Vesicles. *Sci. Rep.* 9 (1), 17574. doi:10.1038/s41598-019-53892-x
- Fehlmann, T., Lehallier, B., Schaum, N., Hahn, O., Kahraman, M., Li, Y., et al. (2020). Common Diseases Alter the Physiological Age-Related Blood microRNA Profile. *Nat. Commun.* 11 (1), 5958. doi:10.1038/s41467-020-19665-1
- Ferrero, G., Cordero, F., Tarallo, S., Arigoni, M., Riccardo, F., Gallo, G., et al. (2018). Small Non-coding RNA Profiling in Human Biofluids and Surrogate Tissues from Healthy Individuals: Description of the Diverse and Most Represented Species. *Oncotarget* 9 (3), 3097–3111. doi:10.18632/oncotarget.23203
- Giraldez, M. D., Spengler, R. M., Etheridge, A., Godoy, P. M., Barczak, A. J., Srinivasan, S., et al. (2018). Comprehensive Multi-center Assessment of Small RNA-Seq Methods for Quantitative miRNA Profiling. *Nat. Biotechnol.* 36 (8), 746–757. doi:10.1038/nbt.4183
- Giraldez, M. D., Spengler, R. M., Etheridge, A., Goicochea, A. J., Tuck, M., Choi, S. W., et al. (2019). Phospho-RNA-seq: a Modified Small RNA-seq Method that Reveals Circulating mRNA and lncRNA Fragments as Potential Biomarkers in Human Plasma. *EMBO J.* 38 (11). doi:10.15252/embj.2019101695
- Godoy, P. M., Bhakta, N. R., Barczak, A. J., Cakmak, H., Fisher, S., MacKenzie, T. C., et al. (2018). Large Differences in Small RNA Composition between Human Biofluids. *Cel Rep.* 25 (5), 1346–1358. doi:10.1016/j.celrep.2018.10.014
- Gogakos, T., Brown, M., Garzia, A., Meyer, C., Hafner, M., and Tuschl, T. (2017). Characterizing Expression and Processing of Precursor and Mature Human tRNAs by Hydro-tRNAseq and PAR-CLIP. *Cel Rep.* 20 (6), 1463–1475. doi:10.1016/j.celrep.2017.07.029
- Griffiths-Jones, S. (2004). The microRNA Registry. *Nucleic Acids Res.* 32 (Database issue), 109D–111D. doi:10.1093/nar/gkh023
- Grimson, A., Farh, K. K.-H., Johnston, W. K., Garrett-Engle, P., Lim, L. P., and Bartel, D. P. (2007). MicroRNA Targeting Specificity in Mammals: Determinants beyond Seed Pairing. *Mol. Cel* 27 (1), 91–105. doi:10.1016/j.molcel.2007.06.017
- Guo, L., and Chen, F. (2014). A challenge for miRNA: Multiple isomiRs in miRNAomics. *Gene* 544 (1), 1–7. doi:10.1016/j.gene.2014.04.039
- Guo, L., and Lu, Z. (2010). Global Expression Analysis of miRNA Gene Cluster and Family Based on isomiRs from Deep Sequencing Data. *Comput. Biol. Chem.* 34 (3), 165–171. doi:10.1016/j.compbiolchem.2010.06.001
- Haseeb, A., Makki, M. S., Khan, N. M., Ahmad, I., and Haqqi, T. M. (2017). Deep Sequencing and Analyses of miRNAs, isomiRs and miRNA Induced Silencing Complex (miRISC)-Associated miRNA in Primary Human Chondrocytes. *Sci. Rep.* 7 (1), 15178. doi:10.1038/s41598-017-15388-4
- Hulstaert, E., Morlion, A., Avila Cobos, F., Verniers, K., Nuytens, J., Vanden Eynde, E., et al. (2020). Charting Extracellular Transcriptomes in the Human Biofluid RNA Atlas. *Cel Rep.* 33 (13), 108552. doi:10.1016/j.celrep.2020.108552
- Ishikawa, T., Haino, A., Seki, M., Terada, H., and Nashimoto, M. (2017). The Y4-RNA Fragment, a Potential Diagnostic Marker, Exists in Saliva. *Non-coding RNA Res.* 2 (2), 122–128. doi:10.1016/j.ncrna.2017.07.002
- Jain, A., and Tuteja, G. (2019). TissueEnrich: Tissue-specific Gene Enrichment Analysis. *Bioinformatics* 35 (11), 1966–1967. doi:10.1093/bioinformatics/bty890
- Kalani, M. Y. S., Alsop, E., Meechoovet, B., Beecroft, T., Agrawal, K., Whitsett, T. G., et al. (2020). Extracellular microRNAs in Blood Differentiate between Ischaemic and Haemorrhagic Stroke Subtypes. *J. Extracellular Vesicles* 9 (1), 1713540. doi:10.1080/20013078.2020.1713540
- Karali, M., Persico, M., Mutarelli, M., Carissimo, A., Pizzo, M., Singh Marwah, V., et al. (2016). High-resolution Analysis of the Human Retina miRNome Reveals isomiR Variations and Novel microRNAs. *Nucleic Acids Res.* 44 (4), 1525–1540. doi:10.1093/nar/gkw039
- Kopkova, A., Sana, J., Fadrus, P., Machackova, T., Vecera, M., Vybihal, V., et al. (2018). MicroRNA Isolation and Quantification in Cerebrospinal Fluid: A Comparative Methodical Study. *PLoS One* 13 (12), e0208580. doi:10.1371/journal.pone.0208580

- Kozomara, A., Birgaoanu, M., and Griffiths-Jones, S. (2019). miRBase: from microRNA Sequences to Function. *Nucleic Acids Res.* 47 (D1), D155–D162. doi:10.1093/nar/gky1141
- Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., et al. (2007). A Mammalian microRNA Expression Atlas Based on Small RNA Library Sequencing. *Cell* 129 (7), 1401–1414. doi:10.1016/j.cell.2007.04.040
- Lee, L. W., Zhang, S., Etheridge, A., Ma, L., Martin, D., Galas, D., et al. (2010). Complexity of the microRNA Repertoire Revealed by Next-Generation Sequencing. *RNA* 16 (11), 2170–2180. doi:10.1261/rna.2225110
- Li, F., Kaczor-Urbanowicz, K. E., Sun, J., Majem, B., Lo, H.-C., Kim, Y., et al. (2018). Characterization of Human Salivary Extracellular RNA by Next-Generation Sequencing. *Clin. Chem.* 64 (7), 1085–1095. doi:10.1373/clinchem.2017.285072
- Liang, T., Yu, J., Liu, C., and Guo, L. (2017). IsomiR Expression Patterns in Canonical and Dicer-independent microRNAs. *Mol. Med. Rep.* 15 (3), 1071–1078. doi:10.3892/mmr.2017.6117
- Londin, E., Lohrer, P., Telonis, A. G., Quann, K., Clark, P., Jing, Y., et al. (2015). Analysis of 13 Cell Types Reveals Evidence for the Expression of Numerous Novel Primate- and Tissue-specific microRNAs. *Proc. Natl. Acad. Sci. USA* 112 (10), E1106–E1115. doi:10.1073/pnas.1420955112
- Love, M. I., Huber, W., and Anders, S. (2014). Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2. *Genome Biol.* 15 (12), 550. doi:10.1186/s13059-014-0550-8
- Luciano, D. J., Mirsky, H., Vendetti, N. J., and Maas, S. (2004). RNA Editing of a miRNA Precursor: FIGURE 1. *RNA* 10 (8), 1174–1177. doi:10.1261/rna.7350304
- Ludwig, N., Leidinger, P., Becker, K., Backes, C., Fehlmann, T., Pallasch, C., et al. (2016). Distribution of miRNA Expression across Human Tissues. *Nucleic Acids Res.* 44 (8), 3865–3877. doi:10.1093/nar/gkw116
- Magee, R., Londin, E., and Rigoutsos, I. (2019). TRNA-derived Fragments as Sex-dependent Circulating Candidate Biomarkers for Parkinson's Disease. *Parkinsonism Relat. Disord.* 65, 203–209. doi:10.1016/j.parkreldis.2019.05.035
- Maleki Dana, P., Mansournia, M. A., and Mirhashemi, S. M. (2020). PIWI-interacting RNAs: New Biomarkers for Diagnosis and Treatment of Breast Cancer. *Cell Biosci.* 10, 44. doi:10.1186/s13578-020-00403-5
- Marco-Puche, G., Lois, S., Benítez, J., and Trivino, J. C. (2019). RNA-seq Perspectives to Improve Clinical Diagnosis. *Front. Genet.* 10, 1152. doi:10.3389/fgene.2019.01152
- Martí, E., Pantano, L., Bañez-Coronel, M., Llorens, F., Miñones-Moyano, E., Porta, S., et al. (2010). A Myriad of miRNA Variants in Control and Huntington's Disease Brain Regions Detected by Massively Parallel Sequencing. *Nucleic Acids Res.* 38 (20), 7219–7235. doi:10.1093/nar/gkq575
- Martínez, V. D., Vucic, E. A., Thu, K. L., Hubaux, R., Enfield, K. S. S., Pikor, L. A., et al. (2015). Unique Somatic and Malignant Expression Patterns Implicate PIWI-Interacting RNAs in Cancer-type Specific Biology. *Sci. Rep.* 5, 10423. doi:10.1038/srep10423
- Max, K. E. A., Bertram, K., Akat, K. M., Bogardus, K. A., Li, J., Morozov, P., et al. (2018). Human Plasma and Serum Extracellular Small RNA Reference Profiles and Their Clinical Utility. *Proc. Natl. Acad. Sci. USA* 115 (23), E5334–E5343. doi:10.1073/pnas.1714397115
- Murillo, O. D., Thistlethwaite, W., Rozowsky, J., Subramanian, S. L., Lucero, R., Shah, N., et al. (2019). exRNA Atlas Analysis Reveals Distinct Extracellular RNA Cargo Types and Their Carriers Present across Human Biofluids. *Cell* 177 (2), 463–477. doi:10.1016/j.cell.2019.02.018
- Nik Mohamed Kamal, N. N. S. B., and Shahidan, W. N. S. (2019). Non-Exosomal and Exosomal Circulatory MicroRNAs: Which Are More Valid as Biomarkers? *Front. Pharmacol.* 10, 1500. doi:10.3389/fphar.2019.01500
- O'Brien, K., Breyne, K., Ughetto, S., Laurent, L. C., and Breakefield, X. O. (2020). RNA Delivery by Extracellular Vesicles in Mammalian Cells and its Applications. *Nat. Rev. Mol. Cell Biol.* 21 (10), 585–606. doi:10.1038/s41580-020-0251-y
- Pan, T. (2018). Modifications and Functional Genomics of Human Transfer RNA. *Cell Res.* 28 (4), 395–404. doi:10.1038/s41422-018-0013-y
- Perera, B. P. U., Tsai, Z. T.-Y., Colwell, M. L., Jones, T. R., Goodrich, J. M., Wang, K., et al. (2019). Somatic Expression of piRNA and Associated Machinery in the Mouse Identifies Short, Tissue-specific piRNA. *Epigenetics* 14 (5), 504–521. doi:10.1080/15592294.2019.1600389
- Qu, A., Wang, W., Yang, Y., Zhang, X., Dong, Y., Zheng, G., et al. (2019). A Serum piRNA Signature as Promising Non-invasive Diagnostic and Prognostic Biomarkers for Colorectal Cancer. *Cmar* Vol. 11, 3703–3720. doi:10.2147/CMAR.S193266
- Rahman, R.-U., Liebhoff, A.-M., Bansal, V., Fiosins, M., Rajput, A., Sattar, A., et al. (2020). SEAwab: the Small RNA Expression Atlas Web Application. *Nucleic Acids Res.* 48 (D1), D204–D219. doi:10.1093/nar/gkz869
- Rodosthenous, R. S., Hutchins, E., Reiman, R., Yeri, A. S., Srinivasan, S., Whitsett, T. G., et al. (2020). Profiling Extracellular Long RNA Transcriptome in Human Plasma and Extracellular Vesicles for Biomarker Discovery. *iScience* 23 (6), 101182. doi:10.1016/j.isci.2020.101182
- Rozowsky, J., Kitchen, R. R., Park, J. J., Galeev, T. R., Diao, J., Warrell, J., et al. (2019). exRpt: A Comprehensive Analytic Platform for Extracellular RNA Profiling. *Cel Syst.* 8 (4), 352–357. doi:10.1016/j.cels.2019.03.004
- Sagi, D., Rak, R., Gingold, H., Adir, I., Maayan, G., Dahan, O., et al. (2016). Tissue- and Time-specific Expression of Otherwise Identical tRNA Genes. *Plos Genet.* 12 (8), e1006264. doi:10.1371/journal.pgen.1006264
- Saugstad, J. A., Lusardi, T. A., Van Keuren-Jensen, K. R., Phillips, J. I., Lind, B., Harrington, C. A., et al. (2017). Analysis of Extracellular RNA in Cerebrospinal Fluid. *J. Extracellular Vesicles* 6 (1), 1317577. doi:10.1080/20013078.2017.1317577
- Savelyeva, A. V., Kuligina, E. V., Bariakin, D. N., Kozlov, V. V., Ryabchikova, E. I., Richter, V. A., et al. (2017). Variety of RNAs in Peripheral Blood Cells, Plasma, and Plasma Fractions. *Biomed. Res. Int.* 2017, 1–10. doi:10.1155/2017/7404912
- Shah, R., Yeri, A., Das, A., Courtright-Lim, A., Ziegler, O., Gervino, E., et al. (2017). Small RNA-Seq during Acute Maximal Exercise Reveal RNAs Involved in Vascular Inflammation and Cardiometabolic Health: Brief Report. *Am. J. Physiology-Heart Circulatory Physiol.* 313 (6), H1162–H1167. doi:10.1152/ajpheart.00500.2017
- Skog, J., Würdinger, T., van Rijn, S., Meijer, D. H., Gainche, L., Curry, W. T., et al. (2008). Glioblastoma Microvesicles Transport RNA and Proteins that Promote Tumour Growth and Provide Diagnostic Biomarkers. *Nat. Cell Biol.* 10 (12), 1470–1476. doi:10.1038/ncb1800
- Srinivasan, S., Yeri, A., Cheah, P. S., Chung, A., Danielson, K., De Hoff, P., et al. (2019). Small RNA Sequencing across Diverse Biofluids Identifies Optimal Methods for exRNA Isolation. *Cell* 177 (2), 446–462. doi:10.1016/j.cell.2019.03.024
- Sun, L., Yu, Y., Niu, B., and Wang, D. (2020). Red Blood Cells as Potential Repositories of MicroRNAs in the Circulatory System. *Front. Genet.* 11, 442. doi:10.3389/fgene.2020.00442
- Tabet, F., Vickers, K. C., Cuesta Torres, L. F., Wiese, C. B., Shoucri, B. M., Lambert, G., et al. (2014). HDL-transferred microRNA-223 Regulates ICAM-1 Expression in Endothelial Cells. *Nat. Commun.* 5, 3292. doi:10.1038/ncomms4292
- Takeshita, T., Kageyama, S., Furuta, M., Tsuboi, H., Takeuchi, K., Shibata, Y., et al. (2016). Bacterial Diversity in Saliva and Oral Health-Related Conditions: the Hisayama Study. *Sci. Rep.* 6, 22164. doi:10.1038/srep22164
- Tang, Y.-T., Huang, Y.-Y., Zheng, L., Qin, S.-H., Xu, X.-P., An, T.-X., et al. (2017). Comparison of Isolation Methods of Exosomes and Exosomal RNA from Cell Culture Medium and Serum. *Int. J. Mol. Med.* 40 (3), 834–844. doi:10.3892/ijmm.2017.3080
- Telonis, A. G., Lohrer, P., Jing, Y., Londin, E., and Rigoutsos, I. (2015). Beyond the One-Locus-One-miRNA Paradigm: microRNA Isoforms Enable Deeper Insights into Breast Cancer Heterogeneity. *Nucleic Acids Res.* 43 (19), 9158–9175. doi:10.1093/nar/gkv922
- Telonis, A. G., Magee, R., Lohrer, P., Chervoneva, I., Londin, E., and Rigoutsos, I. (2017). Knowledge about the Presence or Absence of miRNA Isoforms (isomiRs) Can Successfully Discriminate Amongst 32 TCGA Cancer Types. *Nucleic Acids Res.* 45 (6), 2973–2985. doi:10.1093/nar/gkx082
- Tosar, J. P., and Cayota, A. (2020). Extracellular tRNAs and tRNA-Derived Fragments. *RNA Biol.* 17 (8), 1149–1167. doi:10.1080/15476286.2020.1729584
- Turchinovich, A., Weiz, L., Langheinz, A., and Burwinkel, B. (2011). Characterization of Extracellular Circulating microRNA. *Nucleic Acids Res.* 39 (16), 7223–7233. doi:10.1093/nar/gkr254
- Uhlén, M., Fagerberg, L., Hallström, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., et al. (2015). Tissue-based Map of the Human Proteome. *Science* 347 (6220), 1260419. doi:10.1126/science.1260419
- Umu, S. U., Langseth, H., Bucher-Johannessen, C., Fromm, B., Keller, A., Meese, E., et al. (2018). A Comprehensive Profile of Circulating RNAs in Human Serum. *RNA Biol.* 15 (2), 242–250. doi:10.1080/15476286.2017.1403003

- Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J., and Lötvall, J. O. (2007). Exosome-mediated Transfer of mRNAs and microRNAs Is a Novel Mechanism of Genetic Exchange between Cells. *Nat. Cell Biol.* 9 (6), 654–659. doi:10.1038/ncb1596
- Van Keuren-Jensen, K. R., Malenica, I., Courtright, A. L., Ghaffari, L. T., Starr, A. P., Metpally, R. P., et al. (2016). Micro RNA Changes in Liver Tissue Associated with Fibrosis Progression in Patients with Hepatitis C. *Liver Int.* 36 (3), 334–343. doi:10.1111/liv.12919
- Van Keuren-Jensen, K., Keats, J. J., and Craig, D. W. (2014). Bringing RNA-Seq Closer to the Clinic. *Nat. Biotechnol.* 32 (9), 884–885. doi:10.1038/nbt.3017
- Waller, R., Wyles, M., Heath, P. R., Kazoka, M., Wollff, H., Shaw, P. J., et al. (2017). Small RNA Sequencing of Sporadic Amyotrophic Lateral Sclerosis Cerebrospinal Fluid Reveals Differentially Expressed miRNAs Related to Neural and Glial Activity. *Front. Neurosci.* 11, 731. doi:10.3389/fnins.2017.00731
- Wang, X., Yang, Y., Tan, X., Mao, X., Wei, D., Yao, Y., et al. (2019). Identification of tRNA-Derived Fragments Expression Profile in Breast Cancer Tissues. *Cg* 20 (3), 199–213. doi:10.2174/1389202920666190326145459
- Wei, Z., Batagov, A. O., Schinelli, S., Wang, J., Wang, Y., El Fatimy, R., et al. (2017). Coding and Noncoding Landscape of Extracellular RNA Released by Human Glioma Stem Cells. *Nat. Commun.* 8 (1), 1145. doi:10.1038/s41467-017-01196-x
- Weiland, M., Gao, X.-H., Zhou, L., and Mi, Q.-S. (2012). Small RNAs Have a Large Impact. *RNA Biol.* 9 (6), 850–859. doi:10.4161/rna.20378
- Witwer, K. W. (2015). Circulating microRNA Biomarker Studies: Pitfalls and Potential Solutions. *Clin. Chem.* 61 (1), 56–63. doi:10.1373/clinchem.2014.221341
- Wu, C. W., Evans, J. M., Huang, S., Mahoney, D. W., Duke, B. A., Taylor, W. R., et al. (2018). A Comprehensive Approach to Sequence-Oriented IsomiR Annotation (CASMIR): Demonstration with IsomiR Profiling in Colorectal Neoplasia. *BMC Genomics* 19 (1), 401. doi:10.1186/s12864-018-4794-7
- Xu, H., Yao, J., Wu, D. C., and Lambowitz, A. M. (2019). Improved TGIRT-Seq Methods for Comprehensive Transcriptome Profiling with Decreased Adapter Dimer Formation and Bias Correction. *Sci. Rep.* 9 (1), 7953. doi:10.1038/s41598-019-44457-z
- Yáñez-Mó, M., Siljander, P. R.-M., Andreu, Z., Bedina Zavec, A., Borràs, F. E., Buzas, E. I., et al. (2015). Biological Properties of Extracellular Vesicles and Their Physiological Functions. *J. Extracellular Vesicles* 4, 27066. doi:10.3402/jev.v4.27066
- Yeri, A., Courtright, A., Danielson, K., Hutchins, E., Alsop, E., Carlson, E., et al. (2018). Evaluation of Commercially Available Small RNASeq Library Preparation Kits Using Low Input RNA. *BMC Genomics* 19 (1), 331. doi:10.1186/s12864-018-4726-6
- Yeri, A., Courtright, A., Reiman, R., Carlson, E., Beecroft, T., Janss, A., et al. (2017). Total Extracellular Small RNA Profiles from Plasma, Saliva, and Urine of Healthy Subjects. *Sci. Rep.* 7, 44061. doi:10.1038/srep44061
- Yu, F., Pillman, K. A., Neilsen, C. T., Toubia, J., Lawrence, D. M., Tsykin, A., et al. (2017). Naturally Existing Isoforms of miR-222 Have Distinct Functions. *Nucleic Acids Res.* 45 (19), 11371–11385. doi:10.1093/nar/gkx788

Conflict of Interest: KV and SD are members of the scientific advisory board at Dyrnamix. Their involvement in this company had no bearing on the work performed in this manuscript. KV is on the scientific advisory board of HTG. Her involvement has no bearing on this work.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Alsop, Meechoovet, Kitchen, Sweeney, Beach, Serrano, Hutchins, Ghiran, Reiman, Syring, Hsieh, Courtright-Lim, Valkov, Whitsett, Rakela, Pockros, Rozowsky, Gallego, Huentelman, Shah, Nakaji, Kalani, Laurent, Das and Van Keuren-Jensen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Extracellular Vesicle-Mediated Delivery of Ultrasmall Superparamagnetic Iron Oxide Nanoparticles to Mice Brain

Naseer A. Kutchy^{1,2}, Rong Ma^{1,3}, Yutong Liu⁴, Shilpa Buch¹ and Guoku Hu^{1*}

¹Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE, United States, ²Department of Anatomy, Physiology, and Pharmacology, School of Veterinary Medicine, St. George's University, St. George's, Grenada, ³Department of Pharmacology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, ⁴Department of Radiology, University of Nebraska Medical Center, Omaha, NE, United States

OPEN ACCESS

Edited by:

Suman Dutta,
International Institute of Innovation and
Technology, India

Reviewed by:

Bahnishikha Barman,
Vanderbilt University, United States
Ibrar Siddique,
UCLA Health System, United States
Roy McReynolds III,
University of California, Los Angeles,
United States, in collaboration with
reviewer IS

*Correspondence:

Guoku Hu
guoku.hu@unmc.edu

Specialty section:

This article was submitted to
Experimental Pharmacology and Drug
Discovery,
a section of the journal
Frontiers in Pharmacology

Received: 21 November 2021

Accepted: 07 March 2022

Published: 07 April 2022

Citation:

Kutchy NA, Ma R, Liu Y, Buch S and
Hu G (2022) Extracellular Vesicle-
Mediated Delivery of Ultrasmall
Superparamagnetic Iron Oxide
Nanoparticles to Mice Brain.
Front. Pharmacol. 13:819516.
doi: 10.3389/fphar.2022.819516

Extracellular vesicles (EVs) are small lipid membrane-bound vesicles that can pass the blood–brain barrier. Therefore, EVs could be used for the delivery of therapeutics to the brain. Herein, we investigated the biodistribution of intranasal perfusion of ultrasmall superparamagnetic iron oxide (USPIO)-labeled astrocyte-derived EVs (ADEVs) in mice. We used Western blotting, transmission electron microscopy (TEM), and nanoparticle uptake assay to characterize ADEVs. In addition, intranasal perfusion coupled with magnetic resonance imaging (MRI) was employed to determine the distribution of USPIO-labeled ADEVs in mice. Our results showed the uptake of USPIO by mouse astrocytes and ADEVs. In addition, we confirmed the biodistribution of ADEVs in the brain and other internal organs, including the kidneys, liver, and spleen. Our results suggest that USPIO did not affect mouse astrocyte cell survivability and EV release. Therefore, intranasal delivery of therapeutic loaded EVs could be used for the treatment of various brain disorders.

Keywords: brain drug delivery, extracellular vesicles, magnetic resonance imaging, nanoparticles, intranasal perfusion

INTRODUCTION

Extracellular vesicles (EVs) are small lipid membrane-bound vesicles and are heterogeneous in nature. They were initially thought of as secreted debris of platelets and have attracted research interest enormously in the past decade. EVs are subdivided into several subtypes as per their biogenesis pathways, including small EVs (diameter of 40–150 nm), which are present inside multi-vesicular bodies (MVBs) and released into the extracellular space by the fusion of MVBs with the cell membrane, and large EVs (diameter of 150–2,000 nm) originating from the plasma membrane (EL Andaloussi et al., 2013; Raposo and Stoorvogel, 2013; Théry et al., 2018; Mathieu et al., 2019; Pegtel and Gould, 2019). EVs released from almost all cell types are recognized as messengers in intercellular communication using stored cargoes such as proteins, lipids, and RNA molecules, including miRNA, mRNA, and tRNA (Ratajczak et al., 2006; Valadi et al., 2007; Al-Nedawi et al., 2008; Peinado et al., 2012; EL Andaloussi et al., 2013; Chivero et al., 2021). Cells treated with nanoparticles have been shown to be generating EVs with nanoparticles in them (Busato et al., 2016). Furthermore, EVs have also been utilized successfully to deliver siRNAs in rodents (van den Boorn

et al., 2011). EVs can cross the blood–brain barrier (BBB) easily; therefore, intranasal administration of EVs is considered a preferred non-invasive method for rapid delivery of EV-encapsulated drug(s) to the brain (Visweswaraiiah et al., 2002; Lakhal and Wood, 2011; Zhuang et al., 2011; Grassin-Delyle et al., 2012). Manipulating EVs and their cargo *ex vivo* can thus be envisioned as an efficient means for delivery to target organs.

The labeling of EVs with ultrasmall superparamagnetic iron oxide nanoparticles (USPIO, 4–6 nm) provides an advantage in that they do not alter the morphology and physiology of cells (Busato et al., 2016). The USPIO nanoparticles are approved by the U.S. Food and Drug Administration (FDA) and the European Commission for use as MRI contrast agents (Daldrup-Link, 2017). Nanoparticles could be loaded with different compounds like paclitaxel (PTX), a microtubule-stabilizing agent and a potent antineoplastic against small-cell lung carcinoma and breast cancer (Weaver, 2014; Ganipineni et al., 2019). The distribution of the nanoparticle-loaded EVs could be traced by magnetic resonance imaging (MRI) techniques, which are non-invasive methods of visualization. MRI is emerging as a functional probe system, the stimulus-responsive MRI-monitored drug delivery system, pH-responsive release, and thermo-responsive release system (Kim et al., 2013; Gupta et al., 2016; Park et al., 2016; Zheng et al., 2018). Therefore, in this study, we generated USPIO-labeled astrocyte-derived EVs (ADEVs) and tracked their biodistribution in mice using MRI.

Intranasal drug administration is a non-invasive method of delivering therapeutic agents to the brain and spinal cord (Hanson and Frey, 2008). This method is efficient in delivering drugs to the central nervous system (CNS) and replaces the invasive delivery methods that lead to unwanted side effects (Sil et al., 2020; Lombardo et al., 2021). In intranasal perfusion, the delivery to the CNS is fast and takes minutes to pass the BBB and reach the CNS along the olfactory and trigeminal neural pathways (Hanson and Frey, 2008) (Thorne et al., 1995; Chen et al., 1998).

This study demonstrated that USPIO exposure results in labeling EVs with USPIO released from astrocytes but does not affect cellular morphology, survivability, and EV release. Furthermore, intranasal perfusion of USPIO-labeled ADEVs showed that labeled EVs were localized in the brain, kidneys, and liver, as evidenced by MRI scanning.

MATERIALS AND METHODS

Animals

C57BL/6N wild-type mice (male, 6–8 weeks) used in this study were purchased from Charles River Laboratories, Inc. (Wilmington, MA, United States) and housed in the animal facility of the University of Nebraska Medical Center (UNMC) with free access to water/food and 12-h light/dark cycle and controlled temperature and humidity. Previous studies have demonstrated gender difference effects of intranasal delivery nanoparticle drugs in aged mice but not in young mice (Ma et al., 2016; Xu et al., 2020). This study aimed to test the concept of ADEV delivery to the brain; to reduce the variables, we used only male mice. All animal protocols were reviewed and approved by

the Institutional Animal Care and Use Committee (IACUC) at the UNMC.

Cell Cultures

The mouse astrocytic cell line C8D1A [ATCC® CRL-2541™; American Type Culture Collection (ATCC), Manassas, VA, United States] was cultured as described previously (Sturdivant et al., 2016) and maintained in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL). C8D1A cells were used within ten passages. Cells were serum-starved for 12 h, before being treated with USPIO.

Labeling of mouse astrocytes with ultrasmall superparamagnetic iron oxide nanoparticles (USPIO)

The commercial USPIO (magnetite Fe₃O₄; Sigma-Aldrich Co., St Louis, MO, United States; catalog #725331, particle size 4–6 nm, stock solution 5 mg Fe/mL) were used to label mouse astrocytic cells. The cells were incubated with an increasing concentration of USPIO nanoparticles (50, 100, 200, 300, 400 µg/mL) for 12 h, and USPIO were diluted in high-glucose DMEM supplemented with 10% heat-inactivated FBS, glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL). After incubation, the cells were washed twice with PBS, trypsinized, and counted, and cell cytotoxicity of nanoparticles was determined by automated cell counters (Thermo Fisher Scientific).

Nanoparticle Uptake Assay

Prussian blue staining was utilized to visibly assess the iron uptake by astrocytes that were treated with 100 µg/mL USPIO nanoparticles. Cells were seeded on coverslips kept in 12-well plates at a density of 1×10^6 cells and incubated with USPIO at 100 µg/mL for 12 h. Cells were then fixed with a 2% solution of paraformaldehyde for 30 min at room temperature and washed gently with PBS thrice. The cells were incubated for 40 min in a solution of 1% HCl and 2% potassium ferrocyanide, washed twice with distilled water, and counterstained using Nuclear Fast Red for 15 min. The cells were then washed twice with distilled water and finally embedded in a mounting medium (Dako Mounting Medium, Agilent Technologies, Santa Clara, CA, United States). The nanoparticle internalization was investigated using a light microscope. Iron nanoparticles appeared as blue spots inside the cells, while the nucleus appeared red. The intensity was measured using Zen 3.4 (Blue edition). The background intensity (control) was subtracted from the USPIO-treated cells.

Immunofluorescence

Cells cultured on slides or coverslips were fixed with 4% paraformaldehyde for 15 min at room temperature, followed by permeabilization with 0.3% Triton X-100 in PBS, and incubated with a blocking buffer containing 10% normal goat serum in PBS for 1 h at room temperature. The cells were then incubated with CoraLite®488-conjugated GFAP antibody (Proteintech, Rosemont, IL, United States) overnight at 4°C. The nuclei were labeled with DAPI. The slides were covered with a coverslip with ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA, United States) and allowed to dry

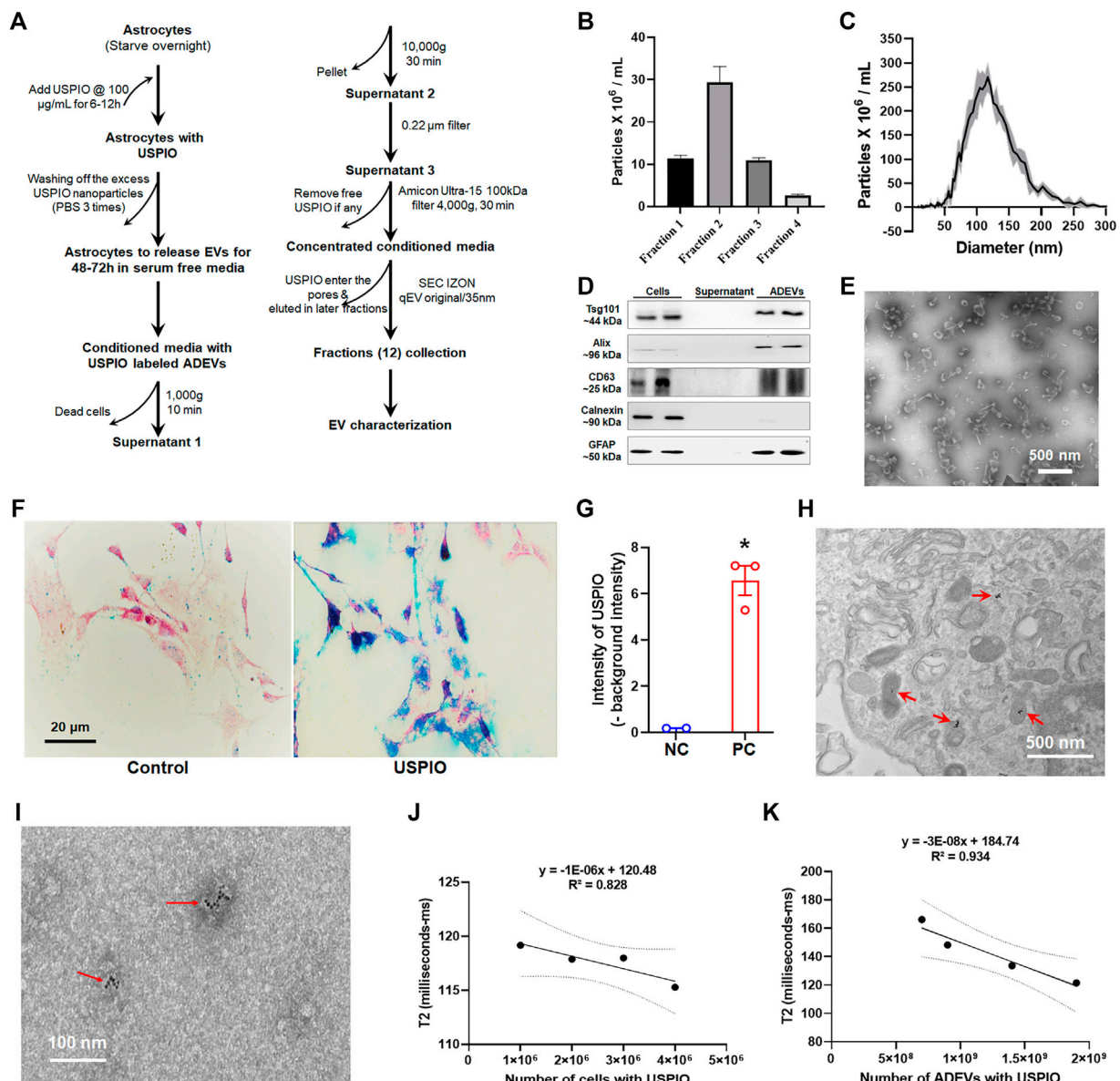


FIGURE 1 | Uptake of USPIO by mouse astrocytes and characterization of EVs. **(A)** Schematic diagram of the procedure used for separating EVs from astrocyte culture by size exclusion chromatography (SEC). **(B,C)** Number and size distribution plots of EVs from selected fractions by ZetaView. **(D)** The four fractions of the qEV column with maximum EVs were pooled together for detection of EV markers using Western blotting. EV proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electroblotted onto a nitrocellulose membrane. Calnexin (non-exosomal protein) served as negative control. **(E)** TEM image of EVs isolated from mouse astrocytes via a qEV column (IZON Science). **(F)** Cells were treated with 100 µg/ml of USPIO, and uptake of USPIO nanoparticles was determined by nanoparticle uptake assay. **(G)** Blue signal intensity in **(F)** measured using ZEN (Blue edition) software. **(H)** TEM image of astrocytes treated with USPIO. **(I)** TEM image of EVs isolated from USPIO-treated astrocytes. **(J,K)** Decrease in T2-weighted data acquired using MRI showing a strong linear correlation between R2 values and numbers of cells ($R^2 = 0.828$), and numbers of ADEVs ($R^2 = 0.934$). All experiments were repeated at least three times.

for 24 h at room temperature. Images were captured with a 20X objective.

Real-Time PCR

To determine the expression of GAPDH, IL-6, and TNF α , cDNA was synthesized using a Verso cDNA kit (AB-1453/B; Thermo Fisher Scientific) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Green ROX qPCR Master Mix

(QIAGEN, Valencia, CA, United States). The primers were as follows: mouse GAPDH: 5'-TGCACCACCAACTGCTTAGC-3' and 5'-ATGCCAGTGAGCTTCCCGTT-3'; IL6: 5'-CCCAATTTCCAA TGCTCTCCT-3' and 5'-CCACAGTGAGGAATGTCCACA-3'; TNF α : 5'-CGAATTCAGTGGAGCCTCGAA-3' and 5'-TGTGAG GAAGGCTGTGCATTG-3'. The comparative cycle threshold (Ct) method ($2\Delta\Delta Ct$) was used to calculate the relative level of gene expression. The Ct values were normalized to GAPDH.

Characterization of Astrocyte-Derived Extracellular Vesicles and Separation Using Size Exclusion Chromatography

EVs were isolated from serum-free conditioned medium (FBS depleted) of astrocytes using a qEV column (Izon Science, Christchurch, New Zealand). In brief, the conditioned medium was harvested, centrifuged at 1,000 X g for 10 min to eliminate cells, and again spun at 10,000 X g for 30 min. The supernatant was filtered through a 0.22- μ m filter to remove cell debris and large vesicles. The collected supernatants were then concentrated using Amicon Ultra-15 100-kDa filter units and then subjected immediately to SEC on qEV original/35 nm columns (IZON Science, Christchurch, New Zealand). The columns were first rinsed with 1x filtered PBS, and 0.5 ml of supernatant was applied on top of a qEV column (columns allowed to remove all free USPIO nanoparticles, Izon Science), and twelve 0.5 ml fractions were collected (**Figure 1A**). Out of 12 fractions analyzed by nanoparticle tracking analysis (NTA), four EV-rich fractions (fractions 1–4), as well as 0.5 ml EV devoid supernatant of the same four fractions, were pooled and concentrated by evaporation. The EV and EV devoid supernatant samples were resuspended in the protein lysis buffer to detect EV markers—Alix, TSG101, and CD63—by Western blotting. The EV number and size distribution were analyzed using a ZetaView Particle Metrix, as previously reported (Ma et al., 2021).

Western Blotting

Cells and EVs were lysed using a mammalian cell lysis kit (Sigma-Aldrich, St. Louis, MO, United States), as described previously (Liao et al., 2019). Proteins were separated in sodium dodecyl sulfate (SDS)–polyacrylamide gel, followed by transfer to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 3% non-fat dry milk and 0.05% Tween 20 in Tris-buffered saline for 1 h at room temperature. The membrane was then probed with a primary antibody in 5% non-fat milk overnight at 4°C. Primary antibodies specific for TSG101 (Proteintech; Rosemont, IL, United States), Alix (Proteintech; Rosemont, IL, United States), CD63 (Abcam, Cambridge, MA, United States), Calnexin (Proteintech, Rosemont, IL, United States), and GFAP (Abcam, Cambridge, MA, United States) were used in this study. The next day, the membrane was washed three times with TTBS for 10 min each and subsequently incubated with a secondary antibody—alkaline phosphatase-conjugated to goat anti-mouse/rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, United States)—for 1 h at room temperature. The membrane was washed three times with TTBS for 10 min each and then developed using a West Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, United States). All experiments were repeated at least three times, and the representative blots are presented in the figures.

Transmission Electron Microscopy

EVs were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M PBS. Then 6 μ l of EVs were gently placed on a 200-mesh formvar-coated copper grid, allowed to adsorb for 5 min, and processed for standard uranyl acetate staining. The grid was then washed with PBS three times and allowed to semi-

dry for 2 min at room temperature before observation under an FEI Tecnai G2 Spirit Transmission Electron Microscope in the Electron Microscopy Core Facility at the University of Nebraska Medical Center. For the ultrastructural morphology of cells, cell pellets were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h. Transmission electron microscopy (TEM) images were acquired with a Hitachi Transmission EM in the Central Electron Microscopy Facility at the University of Connecticut Health Center.

Labeling of Ultrasmall Superparamagnetic Iron Oxide in Astrocyte-Derived Extracellular Vesicles

Astrocytes were seeded in a T75 flask at $1-2 \times 10^6$ cells. When reaching 80–90% confluency, cells were treated with 100 μ g/ml USPIO for 12 h. After that, the cells were washed three times with 1x PBS and incubated in a serum-free medium for 48 h. Then, the supernatant was collected and centrifuged at 1,000 X g for 10 min to eliminate cells and again spun at 10,000 X g for 30 min. The supernatant was filtered through a 0.22- μ m filter to remove cell debris and large vesicles. The collected supernatants were then subjected to qEV columns (Izon Science). The columns were first rinsed with 1x filtered PBS, and 0.5 ml of supernatant was applied on top of a qEV column, and 0.5 ml fractions were collected. Out of the 12 fractions obtained from the qEV column, four fractions (1–4) having EVs with USPIO were pooled and used for intranasal perfusion. The EV number and size distribution were analyzed using the ZetaView Particle Metrix, as previously reported (Hu et al., 2013; Hu et al., 2018).

Relaxivity of Mouse Astrocytes and Astrocyte-Derived Extracellular Vesicles

Mouse astrocytes were labeled with USPIO, as previously described, and were homogeneously distributed in a gel matrix (agarose low electroendosmosis; PanReac AppliChem; at 0.5% w/w) for *in vitro* T2 relaxation time evaluation. Cells (1×10^6 to 5×10^6) loaded with USPIO and USPIO-loaded ADEVs (5×10^8 to 2×10^9) were analyzed by MRI (Bruker BioSpec 70/20). In order to assess the *in vitro* detectability, USPIO-labeled astrocytes and ADEVs were immobilized in a gel matrix; MRI was performed using T2-weighted imaging and T2 mapping. *In vitro* T2 relaxivity (R_2) was calculated from T2 mapping. $\Delta R_2 = R_{2EVs} - R_{2Base}$ (Eqn (EL Andaloussi et al., 2013)) was calculated on each pixel, where R_{2EVs} is post-EVs administration R_2 , and R_{2Base} is the baseline (cells without USPIO and EVs without USPIO) R_2 .

Magnetic Resonance Imaging of Astrocyte-Derived Extracellular Vesicles With Ultrasmall Superparamagnetic Iron Oxide in Mouse Brain

Mice were intranasally administered with USPIO-labeled EVs (2×10^{12} EVs per perfusion) for 4 days, as illustrated in **Table 1**. MRI was performed 24 h after each EV

TABLE 1 | Intranasal perfusion of USPIO-loaded mouse astrocyte-derived EVs.

Day	Intranasal perfusion of ADEVs and MRI scan
Day 0	• MRI pre-scan on 6 mice • Wait 1 h and intranasal perfusion of 2×10^{12} USPIO-labeled ADEVs in 50 μL PBS
Day 1	• Intranasal perfusion of 2×10^{12} USPIO-labeled ADEVs in 50 μL PBS • Wait 1 h and MRI scan
Day 2	• Intranasal perfusion of 2×10^{12} USPIO-labeled ADEVs in 50 μL PBS • Wait 1 h and MRI scan
Day 3	• Intranasal perfusion of 2×10^{12} USPIO-labeled ADEVs in 50 μL PBS • Wait 1 h and MRI scan
Day 4	• Intranasal perfusion of 2×10^{12} USPIO-labeled ADEVs in 50 μL PBS • Wait 1 h and MRI scan

administration. For MRI, animals were anesthetized by 1% isoflurane inhalation in a mixture of oxygen and nitrogen and were placed in an MRI system (Bruker BioSpec 70/20) on a heated bed. T2-weighted images were acquired using TurboRARE with TR/TE = 3,600/40 ms, RARE factor = 4, averaging number = 4, matrix size = 256×256 , FOV = 20×20 mm², 30 slices, and slice thickness = 0.5 mm. T2 mapping was performed using MSME with TR = 4,600 ms, 20 echos from 7 to 140 ms with 7 ms echo spacing, matrix size = 192×192 , FOV = 20×20 mm², 30 slices, and slice thickness = 0.5 mm. *In vivo* T2 relaxivity (R₂) was calculated from T2 mapping. Eqn (EL Andaloussi et al., 2013) was calculated on each pixel. ΔR_2 heatmaps were superimposed on anatomical (T2-weighted) images. Increased ΔR_2 values indicate EV entry into the brain. Baseline MRI of all animals before the administration of USPIO-labeled EVs was used as the respective control. The same methodology was used to take body MRI scans of the kidneys, liver, and spleen.

Statistical Analyses

All the data were expressed as mean $\pm$ SEM, and appropriate statistical significance was determined based on the experimental strategy using GraphPad Prism version 9.1.2. The precise statistical analyses and experimental designs, including tests performed, exact *p* values, and sample sizes, are provided in the figure legends. Non-parametric Kruskal–Wallis one-way ANOVA, followed by Dunn's post hoc test, was used to determine the statistical significance between multiple groups, and an unpaired Student's *t* test was used to compare between two groups.

RESULTS

Characterization of Extracellular Vesicles Separated From Mouse Astrocytes

ADEVs were separated into 12 fractions (qEV columns allowed to separate free USPIO nanoparticles from EVs; **Figure 1A**). As shown in **Figures 1B,C**, fractions 1–4 contained maximum ADEVs, with sizes ranging from 50 to 250 nm. The ADEVs were further characterized by Western blotting for EV

markers—TSG101, Alix, and CD63. As shown in **Figure 1D**, ADEVs were positive for all of the detected EV markers and astrocyte marker—GFAP, while the supernatants were negative for the EV markers. Calnexin (non-EV protein) served as a negative control. TEM was used to confirm the morphology and size of ADEVs further. As shown in **Figure 1E**, ADEVs were around 100 nm in diameter.

Uptake of Ultrasmall Superparamagnetic Iron Oxide by Astrocytes

Astrocytes were treated with 50 μ g/ml to 400 μ g/ml of USPIO, followed by assessing cell survivability. As shown in **Supplementary Figure S1A–D**, USPIO treatment did not affect cell survivability, morphology, and response to LPS stimulation. We next sought to determine the uptake of USPIO in astrocytes using the nanoparticle uptake assay. As shown in **Figure 1F**, cells treated with 100 μ g/ml of USPIO based on the previous study (Busato et al., 2016) showed an uptake of USPIO nanoparticles by astrocytes (**Figures 1G,H**).

Relaxivity of Ultrasmall Superparamagnetic Iron Oxide-Labeled Astrocyte and Astrocyte-Derived Extracellular Vesicles

We next sought to confirm the presence of USPIO in astrocytes and ADEVs using TEM and MRI. TEM assays revealed that USPIO nanoparticles were present inside 85.7% of the cells (**Figure 1H**) and the presence of USPIO nanoparticles in 45% of ADEVs (**Figure 1I**). To evaluate the detection limit of astrocytes and ADEVs with USPIO in MRI, we used 1×10^6 to 5×10^6 astrocytes and 5×10^8 to 2×10^9 ADEVs immobilized in different gel tubes (agarose at 0.5% w/w, 1.5 ml). Unlabeled astrocytes and ADEVs, as well as free agarose gel, were used as negative controls. Relaxivity data were acquired using MRI. As T₂ (transverse relaxation time) is the inverse of R₂ (transverse relaxivity) and USPIO is a T₂-shortening contrast agent, the lower values of T₂ represent the higher levels of USPIO in the samples. As shown in **Figures 1J,K**, the linear correlations between R₂ values of USPIO in astrocytes and the cell concentrations ($R^2 = 0.828$), and USPIO in ADEV and ADEV concentrations ($R^2 = 0.934$) suggest the uptake of USPIO by astrocytes and ADEVs. We also found that USPIO treatment did not significantly affect EV release (**Supplementary Figure S1E**).

Magnetic Resonance Imaging of Mice Administrated With Astrocyte-Derived Extracellular Vesicles labeled with Ultrasmall Superparamagnetic Iron Oxide

To examine the biodistribution of ADEVs loaded with USPIO *in vivo*, we intranasally perfused C57BL/6 mice with USPIO-labeled ADEVs. The mice were pre-scanned using MRI on day 0 to set the baseline, followed by intranasal perfusion with 2×10^{12} USPIO-ADEVs per mouse and another perfusion on day 1. The mice were then scanned 1 hour post-ADEV perfusion on day 1. This procedure was repeated for 4 days (**Table 1**). As shown in **Figure 2A**, ΔR_2

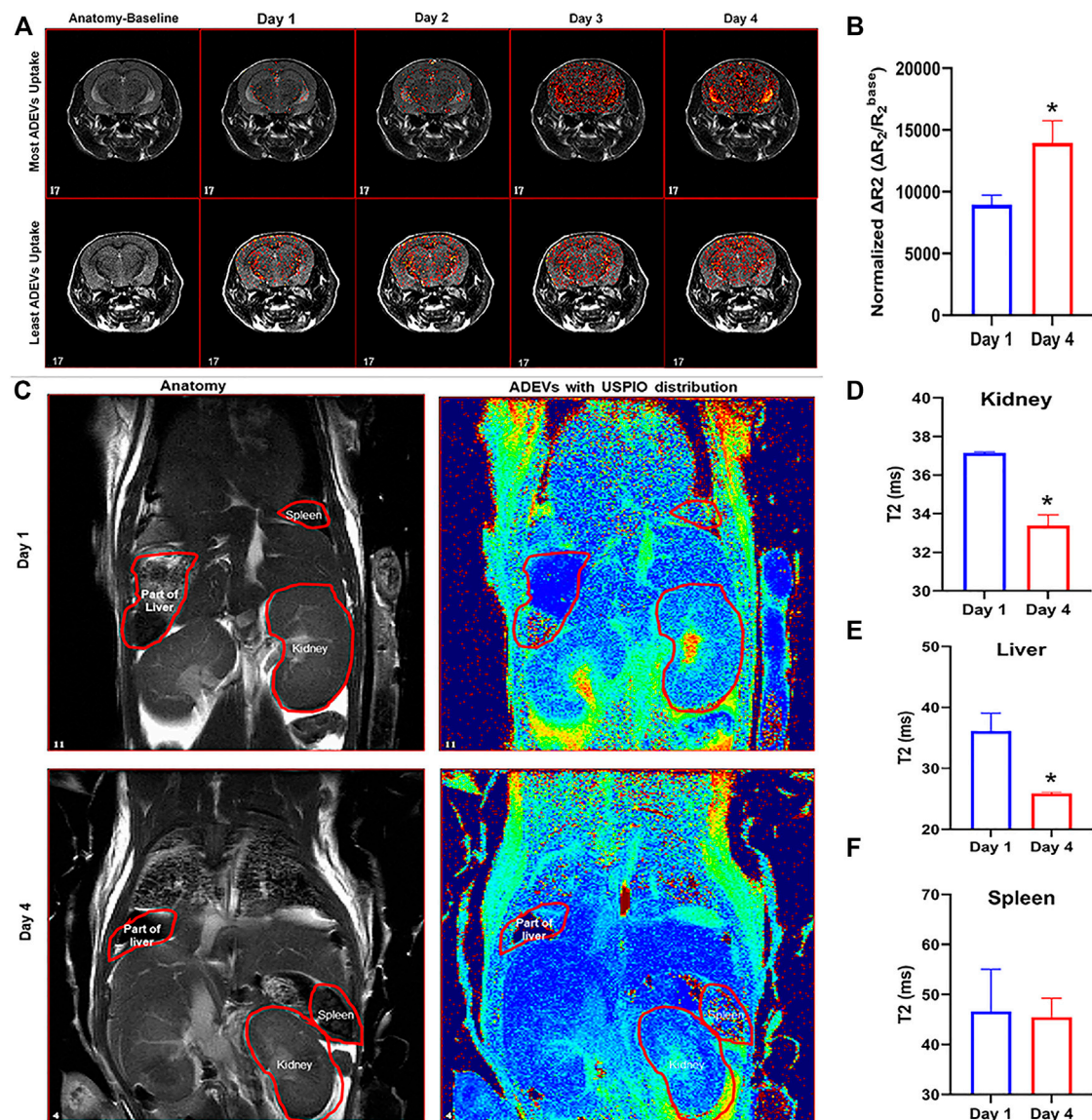


FIGURE 2 | Biodistribution of USPIO-labeled ADEVs in mice. **(A)** MRI T2-weighted images showing the USPIO-labeled ADEVs were present in the brain, and their abundance increased with each intranasal perfusion. **(B)** Quantification of the USPIO-labeled ADEVs in the brain; the bar graph shows the sum of normalized ΔR_2 (i.e., $\Delta R_2/R_2^{base}$) of all pixels in brain R_2 maps, and value increases along with time. **(C)** MRI T2-weighted images showing the USPIO-labeled ADEVs were present in the **(D)** kidneys, **(E)** liver, and **(F)** spleen.

heatmaps were superimposed on anatomical (T2-weighted) images. USPIO-labeled ADEVs could be found in the brain 1 day after intranasal perfusion. The amount of USPIO-labeled ADEVs was increased with each intranasal perfusion in the brain (**Figure 2B**). Moreover, USPIO-labeled ADEVs could also be found in the kidneys (**Figures 2C,D**), liver (**Figures 2C,E**), and the spleen (**Figures 2C,F**).

DISCUSSION

This study developed a method of loading ADEVs with USPIO nanoparticles and tracked them *in vivo* using MRI. Nanoparticle uptake assay was used to determine the uptake of USPIO by

mouse astrocytes without disrupting their morphology, normal growth, and release of EVs. ADEVs were separated using IZON qEV columns, followed by the characterization for EV markers by Western blotting, morphology by TEM, and number and size distribution by ZetaView. USPIO-labeled ADEVs were intranasally delivered to mice, followed by MRI scanning. MRI images suggested that USPIO-labeled ADEVs were delivered to the brain, kidneys, liver, and, to some extent, the spleen.

EVs have a pivotal role in intercellular communication under normal and diseased conditions (Patters and Kumar, 2018; Ma et al., 2021). Moreover, EVs have tremendous potential to be used as delivery vehicles for therapeutics as they can cross the BBB (Alvarez-Erviti et al., 2011; Yang et al., 2015). EVs are safer in terms of their

transplantation (loss in transplanted cells), transformation to become malignant, or immune rejection (Alvarez-Erviti et al., 2011). In addition, EVs are stable and could be scaled up for therapeutics relatively easily in a stepwise process (György et al., 2015). Indeed, studies have demonstrated that delivery of EVs loaded with small RNAs such as siRNA and miRNA to rodents can reach various organs and regulate the expression of the target genes (O'Brien et al., 2020; Chivero et al., 2020; Liao et al., 2020). The current study aimed to determine the biodistribution of ADEV in live animals using a sensitive method. Herein, we successfully loaded ADEVs with USPIO nanoparticles and tracked them in mice using MRI (Busato et al., 2016). MRI results suggest that USPIO-labeled ADEVs can reach the brain, liver, and kidneys within 1 day after intranasal delivery.

Previous studies demonstrated that intranasal delivery of EVs isolated from bone marrow mesenchymal stem cells (MSCs) could suppress neurogenesis and memory dysfunction in status epilepticus mice (György et al., 2015). It has been reported that EVs were able to suppress neuroinflammation and reduce cognitive impairments in traumatic brain-injured mice (O'Brien et al., 2020). Furthermore, intranasal delivery of catalase-loaded EVs has shown significant neuroprotective effects in the Parkinson's disease model by reducing stress-induced neuronal death (Long et al., 2017). Curcumin-encapsulated EVs could also suppress IL-6 and TNF- α expression in LPS-induced septic shock model animals (Sun et al., 2010). In line with these studies, our results suggest that USPIO-labeled EVs delivered through the intranasal route can be found in the brain, as well as the kidneys and liver.

ADEVs are endogenous brain vesicles that play a profound role in brain development, synapse formation, control of neurotransmitter release and uptake, making of trophic factors, and regulation of neuronal survivability (Ma et al., 2016; Venturini et al., 2019; Zhao et al., 2021). ADEVs thus could serve as ideal drug delivery vehicles for the treatment of brain disorders. For example, ADEVs have been used as siRNA delivery vehicles to treat LPS-mediated neuroinflammation (Liao et al., 2019). The results suggest that intranasal delivery of siRNA-loaded ADEVs significantly reversed the expression of target genes in microglia in LPS-administered mice. Efforts to engineer the ADEV for targeting specific organs and cells are ongoing and remain a major research focus. Intranasal delivery of USPIO-labeled ADEVs provides a non-invasive tool to visualize and track the engineered ADEVs in future studies.

CONCLUSION

In summary, our data suggest USPIO does not show significant effects on astrocytes and the release of EVs. Our results show that USPIO can be taken up by astrocytes and released in EVs. We further demonstrated intranasal administration of ADEVs can deliver their cargo to the brain, kidneys, and liver. Therefore, engineered EVs could be harnessed to deliver therapeutics for the treatment of brain, kidney, and liver disorders.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at University of Nebraska Medical Center.

AUTHOR CONTRIBUTIONS

NAK, RM, YL, and GH designed and performed the experiments, and collected, analyzed, and discussed the data. NAK, RM, and GH drafted the manuscript. NAK, RM, YL, SB, and GH revised the manuscript and approved the final manuscript.

FUNDING

This work was supported by startup funds from the University of Nebraska Medical Center and the National Institute of Health (NIH) (grants DA046831, DA042704, DA043138, and MH112848). This project was also supported by the NIH grant 2P30MH062261. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

ACKNOWLEDGMENTS

We would like to thank the editor and reviewers for their constructive suggestions and critical comments on the manuscript. We are grateful to Yunlong Huang for his outstanding technical assistance and insightful discussions. We would like to thank Melissa Mellon and Lirong Xu from the Bioimaging Core (small animal MRI) for training Naseer with intranasal perfusion and MRI scanning. We would like to thank the Nebraska Center for Substance Abuse Research (NCSAR) for support. We would also like to thank Tom Bargar and Nicholas Conoan of the Electron Microscopy Core Facility (EMCF) at the University of Nebraska Medical Center for technical assistance. The EMCF is supported by state funds from the Nebraska Research Initiative (NRI) and the University of Nebraska Foundation and institutionally by the Office of the Vice-Chancellor for Research.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2022.819516/full#supplementary-material>

Supplementary Figure S1 | Effect of USPIO on cell survivability and EV release, and distribution of USPIO labeled ADEVs in the brain. **(A)** Viability of cells treated with various concentrations of USPIO. **(B)** Immunostaining for GFAP in astrocytes exposed to 100 μ g/ml USPIO. **(C,D)** Real-time PCR for IL6 and TNF α in cells treated with 100 μ g/ml USPIO for 12 h followed by LPS (100 ng/ml) stimulation for 6 h. **(E)** Number of EVs isolated from 100 μ g/ml USPIO-treated astrocytes for 48 h. All experiments were repeated at least three times.

REFERENCES

- Al-Nedawi, K., Meehan, B., Micallef, J., Lhotak, V., May, L., Guha, A., et al. (2008). Intercellular Transfer of the Oncogenic Receptor EGFRvIII by Microvesicles Derived from Tumour Cells. *Nat. Cel Biol* 10, 619–624. doi:10.1038/ncb1725
- Alvarez-Erviti, L., Seow, Y., Yin, H., Betts, C., Lakhal, S., and Wood, M. J. (2011). Delivery of siRNA to the Mouse Brain by Systemic Injection of Targeted Exosomes. *Nat. Biotechnol.* 29, 341–345. doi:10.1038/nbt.1807
- Busato, A., Bonafede, R., Bontempi, P., Scambi, I., Schiaffino, L., Benati, D., et al. (2016). Magnetic Resonance Imaging of Ultrasmall Superparamagnetic Iron Oxide-Labeled Exosomes from Stem Cells: a New Method to Obtain Labeled Exosomes. *Int. J. Nanomedicine* 11, 2481–2490. doi:10.2147/IJN.S104152
- Chen, X. Q., Fawcett, J. R., Rahman, Y. E., Ala, T. A., and Frey II, W. H. (1998). Delivery of Nerve Growth Factor to the Brain via the Olfactory Pathway. *J. Alzheimers Dis.* 1, 35–44. doi:10.3233/jad-1998-1102
- Chivero, E. T., Dagur, R. S., Peeples, E. S., Sil, S., Liao, K., Ma, R., et al. (2021). Biogenesis, Physiological Functions and Potential Applications of Extracellular Vesicles in Substance Use Disorders. *Cell Mol Life Sci* 78, 4849–4865. doi:10.1007/s00018-021-03824-8
- Chivero, E. T., Liao, K., Niu, F., Tripathi, A., Tian, C., Buch, S., et al. (2020). Engineered Extracellular Vesicles Loaded with miR-124 Attenuate Cocaine-Mediated Activation of Microglia. *Front Cel Dev Biol* 8, 573. doi:10.3389/fcell.2020.00573
- Daldrup-Link, H. E. (2017). Ten Things You Might Not Know about Iron Oxide Nanoparticles. *Radiology* 284, 616–629. doi:10.1148/radiol.2017162759
- EL Andaloussi, S., Mäger, I., Breakefield, X. O., and Wood, M. J. (2013). Extracellular Vesicles: Biology and Emerging Therapeutic Opportunities. *Nat. Rev. Drug Discov.* 12, 347–357. doi:10.1038/nrd3978
- Ganipineni, L. P., Ucakar, B., Joudiou, N., Riva, R., Jérôme, C., Gallez, B., et al. (2019). Paclitaxel-loaded Multifunctional Nanoparticles for the Targeted Treatment of Glioblastoma. *J. Drug Target.* 27, 614–623. doi:10.1080/1061186X.2019.1567738
- Grassin-Delye, S., Buenestado, A., Naline, E., Faisy, C., Blouquit-Laye, S., Couderc, L. J., et al. (2012). Intranasal Drug Delivery: an Efficient and Non-invasive Route for Systemic Administration: Focus on Opioids. *Pharmacol. Ther.* 134, 366–379. doi:10.1016/j.pharmthera.2012.03.003
- Gupta, J., Mohapatra, J., Bhargava, P., and Bahadur, D. (2016). A pH-Responsive Folate Conjugated Magnetic Nanoparticle for Targeted Chemo-thermal Therapy and MRI Diagnosis. *Dalton Trans.* 45, 2454–2461. doi:10.1039/c5dt04135j
- György, B., Hung, M. E., Breakefield, X. O., and Leonard, J. N. (2015). Therapeutic Applications of Extracellular Vesicles: Clinical Promise and Open Questions. *Annu. Rev. Pharmacol. Toxicol.* 55, 439–464. doi:10.1146/annurev-pharmtox-010814-124630
- Hanson, L. R., and Frey, W. H., 2nd (2008). Intranasal Delivery Bypasses the Blood-Brain Barrier to Target Therapeutic Agents to the central Nervous System and Treat Neurodegenerative Disease. *BMC Neurosci.* 9 Suppl 3 (Suppl. 3), S5. doi:10.1186/1471-2202-9-S3-S5
- Hu, G., Gong, A. Y., Roth, A. L., Huang, B. Q., Ward, H. D., Zhu, G., et al. (2013). Release of Luminal Exosomes Contributes to TLR4-Mediated Epithelial Antimicrobial Defense. *Plos Pathog.* 9, e1003261. doi:10.1371/journal.ppat.1003261
- Hu, G., Liao, K., Niu, F., Yang, L., Dallon, B. W., Callen, S., et al. (2018). Astrocyte EV-Induced lincRNA-Cox2 Regulates Microglial Phagocytosis: Implications for Morphine-Mediated Neurodegeneration. *Mol. Ther. Nucleic Acids* 13, 450–463. doi:10.1016/j.omtn.2018.09.019
- Kim, D. H., Vitol, E. A., Liu, J., Balasubramanian, S., Gosztola, D. J., Cohen, E. E., et al. (2013). Stimuli-responsive Magnetic Nanomicelles as Multifunctional Heat and Cargo Delivery Vehicles. *Langmuir* 29, 7425–7432. doi:10.1021/la3044158
- Lakhal, S., and Wood, M. J. (2011). Intranasal Exosomes for Treatment of Neuroinflammation? Prospects and Limitations. *Mol. Ther.* 19, 1754–1756. doi:10.1038/mt.2011.198
- Liao, K., Niu, F., Dagur, R. S., He, M., Tian, C., and Hu, G. (2020). Intranasal Delivery of lincRNA-Cox2 siRNA Loaded Extracellular Vesicles Decreases Lipopolysaccharide-Induced Microglial Proliferation in Mice. *J. Neuroimmune Pharmacol.* 15, 390–399. doi:10.1007/s11481-019-09864-z
- Liao, K., Niu, F., Dagur, R. S., He, M., Tian, C., and Hu, G. (2019). Intranasal Delivery of lincRNA-Cox2 siRNA Loaded Extracellular Vesicles Decreases Lipopolysaccharide-Induced Microglial Proliferation in Mice. *J. Neuroimmune Pharmacol.* doi:10.1007/s11481-019-09864-z
- Lombardo, R., Musumeci, T., Carbone, C., and Pignatello, R. (2021). Nanotechnologies for Intranasal Drug Delivery: an Update of Literature. *Pharm. Dev. Technol.*, 1–22. doi:10.1080/10837450.2021.1950186
- Long, Q., Upadhy, D., Hattiangady, B., Kim, D. K., An, S. Y., Shuai, B., et al. (2017). Intranasal MSC-Derived A1-Exosomes Ease Inflammation, and Prevent Abnormal Neurogenesis and Memory Dysfunction after Status Epilepticus. *Proc. Natl. Acad. Sci. U S A.* 114, E3536–E3545. doi:10.1073/pnas.1703920114
- Ma, R., Kutchy, N. A., and Hu, G. (2021). Astrocyte-Derived Extracellular Vesicle-Mediated Activation of Primary Ciliary Signaling Contributes to the Development of Morphine Tolerance. *Biol. Psychiatry* 90, 575–585. doi:10.1016/j.biopsych.2021.06.009
- Ma, X. C., Liu, P., Zhang, X. L., Jiang, W. H., Jia, M., Wang, C. X., et al. (2016). Intranasal Delivery of Recombinant AAV Containing BDNF Fused with HA2TAT: a Potential Promising Therapy Strategy for Major Depressive Disorder. *Sci. Rep.* 6, 22404. doi:10.1038/srep22404
- Mathieu, M., Martin-Jaular, L., Lavieu, G., and Théry, C. (2019). Specificities of Secretion and Uptake of Exosomes and Other Extracellular Vesicles for Cell-To-Cell Communication. *Nat. Cel Biol* 21, 9–17. doi:10.1038/s41556-018-0250-9
- O'Brien, K., Breyne, K., Ughetto, S., Laurent, L. C., and Breakefield, X. O. (2020). RNA Delivery by Extracellular Vesicles in Mammalian Cells and its Applications. *Nat. Rev. Mol. Cel Biol* 21, 585–606. doi:10.1038/s41580-020-0251-y
- Park, W., Chen, J., Cho, S., Park, S. J., Larson, A. C., Na, K., et al. (2016). Acidic pH-Triggered Drug-Eluting Nanocomposites for Magnetic Resonance Imaging-Monitored Intra-arterial Drug Delivery to Hepatocellular Carcinoma. *ACS Appl. Mater. Inter.* 8, 12711–12719. doi:10.1021/acsami.6b03505
- Patters, B. J., and Kumar, S. (2018). The Role of Exosomal Transport of Viral Agents in Persistent HIV Pathogenesis. *Retrovirology* 15, 79. doi:10.1186/s12977-018-0462-x
- Pegtel, D. M., and Gould, S. J. (2019). Exosomes. *Annu. Rev. Biochem.* 88, 487–514. doi:10.1146/annurev-biochem-013118-111902
- Peinado, H., Alečković, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Moreno-Bueno, G., et al. (2012). Melanoma Exosomes Educate Bone Marrow Progenitor Cells toward a Pro-metastatic Phenotype through MET. *Nat. Med.* 18, 883–891. doi:10.1038/nm.2753
- Raposo, G., and Stoorvogel, W. (2013). Extracellular Vesicles: Exosomes, Microvesicles, and Friends. *J. Cel Biol* 200, 373–383. doi:10.1083/jcb.201211138
- Ratajczak, J., Miekus, K., Kucia, M., Zhang, J., Reca, R., Dvorak, P., et al. (2006). Embryonic Stem Cell-Derived Microvesicles Reprogram Hematopoietic Progenitors: Evidence for Horizontal Transfer of mRNA and Protein Delivery. *Leukemia* 20, 847–856. doi:10.1038/sj.leu.2404132
- Sil, S., Dagur, R. S., Liao, K., Peeples, E. S., Hu, G., Periyasamy, P., et al. (2020). Strategies for the Use of Extracellular Vesicles for the Delivery of Therapeutics. *J. Neuroimmune Pharmacol.* 15, 422–442. doi:10.1007/s11481-019-09873-y
- Sturdivant, N. M., Smith, S. G., Ali, S. F., Wolchok, J. C., and Balachandran, K. (2016). Acetazolamide Mitigates Astrocyte Cellular Edema Following Mild Traumatic Brain Injury. *Sci. Rep.* 6, 33330. doi:10.1038/srep33330
- Sun, D., Zhuang, X., Xiang, X., Liu, Y., Zhang, S., Liu, C., et al. (2010). A Novel Nanoparticle Drug Delivery System: the Anti-inflammatory Activity of Curcumin Is Enhanced when Encapsulated in Exosomes. *Mol. Ther.* 18, 1606–1614. doi:10.1038/mt.2010.105
- Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., et al. (2018). Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): a Position Statement of the International Society for Extracellular Vesicles and Update of the MISEV2014 Guidelines. *J. Extracell Vesicles* 7, 1535750. doi:10.1080/20013078.2018.1535750
- Thorne, R. G., Emory, C. R., Ala, T. A., and Frey, W. H., 2nd (1995). Quantitative Analysis of the Olfactory Pathway for Drug Delivery to the Brain. *Brain Res.* 692, 278–282. doi:10.1016/0006-8993(95)00637-6

- Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J., and Lötvall, J. O. (2007). Exosome-mediated Transfer of mRNAs and microRNAs Is a Novel Mechanism of Genetic Exchange between Cells. *Nat. Cel Biol* 9, 654–659. doi:10.1038/ncb1596
- van den Boorn, J. G., Schlee, M., Coch, C., and Hartmann, G. (2011). SiRNA Delivery with Exosome Nanoparticles. *Nat. Biotechnol.* 29, 325–326. doi:10.1038/nbt.1830
- Venturini, A., Passalacqua, M., Pelassa, S., Pastorino, F., Tedesco, M., Cortese, K., et al. (2019). Exosomes from Astrocyte Processes: Signaling to Neurons. *Front. Pharmacol.* 10, 1452. doi:10.3389/fphar.2019.01452
- Visweswaraiah, A., Novotny, L. A., Hjemdahl-Monsen, E. J., Bakaletz, L. O., and Thanavala, Y. (2002). Tracking the Tissue Distribution of Marker Dye Following Intranasal Delivery in Mice and Chinchillas: a Multifactorial Analysis of Parameters Affecting Nasal Retention. *Vaccine* 20, 3209–3220. doi:10.1016/s0264-410x(02)00247-5
- Weaver, B. A. (2014). How Taxol/paclitaxel Kills Cancer Cells. *Mol. Biol. Cel* 25, 2677–2681. doi:10.1091/mbc.E14-04-0916
- Xu, J., Chen, Z., Yu, F., Liu, H., Ma, C., Xie, D., et al. (2020). IL-4/STAT6 Signaling Facilitates Innate Hematoma Resolution and Neurological Recovery after Hemorrhagic Stroke in Mice. *Proc. Natl. Acad. Sci. U S A.* 117, 32679–32690. doi:10.1073/pnas.2018497117
- Yang, T., Martin, P., Fogarty, B., Brown, A., Schurman, K., Phipps, R., et al. (2015). Exosome Delivered Anticancer Drugs across the Blood-Brain Barrier for Brain Cancer Therapy in Danio rerio. *Pharm. Res.* 32, 2003–2014. doi:10.1007/s11095-014-1593-y
- Zhao, S., Sheng, S., Wang, Y., Ding, L., Xu, X., Xia, X., et al. (2021). Astrocyte-derived Extracellular Vesicles: A Double-Edged Sword in central Nervous System Disorders. *Neurosci. Biobehav Rev.* 125, 148–159. doi:10.1016/j.neubiorev.2021.02.027
- Zheng, X. C., Ren, W., Zhang, S., Zhong, T., Duan, X. C., Yin, Y. F., et al. (2018). The Theranostic Efficiency of Tumor-specific, pH-Responsive, Peptide-Modified, Liposome-Containing Paclitaxel and Superparamagnetic Iron Oxide Nanoparticles. *Int. J. Nanomedicine* 13, 1495–1504. doi:10.2147/IJN.S157082
- Zhuang, X., Xiang, X., Grizzle, W., Sun, D., Zhang, S., Axtell, R. C., et al. (2011). Treatment of Brain Inflammatory Diseases by Delivering Exosome Encapsulated Anti-inflammatory Drugs from the Nasal Region to the Brain. *Mol. Ther.* 19, 1769–1779. doi:10.1038/mt.2011.164

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Kutchy, Ma, Liu, Buch and Hu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



A Triple High Throughput Screening for Extracellular Vesicle Inducing Agents With Immunostimulatory Activity

Nikunj M. Shukla^{1†}, Fumi Sato-Kaneko^{1†}, Shiyin Yao¹, Minya Pu², Michael Chan¹, Fitzgerald S. Lao¹, Yukiya Sako¹, Tetsuya Saito^{1,3}, Karen Messer², Tomoko Hayashi¹, Howard B. Cottam¹, Maripat Corr⁴ and Dennis A. Carson^{1*}

¹Moores Cancer Center, University of California San Diego, La Jolla, CA, United States, ²The Herbert Wertheim School of Public Health and Longevity, University of California San Diego, La Jolla, CA, United States, ³Department of Rheumatology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University (TMDU), Tokyo, Japan, ⁴Department of Medicine, University of California San Diego, La Jolla, CA, United States

OPEN ACCESS

Edited by:

Satish Balasaheb Nimse,
Hallym University, South Korea

Reviewed by:

Baharak Hosseinkhani,
University of Hasselt, Belgium
Ricardo Neves,
University of Coimbra, Portugal

*Correspondence:

Dennis A. Carson
dcarson@health.ucsd.edu

[†]These authors have contributed
equally to this work and share first
authorship

Specialty section:

This article was submitted to
Experimental Pharmacology and Drug
Discovery,
a section of the journal
Frontiers in Pharmacology

Received: 04 February 2022

Accepted: 22 March 2022

Published: 11 April 2022

Citation:

Shukla NM, Sato-Kaneko F, Yao S,
Pu M, Chan M, Lao FS, Sako Y,
Saito T, Messer K, Hayashi T,
Cottam HB, Corr M and Carson DA
(2022) A Triple High Throughput
Screening for Extracellular Vesicle
Inducing Agents With
Immunostimulatory Activity.
Front. Pharmacol. 13:869649.
doi: 10.3389/fphar.2022.869649

Extracellular vesicles (EVs) play an important role in intercellular communication and regulation of cells, especially in the immune system where EVs can participate in antigen presentation and may have adjuvant effects. We aimed to identify small molecule compounds that can increase EV release and thereby enhance the immunogenicity of vaccines. We utilized a THP-1 reporter cell line engineered to release EV-associated tetraspanin (CD63)-Turbo-luciferase to quantitatively measure EVs released in culture supernatants as a readout of a high throughput screen (HTS) of 27,895 compounds. In parallel, the cytotoxicity of the compounds was evaluated by PrestoBlue dye assay. For screening immunostimulatory potency, we performed two additional independent HTS on the same compound library using NF- κ B and interferon-stimulated response element THP-1 reporter cell lines. Hit compounds were then identified in each of the 3 HTS's, using a "Top X" and a Gaussian Mixture Model approach to rule out false positive compounds and to increase the sensitivity of the hit selection. Thus, 644 compounds were selected as hits which were further evaluated for induction of IL-12 in murine bone-marrow derived dendritic cells (mBMDCs) and for effects of cell viability. The resulting 130 hits were then assessed from a medicinal chemistry perspective to remove compounds with functional group liabilities. Finally, 80 compounds were evaluated as vaccine adjuvants *in vivo* using ovalbumin as a model antigen. We analyzed 18 compounds with adjuvant activity for their ability to induce the expression of co-stimulatory molecules on mBMDCs. The full complement of data was then used to cluster the compounds into 4 distinct biological activity profiles. These compounds were also evaluated for quantitation of EV release and spider plot overlays were generated to compare the activity profiles of compounds within each cluster. This tiered screening process identified two compounds that belong to the 4-thieno-2-thiopyrimidine scaffold with identical screening profiles supporting data reproducibility and validating the overall screening process. Correlation patterns in the adjuvanticity data suggested a role for CD63 and NF- κ B pathways in potentiating antigen-specific antibody production. Thus, our three independent cell-based HTS campaigns led to identification of immunostimulatory compounds that release EVs and have adjuvant activity.

Keywords: exosome (vesicle), NF κ B, HTS, compounds, immune, extracellular, adjuvant, reporter cells

INTRODUCTION

Although vaccination against common pathogens is gaining broader acceptance, there remains an unmet need for widely effective adjuvants that can elicit sustained immune responses to targeted antigens (Reed et al., 2013). Vaccine adjuvants act as immunopotentiators that are co-administered with subunit, inactivated or attenuated antigens (Tregoning et al., 2018). In the past decade, there have been advances with adjuvants that have improved the response to varicella, influenza and hepatitis B vaccines in populations with reduced immune responses (Tregoning et al., 2018). Although the adjuvants boost protective efficacy of the vaccines, they often elicit local inflammation at the site of injection in some cases accompanied by flu-like symptoms, reduce patient acceptance especially for vaccines that require annual or booster injections (Petrovsky, 2015; Nanishi et al., 2020). Adjuvants that utilize intracellular communication pathways to enhance antigen presentation and the needed cognate cellular interactions could potentially activate the immune system in a manner that is not as abruptly inflammatory.

Extracellular vesicles (EVs) act as a carrier of cell-type-specific molecules including those involved in innate immune responses, such as cytokines, chemokines, adhesion molecules, other proteins, lipids, peptides, coding and non-coding RNAs (including microRNAs), and DNA fragments (Valadi et al., 2007; Skog et al., 2008; Cossetti et al., 2014; Yanez-Mo et al., 2015). Adhesion molecules integrated into the EV outer surface membrane direct binding to potential target cells while other molecules act as ligands to cellular receptors. EVs can also encapsulate additional proteins or nucleic acids that can convey specific intercellular communications. These properties enable EVs to play modulating roles in mediating immune responses to pathogens and tumors (Campos et al., 2015; Wang et al., 2017). Hence, we hypothesized that small molecule compounds which can simultaneously enhance innate immune responses and EV biogenesis and release (**Figure 1A**) could add immunomodulation modalities, and potentially increase antigen delivery to distal lymphoid organs, leading to improved vaccine efficacy and reduced toxicity.

Recently, we developed and characterized a human monocytic leukemia THP-1 reporter cell line engineered with a fusion construct for the expression of EV-associated tetraspanins (CD63 and CD9) linked to Turbo-luciferase (Tluc) and Emerald Green Fluorescent Protein (EmGFP) (CD63 Tluc-CD9-EmGFP THP-1 cells) to quantitatively measure release of EVs in culture supernatants (Shpigelman et al., 2021). Using this reporter cell line, we described that Tluc activity levels correlated with concentrations of released EVs in the culture supernatant as measured by nanoparticle tracking (Shpigelman et al., 2021). Here, we utilized this cell line for a high throughput screening (HTS) of a library of 27,895 compounds. Additionally, we screened the same library with two additional THP-1 reporter cell lines for NF- κ B and interferon-stimulated response element

(ISRE) activation, respectively. Based on “Top X” and “Gaussian mixture model” (GMM) hit detection methods, 644 compounds were identified as hits. Further studies probing into the immunological properties as well as selection based on chemical structural features narrowed the selection to 80 compounds that were assessed *in vivo* for adjuvant activity. All these studies led to the identification of distinct chemotypes that display immunostimulatory effects and enhance the production of EVs.

RESULTS

High Throughput Screenings

In prior work we identified compounds by HTS that induce NF- κ B activation or prolong the activation of NF- κ B and/or ISRE, using a library from a small molecule diversity collection (SMDC, UCSF) consisting of about 170,000 compounds (Pu et al., 2012; Chan et al., 2017a; Shukla et al., 2018). However, EV release assay was much more complex and required the use of expensive exosome-depleted media under precise incubation conditions. Thus, performing CD63 HTS in this large compound library, for which we had the NF- κ B induction data (Pu et al., 2012) was difficult to achieve. We were therefore interested to obtain smaller compound libraries with extensive chemical space diversity. Thus, we selected commercial libraries developed by Maybridge (Leeds, United Kingdom) consisting of two subset libraries which are representative of the diversity of the two very different compound collections: 1) the Maybridge HitFinder library of 14,400 compounds representative of the entire Maybridge Screening Collection of over 53,000 members, and 2) the Maybridge HitCreator library of 14,000 compounds representative of the diversity of a collection of 550,000 compounds (**Supplementary Table S1**). Another benefit of the Maybridge library is a lack of CAS registry numbers for a large portion of compounds allowing for freedom of intellectual property. Compound purchase, transfer, acquisition, export and quality control led to elimination of 505 compounds (<2%) thus obtaining a final list of 27,895 compounds for the HTS (**Supplementary Table S1**).

The overall HTS workflow strategy is shown in **Figure 1B**. To identify compounds that induce both immune responses and EV release, three sets of screens were performed using the following reporter cell lines: NF- κ B-*bla*, ISRE-*bla*, and CD63-Tluc-CD9-EmGFP THP-1 cells (**Supplementary Table S2**). In order to verify the feasibility of these assays, about 2,211 test compounds (8% of all library compounds) were randomly selected and assessed in a pilot screen for CD63 (Shpigelman et al., 2021), NF- κ B and ISRE. Each screen was done in duplicate as two independent experiments (experiment 1 and 2) run on different days. The duplicate screening format allowed us to better understand the reproducibility of the activity response in the NF- κ B and ISRE assays as we had found that these FRET based assay readouts have been historically less reproducible (Pu et al.,

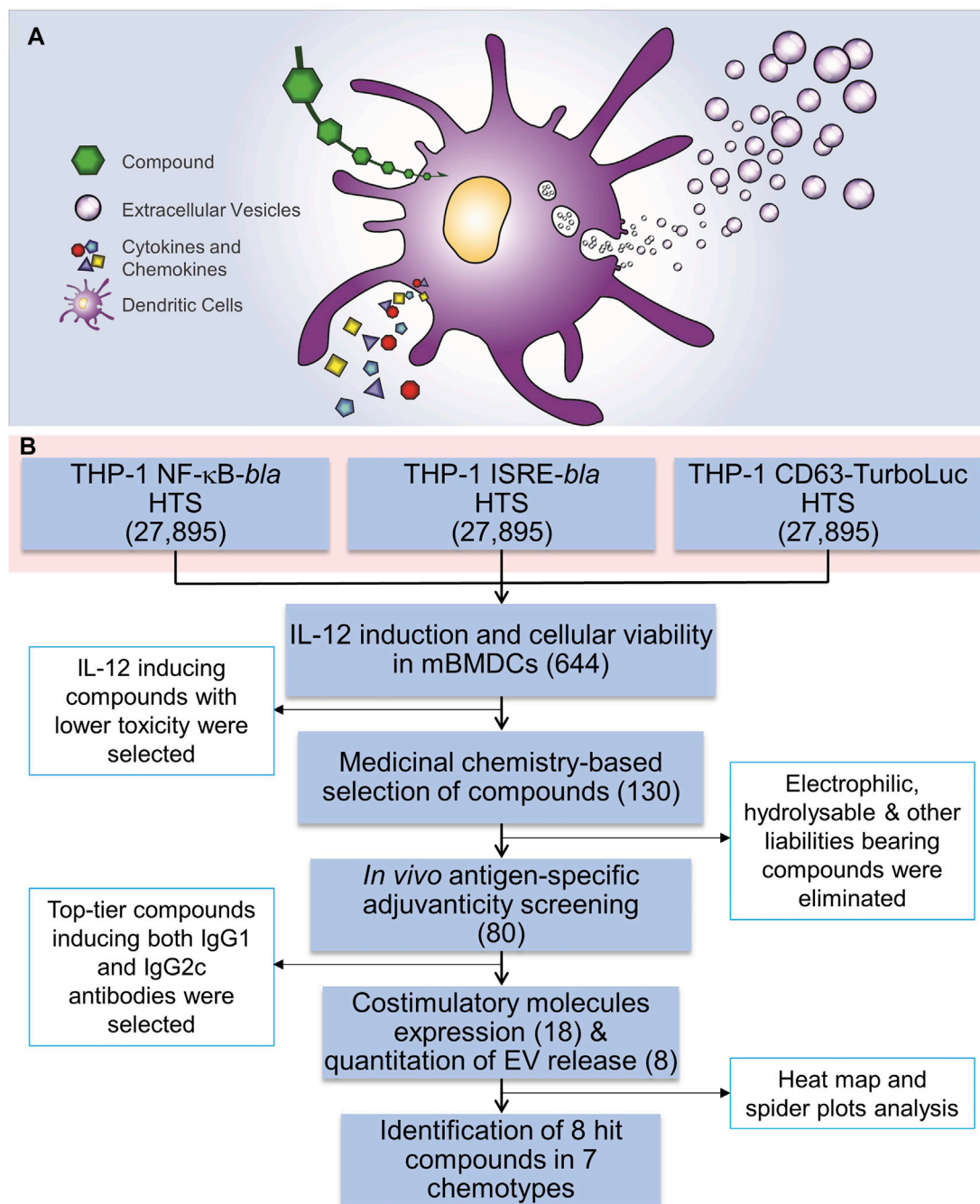


FIGURE 1 | HTS for immunostimulatory compounds that enhance EV release and the HTS workflow. **(A)** A cartoon depicting the rationale for identification of compounds that enhance EV release as well as induction of cytokines and chemokines. **(B)** Three independent THP-1 cell-based high throughput screenings were performed using NF- κ B-beta-lactamase (*bla*), ISRE-*bla* and CD63-Tluc-CD9-EmGFP reporter cells. These assays evaluated 27,895 compounds in duplicate and 644 compounds were identified as hits using two different statistical methods. These compounds were cherry-picked and were subjected to screening for immune stimulating activity, including induction of cytokine IL-12 and evaluation of cytotoxicity using MTT assay in mBMDCs which identified 130 compounds. Further medicinal chemistry approaches eliminated 50 compounds and the remaining 80 compounds were subjected to *in vivo* adjuvanticity screening, followed by co-stimulatory molecule expression screening and quantitation of EVs released from BMDCs. Eight distinct compounds were identified that belonged to 7 different chemotypes. The number in parentheses corresponds to the number of compounds.

2012). Also, in the case of CD63 HTS, the pilot screen helped validate the assay (Shpigelman et al., 2021) and formed the basis for evaluating methods to be utilized for hit selection.

Hit Selection Methods

One of the most common methods utilized in selection of hits from HTS is Top X (McFayden et al., 2005). Thus, we initially employed

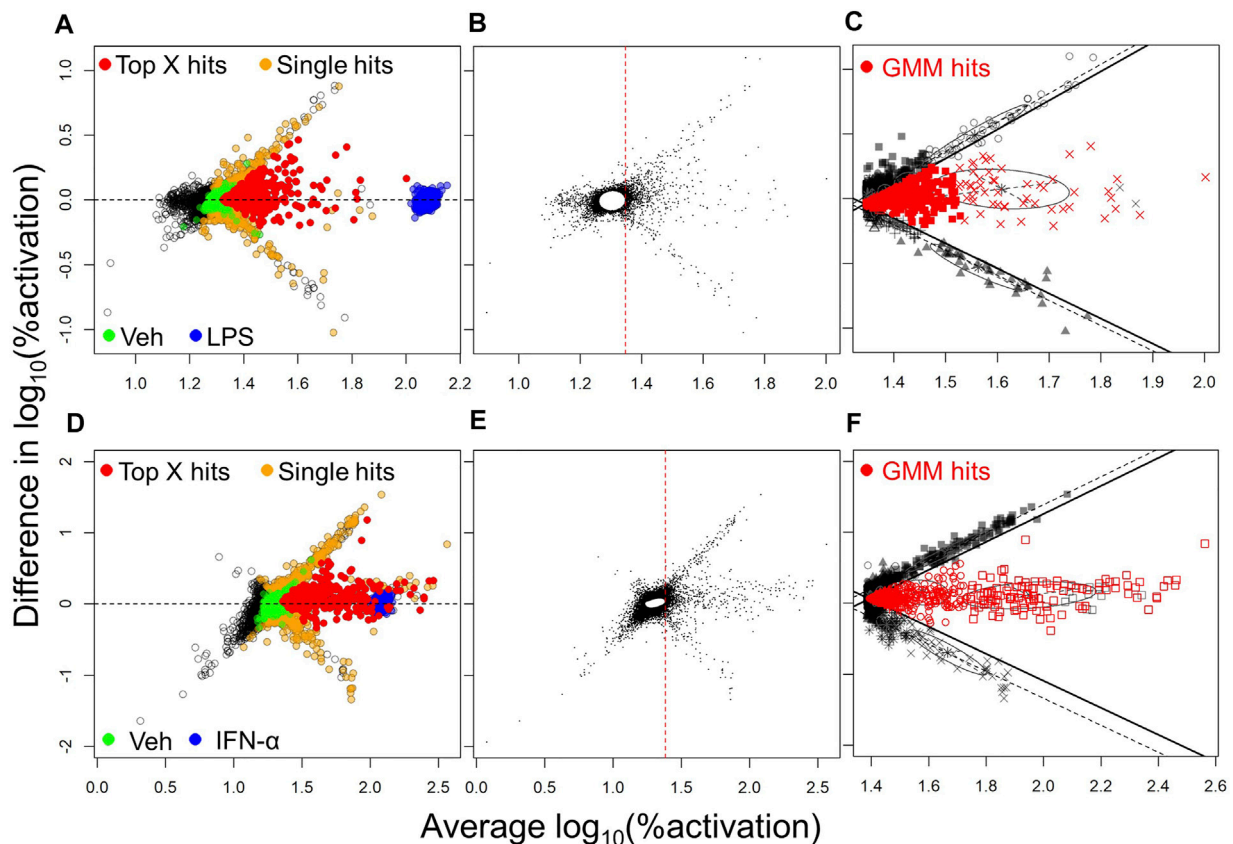


FIGURE 2 | Hit selection methods from the NF- κ B and ISRE HTS. The hit selection process for NF- κ B HTS (**A,B,C**) and ISRE HTS (**D,E,F**) are depicted: (**A,D**) MA plots of $\log_{10}$ transformed %activation for all compounds identified as hits in one experiment (orange spheres) or in both experiments as Top X hits (red spheres). The positive (LPS for NF- κ B HTS; IFN- α for ISRE HTS) and negative (Veh, 0.5% DMSO) controls used in the assay are shown as blue and green spheres, respectively. Panels (**B,E**) represent first steps towards the mixture model method and involved the identification of a null cluster and elimination of compounds to the left side of the red vertical dotted line. (**C,F**) The next step involved identification of linear boundaries based on the apparent false-positive clusters (black symbols) to identify GMM hits (red symbols) that included all compounds within these linear boundaries.

this method for hit selection. Because we evaluated each compound in 2 independent experiments, we were able to eliminate many of the false positives which usually dominate FRET-based screens. To this end, an MA plot (log fold change in activation vs. average activation) was obtained by plotting the difference in the $\log_{10}$ % activation data for the two different experiments on the Y-axis, against the average of these data on X-axis, for each compound. These plots for NF- κ B and ISRE HTS (**Figures 2A,D**, respectively) show the cluster of compounds (yellow circles) which were identified as hits only in one experiment and were thus considered “false positives” while the compounds that were identified as hits in both the experiments (red circles) were marked as “Top X hits.” Since we were working with a very diverse HTS library within a relatively small set of compounds, we sought to increase the sensitivity of hits detection using a Gaussian mixture modeling approach, applied separately to the NF- κ B and ISRE screens. In this approach, the %activation values from the two independent experiments for each test compound were first used to construct a MA plot, and based on the plot we built a bivariate Gaussian mixture model (GMM), and this was used to cluster compounds into hit or non-hit categories. Since this

method was heavily influenced by the large proportion of the non-hits, a null cluster in which the majority of compounds had activity levels similar to those of vehicle (Veh, 0.5% DMSO), was first identified (**Figures 2B,E**) using an initial GMM. The compounds with average %activation values lower than the maximum value of this null cluster (red dotted line, **Figures 2B,E**) were removed from subsequent analysis. GMM were then fitted to the remaining data, where the Bayesian Information Criterion (BIC) was used to determine the optimal number, shape and orientation of clusters. Apparent false positive clusters (grey/black colored compounds clustered along the black lines) were identified to construct linear boundaries (black lines). Compounds in the remaining clusters within these boundaries were considered to be hits (**Figures 2C,F**).

Using these two methods for identification of hits, we identified 398 Top X hits and 497 GMM hits, of which 319 hits were common between both methods, as depicted by different colored spheres in **Figure 3A** (**Supplementary Table S3**). Thus, a total of 576 hits were identified from the NF- κ B HTS. Similarly, for the ISRE screen, we identified 481 Top X hits and 444 GMM hits, of which 383 hits were common between both

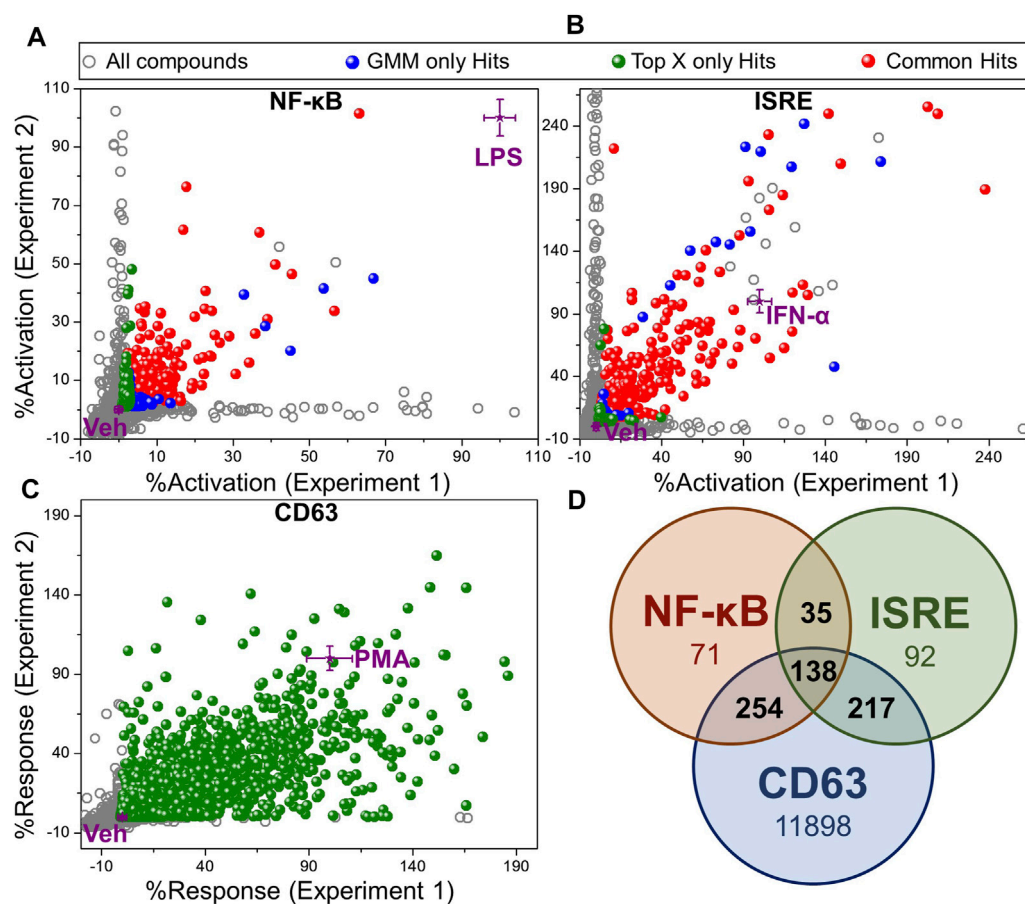


FIGURE 3 | Hit selection from HTS. Scatter plot of activation data for test compounds (evaluated in duplicates) and controls from **(A)** NF-κB HTS, **(B)** ISRE HTS and, **(C)** CD63 HTS. The activation data were calculated as “%activation” based on 2-point normalization between the controls in each plate of the HTS assay. These controls included Veh (0.5% DMSO, negative control, 0%) and LPS (100 ng/ml, 100%) for NF-κB HTS, IFN-α (50 nM, 100%) for ISRE HTS and PMA (10 ng/ml, 100%) for CD63 HTS. Two different statistical methods including Top X and GMM, were utilized for hit identification. All test compounds are shown by grey dots, while the compounds that were identified as hits by Top X only or GMM only methods are shown as green and blue spheres, respectively. The compounds identified as hits by both of these statistical techniques are shown as red spheres. Controls (purple stars) are shown as mean ± standard deviation calculated by intra-assay statistics of the %activation values. **(D)** Venn diagram showing the number of compounds identified as hits in each assay after eliminating toxic compound (<40% viability) identified by PrestoBlue viability assay in CD63 HTS. Compounds confirmed as hits in at least 2 different assays as shown by numbers in the intersections of the Venn diagram were selected for the further bioactivity analysis (total 644 compounds).

(Figure 3B) leading to a total of 542 ISRE hits (Supplementary Table S3). Figures 3A,B show the variability of %activation data between experiments 1 and 2 in both NF-κB and ISRE HTS assays, respectively.

In contrast, the CD63 HTS showed a very good correlation between the two independent experiments. Thus, we used only Top X hits, using an average of the two independent screens. To cover even weak CD63 inducers with good NF-κB or ISRE activity, we chose to use the mean of Veh wells within an assay plate as the threshold to identify Top X hits, which led to 12,954 compounds (Supplementary Table S3). To further narrow down the number of hits to compounds that have activity in a minimum of 2 out of these 3 HTS, we segregated compounds into 161 triple hits (compounds identified as a hit in CD63, NF-κB and ISRE HTS), and 3 sets of dual hits including 296 CD63 and NF-κB hits, 231 CD63 and ISRE hits and 37 NF-κB and ISRE hits (Supplementary Table S4). Then,

based on the cell viability data obtained from the PrestoBlue assay, the number of hits were distributed as shown in Supplementary Table S4 with the number of hits decreasing with increasing cell viability cut-off. However, because cytotoxicity of a compound can often be circumvented by subsequent structure-activity relationship (SAR) studies, we did not want to filter out any potential hits, and thus we kept our cut-off for hit selection as compounds having more than 40% cell viability. A Venn diagram in Figure 3D shows the number of hits selected by each screen based on this 40% viability cut-off in PrestoBlue assay. Thus, a total of 644 compounds which were identified as a hit in at least 2 experiments and had viability of more than 40% by PrestoBlue were selected for further analysis.

An efficiency of compound screening methods and hit identification techniques is determined by hit confirmation rates. Thus, since all the compounds in the pilot screen were part of the HTS, we used the pilot screen as an independent confirmation screen

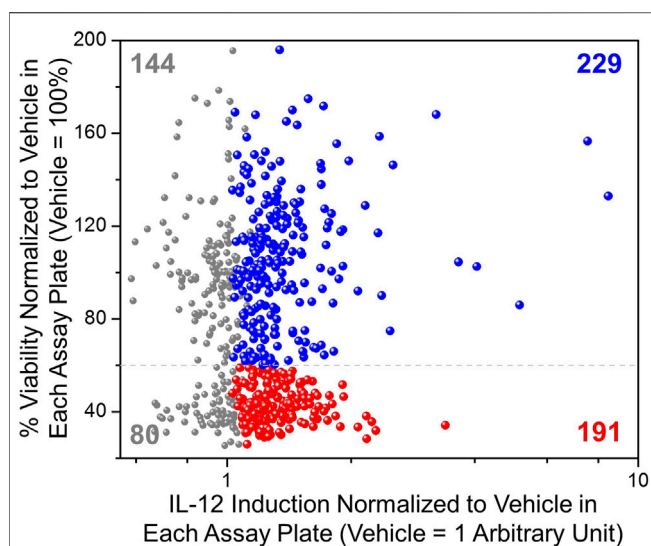


FIGURE 4 | Evaluation of immunostimulatory activity and toxicity in murine BMDCs. Selected hits candidates (644 compounds) were cherry-picked and evaluated in triplicates for induction of IL-12 release and cell viability by MTT assay in mBMDCs. IL-12 induced by test compounds was normalized to IL-12 induced by Veh (0.5% DMSO, 1 Arbitrary unit) in each plate. The cell viability following treatment with each compound was normalized to the viability following treatment of cells with Veh (%viability of Veh = 100%). A scatter plot for all compounds showing normalized IL-12 induction on the X-axis vs. cellular viability on the Y-axis helped for selecting immunostimulatory compounds that were relatively less toxic. All tested compounds are shown in grey, while the compounds that induced IL-12 above mean + 3SD (standard deviation within each assay plate) of the vehicle are shown in color, of which compounds that led to cellular viabilities less than 60% are shown in red spheres (191 compounds) while relatively non-toxic (% viability $\geq 60\%$) and potent IL-12 inducing compounds are shown by blue spheres (229 compounds).

to estimate confirmation rates. A Venn diagram was first generated for both NF- κ B and ISRE screens using the number of hits from the following three sets 1. Pilot screen hits, 2. Hits identified by Top X method in HTS, and 3. Hits identified by GMM method in HTS (Supplementary Figure S1). Based on these numbers, hit confirmation rates were calculated for all three HTS (Supplementary Figure S1). Comparing the combination of Top X and clustering based hit identification methods utilized earlier for NF- κ B HTS to the combination of Top X and GMM utilized here, we found increased confirmation rates from 31.5% in a prior similar HTS (Pu et al., 2012) to 67.6% in the current HTS for Top X method and 79.2% for the GMM method. The high confirmation rates were likely due to increased information available from the evaluation of compounds in duplicate. By using similar methodology, the hit confirmation rates for ISRE HTS were calculated as 57.7% for the Top X method and 60.9% for the GMM method. For CD63 HTS, the hit conformation rate was 52.6% by the Top X method only. The detailed analysis with number of hits, confirmation rates and calculations are shown in Supplementary Figure S1.

Immunostimulatory Cytokine Induction

The selected 644 compounds that consisted of 138 triple hits, 254 CD63 and NF- κ B hits, 217 CD63 and ISRE hits, and 35 NF- κ B and

ISRE hits were cherry-picked from the original HTS source plates to evaluate their immune stimulating activities in primary mBMDCs. These mBMDCs were incubated with compounds (10 μ M, in triplicates) overnight and NF- κ B downstream cytokine IL-12 release in the culture supernatant was measured by ELISA while the remaining cells in the plates were measured for viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. DMSO (0.5%) was used as a Veh control to determine the baseline IL-12 induction, and the IL-12 levels for all the compounds were normalized to Veh (IL-12 induced by Veh = 1). Similarly, the cell viabilities measured by MTT were normalized to Veh as 100%. The scatter plot in Figure 4 demonstrates the relative viability on the Y-axis and the normalized IL-12 inducing activity on the X-axis. We identified compounds that induced IL-12 more than 3 standard deviations above the mean of Veh in each plate and categorized them by cell viability into 2 groups. 229 compounds having viabilities above 60% (blue spheres) and 191 compounds having viabilities below 60% (red spheres), were identified as shown in Figure 4. These 229 hits were then rescreened for IL-12 induction at 5 μ M compound concentration to further confirm IL-12 inducing potency which led to identification of 130 compounds that induce IL-12 more than the mean +SD above Veh in each plate (Supplementary Figure S2).

Medicinal Chemistry Based Elimination of Hits

In an effort to narrow down our selection of hits for further *in vivo* adjuvanticity screening, we evaluated each compound structure for electrophilic characteristics, and presence of reactive and/or unstable functionalities, including Michael acceptors, hydrolyzable esters, reactive thioureas, and other indicators of pan-assay interference compounds (PAINS) (Baell and Holloway, 2010; Baell and Walters, 2014). Of the 130 compounds identified earlier, some of the compounds bearing such functionalities as shown in Supplementary Figure S3 were removed. Thus, the selected 80 compounds were sourced from the vendor and purchased in sufficient quantities (5–10 mg) to perform further bioactivity evaluation in an *in vivo* adjuvanticity screen.

In vivo Adjuvanticity Screening

The selected 80 compounds were first evaluated for purity and identity by HPLC-MS and compounds which were less than 90% pure were purified by Prep-HPLC. These were then evaluated for vaccine adjuvant activity in mice using a model antigen, ovalbumin (OVA). C57BL/6 mice ($n = 3$ for each compound) were immunized with OVA (20 μ g/animal) mixed with 200 nmol/injection compound on days 0 and 21. Monophosphorylated Lipid A (MPLA, 1 μ g/animal/injection) was used as a positive control and 10% DMSO was used as vehicle control (Veh). Sera were collected on day 28 and OVA-specific immunoglobulins IgG1 (Th2 type) and IgG2c (Th1 type) were determined by ELISA (Sato-Kaneko et al., 2020). These data are presented as a scatter plot in Figure 5A that shows the distribution of compounds by their adjuvanticity profiles in inducing Th1 and Th2 responses. The data points are colored

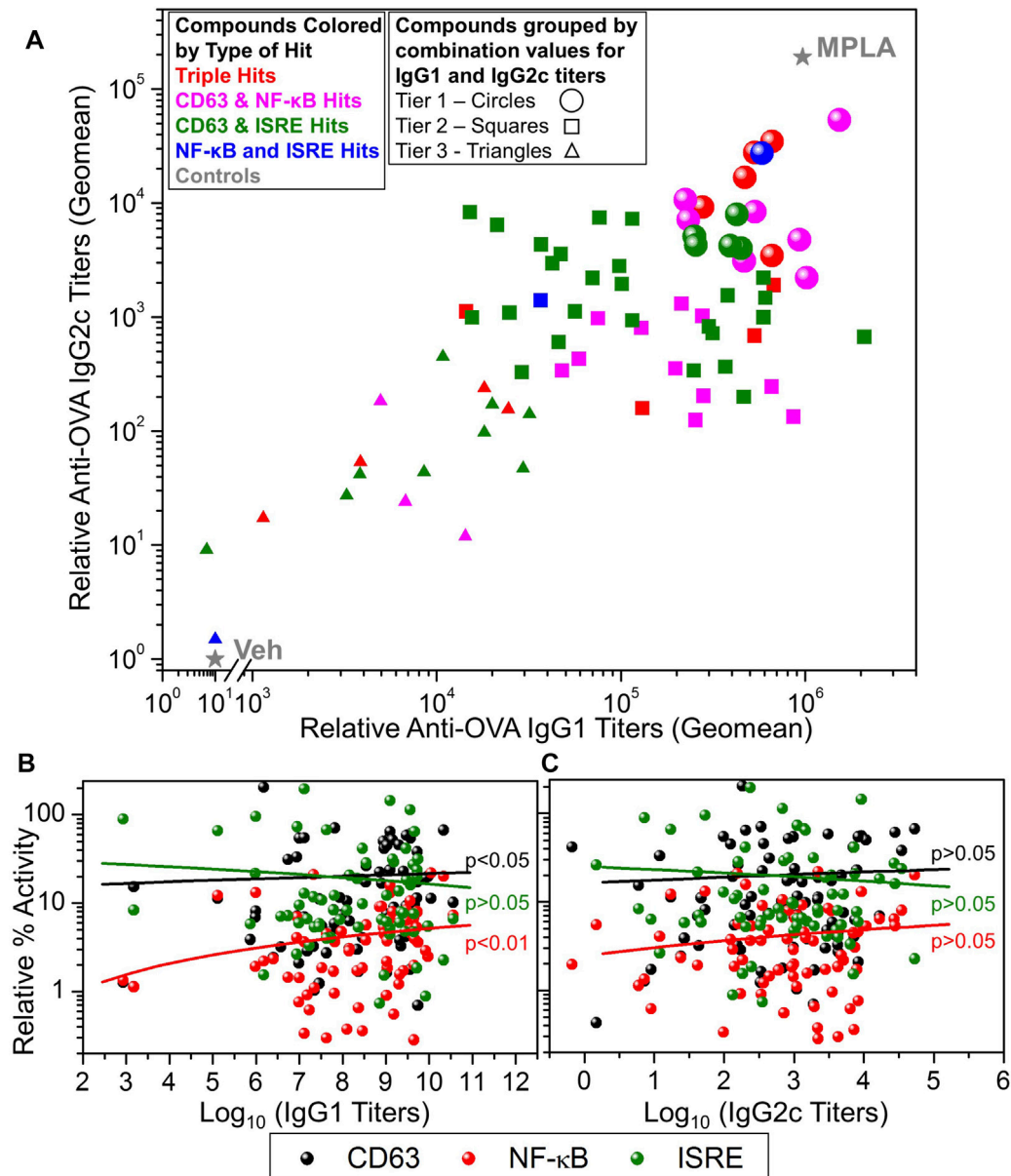
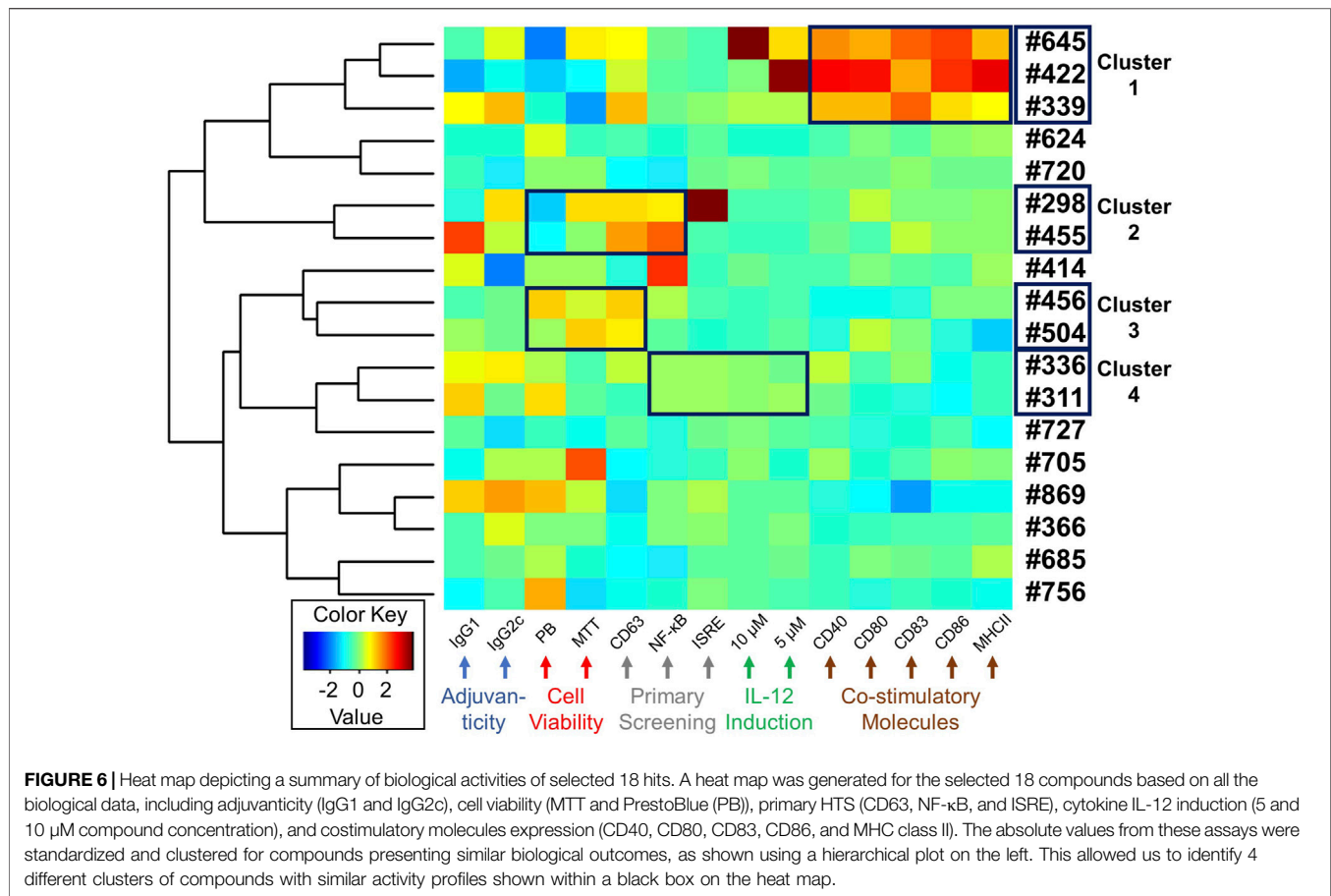


FIGURE 5 | *In vivo* adjuvanticity screening of test compounds with ovalbumin as a model antigen in mice. **(A)** C57BL/6 mice ($n = 3/\text{group}$) were intramuscularly immunized with OVA (20 $\mu\text{g}/\text{mouse}$) as antigen and adjuvanted with test compounds (200 nmoles/mouse), or MPLA (1 $\mu\text{g}/\text{mouse}$), or Veh (10% DMSO) on days 0 and day 21 and bled on day 28 to measure OVA-specific IgG2c and IgG1 levels in sera by ELISA. A scatter plot of the geomean ($n = 3$) values of the IgG2c titers (Y-axis) and IgG1 (X-axis) for each compound was generated to show the adjuvant potency distribution. Compounds were represented by different symbol types based on the type of hit identified, namely, triple hits (red symbols) and dual hits including CD63 and NF- κ B dual hits (magenta symbols), CD63 and ISRE dual hits (green symbols) and NF- κ B and ISRE dual hits (blue symbols). MPLA was the positive control while Veh was used as the negative control shown as grey stars. Based on the potency of compound to enhance the induction of combined IgG1 and IgG2c titers, they were divided into three tiers, Tier 1 (circles), Tier 2 (squares) and Tier 3 (triangles). These tiers were obtained by first calculating $\log_{10}$ transformed values of the IgG1 and IgG2c titers and normalizing these values in between 0 and 10 (10 for MPLA and 0 for Veh). This was followed by averaging these values (for each compound) for IgG1 and IgG2c to obtain a combination value, where Tier 1 compounds had a combination value ≥ 8 , Tier 2 compounds had a combination value ≥ 6 and ≤ 8 , while Tier 3 compounds had a combination value < 6 . Correlation of antigen-specific IgG1 **(B)** and IgG2c **(C)** antibody titers with primary HTS (CD63, NF- κ B, and ISRE) data. The data were analyzed by two-tailed nonparametric correlation (Spearman) with calculated p -values shown.

based on the type of hit (triple hits or three different dual hits) and the shape of the data point represents the adjuvanticity tier (Figure 5A, Tier 1: circles, Tier 2: squares and Tier 3: triangles). These tiers were obtained by first calculating $\log_{10}$

transformed values of the IgG1 and IgG2c titers and normalizing these values in each set for compounds between 0 and 10 (10 for MPLA and 0 for Veh). This was followed by averaging these values for IgG1 and IgG2c to obtain a combination value for each



compound, where Tier 1 compounds had values > 8, Tier 2 compounds had values between 6 and 8 while Tier 3 compounds had this combination value less than 6. The dominant presence of CD63 and NF-κB hits in the Tier 1 compounds suggests the involvement of NF-κB and CD63 activation pathways for adjuvant activities. Thus, we probed the correlation between these primary screening data and the adjuvant activity to understand if these pathways involve any particular common mechanism to induce immunoglobulins. **Figures 5B,C** shows correlation data of primary screening data with IgG1 and IgG2c antibody titers, respectively. The analysis revealed a correlation of IgG1 titers with NF-κB and CD63 assays, but less so with IgG2c titers. Since we aimed to discover compounds that induce good adjuvant activity, we selected Tier 1 compounds (18 compounds, circles in **Figure 5A**) to probe into mechanisms related to immune stimulation.

Co-Stimulatory Molecules Expression

Antigen presenting cells (APCs) such as dendritic cells play important roles in innate immune responses to transduce signals for subsequent humoral immunity (Lichtenstein et al., 2012). Costimulatory molecules, including CD80/86, CD40, and MHC class II molecules, are expressed on APCs and bind to their corresponding receptors on naïve or memory T cells, signalling

T cell proliferation or maturation (Lichtenstein et al., 2012). While we revealed that the selected compounds had cytokine inducing effects and *in vivo* adjuvant activity when used with an antigen, the effect of these compounds on APC function was unknown. Hence, to examine whether these 18 compounds enhance maturation of immature dendritic cells, facilitating antigen-presenting cell function, mBMDCs were treated with 10 μM compound or Veh overnight and the expression of costimulatory molecules (CD40, CD80, CD83, CD86 and MHC class II) on CD11c⁺ cells was examined by flow cytometry (**Figure 6**, **Supplementary Figure S4** and **Supplementary Table S5**). Of all these compounds, #645, #422, and #339 notably enhanced the expression of costimulatory molecules (**Figure 6**, cluster 1).

Heat Map Presentation of Compound Activity Profiles

Starting the HTS with 27,895 compounds, we narrowed down to 18 selected hits. We queried if there was any coherence in biological activity that would have driven the selection. Thus, we clustered the compounds by their activity profiles' including adjuvant activity (IgG1 and IgG2c), cell viability (PrestoBlue and MTT assays), primary HTS (CD63, NF-κB, and ISRE), IL-12 induction (10 and 5 μM compound concentration), and co-

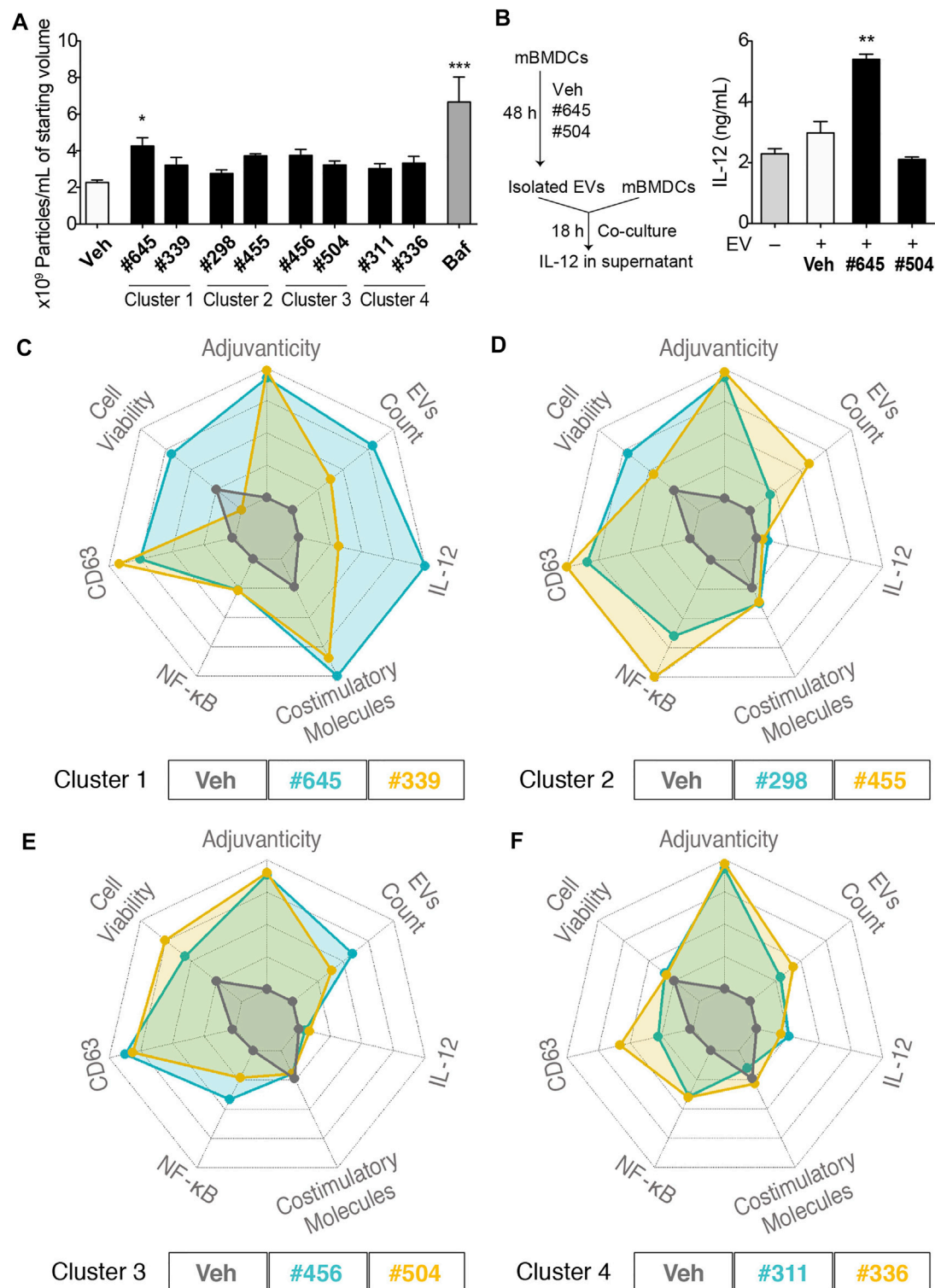


FIGURE 7 | EV characterization and spider plots for selected hits depicting similar biological activities. **(A)** Number of particles per mL of starting culture medium volume assessed by MRPS using the nCS1 instruments with C-400 cartridges. mBMDs were incubated with compound (10 μ M), Veh, or bafilomycin A1 (Baf, positive control) for 48 h and EVs in the supernatant were isolated. The EVs were diluted 100-fold in 1% Tween 20-PBS and quantitated using the nCS1 system. All results were analyzed using the nCS1 Data Analyzer software. Bars indicate means $\pm$ SEM of 3–4 replicates of mBMDC batches. * $p < 0.05$, *** $p < 0.0001$, One-way ANOVA with Dunnett's post hoc test. **(B)** Co-culture of mBMDs with isolated EVs. mBMDs were cultured with 10 μ M #645, #504 or Veh for 48 h and EVs in the culture supernatant were isolated and resuspended in 50 μ L PBS. Freshly prepared mBMDs ($10^5/100 \mu$ L) were mixed with 7 μ L of the EVs and co-cultured for 18 h. IL-12 levels in the (Continued)

FIGURE 7 | supernatant were evaluated by ELISA. Bars indicate means $\pm$ SD of duplicate wells. * $p < 0.05$, One-way ANOVA with Dunnett's post hoc test vs. Veh. Spider plots show selected activity profiles' overlay for compounds within the cluster. These activity profiles include 1. Adjuvanticity as a composite of IgG1 and IgG2c titers, 2. EVs particle concentration, 3. IL-12 induction, 4. Composite value for the expression of co-stimulatory molecules including CD40, CD80, CD83, CD86 and MHC class II, 5. NF- κ B HTS data, 6. CD63 HTS data, and 7. Cell viability by MTT. The figures are shown as overlays of activity profiles of Veh with (C) Cluster 1 compounds #645 and #339; (D) Cluster 2 compounds #298 and #455; (E) Cluster 3 compounds #456 and #504; (F) Cluster 4 compounds #311 and #336. These clustered compounds show similar activity profiles', thus validating the HTS.

stimulatory molecules expression (CD40, CD80, CD83, CD86, and MHC class II). All these data were normalized using negative and positive controls to remove batch effects and each variable was standardized before heat maps were generated (Figure 6). Based on hierarchical clustering (shown on the left in Figure 6), we were able to group compounds into four clusters. Cluster 1 consisted of 3 compounds #645, #422, and #339. This group of compounds showed similar effects on the induction of co-stimulatory molecules in mBMDCs. Next, cluster 2 (#298 and #455) and cluster 3 (#456 and #504) consisted of compounds with similarity in their cell viability profiles as well as in the primary screenings. The last cluster 4 (#336 and #311) also showed similar bioactivity in primary screenings as well as IL-12 induction.

EV Characterization and Spider Plots

To quantitate EV release from mBMDCs, we selected these clustered 8 compounds (compound #422 had very poor aqueous solubility and was not evaluated for EV particle count) and assessed EV particle numbers in the culture supernatants of mBMDCs cultured with 10 μ M compound for 48 h using microfluidic resistive pulse sensing (MRPS) with a nCS1 Instrument (Spectradyn, Signal Hill, CA). The EVs in the culture supernatants were isolated following the multistep differential ultracentrifugation protocol as previously described (Shpigelman et al., 2021). Only compound #645 increased the number of EV particles released in the culture supernatant compared to Veh control (Figure 7A). Immunoblots of isolated EV pellets confirmed enrichment for the tetraspanins CD81 and Tsg101 (Supplementary Figures S5, S6). We also examined if the immunogenicity was from direct compound stimulation of cells or could be transferred by EVs from stimulated cells (Figure 7B). Two compounds were selected as relatively high and low inducers of EV release (#645 and #504, respectively). The EVs from #645-treated cells stimulated higher levels of IL-12 release than those from Veh-treated cells indicating that the EVs from the cells are capable of innate immune stimulation.

To compare activity of the clustered compounds from the heat map above, their multiple bioactivities, including adjuvanticity and co-stimulatory molecules expression, were used to generate spider plot overlays along with the EV particle count data. For cluster 1, compounds #645 and #339 had equipotent activity in CD63 and NF- κ B assays and co-stimulatory molecules expression, although they varied in IL-12 induction and cell viability, which translated as well to quantities of EVs (Figure 7C). Thus, #645 that belongs to a 3-pyridyl-oxadiazole scaffold was revealed as a promising lead chemotype (Supplementary Figure S6). For cluster 2,

compounds #298 and #455, both had moderate effects on induction of cytokine IL-12 and expression of co-stimulatory molecules, but had similar CD63 and NF- κ B activities in the primary HTS and both showed high values (indicating possible cell proliferation) in the MTT assays (Figure 7D). The EV particle counts inversely varied with MTT assay data suggesting distinct properties from cluster 1 compounds and pointed towards mechanisms that may involve increased formation of EVs due to decreased cell numbers (cells breaking down to release EVs). Cluster 3, consisting of compounds #456 and #504, had similar activity profiles as that of cluster 2 compounds in terms of higher MTT values inversely relating to EV particle count size but had much lower NF- κ B inducing activity (Figure 7E). Cluster 4, consisting of compounds #311 and #336, showed very similar activity profiles. Through a rigorous tiered screening process, we discovered that these 2 compounds share structural similarity and belonged to the same 4-thieno-2-thiopyrimidine chemotype (Figure 7F and Supplementary Figure S7).

DISCUSSION

In immune responses, EVs have been reported to play immune-stimulatory and -suppressive roles. As a screening tool we utilized a human cell line from a lineage that has antigen presentation functions. Dendritic cells (DCs) are the most effective cell type for antigen presentation to immune cells and EVs released from DC can stimulate pro-inflammatory responses (Zitvogel et al., 1998; Escudier et al., 2005; Morse et al., 2005; Besse et al., 2016). Moreover, EVs are known to play a role in acquired immunity, as EVs released from macrophages and DCs display major histocompatibility (MHC) class I and II molecules, co-stimulatory molecules (CD80 and CD86), and the adhesion protein ICAM-1 (CD54) on their surface (Admyre et al., 2006; Schorey et al., 2015; Wen et al., 2017; Lindenberg and Stoorvogel, 2018). Antigen presentation to T cells can occur directly by MHC molecules loaded with the antigenic peptide on the surface of EVs or the EVs can be taken up by DCs or macrophages and the antigen processed to be presented by their MHC surface molecules. We developed a HTS to specifically identify chemicals that would increase biogenesis and release of EVs to enhance antigen specific immune responses in an adjuvant role. We identified #645 exhibiting not only intrinsic immunostimulatory activity but also induce release of immunostimulatory EVs (Figures 7A,B). As #645 enhanced the expression of MHC class II and costimulatory molecules on mBMDCs (Figure 6), the EVs isolated from #645-treated mBMDCs may also have these surface proteins, contributing

to antigen presentation and augmenting T cell responses. Furthermore, the *in vitro* co-culture study with EVs and mBMDCs showed that the EV released from #645-treated mBMDCs induced IL-12 release (Figure 7F), suggesting that these EVs may also induce innate immune responses from other surface interactions like PRRs.

The use of EVs as a vaccine platform is under intensive investigation. EVs can be harvested from the supernatants of cells engineered to produce antigens and/or have specific cargos like mRNA (Kanuma et al., 2017; Anticoli et al., 2018; Jafari et al., 2020). In the first case the released EVs can preserve the native conformation of the antigenic proteins for delivery to the lymphoid system. In the second system the vaccine recipient would express the proteins at the site of delivery. The distribution of EVs administered *in vivo* is dependent on the cell source of the EVs and the route of administration (Wiklander et al., 2015). EVs administered intravenously in mice are rapidly cleared from the circulation, with a half-life of 2–4 min with complete clearance after 4 h (Takahashi et al., 2013). These EVs preferentially accumulate in the liver and spleen and are largely taken up by macrophages, which participate in the clearance of EVs (Imai et al., 2015). After half an hour EVs start to be eliminated by hepatic and renal clearance mechanisms which is completed within roughly 6 h (Takahashi et al., 2013; Lai et al., 2014). EVs that are administered subcutaneously have less hepatic uptake and a slower clearance. The long term aim of our strategy is to use a chemically controlled release of EVs at the site of antigen administration, that would enable the recipients' cells to produce EVs continuously over time. This release is likely to be slower than a bolus of EV administration, but the continuous production would potentially overcome the limitation of rapid clearance.

Our CD63 HTS demonstrated that there are many different chemical scaffolds that induce the release of EVs, further reinforcing that the release of EVs is an important intercellular communication mechanism potentially inducing the stimulation of multiple intracellular pathways. To reduce our pool of hit candidates, we performed parallel screens on two other reporter lines, NF- κ B and ISRE-*bla* reporter cells utilizing the same parent THP-1 cells. Compounds from each of the intersecting groups were able to stimulate an *in vivo* immune response to a test antigen above that of the antigen without any adjuvant. This indicates that the use of a triple screen still allowed for a broad ability to capture multiple potential leads without skewing to a single mechanism of action. Choosing Maybridge compound library for HTS enabled us to identify relatively unexplored compounds and thus we could not find any literature precedence for immunomodulatory activities by these identified hits or substructure compounds of these chemotypes using Scifinder and PubChem databases. However, our tiered screening process selected two compounds that belong to 4-thieno-2-thiopyrimidine scaffold having identical screening profiles for cytokine stimulation and cell surface marker induction thus supporting an internal reproducibility and validating our overall screening.

Although we utilized three parallel screens to identify compounds, the initial *in vivo* testing indicated that

compounds with all three activities were effective adjuvants. Among the three HTS, the compounds that had the ability to stimulate EV release and NF- κ B activity with or without ISRE activity were found to be the most potent adjuvants. The compounds that were the most effective that had ISRE activity also had NF- κ B activity and high EV release (triple hits). In contrast, others have identified molecules that stimulate type I interferon release and ISRE activity that resulted in potent activity as adjuvants (Martínez-Gil et al., 2013). Our findings may be relative only to the compounds that were included in the library and may not be generalizable to other systems. In addition, we carefully screened compounds for *in vitro* toxicity with both PrestoBlue and MTT assays. Thus, it is not likely that NF- κ B and CD63 inducing activity was due to toxicity. Although this study did not formally evaluate the safety profiles of the active compounds *in vivo*, externally isolated and administered EVs have been reported as having an acceptable safety profile including after multiple rounds of administration. (Maji et al., 2017; Zhu et al., 2017; Mendt et al., 2018; Saleh et al., 2019).

Here we have described the identification of novel chemical scaffolds that are effective *in vivo* adjuvants. Screening for the release of EVs in addition to the activation of known immunologic signaling pathways added a new additional dimension that identified robust scaffolds for further SAR studies of vaccine adjuvant activity. Chemical induction of EV release may prolong the delivery of antigen to the lymphoid system. Further studies on the induction of long-term immune memory are warranted.

MATERIALS AND METHODS

Cell Lines

CellSensor[®] NF κ B-*bla* human monocytic leukemic THP-1 cell line was purchased from Thermo Fisher Scientific (Waltham, MA). The ISRE-*bla* THP-1 cell line was developed as described earlier (Shukla et al., 2018). These cell lines contain NF- κ B and ISRE reporter constructs that uses a β -lactamase reporter gene which on activation results in beta-lactamase production and shifts the fluorescence emission of the beta-lactamase substrate [LiveBLazerTM-FRET B/G (CCF4-AM), Thermo Fisher Scientific] to favor coumarin (460 nm emission) over fluorescein (530 nm emission).

CD63-Tluc-CD9EmGFP THP-1 reporter cells were prepared by Thermo Fisher Scientific as described previously (Shpigelman et al., 2021). Briefly, a construct with dual reporters consisting of two tetraspanins, CD63 and CD9 reporter constructs; CD63-Tluc and CD9-EmGFP, was transduced into THP-1 cells. The Tluc activities of EVs shed from CD63-Tluc-CD9EmGFP reporter cells in the culture supernatant were quantitatively measured for EV release.

All THP-1 reporter cells were maintained in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered RPMI 1640 medium (#72400, Thermo Fisher Scientific) supplemented with 10% dialyzed FBS (dFBS, #26400044, Thermo Fisher Scientific), 100 U/ml penicillin, 100 μ g/ml streptomycin, 1 mM sodium pyruvate, 1 $\times$ MEM

non-essential amino acids (NEAA), and 5 µg/ml blasticidin at 37°C in 5% CO₂. All the HTS assay validations were carried out in assay medium OptiMEM® I Reduced Serum Medium (#31985-070, Thermo Fisher Scientific) in 384-well plates (#3712, Corning).

Reagents

The Maybridge library series, including the Maybridge HitFinder library (14,303 compounds) and the Maybridge HitCreator library (13,592 compounds) were purchased from Thermo Fisher Scientific (Leeds, United Kingdom) (**Supplementary Table S1**).

LPS used as a positive control for the NF-κB HTS was obtained from Sigma-Aldrich (St. Louis, MO). MPLA was purchased from Invivogen (San Diego, CA). Human IFN-α (#11101-1, PBL Assay Science) was used as a positive control for ISRE-*bla* HTS. Phorbol 12-myristate 13-acetate (PMA, BP685-1, Thermo Fisher Scientific) was used as a positive control for HTS using CD63-Tluc-CD9EmGFP reporter cells. MTT was purchased from Thermo Fisher Scientific. Ovalbumin (OVA) was obtained from Worthington Biochemical Co. (Lakewood, NJ). PBS (#14190, Thermo Fisher Scientific) filtered through a 0.02 µm inorganic membrane filter (#6809-2002, Millipore, Burlington, MA) was used to wash and dilute EVs.

High Throughput Screens and Statistical Analysis

The robotic HTS using the three reporter cells were performed using 384-well plates by the SelectScreen™ service, Thermo Fisher Scientific (Madison, WI) (Pu et al., 2012; Chan et al., 2017b; Shukla et al., 2018). For HTS using NF-κB-*bla* and ISRE-*bla* THP-1 cells, LPS (100 ng/ml) and human IFN-α (50 nM) were used as the positive controls, respectively, while 0.5% DMSO was used as vehicle (Veh). The cells were incubated with compounds (10 µM) for 5 h, and LiveBLazer™ FRET B/G substrate (CCF4-AM) mixture was added. Fluorescence was measured at an excitation wavelength of 405 nm, and emission wavelengths of 465 and 535 nm. The background values were subtracted from the raw values (cell-free wells at the same fluorescence wavelength). Emission ratios were calculated by dividing background-subtracted values from emission wavelength of 465 nm by those from emission wavelength of 535 nm. The response ratio (RR) was calculated as follows (emission ratio of a test well)/(average emission ratio of wells with Veh). Further, for comparison of activity, “%activation” for each compound was computed within the plate as $100 \times (\text{compound RR} - \text{average Veh RR}) / (\text{average LPS RR} - \text{average Veh RR})$.

In the HTS using CD63-Tluc-CD9EmGFP reporter cells (CD63 HTS), PMA (10 ng/ml) was used as a positive control (Shpigelman et al., 2021) and 0.5% DMSO was used as negative control (vehicle, Veh). Briefly, the harvested cells were resuspended in assay media containing 10% exosome-depleted FBS (#A2720801, Thermo Fisher Scientific) and plated at 2×10^5 cells/mL (50 µL/well of 384-well plates) (#3674, Corning). Test compounds were added at a final concentration of 10 µM to cells

and incubated for 48 h at 37°C. Subsequently, the plate was centrifuged, supernatant (25 µL) was transferred, and chemiluminescent was measured as recombinant luciferase activity (RLU) after 10 min incubation with TurboLuc assay reagent (TurboLuc™ One-Step Glow Assay kit, Thermo Fisher Scientific). The response that measures activation and subsequent release of EVs was calculated using the following formula; % response = $100 \times (\text{compound RLU} - \text{average Veh RLU}) / (\text{average PMA RLU} - \text{average Veh RLU})$. The viability of cells was assessed using PrestoBlue reagent (Thermo Fisher Scientific). Briefly, PrestoBlue reagent (2.5 µL) was added to the remaining cells and incubated for 30 min at room temperature, followed by fluorescence readout at (Ex 560 nm/Em 590 nm) which was normalized to fluorescence data of the Veh wells within the plate to obtain “%viability” calculated as $100 \times (\text{compound fluorescence} / \text{average Veh fluorescence})$.

Data and Statistical Analysis for Selection of Hits

In each of the 3 screens, compounds were assayed twice in two replicate HTS experiments (experiments 1 and 2 performed on different days). The HTS readout was evaluated as %activation as mentioned above). For the NF-κB and ISRE HTS, hit compounds were identified using two statistical methods 1) “Top X” threshold approach and 2) “Gaussian mixture model (GMM)” approach. A compound identified by either of these methods was considered a hit. However, for the CD63 HTS, only the Top X method was used to identify hits.

Top X method: In the Top X method, all compounds with % activation values above a given threshold were selected. The threshold was computed for each plate, using the Veh wells (cells treated with 0.5% DMSO), as the mean + 3SD of % activation from these wells. Any selected compound was considered to be a false-positive if both coumarin and fluorescein values were extreme outliers according to the manufacturer’s instruction (Thermo Fisher Scientific). When a test compound was selected as a hit in both of the 2 independent HTS experiments, we reported it as a Top X hit (**Figures 2A,D**). For the CD63 HTS, we used the mean %response of the Veh wells as the per-plate threshold value.

GMM method: Since all compounds were assayed twice in two independent experiments, we could identify hits using a GMM. In this approach %activation data from the two independent experiments were used to construct bivariate GMM (Hastie and Tibshirani, 1996) implemented in the R-mclust package (Scrucca et al., 2016). These models were used to cluster compounds into hit or non-hit categories. Briefly, the arbitrary number of 20 units was added to all % activation values to ensure all values were greater than 0 and the data were log₁₀ transformed. The two independent experiments were visualized using MA (log ratio vs. average) plots, in which the X-axis was the average of log₁₀ (%activation + 20) for two replicate values for each test compound and the Y-axis was the difference in these values. A GMM was then fit to the plotted data, where the Bayesian Information Criterion (BIC) was used to determine the optimal number, shape and orientation of the

gaussian clusters. Since this method was heavily influenced by a large number of the compounds with low %activation values, a two-step approach was employed: at the initial step, a null cluster was identified in which the majority of compounds had %activation levels similar to those from Veh-treated wells (Figures 2B,E). This null cluster was removed, as were all compounds with average activity values lower than the maximum value of this null cluster (red dotted line, Figures 2B,E). At the second step, a second GMM was fitted, using the remaining data. Apparent false-positive clusters were identified and used to construct linear boundaries, and finally compounds from the remaining clusters within these boundaries were considered GMM hits (Figures 2C,F). The GMM method ensured both a larger number of hits and also higher confirmation rate when data from an initial independent pilot screen was used to estimate the hit confirmation rate.

R statistical software (R version 3.6.1, www.r-project.org) was used for selection of hits. For the data other than HTS, Prism 6 (GraphPad Software, San Diego, CA) statistical software was used to obtain *p*-values for comparison between groups (*p* < 0.05 was considered significant) and for Spearman's rank correlation to test for a non-zero correlation between antigen-specific antibodies and HTS data.

Animals

Wild type C57BL/6 mice were purchased from the Jackson Laboratories. All animal experiments received prior approval by the Institutional Animal Care and Use Committee (IACUC) for UC San Diego.

Generation of mBMDCs

mBMDCs were prepared from bone marrow cells harvested from femurs of C57BL/6 mice as previously described (Lutz et al., 1999; Datta et al., 2003). Briefly, murine bone marrow cells were harvested from C57BL/6 mice. The cells were cultured with murine granulocyte-macrophage colony-stimulating factor (GM-CSF, 20 ng/ml) for 7–8 days. Non-adherent cells were harvested and used for experiments.

Cell Viability Assay

mBMDCs (10^5 cells/200 μ L/well) were treated with 10 and 5 μ M of a test compound in 96-well plates overnight. After 18 h of drug treatment, MTT (0.5 mg/ml) was added to each well. The cells were lysed after 6–8 h incubation, and absorbance values at 570 and 650 nm were measured. PrestoBlue reagent (#A13261, Thermo Fisher Scientific) was used for cell viability assay in CD63 HTS as described earlier.

Assessment of Cytokine Levels Using Primary Cells

mBMDCs (10^5 cells/200 μ L/well) were plated in wells of 96-well plates and treated with test compound (5 μ M or 10 μ M) or vehicle (0.5% DMSO) overnight. IL-12 levels in the culture supernatants were assessed by ELISA as previously described (Sato-Kaneko et al., 2021). Antibodies used in ELISA are shown in Supplementary Table S5.

In vivo Assessment of Adjuvanticity of Compounds

C57BL/6 mice were intramuscularly injected with OVA (20 μ g/mouse) mixed with a test compound (200 nmol/mouse) or MPLA (1 μ g/mouse) or Veh (10% DMSO) in 50 μ L total volume on days 0 and 21 and bled on day 28. OVA-specific IgG1 and IgG2c levels in sera were evaluated by ELISA as described previously (Chan et al., 2009).

Flow Cytometric Analysis for Costimulatory Molecules

mBMDCs (10^5 cells/200 μ L/well) were treated with a test compound (10 μ M) or Veh (0.5% DMSO) overnight and then the costimulatory molecule expression on mBMDCs was evaluated using flow cytometry. The cells were stained with antibodies for CD11c, CD80, CD83, CD86, CD40, and MHC class II (antibodies used are listed in Supplementary Table S5). Dead cells (DAPI^{high}) were excluded from the analysis. Percent positive population of CD80, CD83, CD86, CD40, or MHC class II in the gated CD11c population were analyzed (Supplementary Figure S4).

Heat Maps

Variables used to make a heat map were normalized and scaled by subtracting the mean and dividing by the standard deviation. Hierarchical clustering was performed, where the distance measure was the Spearman rank correlation. The R-*gplots* package was used to make heat maps.

EV Isolation by Differential Ultracentrifugation

EVs were isolated following the protocol described in the previous study with minor modifications (Shpigelman et al., 2021). Conditioned culture media (40 ml) was spun at 300 *g* for 10 min to remove debris. Supernatants were subsequently spun at 2,000 *g* for another 10 min followed by the 10,000 *g* step for 30 min. Next, 30 ml of supernatants were transferred to 31.5 ml open-top polypropylene UC tubes (358,126, Beckman Coulter Life Sciences, CA) and spun at 100,000 *g*_{avg} for 3 h in an SW28 rotor (K-Factor: 2,554) by Beckman Optima XL-90 Ultracentrifuge (Beckman Coulter Life Sciences). The supernatants were then gently aspirated (leaving ~50 μ L), and pellets resuspended in 30 ml cold filtered PBS. Re-suspended pellets were then spun under the same conditions as the prior spin, followed by another round of gentle aspiration and resuspension to a final volume of 50 μ L in cold filtered PBS. All centrifugation steps were performed at 4°C, and resultant samples were stored at –80°C until use. All relevant data of our experiments have been submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV220165, <https://evtrack.org/index.php>) (Van Deun et al., 2017).

Evaluation of EV Concentrations Released by mBMDCs

mBMDCs (7.5×10^5 /ml, total 40 ml) were incubated with 10 μ M test compound or vehicle (0.01% DMSO) in RPMI 1640

(#11875, Thermo Fisher Scientific) supplemented with exosome depleted FBS (#A27208, Thermo Fisher Scientific) in a T182 flask (#25–211, Genesee Scientific, San Diego, CA) for 46–48 h and EVs were isolated from culture supernatant by differential centrifugation as described above. EV particle concentrations and particle size/distribution were determined by MRPS technology with nCS1 particle analyzer utilizing C-400 cartridges (Spectradyne, Signal Hill, CA). EV samples were diluted 100-fold in 1% Tween 20-PBS and run on the nCD1 instrument. All results were analyzed using the nCS1 Data Analyzer (Spectradyne). To exclude false particle events, we applied the following peak filters: Transit time (μ s) from 0 to 80, symmetry from 0.2 to 4.0, diameter (nm) from 75 to 400, signal to noise ratio (S/N) at least 10.

Spider Plots

In a spider plot, each axis represents one of the variables to be displayed. To reduce the number of axes and make for a more interpretable visualization, selected assay readouts within the same category were combined by averaging the scaled individual variables. The final derived variables each underwent min-max normalization, i. e., $x_{\text{new}} = (x - \min) / (\max - \min)$, where min and max are minimum and maximum values of variable x , with the min (max) taken over the set of candidate compounds. The innermost net of a spider plot marks the minimum value over all the compounds, whereas the outer most net marks the maximum. R-*fmsb* package was used to make spider plots.

Immunoblotting

mBMDs were lysed with radioimmune precipitation assay buffer (RIPA) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany) and a phosphatase inhibitor (Millipore). The total protein in the samples was quantitated by Pierce micro BCA Protein Assay Kit. Two μ g of cell lysate or EVs were mixed with 4 $\times$ NuPAGE sample buffer (Thermo Fisher Scientific) under reducing condition with dithiothreitol (DTT, Sigma) for Tsg101 or nonreducing condition (without DTT) for CD81. When DTT, a reducing agent, was used, samples were also denatured at 95°C for 5 min prior to loading. After fractionation on NuPAGE 4–12% Bis-Tris Gels (Thermo Fisher Scientific), samples were blotted onto Immobilon-P PVDF membranes (#IPVH00010, Sigma) and blocked for 1 h in 5% BSA-TBS-T at RT. The blots were then incubated with primary antibodies (Ab): anti-CD81 Ab (1:1,000 dilution), anti-Tsg101 Ab (1:500 dilution) overnight at 4°C with gentle agitation. After washing, the membranes were incubated with corresponding secondary antibody for 30 min at RT with gentle agitation. Blots were developed with ProSignal Dura ECL Reagent (Prometheus Protein Biology Products, Genesee Scientific, San Diego, CA) and visualized using a ChemiDoc Imaging System (Bio-Rad Laboratories, Hercules, CA). AccuRuler Prestained Protein Ladder (Lamda

Biotech, St. Louis, MO) was used for the molecular weight markers. Details for antibodies and reagents are shown in **Supplementary Table S5**.

Co-Culture Study With mBMDs and Isolated EVs

mBMDs (7.5×10^5 /ml, total 40 ml in T182 flask) were treated with 10 μ M #645, #504 or Veh (0.01% DMSO) in RPMI 1640 (Thermo Fisher Scientific) supplemented with exosome depleted FBS (10%, Thermo Fisher Scientific) for 46–48 h. The EVs were isolated from the conditioned media and resuspended in 50 μ L filtered PBS at the final step as described above. Freshly prepared mBMDs (10^5 cells/100 μ L) were mixed with 7 μ L of the EVs in a well of 96-well plate and incubated for 18 h. IL-12 levels in the culture supernatants were assessed by ELISA.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) for UC San Diego.

AUTHOR CONTRIBUTIONS

NS and FS-K contributed equally to this work and share first authorship. NS, FS-K, MP, KM, TH, HC, MCorr and DC wrote the manuscript. MP and KM performed statistical calculations and HTS analysis, generation of heat maps and spider plots. FS-K, SY, FL, YS, TS, TH and MCorr planned and performed biological studies. NS, MChan and HC performed medicinal chemistry based compound selection, chemotype and compound clustering analysis.

FUNDING

This study was supported by the National Institute of Health/ National Institute of Allergy and Infectious Diseases under contract 75N93019C00042 (Principal Investigator-DC).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2022.869649/full#supplementary-material>

REFERENCES

- Admyre, C., Johansson, S. M., Paulie, S., and Gabrielsson, S. (2006). Direct Exosome Stimulation of Peripheral Human T Cells Detected by ELISPOT. *Eur. J. Immunol.* 36 (7), 1772–1781. doi:10.1002/eji.200535615
- Anticoli, S., Manfredi, F., Chiozzini, C., Arenaccio, C., Olivetta, E., Ferrantelli, F., et al. (2018). An Exosome-Based Vaccine Platform Imparts Cytotoxic T Lymphocyte Immunity against Viral Antigens. *Biotechnol. J.* 13 (4), e1700443. doi:10.1002/biot.201700443
- Baell, J., and Walters, M. A. (2014). Chemistry: Chemical con Artists Foil Drug Discovery. *Nature* 513 (7519), 481–483. doi:10.1038/513481a
- Baell, J. B., and Holloway, G. A. (2010). New Substructure Filters for Removal of pan Assay Interference Compounds (PAINS) from Screening Libraries and for Their Exclusion in Bioassays. *J. Med. Chem.* 53 (7), 2719–2740. doi:10.1021/jm901137j
- Besse, B., Charrier, M., Lapiere, V., Dansin, E., Lantz, O., Planchard, D., et al. (2016). Dendritic Cell-Derived Exosomes as Maintenance Immunotherapy after First Line Chemotherapy in NSCLC. *Oncimmunology* 5 (4), e1071008. doi:10.1080/2162402X.2015.1071008
- Campos, J. H., Soares, R. P., Ribeiro, K., Andrade, A. C., Batista, W. L., and Torrecilhas, A. C. (2015). Extracellular Vesicles: Role in Inflammatory Responses and Potential Uses in Vaccination in Cancer and Infectious Diseases. *J. Immunol. Res.* 2015, 832057. doi:10.1155/2015/832057
- Chan, M., Ahmadi, A., Yao, S., Sato-Kaneko, F., Messer, K., Pu, M., et al. (2017a). Identification of Biologically Active Pyrimido[5,4-B]indoles that Prolong NF- κ B Activation without Intrinsic Activity. *ACS Comb. Sci.* 19 (8), 533–543. doi:10.1021/acscombsci.7b00080
- Chan, M., Hayashi, T., Kuy, C. S., Gray, C. S., Wu, C. C., Corr, M., et al. (2009). Synthesis and Immunological Characterization of Toll-Like Receptor 7 Agonistic Conjugates. *Bioconjug. Chem.* 20 (6), 1194–1200. doi:10.1021/bc900054q
- Chan, M., Kakitsubata, Y., Hayashi, T., Ahmadi, A., Yao, S., Shukla, N. M., et al. (2017b). Structure-Activity Relationship Studies of Pyrimido[5,4-B]indoles as Selective Toll-Like Receptor 4 Ligands. *J. Med. Chem.* 60 (22), 9142–9161. doi:10.1021/acs.jmedchem.7b00797
- Cossetti, C., Iraci, N., Mercier, T. R., Leonardi, T., Alpi, E., Drago, D., et al. (2014). Extracellular Vesicles from Neural Stem Cells Transfer IFN- γ via Ifngr1 to Activate Stat1 Signaling in Target Cells. *Mol. Cell* 56 (2), 193–204. doi:10.1016/j.molcel.2014.08.020
- Datta, S. K., Redecke, V., Prilliman, K. R., Takabayashi, K., Corr, M., Tallant, T., et al. (2003). A Subset of Toll-Like Receptor Ligands Induces Cross-Presentation by Bone Marrow-Derived Dendritic Cells. *J. Immunol.* 170 (8), 4102–4110. doi:10.4049/jimmunol.170.8.4102
- Escudier, B., Dorval, T., Chaput, N., André, F., Caby, M. P., Novault, S., et al. (2005). Vaccination of Metastatic Melanoma Patients with Autologous Dendritic Cell (DC) Derived-Exosomes: Results of Thefirst Phase I Clinical Trial. *J. Transl. Med.* 3 (1), 10. doi:10.1186/1479-5876-3-10
- Hastie, T., and Tibshirani, R. (1996). Discriminant Analysis by Gaussian Mixtures. *J. R. Stat. Soc. Ser. B (Methodological)* 58 (1), 155–176. doi:10.1111/j.2517-6161.1996.tb02073.x
- Imai, T., Takahashi, Y., Nishikawa, M., Kato, K., Morishita, M., Yamashita, T., et al. (2015). Macrophage-dependent Clearance of Systemically Administered B16BL6-Derived Exosomes from the Blood Circulation in Mice. *J. Extracell. Vesicles* 4, 26238. doi:10.3402/jev.v4.26238
- Jafari, D., Shajari, S., Jafari, R., Mardi, N., Gomari, H., Ganji, F., et al. (2020). Designer Exosomes: A New Platform for Biotechnology Therapeutics. *BioDrugs* 34 (5), 567–586. doi:10.1007/s40259-020-00434-x
- Kanuma, T., Yamamoto, T., Kobiyama, K., Moriishi, E., Masuta, Y., Kusakabe, T., et al. (2017). CD63-Mediated Antigen Delivery into Extracellular Vesicles via DNA Vaccination Results in Robust CD8(+) T Cell Responses. *J. Immunol.* 198 (12), 4707–4715. doi:10.4049/jimmunol.1600731
- Lai, C. P., Mardini, O., Ericsson, M., Prabhakar, S., Maguire, C., Chen, J. W., et al. (2014). Dynamic Biodistribution of Extracellular Vesicles *In Vivo* Using a Multimodal Imaging Reporter. *ACS Nano* 8 (1), 483–494. doi:10.1021/nn404945r
- Liechtenstein, T., Dufait, I., Lanna, A., Breckpot, K., and Escors, D. (2012). Modulating Co-Stimulation during Antigen Presentation to Enhance Cancer Immunotherapy. *Immunol. Endocr. Metab. Agents Med. Chem.* 12 (3), 224–235. doi:10.2174/187152212802001875
- Lindenberg, M. F. S., and Stoorvogel, W. (2018). Antigen Presentation by Extracellular Vesicles from Professional Antigen-Presenting Cells. *Annu. Rev. Immunol.* 36, 435–459. doi:10.1146/annurev-immunol-041015-055700
- Lutz, M. B., Kukulski, N., Ogilvie, A. L., Rössner, S., Koch, F., Romani, N., et al. (1999). An Advanced Culture Method for Generating Large Quantities of Highly Pure Dendritic Cells from Mouse Bone Marrow. *J. Immunol. Methods* 223 (1), 77–92. doi:10.1016/s0022-1759(98)00204-x
- Maji, S., Yan, I. K., Parasramka, M., Mohankumar, S., Matsuda, A., and Patel, T. (2017). *In Vitro* toxicology Studies of Extracellular Vesicles. *J. Appl. Toxicol.* 37 (3), 310–318. doi:10.1002/jat.3362
- Martinez-Gil, L., Goff, P. H., Hai, R., Garcia-Sastre, A., Shaw, M. L., and Palese, P. (2013). A Sendai Virus-Derived RNA Agonist of RIG-I as a Virus Vaccine Adjuvant. *J. Virol.* 87 (3), 1290–1300. doi:10.1128/jvi.02338-12
- McFayden, I., Walker, G., and Alvarez, J. (2005). Enhancing hit quality and diversity within assay throughput constraints. *Chemoinformatics in Drug Discovery*, 141–17.
- Mendt, M., Kamerkar, S., Sugimoto, H., McAndrews, K. M., Wu, C. C., Gagea, M., et al. (2018). Generation and Testing of Clinical-Grade Exosomes for Pancreatic Cancer. *JCI Insight* 3 (8), e99263. doi:10.1172/jci.insight.99263
- Morse, M. A., Garst, J., Osada, T., Khan, S., Hobeika, A., Clay, T. M., et al. (2005). A Phase I Study of Dexosome Immunotherapy in Patients with Advanced Non-Small Cell Lung Cancer. *J. Transl. Med.* 3 (1), 9. doi:10.1186/1479-5876-3-9
- Nanishi, E., Dowling, D. J., and Levy, O. (2020). Toward Precision Adjuvants: Optimizing Science and Safety. *Curr. Opin. Pediatr.* 32 (1), 125–138. doi:10.1097/mop.0000000000000868
- Petrovsky, N. (2015). Comparative Safety of Vaccine Adjuvants: A Summary of Current Evidence and Future Needs. *Drug Saf.* 38 (11), 1059–1074. doi:10.1007/s40264-015-0350-4
- Pu, M., Hayashi, T., Cottam, H., Mulvaney, J., Arkin, M., Corr, M., et al. (2012). Analysis of High-Throughput Screening Assays Using Cluster Enrichment. *Stat. Med.* 31 (30), 4175–4189. doi:10.1002/sim.5455
- Reed, S. G., Orr, M. T., and Fox, C. B. (2013). Key Roles of Adjuvants in Modern Vaccines. *Nat. Med.* 19 (12), 1597–1608. doi:10.1038/nm.3409
- Saleh, A. F., Lázaro-Ibáñez, E., Forsgard, M. A., Shatnyeva, O., Ostekoitea, X., Karlsson, F., et al. (2019). Extracellular Vesicles Induce Minimal Hepatotoxicity and Immunogenicity. *Nanoscale* 11 (14), 6990–7001. doi:10.1039/c8nr08720b
- Sato-Kaneko, F., Yao, S., Lao, F. S., Nan, J., Shpigelman, J., Cheng, A., et al. (2021). Mitochondria-Dependent Synthetic Small-Molecule Vaccine Adjuvants for Influenza Virus Infection. *Proc. Natl. Acad. Sci. U S A.* 118 (23), e2025718118. doi:10.1073/pnas.2025718118
- Sato-Kaneko, F., Yao, S., Lao, F. S., Shpigelman, J., Messer, K., Pu, M., et al. (2020). A Novel Synthetic Dual Agonistic Liposomal TLR4/7 Adjuvant Promotes Broad Immune Responses in an Influenza Vaccine with Minimal Reactogenicity. *Front. Immunol.* 11, 1207. doi:10.3389/fimmu.2020.01207
- Schorey, J. S., Cheng, Y., Singh, P. P., and Smith, V. L. (2015). Exosomes and Other Extracellular Vesicles in Host-Pathogen Interactions. *EMBO Rep.* 16 (1), 24–43. doi:10.15252/embr.201439363
- Scrucca, L., Fop, M., Murphy, T. B., and Raftery, A. E. (2016). Mclust 5: Clustering, Classification and Density Estimation Using Gaussian Finite Mixture Models. *R. J.* 8 (1), 289–317. doi:10.32614/rj-2016-021
- Shpigelman, J., Lao, F. S., Yao, S., Li, C., Saito, T., Sato-Kaneko, F., et al. (2021). Generation and Application of a Reporter Cell Line for the Quantitative Screen of Extracellular Vesicle Release. *Front. Pharmacol.* 12, 668609. doi:10.3389/fphar.2021.668609
- Shukla, N. M., Arimoto, K. I., Yao, S., Fan, J. B., Zhang, Y., Sato-Kaneko, F., et al. (2018). Identification of Compounds that Prolong Type I Interferon Signaling as Potential Vaccine Adjuvants. *SLAS Discov.* 23 (9), 960–973. doi:10.1177/2472555218774308
- Skog, J., Würdinger, T., van Rijn, S., Meijer, D. H., Gainche, L., Sena-Estevés, M., et al. (2008). Glioblastoma Microvesicles Transport RNA and Proteins that Promote Tumour Growth and Provide Diagnostic Biomarkers. *Nat. Cell Biol.* 10 (12), 1470–1476. doi:10.1038/ncb1800
- Takahashi, Y., Nishikawa, M., Shinotsuka, H., Matsui, Y., Ohara, S., Imai, T., et al. (2013). Visualization and *In Vivo* Tracking of the Exosomes of Murine

- Melanoma B16-BL6 Cells in Mice after Intravenous Injection. *J. Biotechnol.* 165 (2), 77–84. doi:10.1016/j.jbiotec.2013.03.013
- Tregoning, J. S., Russell, R. F., and Kinnear, E. (2018). Adjuvanted Influenza Vaccines. *Hum. Vaccin. Immunother.* 14 (3), 550–564. doi:10.1080/21645515.2017.1415684
- Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J., and Lötvall, J. O. (2007). Exosome-Mediated Transfer of mRNAs and microRNAs Is a Novel Mechanism of Genetic Exchange between Cells. *Nat. Cell Biol.* 9 (6), 654–659. doi:10.1038/ncb1596
- Van Deun, J., Van Deun, J., Mestdag, P., Agostinis, P., Akay, Ö., Anand, S., et al. (2017). EV-TRACK: Transparent Reporting and Centralizing Knowledge in Extracellular Vesicle Research. *Nat. Methods* 14 (3), 228–232. doi:10.1038/nmeth.4185
- Wang, J., Sun, X., Zhao, J., Yang, Y., Cai, X., Xu, J., et al. (2017). Exosomes: A Novel Strategy for Treatment and Prevention of Diseases. *Front. Pharmacol.* 8, 300. doi:10.3389/fphar.2017.00300
- Wen, C., Seeger, R. C., Fabbri, M., Wang, L., Wayne, A. S., and Jong, A. Y. (2017). Biological Roles and Potential Applications of Immune Cell-Derived Extracellular Vesicles. *J. Extracell. Vesicles* 6 (1), 1400370. doi:10.1080/20013078.2017.1400370
- Wiklander, O. P., Nordin, J. Z., O'Loughlin, A., Gustafsson, Y., Corso, G., Mäger, I., et al. (2015). Extracellular Vesicle *In Vivo* Biodistribution Is Determined by Cell Source, Route of Administration and Targeting. *J. Extracell. Vesicles* 4, 26316. doi:10.3402/jev.v4.26316
- Yanez-Mo, M., Siljander, P. R., Andreu, Z., Zavec, A. B., Borràs, F. E., Buzas, E. I., et al. (2015). Biological Properties of Extracellular Vesicles and Their Physiological Functions. *J. Extracell. Vesicles* 4, 27066. doi:10.3402/jev.v4.27066
- Zhu, X., Badawi, M., Pomeroy, S., Sutaria, D. S., Xie, Z., Baek, A., et al. (2017). Comprehensive Toxicity and Immunogenicity Studies Reveal Minimal Effects in Mice Following Sustained Dosing of Extracellular Vesicles Derived from HEK293T Cells. *J. Extracell. Vesicles* 6 (1), 1324730. doi:10.1080/20013078.2017.1324730
- Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., Flament, C., Tenza, D., et al. (1998). Eradication of Established Murine Tumors Using a Novel Cell-Free Vaccine: Dendritic Cell-Derived Exosomes. *Nat. Med.* 4 (5), 594–600. doi:10.1038/nm0598-594

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Shukla, Sato-Kaneko, Yao, Pu, Chan, Lao, Sako, Saito, Messer, Hayashi, Cottam, Corr and Carson. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Differential Effects of APOE Genotype on MicroRNA Cargo of Cerebrospinal Fluid Extracellular Vesicles in Females With Alzheimer's Disease Compared to Males

Ursula S. Sandau¹, Trevor J. McFarland¹, Sierra J. Smith¹, Douglas R. Galasko², Joseph F. Quinn^{3,4,5} and Julie A. Saugstad^{1*}

¹Department of Anesthesiology and Perioperative Medicine, Oregon Health and Science University, Portland, OR, United States, ²Department of Neurosciences, University of California, San Diego, La Jolla, CA, United States, ³Department of Neurology, Oregon Health and Science University, Portland, OR, United States, ⁴Parkinson Center and Movement Disorders Program, Oregon Health and Science University, Portland, OR, United States, ⁵Portland VAMC Parkinson's Disease Research, Education, and Clinical Center, Portland, OR, United States

OPEN ACCESS

Edited by:

Suman Dutta,
International Institute of Innovation and
Technology, India

Reviewed by:

Sowmya Yelamanchili,
University of Nebraska Medical
Center, United States
Tina Bilousova,
UCLA Health System, United States

*Correspondence:

Julie A. Saugstad
saugstad@ohsu.edu

Specialty section:

This article was submitted to
Membrane Traffic,
a section of the journal
Frontiers in Cell and Developmental
Biology

Received: 31 January 2022

Accepted: 25 March 2022

Published: 27 April 2022

Citation:

Sandau US, McFarland TJ, Smith SJ,
Galasko DR, Quinn JF and
Saugstad JA (2022) Differential Effects
of APOE Genotype on MicroRNA
Cargo of Cerebrospinal Fluid
Extracellular Vesicles in Females With
Alzheimer's Disease Compared
to Males.
Front. Cell Dev. Biol. 10:864022.
doi: 10.3389/fcell.2022.864022

Multiple biological factors, including age, sex, and genetics, influence Alzheimer's disease (AD) risk. Of the 6.2 million Americans living with Alzheimer's dementia in 2021, 3.8 million are women and 2.4 million are men. The strongest genetic risk factor for sporadic AD is apolipoprotein E-ε4 (APOE-ε4). Female APOE-ε4 carriers develop AD more frequently than age-matched males and have more brain atrophy and memory loss. Consequently, biomarkers that are sensitive to biological risk factors may improve AD diagnostics and may provide insight into underlying mechanistic changes that could drive disease progression. Here, we have assessed the effects of sex and APOE-ε4 on the miRNA cargo of cerebrospinal fluid (CSF) extracellular vesicles (EVs) in AD. We used ultrafiltration (UF) combined with size exclusion chromatography (SEC) to enrich CSF EVs (e.g., Flotillin+). CSF EVs were isolated from female and male AD or controls (CTLs) that were either APOE-ε3,4 or -ε3,3 positive (n = 7/group, 56 total). MiRNA expression levels were quantified using a custom TaqMan™ array that assayed 190 miRNAs previously found in CSF, including 25 miRNAs that we previously validated as candidate AD biomarkers. We identified changes in the EV miRNA cargo that were affected by both AD and sex. In total, four miRNAs (miR-16-5p, -331-3p, -409-3p, and -454-3p) were significantly increased in AD vs. CTL, independent of sex and APOE-ε4 status. Pathway analysis of the predicted gene targets of these four miRNAs with identified pathways was highly relevant to neurodegeneration (e.g., senescence and autophagy). There were also three miRNAs (miR-146b-5p, -150-5p, and -342-3p) that were significantly increased in females vs. males, independent of disease state and APOE-ε4 status. We then performed a statistical analysis to assess the effect of APOE genotype in AD within each sex and found that APOE-ε4 status affects different subsets of CSF EV miRNAs in females vs. males. Together, this study demonstrates the complexity of the biological factors associated with AD risk and the impact on EV miRNAs, which may contribute to AD pathophysiology.

Keywords: Alzheimer's disease, cerebrospinal fluid (CSF), extracellular vesicle (EV), microRNA, apolipoprotein E allele (APOE), sex difference

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia, the sixth leading cause of death in the United States, and the fifth leading cause of death for those age 65 and older (Alzheimer's Association, 2021). In 2021, an estimated 6.2 million Americans age 65 and older are living with Alzheimer's dementia, a number that is projected to reach 13.8 million by 2060 (Alzheimer's Association, 2021). The costs of health- and long-term care for individuals with Alzheimer's dementias are substantial: total payments in 2021 for all individuals with Alzheimer's or other dementias are estimated at \$355 billion, not including value for informal caregiving, and these costs will increase by \$1 billion each year (Alzheimer's Association, 2021). As AD is a global disease, these projections make dementia one of the costliest conditions to society (Alzheimer's Association, 2021).

There are multiple biological factors, including age, sex, and genetics, that influence AD risk (Alzheimer's Association, 2021). Of the 6.2 million Americans living with Alzheimer's dementia, 3.8 million are women and 2.4 million are men (Rajan et al., 2021). Thus, more women than men have Alzheimer's or other dementias, and almost two-thirds of Americans with AD are women (Rajan et al., 2021). The most important genetic risk factor for sporadic AD is apolipoprotein E (APOE), a major lipoprotein in the central nervous system (CNS) that is associated with triglyceride-rich lipoproteins and mediates the clearance of these lipoproteins from the plasma (Corder et al., 1993; Levy and Levy, 2021). Of the three major APOE gene alleles (e2, e3, and e4), the APOE-e4 allele is the strongest risk factor for AD. Importantly, while the APOE-e2 allele is relatively rare and may provide some protection against AD (Reiman et al., 2020), people who inherit one copy of the APOE-e4 allele have an increased chance of developing the disease; those who inherit two copies of the allele are at even greater risk (Sando et al., 2008; Reiman et al., 2020). In addition, female APOE-e4 carriers are more likely to progress from mild cognitive impairment (MCI) to AD, have more brain atrophy and memory loss, and develop AD more frequently than age-matched males (Fleisher et al., 2008; Altmann et al., 2014; Sampedro et al., 2015). Consequently, biomarkers that are sensitive to biological risk factors may improve diagnostics and provide insight to underlying mechanistic changes that could drive AD progression.

In our prior studies, we focused on the utility of extracellular miRNAs as biomarkers for AD in total CSF that was not fractionated to separate and enrich for EVs. We initially discovered a set of 36 miRNAs in CSF from living donors that could classify AD patients from healthy controls (CTLs) (Lusardi et al., 2017). Our validation study in CSF from a new and independent cohort of AD patients and CTLs showed that 25 of the 36 biomarker candidates serve as classifiers for AD (Wiedrick et al., 2019). We then assessed whether any of the validated CSF miRNAs are sensitive to early-stage pathology as exemplified by MCI diagnosis. We observed that five miRNAs showed a linear trend of decreasing median expression across the ordered diagnoses (CTL to MCI to AD) (Sandau et al., 2020b). Importantly, three of these five trending miRNAs (miR-142-3p, -146a-5p, and -146b-5p) have been identified as candidate

biomarkers for MCI and/or AD in total CSF in other studies (Cogswell et al., 2008; Alexandrov et al., 2012; Kiko et al., 2014; Denk et al., 2015; Nagaraj et al., 2017; Park et al., 2019).

Extracellular miRNAs in CSF have multiple carrier types, including extracellular vesicles (EVs), RNA-binding proteins, and high-density lipoproteins (Vickers and Remaley, 2012; Mori et al., 2019). EVs are membrane-bound spheres that carry complex cargos, including lipids, proteins, and nucleic acids (Colombo et al., 2013). The release of EVs is a universally conserved cellular process that occurs in all eukaryotes and prokaryotes and in every biofluid examined. EVs released from CNS cells contribute to cell-to-cell communication throughout the CNS and the periphery (Chivet et al., 2012; Dickens et al., 2017; Zhang and Yang, 2018) in normal and pathological processes (Yuyama and Igarashi, 2016; Neven et al., 2017). Thus, there is interest in exploring the molecular cargo of EVs in biofluids as biomarkers for AD that may 1) aid in tracking disease progression including diagnosis during the prodromal stage, 2) differentiate AD patients from other neurodegenerative disorders that also secrete EVs, and 3) identify new therapeutic targets (Rastogi et al., 2021).

Cells produce three main types of EVs classified by their size and mode of release from cells (Raposo and Stoorvogel, 2013; Kim et al., 2017). Apoptotic bodies are ~500–2000 nm in diameter and are released *via* blebbing of the plasma membrane, while microvesicles (MVs) are ~150–1,000 nm and are released *via* budding of the plasma membrane. In contrast, exosomes measure ~40–150 nm and arise from the endosomal pathway, which forms intracellular multivesicular bodies (MVB) that are secreted as vesicles into the extracellular space. Two additional nanoparticle types, exomeres and supermeres, have also been recently identified in cultured cells (Zhang et al., 2018; Zhang et al., 2021). Importantly, to date all of the EV subtypes and small nanoparticles have distinct RNA profiles (Crescitelli et al., 2013; Mori et al., 2019; Zhang et al., 2021). Furthermore, EVs have protein cargo and surface marker profiles that are indicative of their respective biogenesis routes and cellular origin (e.g., neuron vs. astrocyte). Importantly, in AD, disruptions in the endolysosomal pathway affect exosome biogenesis, including the RNA and protein cargo, which can consequently be exploited as biomarkers (Mathews and Levy, 2019). While only four studies have profiled CSF EV miRNAs by either RNA sequencing or comprehensive qPCR arrays (Gui et al., 2015; Riancho et al., 2017; McKeever et al., 2018; Jain et al., 2019), the expression levels for two (miR-16-5p and -125b-5p) of our 25 validated biomarkers were altered in CSF EVs of AD participants, relative to CTLs (Gui et al., 2015; McKeever et al., 2018; Wiedrick et al., 2019). There is also evidence that APOE-e4 mediates disruptions in endolysosomal pathways and reduces brain exosome levels in aged, non-AD human brain (Peng et al., 2019). However, broad profiles of the types of EV and their cargos are yet to be defined in cerebrospinal fluid (CSF) from AD participants while also taking into account the APOE-e4 status and sex.

Here, we sought to establish the effect of AD on the miRNA cargo of CSF EVs and determine whether AD risk factors (sex and APOE genotype) also impact EV miRNA expression. We first established a protocol using living donor's CSF and a combined

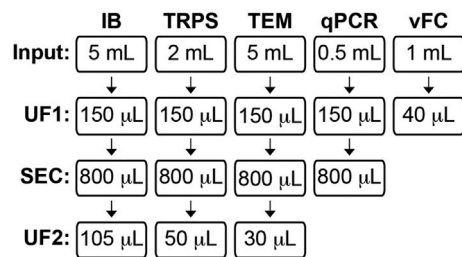


FIGURE 1 | Human CSF EV study workflow. Human CSF was assessed using five independent methods: immunoblot (IB), tunable resistive pulse sensing (TRPS), transmission electron microscopy (TEM), qPCR for miRNA analysis, and vesicle flow cytometry (vFC). The CSF volume input and subsequent processing step(s) prior to assaying are shown for each technique. CSF for IB, TRPS, and TEM was processed by ultrafiltration (UF1), followed by size exclusion chromatography (SEC) to collect an 800 µL pool of fractions (Fxs) 6–9, and post-SEC UF (UF2) to concentrate the pool. CSF for miRNA qPCR was processed by UF1 and then SEC, and RNA was isolated from an 800 µL pool of Fxs 6–9. CSF for vFC was processed by UF-only to concentrate the biofluid prior to being assayed.

approach of ultrafiltration (UF) plus size exclusion chromatography (SEC) to enrich EVs positive for canonical markers, such as Flotillin, TSG101, and CD81, while also depleting a majority of proteins and lipoproteins not associated with EVs. We then used UF plus SEC to isolate EVs from females and males that were either AD or CTLs and were either APOE-ε3,4 or APOE-ε3,3 positive. We quantified miRNA expression levels in the isolated EVs using a custom TaqMan™ qPCR array with probes for 190 miRNAs previously found in CSF, including the 25 miRNAs we validated as candidate biomarkers for AD (Wang et al., 2017; Wiedrick et al., 2019). We identified EV miRNAs whose expression levels were affected by both AD and sex of the participants. Furthermore, we found that APOE-ε4 status affects different subsets of CSF EV miRNAs in females vs. males. Thus, the miRNA cargo of CSF EVs is informative for neurological disorders and sensitive to both sex and genotype. Together, these studies demonstrate the complexity of the biological factors associated with AD risk, and their impact the EV cargo, which may play a mechanistic role in AD pathophysiology.

MATERIALS AND METHODS

Study Participants

For experiments that characterized CSF EVs by immunoblot, tunable resistive pulse sensing (TRPS), transmission electron microscopy (TEM), and single vesicle flow cytometry (vFC) (Figure 1), we pooled human CSF from neurologically normal male and female participants obtained from the Oregon Health & Science University (OHSU) Oregon Alzheimer's Disease Research Center (OADC), core program of the OHSU Layton Aging & Alzheimer's Disease Center or purchased from BioChemex (Winchester, VA). For CSF EV miRNA quantification by qPCR, we used individual human CSF samples from 28 CTL and 28 AD participants (Figure 1;

TABLE 1 | CSF participant characteristics. The table includes sex, age, and MMSE measures of the 56 participants in this study. The ratios of APOE genotype (ε3,3 or ε3,4) and sex was matched across all groups.

	CTL ε-3,3	CTL ε-3,4	AD ε-3,3	AD ε-3,4
SEX				
Female	7 (50%)	7 (50%)	7 (50%)	7 (50%)
Male	7 (50%)	7 (50%)	7 (50%)	7 (50%)
Total	14	14	14	14
AGE (years) ^a				
	Mean ± SD			
Female	71.0 ± 5.8	70.9 ± 3.7	73.4 ± 12.4	73.3 ± 5.1
Male	72.6 ± 8.5	73.3 ± 9.5	72.4 ± 7.9	73.1 ± 4.5
Total	71.8 ± 7.0	72.1 ± 7.0	72.9 ± 10.0	73.2 ± 4.6
MMSE ^a				
	Mean ± SD			
Female	29.6 ± 0.8	29.6 ± 0.8	21.9 ± 4.0	21.3 ± 2.5
Male	29.4 ± 0.8	28.6 ± 2.2	21.8 ± 3.6	21.1 ± 2.7
Total	29.5 ± 0.8	29.1 ± 1.7	21.8 ± 3.6	21.2 ± 2.2

^aAge and MMSE values represent data at the time of CSF collection.

Table 1) obtained from the OADC or from the University of California San Diego Shiley-Marcos Alzheimer's Disease Research Center (UCSD ADRC). All procedures were approved by the Institutional Review Boards of OHSU (IRB 6845) and UCSD (IRB 80012). All participants at both the institutions provided written informed consent and underwent detailed evaluations consisting of medical history, physical and neurological examinations, laboratory tests, and neuropsychological assessments, including cognitive tests and interview with a collateral historian. CTL participants were volunteers in good health with no symptoms of cognitive impairment or neurological disease and had normal performance on a detailed battery of neuropsychological tests. Sex, age, MMSE, and APOE genotype (ε3,3 or ε3,4) status for all 56 participants in this study are shown in Table 1.

CSF Collection

The OADC and USCD ADRC both used a standardized CSF collection protocol corresponding to that of other AD centers engaged in biomarker research (Shi et al., 2011). Lumbar punctures were done in the morning under fasting conditions in the lateral decubitus position with a 24-gauge Sprotte spinal needle. The OADC sends the first 3–5 ml of CSF collected to the clinical lab for cell counts and determination of glucose and total protein levels, and the UCSD ADRC sends the first 2 ml of CSF collected to the clinical lab for testing. At both sites, serial syringes with 5 ml of CSF were collected, mixed, and transferred to polypropylene tubes in 0.5-ml aliquots, and the tubes were numbered to account for any gradient effect in subsequent experiments. The tubes were labeled with a subject number, but no other identifying information was mentioned. The CSF aliquots were flash frozen on dry ice and stored at –80°C. All CSF samples were only thawed once on ice prior to use.

APOE-e4 Genotyping

APOE-e4 genotypes at OHSU were determined by sequencing the amplicon of APOE exon 4 (e4 allele). Genomic DNA was isolated from blood and amplified by Touchdown PCR with 250 μ M dNTPs, 1 unit Taq DNA polymerase, buffer, 1X Q-solution (Qiagen, Valencia, CA), and 0.5 μ M forward (5'-GGCGCTGATGGACGAGACC-3') and reverse (5'-CCTGGG CCGCTCCTGTAG-3') primers to amplify the APOE exon 4. A product size of 443 nucleotides identified on a 1% agarose 1X TBE gel was excised, cleaned with ExoSAP-IT reagent (Affymetrix), and sequenced on a model 377 automated fluorescence sequencer (Applied Biosystems). Chromatogram traces were examined, and nucleotide sequences were determined using FinchTV (Geospiza, Inc.). APOE-e4 genotypes at UCSD were determined by PCR amplification and restriction fragment length polymorphism (RFLP) (Hixson and Vernier, 1990), as previously described (Wierenga et al., 2012). Venous blood was drawn from participants and genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen) followed by PCR amplification. The APOE-e4 gene sequences were amplified using forward (5'-ACGCGGGCACGG CTGTCCAAGGA-3') and reverse (5'-GCGGGCCCCGGCCTG GTACAC-3') primers. The amplification products were digested with *HhaI* (restriction enzyme site GCGC) and then subjected to electrophoresis on polyacrylamide gels; the gels were stained with ethidium bromide, and the digested fragments were visualized by ultraviolet illumination. A unique combination of *HhaI* fragment sizes enabled typing of all homozygotic and heterozygotic combinations: *HhaI* cleaves at GCGC encoding 112arg (e4) and 158arg (e3, e4) but does not cut at GTGC-encoding 112cys (e2, e3) and 158cys (e2).

Single vFC of CSF

For single vFC studies, we examined total CSF that was not concentrated as well as 1.0 ml of CSF that was concentrated by UF using Microcon®-100 kDa Centrifugal Filters (MPE100025, Millipore Sigma, Burlington, MA) to a final volume of 40 μ L (25x). We analyzed the CSF by single vFC using fluorescence to estimate vesicle size, concentration, and surface cargo with a commercial flow cytometer (Beckman Coulter CytoFlex S, Brea, CA) and an assay kit (vFC™ EV Analysis kit, Cellarcus Biosciences, San Diego, CA). In brief, 5 μ L each of the unconcentrated and 25x concentrated CSF were stained according to the manufactures recommendations with a membrane stain (vFRed) and pool of fluorescence-labeled antibodies against tetraspanins (TS PE mix: CD9, CD63, and CD81) for 1 h at room temperature. The stained samples were diluted 1:1,000 prior to reading on the flow cytometer and detected using fluorescence triggering (excitation: 488 nm; emission: 690/50 nm). To demonstrate the vesicular nature of the vFRed and/or TS-positive (TS+) events, an aliquot of the stained, concentrated 25x CSF was detergent treated (0.05% Triton X-100) for 1 min prior to being assayed. Data analysis was performed using FCS Express (Version 7, De Novo Software, Pasadena, CA). Events were gated with respect to time (to eliminate spurious background that occurs at the start of each sample), vFRed pulse shape (to eliminate short pulse width

background events), and violet side scatter (VSSC) vs. vFRed fluorescence (to include events with characteristic membrane fluorescence and light scatter) (**Supplementary Figure S4**). NanoRainbow beads (Cellarcus Biosciences) were used to characterize critical performance metrics that enable the evaluation of laser alignment and fluorescence resolution (**Supplementary Figure S1**). Membrane fluorescence was calibrated in terms of vesicle size (surface area) using a synthetic vesicle size standard (Lipo100 beads, Cellarcus Biosciences), and diameter was calculated assuming a spherical shape, resulting in an acceptable size distribution, in accordance to Cellarcus Biosciences (**Supplementary Figure S3A**). Standardized preparations of platelet EVs were used as reference samples for surface marker immunofluorescence (**Supplementary Figure S3B**). Particle concentrations were determined by subtracting the background values of buffer control containing vFRed and TS PE Mix (without CSF) from the experimental sample values.

CSF EV Isolation by UF and SEC

EVs were isolated from CSF by a combined approach of UF and SEC for downstream use in immunoblots, TRPS, TEM, and miRNA qPCR arrays according to **Figure 1**. Depending on the downstream application, aliquots of 0.5, 2.0, or 5.0 ml of CSF were thawed on ice, pooled, and then concentrated by UF using a 0.5-ml Microcon®-30 kDa Centrifugal Filters (MRCF0R030, Millipore Sigma) and centrifugation at 14,000 $\times$ g for 15 min at 4°C. Concentrated samples were recovered by inverting the centrifugal filter into a clean collection tube and then centrifuged at 2,000 $\times$ g for 3 min at 4°C. The concentrated CSF was then brought to a final volume of 150 μ L with 0.22- μ m-filtered PBS. CSF EVs were isolated from the concentrated CSF by SEC using qEV single 35 nm or 70 nm columns (IZON Science, Christchurch, New Zealand), according to the manufacturer's recommendations. Prior to use the qEV columns were brought to room temperature and equilibrated with 0.22- μ m-filtered PBS. The void volume of the column consisting of fractions (Fxs) 1–5 (1 ml) and pools of subsequent Fxs 6–9, 10–13, and 14–17 (800 μ L/pool) were collected. For immunoblot, TRPS, and TEM, each pool was then UF using a 0.5-ml Microcon®-30 kDa Centrifugal Filter to a final volume of 105 μ L for immunoblot, 50 μ L for TRPS, and 30 μ L for TEM, as described before. For qPCR, the 800 μ L pools were directly processed for RNA isolation without a second UF. For immunoblot, TRPS, and qPCR, the pools were frozen at –80°C until use. For TEM, the pools were stored on wet ice or 4°C until processing within 6 h. All centrifugation steps in this subsection of the methods were conducted using the Microfuge 22R centrifuge equipped with an F241.5P fixed angle rotor (Beckman Coulter).

Immunoblot Evaluation of EV Markers in Pools of SEC Fxs

Protein concentrations were measured using the Qubit protein assay kit and Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, MA) for the concentrated UF-SEC pools using 5 μ L of

the void and Fxs 6–9, 1 μ L of Fxs 10–13, and 1 μ L of 1:10 diluted Fxs 14–17. The remaining volume of each pool was prepared for SDS-PAGE by diluting in 4x NuPAGE™ LDS sample buffer (Thermo Fisher Scientific). To assess the separation of EV associated proteins from vesicle-free proteins and lipoproteins, we performed a total protein stain and immunoblots for APOA1, APOE, and albumin by loading equal volumes (37 μ L) of the void, Fxs 6–9, 10–13, and 14–17 on a NuPAGE™ 4–12% Bis-Tris 1.5 mm x 10 well gels (Thermo Fisher Scientific). To identify the SEC Fxs that contained an enrichment of EV markers, equal concentrations (0.1 μ g) of void, Fxs 6–9, 10–13, 14–17, and neurologically normal human frontal cortex lysate (positive control) were run on 4–12% gels, as described above, and immunoblotted for CD9, CD63, CD81, flotillin, TSG101, annexin V (AnnV), synaptophysin (SYP), NCAM-1, GLAST, CD11b, and TMEM119 proteins. In addition, gels loaded with 1.0 μ g of void, Fxs 6–9, 10–13, 14–17, and human frontal cortex were immunoblotted for SYP and CD11b. Following electrophoresis, proteins were transferred to PVDF membranes and kept overnight at 4°C (30 V constant current). Membranes were incubated for 1 h with a blocking buffer (5% non-fat milk in TBS with 0.05% Tween 20 (TBST)) and then incubated with primary antibody diluted with TBST overnight at 4°C, and for 1 h with secondary antibody (1:10,000 TBST) at room temperature. Membranes were developed using the West Dura, Pico, or Femto chemiluminescence kits (Thermo Fisher Scientific) and imaged using the ChemiDoc Touch imaging system (Bio-Rad, Hercules, CA). The following antibodies were used for immunoblotting: albumin 1:1,000 (#4929, Cell Signaling Technology, Danvers, MA), APOA1 (12C8) 1:200 (sc-080551, Santa Cruz Biotechnology, Dallas, TX), APOE 1:2000 (50A-G1A, Academy Bio-medical Company, Inc., Houston, TX), AnnV 1:5,000 (GTX103250, GeneTex, Irvine, CA), CD9 (C-4) 1:200 (sc-13118, Santa Cruz Biotechnology), CD11b 1:1,000 (ab133357, Abcam, Cambridge, United Kingdom), CD63 1:1,000 (ab134045, Abcam), CD81 (B-11) 1:100 (sc-166029, Santa Cruz Biotechnology), flotillin 1:10,000 (ab133497, Abcam), GLAST 1:500 (NB100-1869, Novus Biologicals, Littleton, CO), NCAM-1 1:125 (NBP2-38452, Novus Biologicals), SYP 1:1,000 (#36406, Cell Signaling Technology), TMEM119 (#66948-1-Ig, Proteintech, Rosemont, IL), and TSG101 1:1,000 (ab125011, Abcam). Secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA) and included horseradish peroxidase (HRP)-conjugated donkey anti-mouse (#715-035-150), donkey anti-rabbit (#711-035-152), and donkey anti-goat (#705-035-003).

TRPS of SEC Pools

Pools of the void volume (Fxs 1–5) and Fxs 6–9 were analyzed for particle size and concentration by TRPS using a qNANO gold instrument fitted with a NP80 nanopore (IZON Science). Prior to TRPS, 800 μ L SEC pools were subjected to UF using Microcon®-30 kDa centrifugal filters and then brought to a final volume of 50 μ L in 2x PBS (**Figure 1**). TRPS measurements were recorded with the NP80 stretched to 47.50 mm and using 0.34 V at a pressure reading of 5 (mbar). Each sample was analyzed until at least 500 particles were recorded or until the maximum recording

time of 10 min elapsed. Data were acquired and analyzed using the IZON Control Suite version 3.4 software (IZON Science). The size (nm) and concentration (particles/mL) of particles measured in the SEC pools were calibrated against CPC100 calibration beads (IZON Science).

TEM of SEC Pools

Pools of CSF SEC fractions including the void, Fxs 6–9, 10–13, and 14–17 were concentrated by UF to a final volume of 30 μ L and then stored on wet ice until processing for TEM within 6 h (**Figure 1**) by the OHSU Multiscale Microscopy Core. A volume of 5 μ L of the SEC preparations were deposited on glow-discharged (120 s 15 mAmp, negative mode) carbon formvar 400 mesh copper grids (01822-F, Ted Pella, Inc., Redding, CA) for 3 min, rinsed 15 s in water, wicked on Whatman filter paper 1, stained for 60 s in filtered 1.33% (w/v) uranyl acetate in water, wicked again, and air dried. Samples were imaged by using the Multiscale Microscopy Core at 120 kV on a FEI Tecnai™ Spirit TEM system (Thermo Fisher Scientific Electron Microscopy, Hillsboro, OR). Images were acquired as 2048 × 2048 pixel, 16-bit gray scale files using the FEI's TEM Imaging & Analysis interface on an Eagle™ 2K CCD multiscan camera.

CSF EV RNA Isolation and miRNA Arrays

EVs were isolated from 500 μ L of CSF that was first concentrated by UF to 150 μ L then fractionated by SEC (**Figure 1**). Fxs 6–9, 10–13, and 14–17 were pooled to final volume of 800 μ L, and total RNA was isolated using the mirVana™ PARIS™ RNA and a Native Protein Purification Kit (AM1556, Thermo Fisher Scientific), with modification (Burgos et al., 2014), as we have previously reported for CSF and plasma EVs (Lusardi et al., 2017; Wiedrick et al., 2019; Sandau et al., 2020a; Sandau et al., 2020b; Lusardi et al., 2021). Isolated RNA was then stored at –80°C until use. For qPCR, the RNA samples were concentrated (R1013, RNA Clean and Concentrator™-5 Kit, Zymo Research, Irvine, CA) and then eluted into 6 μ L of RNase/DNase-free water. Total RNA was assayed on Custom TaqMan™ Advanced Array Cards (192a Format) that include probes for 191 human CSF miRNAs, plus five endogenous non-changing miRNA controls used for normalization: miR-191-5p, -204-3p, -204-5p, -342-3p, and -574-3p (**Supplementary Table S1**). Using a pool of Custom RT Primers for the CSF array (Thermo Fisher Scientific) and MultiScribe™ Reverse Transcriptase (4, 311, 235, Thermo Fisher Scientific), 3.2 μ L of concentrated RNA was reverse transcribed in a total reaction volume of 7.5 μ L. A volume of 5 μ L of cDNA was pre-amplified (PreAmp) for 14 cycles using Custom PreAmp Primers for the CSF array (Thermo Fisher Scientific) and TaqMan™ PreAmp Master Mix (4, 391, 128, Thermo Fisher Scientific) in a final reaction volume of 25 μ L. Reverse transcription and PreAmp reactions were run with a Bio-Rad T100 thermal cycler (Thermo Fisher Scientific), following the manufacturer's instructions, for detection of miRNAs with PreAmp. All cDNA was stored at –20°C. Prior to qPCR the PreAmp cDNA was diluted to 1:2 in RNase/DNase-free water and 18 μ L was mixed with TaqMan™ Universal Master Mix II, no UNG (444,047, Thermo Fisher Scientific). The samples were loaded on the custom CSF arrays, and the qPCR

amplifications and data acquisition were carried out on a QuantStudio™ 12K Flex real-time PCR system (Thermo Fisher Scientific).

CSF EV MiRNA Expression Analysis

MiRNAs were analyzed using relative quantification ($\Delta\Delta Cq$) based on Applied Biosystems recommendations (Thermo Fisher Scientific, Part Number 4371095 Rev B). Cq values were calculated using automatic baseline, and threshold values were determined by ExpressionSuite Software v.1.3 (Thermo Fisher Scientific). The Cq value for each well was reported along with the amplification score (AmpScore) and a Cq confidence (CqConf), which are metrics for the quality of each amplification, as we have previously reported (Lusardi et al., 2017; Wiedrick et al., 2019; Sandau et al., 2020a; Sandau et al., 2020b; Lusardi et al., 2021). Prior to data analysis, amplifications were filtered according to each well's Cq value, AmpScore, and CqConf: 1) PCR products with a $Cq > 34$, or reported as "Undetected", were considered below the detection threshold and assigned a Cq value of "34"; 2) amplifications with a $Cq \leq 34$ and an AmpScore < 1.0 or a CqConf < 0.8 were excluded from analysis. Based on these criteria, miRNAs with a $Cq \leq 34$, AmpScore > 1.0 , and CqConf > 0.8 were deemed considered for further analysis. Next, we excluded miRNAs that were not expressed in at least 20% of the samples (11 out of 56), which would enable analysis of miRNAs differentially detected in one out of the eight subgroups (e.g., APOE- $\epsilon 3,4$ females with AD). We also performed qPCR reactions with water only (no RNA—no template control) as a control for spurious PCR amplifications (Supplementary Table S2). MiRNAs that had good quality amplifications ($Cq \leq 34$, AmpScore > 1.0 , and CqConf > 0.8) in the water only control that were within 1 Cq of the average Cq for the 56 experimental samples were excluded from downstream analysis. For miRNAs that did meet all of the inclusion criteria, we then used the following formula for calculating the $\Delta\Delta Cq$ for each miRNA: $\Delta\Delta Cq = \text{mean } \Delta Cq \text{ of the test samples (e.g., AD)} - \text{mean } \Delta Cq \text{ of reference samples (e.g., CTL)}$. Within each sample, the ΔCq for a miRNA was calculated by the following formula: $\Delta Cq = \text{miRNA } Cq - \text{mean } Cq \text{ of endogenous control miRNAs}$. MiRNAs selected as endogenous control normalizers showed 1) stable good quality expression values in all samples regardless of experimental group and 2) best endogenous control scores identified in ExpressionSuite. For each miRNA, the fold change (RQ value) was calculated by the $2^{-\Delta\Delta Cq}$ method, with $RQ > 1$ indicating increased miRNA expression in either AD vs. CTL or females vs. males. Conversely, $RQ < 1$ indicates decreased expression in AD or females, compared to their respective reference group. The miRNA microarray data is MIAME compliant and submitted to the Gene Expression Omnibus site: ncbi.nlm.nih.gov/geo/.

CSF EV MiRNA Statistical Analysis

Data were analyzed with GraphPad Prism software v9.3.1 (GraphPad Software, Inc., San Diego, CA). Data are shown as either a Volcano plot ($-\log_{10}$ (q-value) vs. the $\log_2$ (Fold change)) or the ΔCq mean $\pm$ standard error mean (SEM). Where possible the biological replicates are displayed as

individual symbols. To assess the effects of AD on miRNA levels, participants were categorized as either AD ($n = 28$) or CTL ($n = 28$) and analyzed by Welch's unpaired t -test with a Benjamini–Hochberg false discovery rate (B-H FDR) of 0.20, a cutoff that is in line with a hypothesis-generating study, and the preference for some false positive findings in exchange for a larger list of potentially interesting true findings (Efron, 2010). To assess the effects of sex on miRNA levels, data generated from the participants were re-analyzed for females ($n = 28$) vs. males ($n = 28$) and analyzed by Welch's unpaired t -test with a B-H FDR of 0.20. To assess the effect of disease state and APOE status, data were analyzed within each sex using a two-way ANOVA (disease $\times$ APOE genotype) followed by a Tukey's multiple comparisons post hoc tests ($n = 7$ /group). Given the small number of participants in this study, a covariate analysis of disease by sex by APOE genotype was not conducted. Thus, conclusions drawn from the analysis assume no interaction between all three variables.

MiRNA Target Prediction and Pathway Analysis

We used TargetScan 7.2 (Agarwal et al., 2015) and miRDB (Liu and Wang, 2019; Chen and Wang, 2020) to predict targets of the four CSF EV miRNAs significantly associated with AD, as these programs are widely used and frequently updated. As pathway analysis is most effective for predictions generated from a limited gene set, predicted targets were excluded if they had a Cumulative Weighted Context Score (CWCS) > -0.3 in TargetScan or a target score of < 60 in miRDB. To determine whether to use a union, an intersection, or an individual target list in subsequent pathway analysis, we calculated sensitivity, specificity, and precision values using validated miRNA and target pairs from miRTarBase, an experimentally validated miRNA–target interaction database (Chou et al., 2018). The values in miRTarBase range from 0–1, with high-quality results closer to 1 (Fan and Kurgan, 2015; Oliveira et al., 2017). The union of TargetScan and miRDB showed the highest values of sensitivity, specificity, and precision with values of 0.35, 0.83, and 0.98, respectively. The use of a union between the two target prediction algorithm outputs increases the sensitivity of the targets predicted and the chance of predicting a novel target, as opposed to an intersection where only targets predicted by both algorithms are included in analysis (Fan and Kurgan, 2015; Oliveira et al., 2017). Pathway analysis was then performed on the union set of 2,319 unique targets using the ingenuity pathway analysis (IPA; QIAGEN Inc., qiagenbioinformatics.com/products/ingenuity-pathway-analysis). For this analysis, we excluded cancer-related tissues and cell lines to avoid knowledge bias toward cancer in IPA and applied a B-H FDR of 0.01.

MISEV 2018, MIFlowCyt-EV, and EV-TRACK

The isolation and characterization of CSF EVs followed recommendations from the Minimal Information of Studies

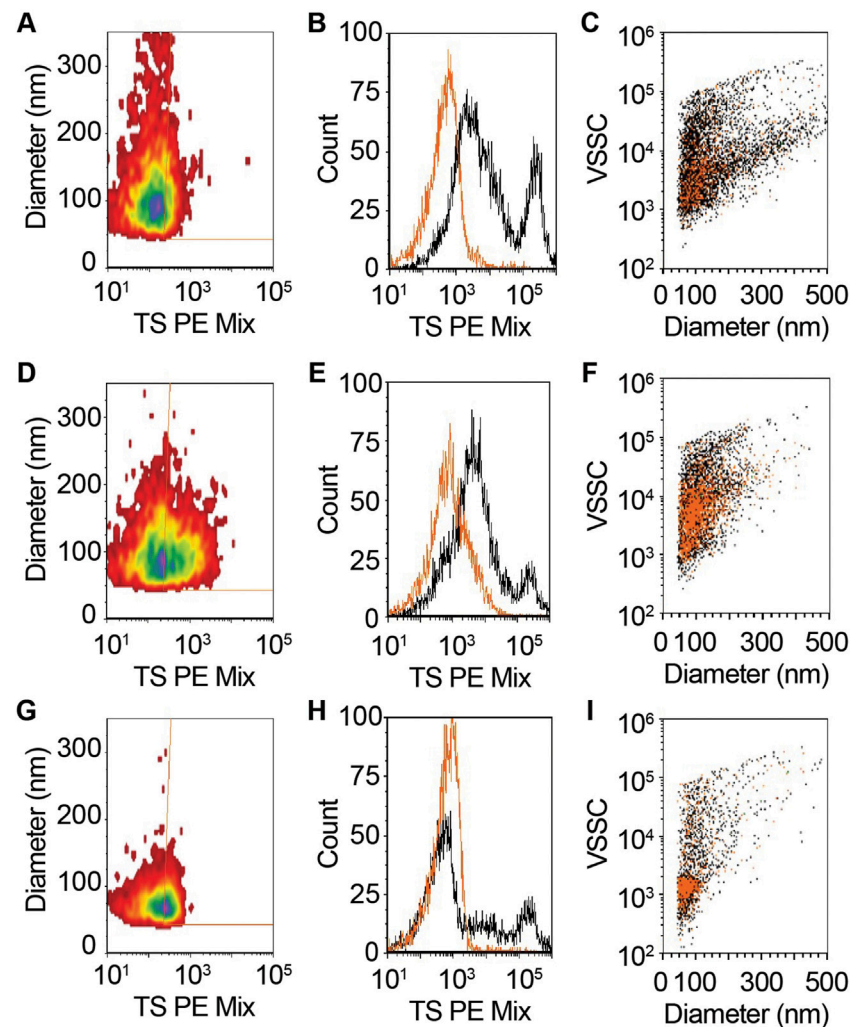


FIGURE 2 | CSF contains tetraspanin (CD9, CD63, and CD81)-positive EVs. **(A–C)** Unconcentrated and **(D–F)** 25x concentrated CSF was stained with vFRed, plus a pool of PE conjugated antibodies to CD9, CD63, and CD81 (TS PE mix). **(G–I)** Treatment of stained 25x CSF with 0.1% triton X100 demonstrated the vesicular nature of the measured positive events in panels **(D–F)**. **(A,D,G)** Diameter vs. fluorescence distributions of TS + EVs. **(B,E,H)** One parameter histogram overlays of CSF with (orange) and without (black) TS + staining. **(C,F,I)** Staining events backgated onto diameter vs. violet side scatter (VSSC) for TS PE. vFRed + events are black and TS + events are orange.

of Extracellular Vesicles 2018 (MISEV 2018), a position study from the International Society of Extracellular Vesicles (ISEV) (Théry et al., 2018). The vFC experiments followed recommendations from MIFlowCyt-EV, a framework for standardized reporting of EV flow cytometry experiments from ISEV (Welsh et al., 2020). Relevant experimental parameters can be accessed in the EV-TRACK (evtrack.org) knowledgebase (EV-Track et al., 2017).

RESULTS

Study Participant Characteristics

CTL CSF was pooled and used for vFC, immunoblot, TRPS, and TEM experiments, while individual CSF samples from CTL and AD participants were used for the miRNA qPCR

assays. Characteristics for the 28 AD and 28 CTL participants in this study (56 total participants) are shown in **Table 1**. The ratio of participants was 1:1 female to male overall, with equal numbers of females and males in the AD and CTL groups. Furthermore, the APOE-ε3,ε3 to APOE-ε3,ε4 ratio was 1:1 within each sex to obtain a total of eight groups (disease by sex by genotype) with $n = 7/\text{group}$. Participants were well matched for age at the time of lumbar puncture across all the groups with no significant differences. CTL participants were in good health with a mean MMSE (Folstein et al., 1983) score of 29.3 ± 1.3 . AD patients were diagnosed with probable AD according to ADRDA-NINDS criteria (McKhann et al., 1984; McKhann et al., 2011), with a mean MMSE score of 21.5 ± 3.1 . There was a significant effect of disease state on the MMSE score $F_{(1,48)} = 139.8, p < 0.0001$, but no significant interactions with disease state and sex and/or genotype.

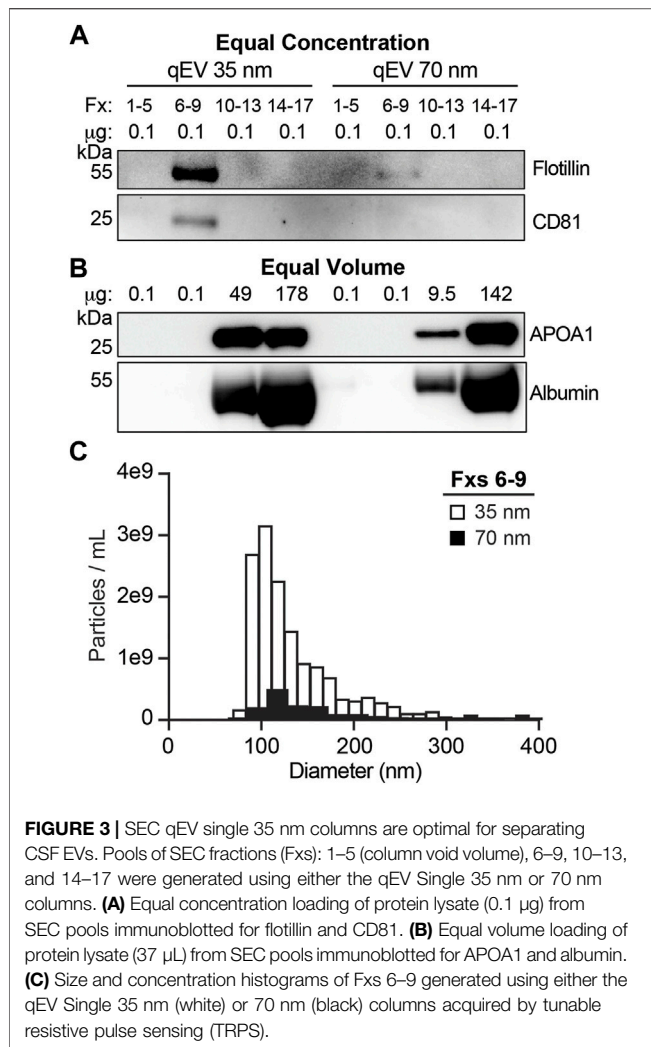


FIGURE 3 | SEC qEV single 35 nm columns are optimal for separating CSF EVs. Pools of SEC fractions (Fxs): 1–5 (column void volume), 6–9, 10–13, and 14–17 were generated using either the qEV Single 35 nm or 70 nm columns. **(A)** Equal concentration loading of protein lysate (0.1 μg) from SEC pools immunoblotted for flotillin and CD81. **(B)** Equal volume loading of protein lysate (37 μL) from SEC pools immunoblotted for APOA1 and albumin. **(C)** Size and concentration histograms of Fxs 6–9 generated using either the qEV Single 35 nm (white) or 70 nm (black) columns acquired by tunable resistive pulse sensing (TRPS).

CSF Contains a Low Concentration of Membrane-Associated TS + EVs

To measure the size and concentration of EVs in total CSF, we performed single vFC, which uses sensitive measurements of light scatter from a fluorescent lipid probe (vFRed) and surface immunofluorescence from staining with a pool of phycoerythrin (PE)-conjugated TS antibodies (CD9, CD63, and CD81; TS PE mix). In our prior studies on plasma, we diluted the sample 150-fold prior to staining to enable detection of TS + membranous particles in the diameter range of ~75–600 nm at mean concentration of $1.3 \times 10^{10}/\text{mL}$ (Sandau et al., 2020a). However, when we performed vFC with undiluted total CSF that was stained with vFRed and the TS PE Mix, we detected TS + membranous particles just above background levels with mean size of 136 nm (range: ~75–400 nm) and at a concentration of $1.7 \times 10^6/\text{mL}$ (Figures 2A–C). To increase counts for the CSF assay, we subsequently used UF to concentrate 1 ml of CSF to 40 μL prior to staining with vFRed and TS PE Mix (Figure 1). The 25x concentrated CSF yielded more robust results with $1.5 \times 10^7/\text{mL}$ TS + particles and

mean particle size of 115 nm (range: ~75–275 nm) (Figures 2D,E). Next, we detergent treated an aliquot of the stained, 25x concentrated CSF, which resulted in loss of >72% of gated events (Figures 2F,G), indicating that the majority of detected events were detergent-labile, as expected for EVs. Together, these data demonstrate that CSF contains a low concentration of TS + EVs compared to plasma.

Small Resin Pore Size is Optimal for CSF EV Isolation by SEC

To isolate EVs from human CSF, we first performed a series of SEC protocol refinement experiments. Using CSF pooled from multiple CTL samples, we tested 35 and 70 nm SEC columns for EV enrichment as indexed by the presence of established EV protein markers (Figure 3A) and depletion of vesicle-free lipoproteins and proteins (Figure 3B). To overcome the limitations of the low concentration of EVs in total CSF (Figure 2), we started with 5 ml of CSF that was concentrated by UF to 150 μL prior to fractionating the samples into SEC pools. We collected the void volume of the column (Fxs 1–5), Fxs 6–9, 10–13, and 14–17 (Figure 1). EVs are expected to elute in Fxs 6–9 based on the manufacturer's recommendations and our prior studies using human plasma (Sandau et al., 2020a). The SEC pools were subsequently concentrated by UF to 105 μL then assayed by immunoblot. Gels loaded by equal concentration of protein (0.1 μg) showed a strong enrichment of flotillin and CD81 in Fxs 6–9 when using the 35 nm columns. In comparison, trace amounts of flotillin were detected in Fxs 6–9 of the 70 nm columns and CD81 was undetectable (Figure 3A). Equal volume loading of concentrated SEC pools demonstrated that both the 35 and 70 nm columns effectively depleted albumin and APOA1 (vesicle free-protein and lipoprotein, respectively) from Fxs 6–9 (Figure 3B). We next used CSF fractionated by SEC on 35 and 70 nm columns to obtain size and concentration measurements by TRPS (Figure 1). The void volume for the 35 and 70 nm columns both had <10 particles of the expected size that were detected during the entirety of the maximum recoding time of 10 min (data not shown). Fxs 6–9 from the 35 nm column had 1.45×10^{10} particles/mL with a mean size of 138 nm (range: 76–620 nm), while the 70 nm column had 1.54×10^9 particles/mL with a mean size of 151 nm (range: 86–360 nm) (Figure 3C). The increased abundance of smaller-sized particles obtained in Fxs 6–9 of the 35 nm column is in agreement with the immunoblot results. Together, these results demonstrate that the 35 nm SEC column is optimal for CSF EV enrichment.

Isolated CSF EVs Have a Lipid Membrane and are Enriched for Markers of the Endolysosomal Pathway, MVs, and Astrocyte-Derived EVs

We further characterized CSF fractionated using the 35 nm SEC columns by TEM and immunoblots (Figure 1). Figure 4 shows representative wide and narrow field of view TEM images of the SEC pools. The images revealed that the void contained a small amount of diffuse material that ranges in size from 30–100 nm

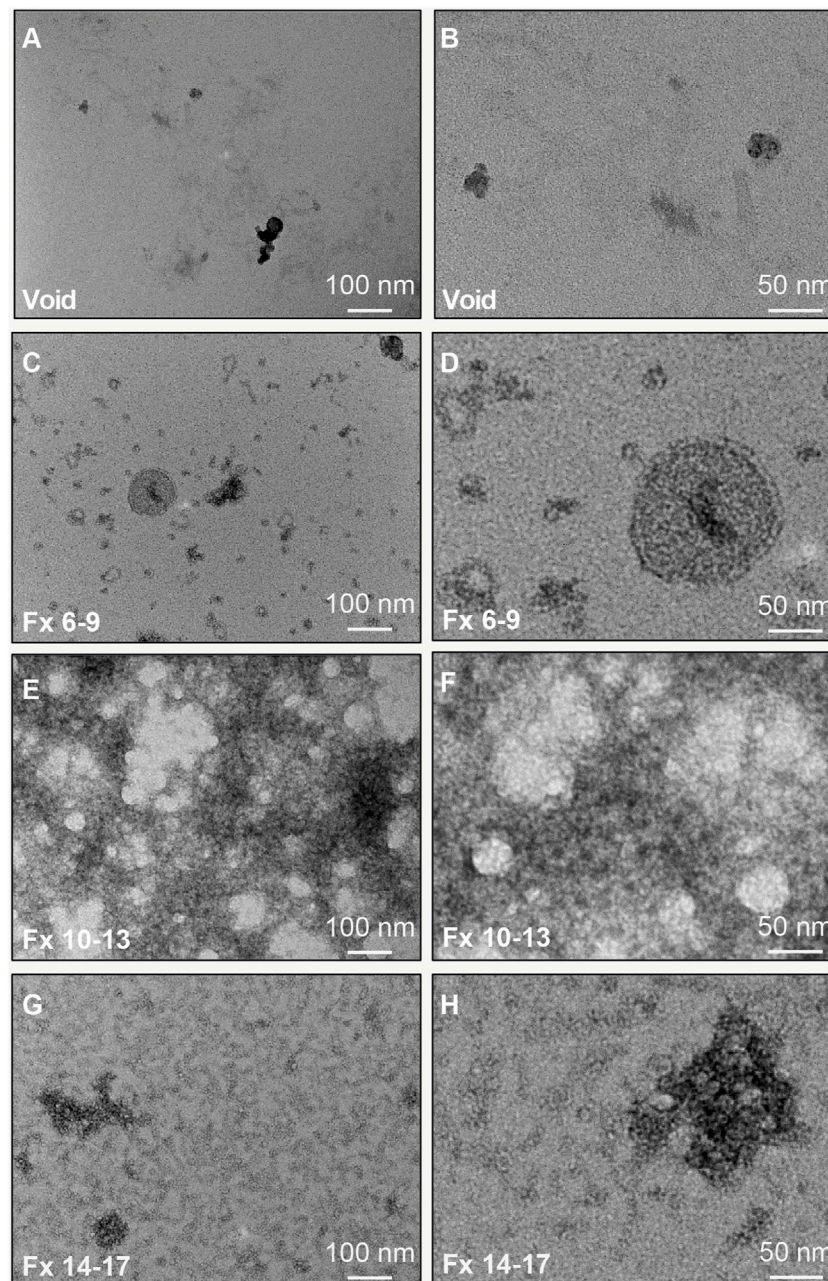


FIGURE 4 | TEM characterization of CSF EVs isolated by SEC. Representative TEM images of pools of SEC fractions (Fxs): 1–5 (**A,B**; column void volume), 6–9 (**C,D**), 10–13 (**E,F**), and 14–17 (**G,H**). Note: Fxs 6–9 show membrane bound vesicles at a size range of ~30–100 nm, with depletion of proteins and lipoproteins, which are present in Fxs 10–13 and 14–17. Panels (**A,C,E,G**) are wide field of view (scale bars = 100 nm) and panels (**B,D,F,H**) are close up views (scale bars = 50 nm).

(**Figures 4A,B**). In line with our TRPS results, EVs with a lipid membrane of the expected size range for exosomes (~40–150 nm) or small MVs (~150–1,000) were detectable in Fxs 6–9. Furthermore, there was a greater proportion of smaller nanoparticles (<40 nm), which are the expected size range for exomeres (~30–50 nm), supermeres (~25–30 nm), or vesicle-free lipoproteins (~5–35 nm) (Zhang et al., 2018; Zipkin, 2019; Zhang et al., 2021). Fxs 6–9 showed negligible levels of background protein, as expected for SEC isolates (**Figures 4C,D**). TEM

images from Fxs 10–13 and 14–17 showed minimal numbers of EVs discernible by the presence of lipid membrane. However, there was a high degree of background, consistent with increasing amounts of vesicle-free lipoproteins and proteins that elute later (**Figures 4E–H**). Next, we used total protein stain and immunoblots to show a depletion of bulk proteins, albumin, and APOA1 when SEC pools were loaded by equal volume (**Figure 5A**). We also found that while a majority of APOE was present in Fxs 10–13 and 14–17, APOE was also detectable in

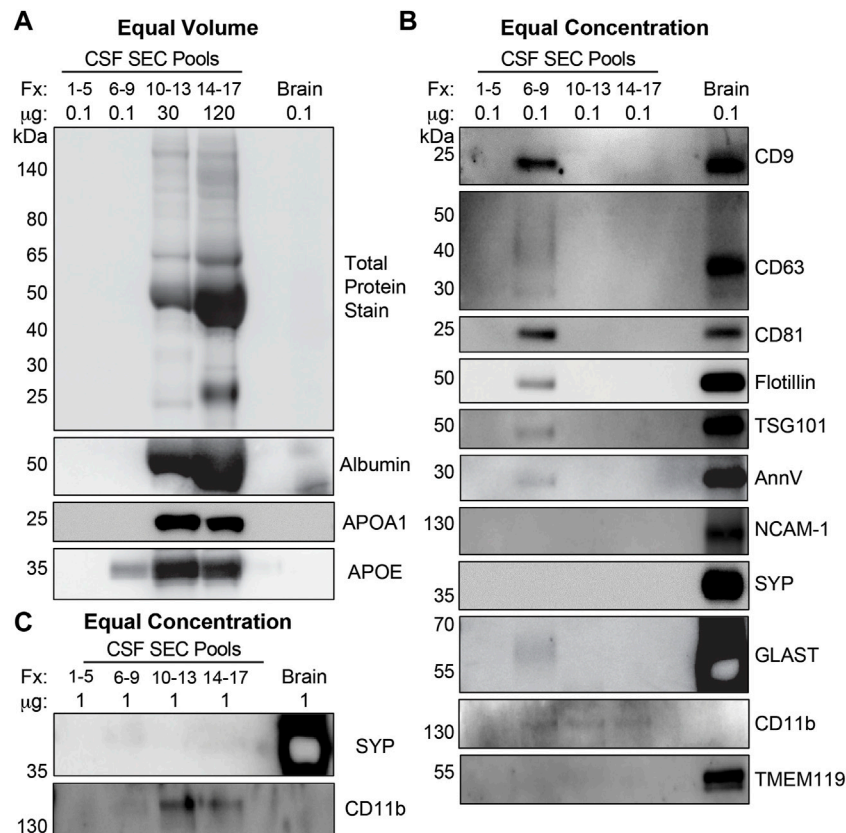


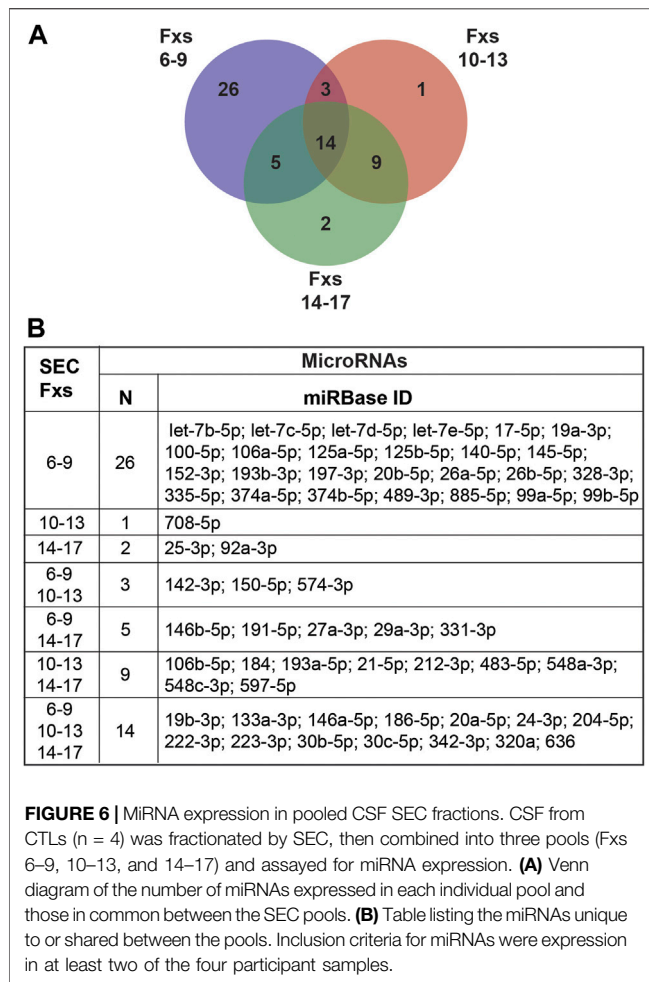
FIGURE 5 | CSF EVs isolated by SEC are enriched for exosome and MV markers. **(A)** Equal volume loading of protein lysate (37 μ L) from pools of SEC Fractions (Fxs): 1–5 (column void volume), 6–9, 10–13, and 14–17 stained for total protein, and immunoblotted for albumin, APOA1, and APOE. **(B)** Equal concentration loading of protein lysate (0.1 μ g) from pools of Fxs 1–5, 6–9, 10–13, and 14–17 immunoblotted for CD9, CD63, CD81, flotillin, TSG101, AnnV, SYP, NCAM-1, GLAST, CD11b, and TMEM119. **(C)** Equal concentration loading of (1 μ g) from pools of Fxs 1–5, 6–9, 10–13, and 14–17 immunoblotted for SYP and CD11b. Postmortem human cerebral cortex protein lysate (0.1 μ g and 1 μ g) was run as a positive control for each gel **(A–C)**: Brain).

the Fxs 6–9 (**Figure 5A**). Equal concentration loading of the SEC pools showed an enrichment of multiple EV protein markers that are associated with the endolysosomal pathway including CD9, CD63, CD81, flotillin, and TSG101 (**Figure 5B**) (Jeppesen et al., 2019). Furthermore, we identified an enrichment of AnnV (**Figure 5B**), which is associated with MVs and/or apoptotic bodies (Cocucci et al., 2009; Gouwens et al., 2018; Jeppesen et al., 2019). We next sought to determine if putative markers for brain-derived EVs were enriched in the CSF SEC pools. After loading 0.1 μ g of CSF SEC Fx protein lysate, we detected an enrichment of GLAST, which is a marker for astrocyte-derived EVs (**Figure 5B**) (You et al., 2020). However, we were unable to detect NCAM-1 and SYP, which are markers for neuronal-derived EVs; as well as TMEM119, which is a microglia-derived EV marker (**Figure 5B**) (Togashi et al., 2009; Satoh et al., 2016; Sathe et al., 2019; Vukojevic et al., 2020; Cohn et al., 2021; Crews et al., 2021; Ruan et al., 2021; You et al., 2022). We also performed an immunoblot assay using 0.1 μ g protein for CD11b, which is another marker for microglia-derived EVs (Gu et al., 2021), and detected faint bands in Fxs 6–9, 10–13, and 14–17 (**Figure 5B**). To further assess whether low concentrations of neuron- and/or microglia-derived EVs are present in CSF, we performed additional immunoblots with 1.0 μ g of protein and probed for SYP and CD11b

(**Figure 5C**). Despite a 10x increase in the amount of protein loaded, we were still unable to detect SYP in any of the CSF Fxs (**Figure 5C**). However, we did detect CD11b in Fxs 10–13 and 14–17, but just a faint CD11b band in Fxs 6–9 (**Figure 5C**). Together, these data support that using a combination of UF and SEC enriches for CSF EVs that contain markers for glial-derived EVs. However, we cannot definitively state that Fxs 6–9 are void of vesicle free lipoproteins and/or proteins based on the presence of APOE in these Fxs (**Figure 5A**).

SEC Fractions Enriched for CSF EVs Contain the Greatest Number of miRNAs

Considering that extracellular miRNAs are associated with exosomes, MVs, apoptotic bodies, RNA binding proteins, and high-density lipoproteins (Lasser, 2019; Mori et al., 2019), we sought to assess the miRNA profile of CSF fractionated by SEC. We concentrated 500 μ L of CSF ($n = 4$) to 150 μ L by UF; performed SEC to collect pools of Fxs 6–9, 10–13, and 14–17; and assayed miRNAs previously shown to be expressed in CSF and miRNAs validated as candidate biomarkers for AD (Wang et al., 2017; Wiedrick et al., 2019). The experiment was performed with CTL



CSF balanced for sex and APOE genotype (n = 4): female APOE-e3,3, female APOE-e3,4, male APOE-e3,3, and male APOE-e3,4 (Table 1). MiRNAs were excluded from the analysis if they were not expressed in at least two of the four participants. In total, Fxs 6–9, which is enriched for CSF EVs, expressed the greatest number of miRNAs (48 total), compared to Fxs 10–13 (27 total) and 14–17 (30 total) (Figure 6). Furthermore, 26 of the 48 miRNAs that were expressed in the CSF EVs (Fxs 6–9) were not detectable in the other SEC pools, while only a few miRNAs were exclusively expressed in Fxs 10–13 and 14–17. Of the 48 miRNAs expressed in Fxs 6–9, 12 were our candidate AD biomarkers (miR-125-5p, -140-5p, -142-3p, -145-5p, -146a-5p, -146b-5p, -19b-3p, -223-3p, -24-3p, -29a-3p, -328-3p, and -331-3p) (Wiedrick et al., 2019). Together, these data demonstrate that a majority of the miRNAs expressed in CSF, including AD biomarkers, are detectable in SEC fractions enriched for EVs.

MiRNA Cargo in AD CSF EVs is Altered and Predicts Gene Targets Relevant to AD Pathophysiology

Next, we assessed the impact of AD on the miRNA cargo of CSF EVs. MiRNAs were assayed in CSF Fxs 6–9 from eight biological

groups that included AD vs. CTL, female vs. male, and APOE-e3,3 vs. APOE-e3,4 participants (Table 1, n = 56 total). We identified 71 miRNAs expressed in the AD (n = 28) and/or CTL (n = 28) participant samples (Figure 7A). Of these four miRNAs were (miR-16-5p, -331-3p, -409-3p, and -454-3p) significantly increased by at least a 1.5-fold in AD relative to CTL (Figures 7A–E and Supplementary Table S3), two of which we previously identified and validated as candidate AD biomarkers (miR-16-5p and -331-3p) (Wiedrick et al., 2019). There were also four miRNAs (let-7d-5p, miR-100-5p, -374a-3p, and -378e) that increased by at least a 1.5-fold change in expression with $p < 0.05$ but were not considered significant based on the FDR (Figure 7A, pink). Next, we used the four significant miRNAs (miR-16-5p, -331-3p, -409-3p, and -454-3p) in our established target prediction pipeline using TargetScan 7.2 (Agarwal et al., 2015) and miRDB (Liu and Wang, 2019; Chen and Wang, 2020) as depicted in Figure 7F. Target prediction returned a total of 272 mRNAs by TargetScan (Supplementary Table S4) and 2,245 mRNAs by miRDB (Supplementary Table S5). Of these, 198 targets were predicted by both TargetScan and miRDB. Thus, the total number of unique predicted mRNA targets is 2,319, which were used in IPA (Qiagen) to identify pathways that have a significant overrepresentation of the predicted targets and potentially regulated by the miRNAs. IPA identified 189 significant canonical pathways (Supplementary Table S6). Of these, 10 of the top 17 pathways are known to be relevant to AD, including the top three overall pathways: senescence, TGF- β signaling, and epithelial adherens junction signaling (Figure 7G) (Caraci et al., 2018; Pearson et al., 2020; Saez-Atienzar and Masliah, 2020).

CSF EV MiRNA Cargo is Affected by Sex and APOE Genotype

Considering that female APOE-e4 carriers are more likely to progress from MCI to AD, develop AD more frequently than age-matched males, and have more brain atrophy and memory loss (Fleisher et al., 2008; Altmann et al., 2014; Sampedro et al., 2015), we next sought to determine if these biological factors impact CSF EV miRNA cargo. First, we assessed the effect of sex on CSF EV miRNAs, independent of AD and APOE genotype. We identified 71 miRNAs expressed in female (n = 28) and/or male (n = 28) participant samples (Figure 8A). Of these, three miRNAs (miR-146b-5p, -150-5p, and -342-3p) were significantly increased by at least 1.5-fold in females relative to males (Figures 8A–D and Supplementary Table S3), and we had previously identified miR-146b-5p as a candidate AD biomarker (Wiedrick et al., 2019). There were also seven miRNAs (miR-19b-3p, -188-5p, -223-3p, -320c, -320d, -483-5p, and -92a-3p) that were decreased by at least a 1.5-fold in females relative to males and $p < 0.05$ but were not considered statistically significant based on the FDR (Figure 8A, light blue).

We next sought to determine if there was an interaction between AD and APOE-e4 status within a sex. First, in females, we analyzed the 71 CSF EV miRNAs for an effect of disease state and/or genotype status and identified 16 significant miRNAs (Supplementary Table S7). Of these, multiple comparisons testing identified five miRNAs with a significant group effect (Figure 9). Let-7d-5p was increased in AD APOE-e3,3 females vs. CTL APOE-e3/e4 females (Figure 9A).

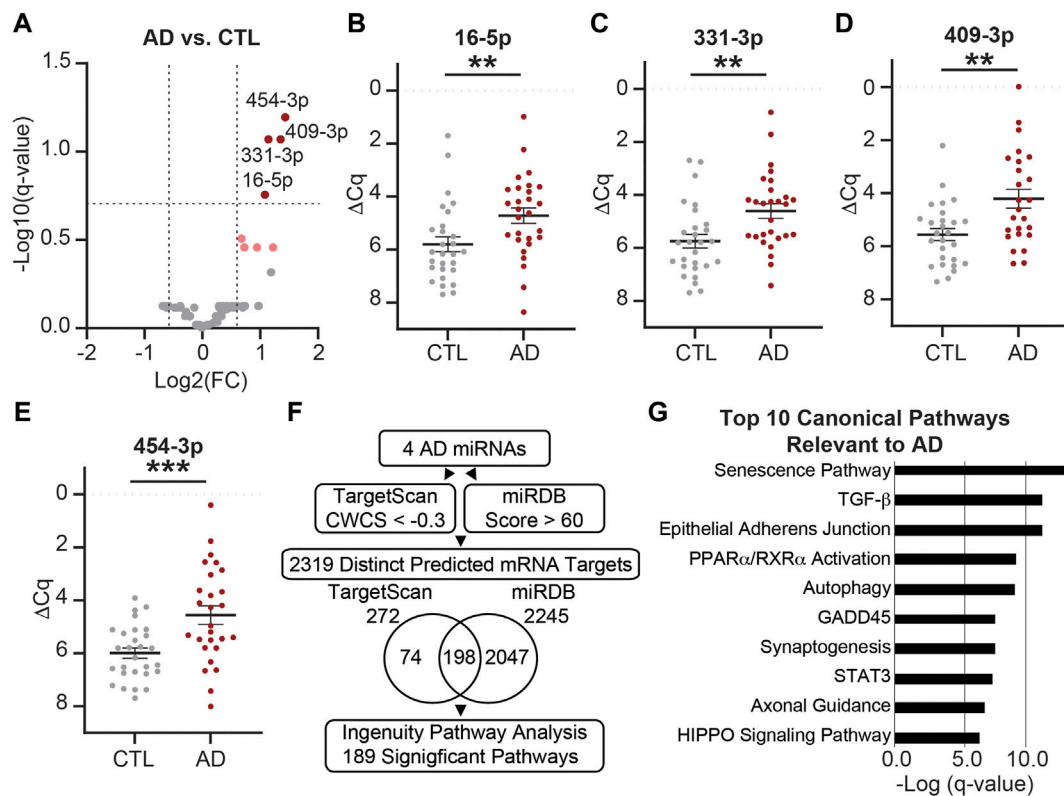


FIGURE 7 | MiRNAs are differentially expressed in AD vs. CTL CSF EVs. **(A)** Volcano plot shows fold change (FC) of 71 miRNAs expressed in AD CSF EVs ($n = 28$) vs. CTL CSF EVs ($n = 28$). The vertical dashed lines correspond to 1.5 FC, and the horizontal dashed line designates the cutoff for statistically significant miRNAs (Welch's unpaired t -test; p -value < 0.011 based on Benjamini–Hochberg false discovery rate (B-H FDR) 0.20). The miRNAs designated by dark red circles are significantly increased by at least 1.5 FC in AD. MiRNAs in pink have a 1.5 FC in expression with p -value < 0.05, but are below the B-H FDR threshold. **(B–E)** Normalized Cq (ΔCq) values for the four miRNAs that are significantly increased in AD vs. CTL. Data shown as the mean $\pm$ SEM and analyzed using Welch's unpaired t -tests, ** $p < 0.01$ and *** $p < 0.001$. **(F)** Workflow of the target prediction analysis for the four significant miRNAs: miR-16-5p, -331-3p, -409-3p, and -454-3p. MiRNAs were queried using TargetScan 7.2 with a CWCS of < -0.3 (272 MiRNAs total) and miRDB with a target score > 60 (2,245 MiRNAs total). The Venn diagram shows the overlap in miRNA targets predicted by TargetScan 7.2 and miRDB. Of the 2,319 predicted mRNA targets (74 from TargetScan + 2047 from miRDB + 198 in common to both), we identified 189 significant pathways (B-H FDR 0.01). **(G)** Top 10 canonical pathways predicted by the mRNA targets, all of which are relevant to AD. Note all 10 pathways were in the top 17 of all 189 significant pathways, and senescence, TGF- β signaling, and epithelial adherens junction signaling pathways were the top three overall.

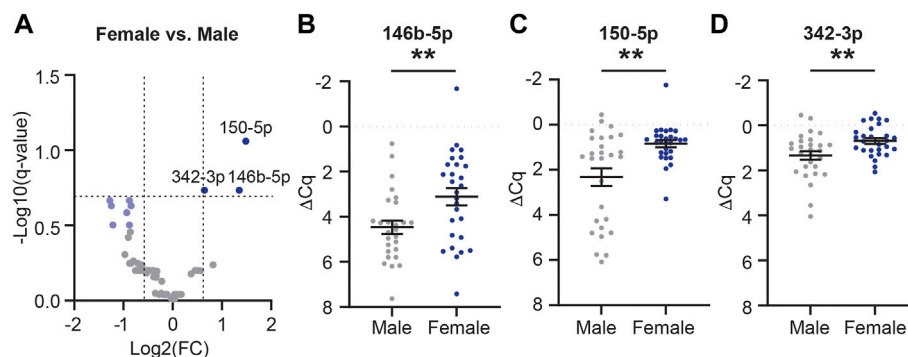


FIGURE 8 | MiRNAs are differentially expressed in female vs. male CSF EVs. **(A)** Volcano plot shows fold change (FC) of 71 miRNAs expressed in CSF EVs in females ($n = 28$) vs. males ($n = 28$). The vertical dashed lines correspond to 1.5 FC, and the horizontal dashed line designates the cutoff for statistically significant miRNAs (Welch's unpaired t -test; p -value < 0.011 based on Benjamini–Hochberg false discovery rate (B-H FDR) 0.20). The miRNAs designated by dark blue circles are significantly increased by at least 1.5 FC in females. MiRNAs in light blue have a 1.5 FC in expression with p -value < 0.05 but are below the B-H FDR threshold. **(B–D)** Normalized Cq (ΔCq) values for the three miRNAs that are significantly increased in females vs. males. Data shown as the mean $\pm$ SEM and analyzed using Welch's unpaired t -tests, ** $p < 0.01$ and *** $p < 0.001$.

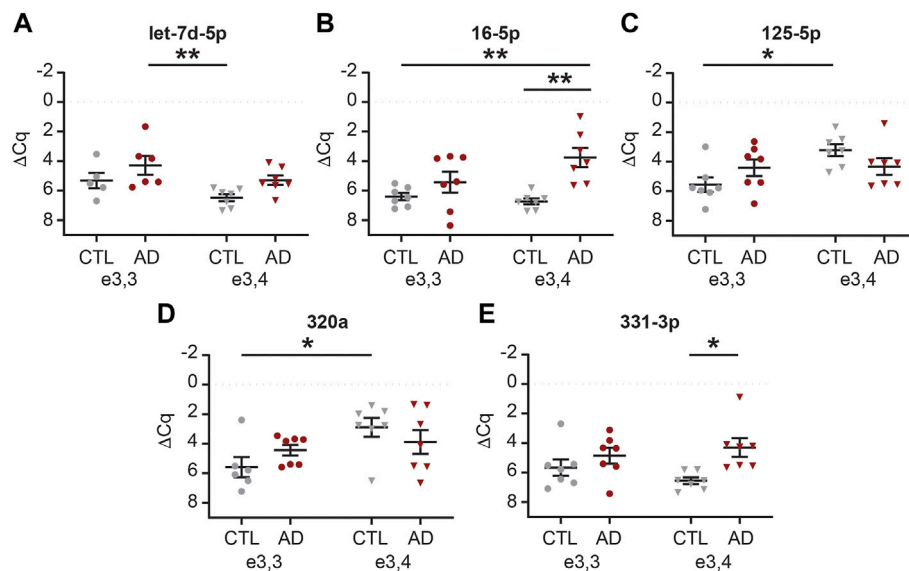


FIGURE 9 | MiRNAs are differentially expressed based on APOE status and disease state in female CSF EVs. Normalized Cq (ΔCq) values for five miRNAs that demonstrate a significant effect of APOE genotype on expression levels within AD and/or CTL female CSF EVs. **(A)** Let-7d-5p was significantly increased in AD APOE-e3,3 vs. CTL APOE-e3,4. **(B)** miR-16-5p was significantly increased in AD APOE-e3,4 vs. CTL APOE-e3,3 and -e3,4. **(C)** miR-125b-5p, and **(D)** miR-320a were significantly increased in CTL APOE-e3,4 vs. CTL APOE-e3,3. **(E)** miR-331-3p was significantly increased in AD APOE-e3,4 vs. CTL APOE-e3,4. Data shown as the mean $\pm$ SEM and analyzed by two-way ANOVA followed by Tukey's multiple comparisons post hoc tests, * $p < 0.05$ and ** $p < 0.01$.

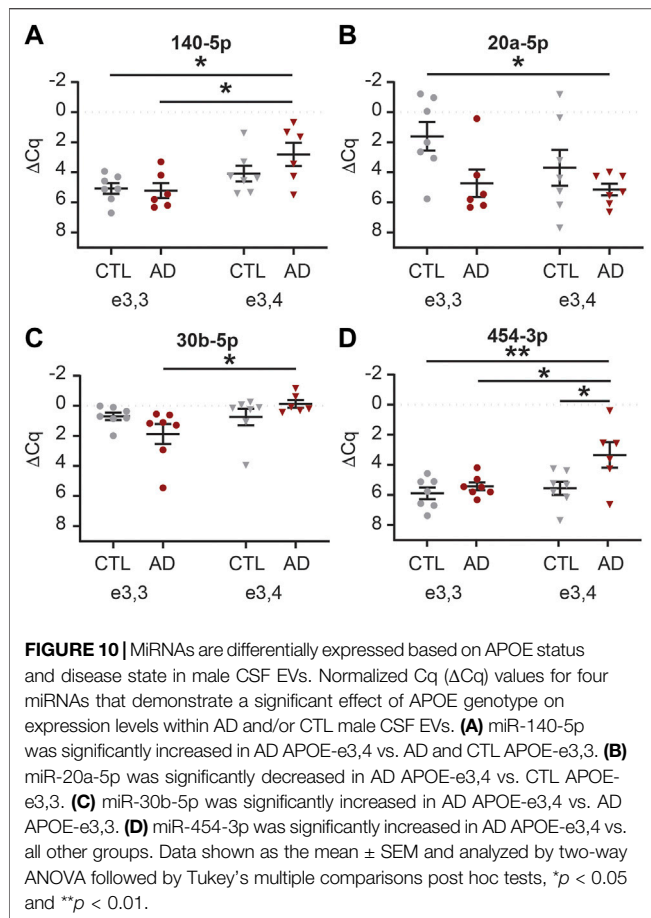
miR-16-5p was increased in AD APOE-e3,4 females vs. CTL APOE-e3,3 and -e3,4 females (**Figure 9B**). miR-125b-5p and -320a were both increased in CTL APOE-e3,4. CTL APOE-e3,3 (**Figures 9C,D**). miR-331-3p was increased in AD APOE-e3,4 vs. CTL APOE-e3,4 (**Figure 9E**). Of these five miRNAs, we previously identified three (miR-16-5p, -125b-5p, and -331-3p) as candidate AD biomarkers (Wiedrick et al., 2019). Next, our analysis of the 71 CSF EV miRNA levels in males identified five miRNAs that had a significant effect of disease state and/or genotype status (**Supplementary Table S7**). Of these, multiple comparisons testing identified four miRNAs with a significant change in expression (**Figure 10**). miR-140-5p was increased in AD APOE-e3,4 males vs. AD and CTL APOE-e3,3 males (**Figure 10A**). miR-20a-5p was decreased in AD APOE-e3,4 males vs. CTL APOE-e3,3 males (**Figure 10B**). miR-30b-5p was increased in AD APOE-e3,4 males vs. AD APOE-e3,3 males (**Figure 10C**). miR-454-3p was increased in AD APOE-e3,4 males vs. all other groups (**Figure 10D**). Of these four miRNAs, we previously identified miR-140-5p as a candidate AD biomarker (Wiedrick et al., 2019). Together, these data demonstrate that biological factors such as sex and genotype can impact the levels of miRNA cargo of CSF EVs. Note that due to the low sample size we were unable to perform a statistical analysis to assess the effect of AD by sex by APOE genotype.

DISCUSSION

The primary goal of this study was to determine the effect of AD and its biological risk factors on the levels of miRNA in human CSF EVs. We used a combined approach of UF plus SEC to isolate EVs from the CSF of AD and CTL participants that were balanced

for sex and APOE genotype (**Table 1**). In line with prior reports that demonstrated an effect of AD on the CSF EV miRNAs (Liu et al., 2014; Gui et al., 2015; Riancho et al., 2017; McKeever et al., 2018; Schneider et al., 2018; Jain et al., 2019; Kumar and Reddy, 2021; Tan et al., 2021), we identified that miR-16-5p, -331-3p, -409-3p, and -454-3p had a significant 1.5 fold increase in expression in AD vs. CTL (**Figure 7**). Next, our pathway analysis of these four miRNAs altered in AD CSF EVs revealed that the top canonical pathways included senescence and autophagy, which are affected by EVs in AD (Misawa et al., 2020; Vassileff et al., 2020). We then investigated the effect of sex and APOE genotype status on CSF EV miRNAs and found an increase in miR-146b-5p, -150-5p, and -342-3p in females relative to males (**Figure 8**). We also found that APOE genotype status affects different subsets of CSF EV miRNAs in females vs. males (**Figures 9, 10**). Together, our data demonstrate that the miRNA cargo of CSF EVs is informative for AD and sensitive to both sex and APOE-e4 genotype. They also reveal that biological factors associated with AD risk impact the EV miRNA cargo and should be taken into consideration for mechanistic and biomarker studies.

Over the past decade, there has been great interest in exploring the utility of circulating RNAs, especially miRNAs, as biomarkers for human disease (Quinn et al., 2015). Through the Extracellular RNA Communication Consortium (ERCC), RNA biomarkers across a spectrum of biofluids and disease states have been identified including, but not limited to, glioblastoma, vascular inflammation and cardiometabolic health, and multiple sclerosis (Ainsztein et al., 2015; Regev et al., 2016; Akers et al., 2017; Shah et al., 2017a; Shah et al., 2017b; Klingenberg et al., 2017;



Nonaka and Wong, 2017). In our prior ERCC studies, we focused on the utility of extracellular miRNAs as biomarkers for AD. We performed a series of discovery and validation studies to identify 25 biomarker candidates that classify AD from CTLs (Lusardi et al., 2017; Wiedrick et al., 2019). We also demonstrated that five (miR-142-3p, -146a-5p, -146b-5p, -193a-5p, and -365a-3p) of these 25 CSF miRNAs are sensitive to early-stage pathology as exemplified by MCI diagnosis (Sandau et al.). These five miRNAs jointly predicted AD with area under the curve (AUC) of 0.770 and MCI with AUC of 0.705. We further showed that while the ratio of CSF $A\beta_{42}$:total-Tau (clinical markers for AD diagnosis (Maddalena et al., 2003)) predicted MCI with AUC of 0.758, $A\beta_{42}$:total-Tau plus the five miRNAs improved the AUC to 0.813 (Sandau et al., 2020b). In addition, the five miRNAs plus APOE-e4 status improved classification performance for both AD and MCI relative to CTL (Sandau et al., 2020b). Aside from our studies, three (miR-142-3p, -146a-5p, and -146b-5p) of these miRNAs have been identified by others as candidate biomarkers for MCI and/or AD in total CSF (Cogswell et al., 2008; Alexandrov et al., 2012; Kiko et al., 2014; Denk et al., 2015; Nagaraj et al., 2017; Park et al., 2019). Together, these data support that CSF miRNAs carry novel information relevant to AD, even in MCI.

Extracellular miRNAs have multiple carrier types including exosomes, exomeres, supermeres, MVs, apoptotic bodies, RNA-binding proteins, and high-density lipoproteins (Vickers and Remaley, 2012; Mori et al., 2019; Zhang et al., 2021). In AD, there are disruptions in the endolysosomal pathway within both neurons and glia that affect exosome biogenesis, including RNA and protein cargo (Asai et al., 2015; Goetzl et al., 2016; Mathews and Levy, 2019; Li et al., 2020; Guo et al., 2021). EVs released from CNS cells contribute to cell-to-cell communication throughout the CNS and the periphery (Chivet et al., 2012; Dickens et al., 2017; Zhang and Yang, 2018) in normal and pathological processes (Yuyama and Igarashi, 2016; Neven et al., 2017). Thus, there is interest in exploring the molecular cargo of EVs as a biomarker for disease. However, to the best of our knowledge only a limited number of studies have assayed miRNA expression of CSF EVs in AD (Liu et al., 2014; Gui et al., 2015; Riancho et al., 2017; McKeever et al., 2018; Schneider et al., 2018; Jain et al., 2019; Kumar and Reddy, 2021; Tan et al., 2021). Furthermore, differences in EV isolation techniques can affect outcomes and render data comparison between studies more challenging.

Prior studies that investigated the miRNA cargo of CSF EVs in AD used precipitation, ultracentrifugation, or density gradient ultracentrifugation-based methods to fractionate CSF (Liu et al., 2014; Gui et al., 2015; Riancho et al., 2017; McKeever et al., 2018; Schneider et al., 2018; Jain et al., 2019; Kumar and Reddy, 2021; Tan et al., 2021). EV precipitation methods have high yields, but low specificity for isolating EVs away from vesicle-free proteins, lipoproteins, and their associated miRNAs (Furi et al., 2017; Stranska et al., 2018; Thery et al., 2018; Karttunen et al., 2019; Ter-Ovanesyan et al., 2021). Isolating EVs by ultracentrifugation results in intermediate yields and purity with potential contamination by non-vesicular macromolecules (Furi et al., 2017; Thery et al., 2018). However, density gradient ultracentrifugation fractionation of CSF results in low yield, but higher purity than other fractionation techniques (Furi et al., 2017; Thery et al., 2018). Here, we isolated EVs from CSF using a combined approach of UF and SEC, which is reported to have an intermediate yield and potential contamination with vesicle free-proteins and lipoproteins, albeit to a lesser degree than precipitation-based methods (Boing et al., 2014; Furi et al., 2017; Stranska et al., 2018; Thery et al., 2018; Ter-Ovanesyan et al., 2021). The increased yield is a key factor considering the low amount of EVs in CSF, given that the total protein content of CSF is 50–100 times lower than plasma (Yuan and Desiderio, 2005). In line with this, our vFC calculated $1.5e7/\text{mL}$ TS + EVs in CSF (Figure 2) vs. $1.3e10/\text{mL}$ TS + EVs in plasma (Sandau et al.). In order to increase the yield of CSF EVs, we tested two SEC column resin sizes (35 and 70 nm). The 35 nm SEC columns showed optimal enrichment for CSF EVs based on the protein markers flotillin and CD81 as well as a 10-fold increase in the concentration of isolated particles measured by TRPS (Figure 3). These data are consistent with a recent study

that also reported a smaller resin size increases SEC yield of CSF EVs (Ter-Ovanessian et al., 2021).

While our goal was to increase yield, we also wanted to minimize vesicle-free lipoproteins and proteins, which can carry miRNAs (Vickers and Remaley, 2012; Mori et al., 2019). Our CSF EV isolation method demonstrated that SEC Fxs 6–9 were enriched for EVs (~40–150 nm) as well as smaller nanoparticles < 40 nm based on TEM (Figure 4). These smaller particles are of the expected size for exomeres (~30–50 nm), supermeres (~25–30 nm), and vesicle-free lipoproteins (~5–35 nm) (Zhang et al., 2018; Zipkin, 2019; Zhang et al., 2021). By immunoblot, Fxs 6–9 showed an enrichment of exosome protein markers associated with the vesicle membrane (CD9, CD63, and CD81) and cytosol (flotillin-1 and TSG101) (Figure 5) (Thery et al., 2018; Jeppesen et al., 2019). Fxs 6–9 were also depleted in the APOA1 lipoprotein and albumin (Figure 5), which are not-associated with EVs, but are abundant in plasma and serum and can be a source of contamination in CSF and CSF EV preparations (Thery et al., 2018; Jeppesen et al., 2019). However, while a majority of APOE was in the later fraction, Fxs 6–9 also contained the lipoprotein (Figure 5). Prior studies have shown that the protein cargo of both neuronal- and astrocyte-derived EVs includes APOE (Nikitidou et al., 2017; Zheng et al., 2018), which is also increased in the secreted EVs following A β ₄₂ treatment (Nikitidou et al., 2017). Together, our data support that using a combination of UF and SEC does enrich for CSF EVs; however, we cannot state the identity of the small nanoparticles that are also present in Fxs 6–9. Additional experiments using immunoTEM in SEC fractions with markers associated with exosomes (CD81), exomeres (Ago1/2), supermeres (ENO2, Ago1/2), and APOE are needed (Zhang et al., 2021). Furthermore, experiments that combine proteinase K treatment with immunoaffinity capture or immunoTEM are also needed to determine if APOE is cargo for EVs or associated with the EV protein corona (Toth et al., 2021).

We also used protein markers to identify the types of EVs that are present in CSF fractionated by SEC (Figure 5). Our detection of CD9, CD63, CD81, flotillin-1, and TSG101 supports the presence of exosomes in CSF, as these proteins are membrane markers for exosomes and mediators of exosome biogenesis *via* the endolysosomal pathways (Jeppesen et al., 2019). The enrichment of AnnV suggests that MVs are also present in CSF, as AnnV is a marker for MVs and/or apoptotic bodies (Cocucci et al., 2009; Gouwens et al., 2018; Jeppesen et al., 2019). While the TRPS and TEM size estimates of our CSF EV fractions (Figures 3, 4) are more in line with the enrichment of MVs (~150–1,000 nm) as opposed to apoptotic bodies (~500–2000 nm) (Crescitelli et al., 2013), additional experiments to rule out the presence of apoptotic bodies are needed. We also assessed the enrichment for markers of neuron- (NCAM-1 and Synaptophysin), astrocyte- (GLAST), and microglia-derived EVs (TMEM119 and CD11b) (Pouget et al., 1971; Togashi et al., 2009; Satoh et al., 2016; Sathe et al., 2019; Vukojevic et al., 2020; Zhou et al., 2020; Gu et al., 2021; Kumar et al., 2021; You et al., 2022). Analysis of 0.1 μ g of pooled CTL CSF SEC fractions revealed astrocyte-derived (GLAST+) EVs, but not neuronal- (NCAM-1+ and SYP+) or microglial- (TMEM119+ and CD11b+) derived EVs. It is important to note that neuron- or microglial-derived EVs may be present in CSF at low concentrations that are below the detection limits of immunoblot using 0.1 μ g of protein. Thus, to address this,

we performed additional SYP and CD11b immunoblots with 1 μ g of protein, which demonstrated undetectable levels of neuronal-derived EV markers, but the presence of very low levels microglial-derived EV markers. Together, these data demonstrated that performing experiments with more sensitive techniques (e.g., vFC) may be necessary to assay low abundance populations of cell-specific EVs. It is also important to note that our immunoblot assays were limited to two markers each for neuronal or microglial-EVs. Thus, additional markers may be better suited to identify neuronal (e.g., ATP1A3) or microglial (e.g., LCP1) EVs in CSF (You et al., 2022). L1CAM has been used for the enrichment of neuronal-derived EV from plasma (Winston et al., 2016; Goetzl et al., 2019; Pulliam et al., 2019; Goetzl et al., 2020; Nogueras-Ortiz et al., 2020; Anastasi et al., 2021; Cressatti et al., 2021; Saeedi et al., 2021; Yao et al., 2021). However, the use of L1CAM as a neuronal EV marker is an ongoing debate in the EV community, in part due to a recent and comprehensive study demonstrated that L1CAM in CSF is not a marker of neuronal EVs (Norman et al., 2021). Another, important limitation of our study is that the CSF EV profile is based on a pool of CTL samples and not individual participants that reflect disease state. Thus, our future experiments include comprehensive profiling of CSF EVs in both AD and CTLs to identify and assess the relative abundance of cell-type specific markers for neurons, astrocytes, and microglia by both immunoblot and vFC.

Considering that extracellular RNAs can be transported by EVs, exomeres, supermeres, lipoproteins, and RNA-binding proteins (Vickers and Remaley, 2012; Mori et al., 2019; Zhang et al., 2021) and that Fxs 6–9 showed an enrichment of EVs and smaller nanoparticles, while Fxs 10–13 and 14–17 contained a majority of the vesicle-free lipoproteins and proteins we sought to determine the miRNA profiles of each pool (Figure 6). The largest total number miRNAs were detected in Fxs 6–9, including 26 miRNAs that were unique to these fractions, and 12 miRNAs that we previously identified as candidate biomarkers for AD in total CSF (miR-125b-5p, -140-5p, -142-3p, -145-5p, -146a-5p, -146b-5p, -19b-3p, -223-3p, -24-3p, -29a-3p, -328-3p, and -331-3p), with five being unique to Fxs 6–9 (miR-125b-5p, -140-5p, -145-5p, -19b-3p, and -328-3p). In comparison, we identified very few miRNAs that were unique to the fractions that contained a majority of the vesicle-free lipoproteins and proteins (Fxs 10–13 and 14–17). However, we did observe 14 miRNAs that were in common between all three pooled fractions, including five AD CSF biomarkers (miR-146a-5p, -19b-5p, -24-3p, and -224-3p). Also, there were two AD biomarkers detected only in the vesicle-free lipoprotein and protein fractions (miR-19b-3p and 193a-5p). Note that some miRNAs included in the AD, sex, and APOE analyses were excluded from this analysis (e.g., miR-16-5p) because of differential detection in AD only and not expressed in at least two of the four CTL samples used in this experiment. Together, these data demonstrate that SEC fractions, which contain EVs and small nanoparticles, also contain a majority of the miRNAs that are informative about disease state. However, miRNAs carried by lipoproteins and RNA-binding proteins also have biomarker potential. Thus, more comprehensive studies are needed to fully elucidate the effects of AD on miRNA transport in CSF.

Our analysis of CSF EV miRNA expression between AD and CTL participants identified four miRNAs that were significantly increased

in AD (**Figure 7**). We had previously identified two (miR-16-5p and -331-3p) of these four miRNAs as biomarkers for AD using total CSF (Wiedrick et al., 2019). Two independent studies that have also shown differential expression of miR-16-5p in CSF EVs from AD participants, compared to CTLs. In contrast to our findings, both studies reported a decrease in miR-16-5p expression levels in AD CSF EVs (Gui et al., 2015; McKeever et al., 2018). The differences may be associated with participant age at time of lumbar puncture. For our study, the average age of AD participants was 73 years old, while the average ages from the other studies were 61 and 63 years old (Gui et al., 2015; McKeever et al., 2018). Together, these data suggest that miR-16-5p expression levels may be sensitive to disease stage, which is further supported by studies in human postmortem AD tissue that show increased miR-16-5p in the prefrontal cortex and hippocampus of postmortem AD brain compared to CTL (Lau et al., 2013; Muller et al., 2014). Of the other miRNAs, two that were significantly increase in AD CSF EVs (miR-409-3p and -454-3p, **Figure 7**) have also been shown to be differentially expressed in postmortem AD prefrontal cortex compared to CTL (Lau et al., 2013). Together, these data demonstrate that the miRNA cargo of EVs is sensitive to neuropathological effects of AD.

Our target prediction analysis (**Figure 6**) found that 10 of the top 17 significant pathways are involved in AD. The top pathway overall was senescence, the permanent arrest of a proliferative cell (Saez-Atienzar and Masliah, 2020). In a study using AD brains, immunostaining showed the accumulation of senescent astrocytes (Bhat et al., 2012). Additionally, astrocyte senescence has been shown to promote glutamate excitotoxicity in cortical neurons, which may lead to neurodegeneration (Limbad et al., 2020). Within the senescence pathway, we identified SMAD2 and TGFB2 as predicted gene targets of miR-409-3p and -454-3p, respectively (**Supplementary Table S5**). In an APP/APOE knockout mouse, inhibition of the TGFB/SMAD2 pathway activity in astrocytes has been shown to increase amyloid plaque formation and cognitive impairment (Zheng et al., 2017). This could suggest that EV miRNAs may act to suppress SMAD2 and TGFB2 translation in astrocytes and contribute to disease pathogenesis. Since our CSF EVs were enriched for GLAST and likely associated with astrocytes, the miRNAs may be part of an astrocyte-derived mechanism that drives senescence and subsequently neurodegeneration. The top 10 pathways also included autophagy, which is essential to maintain the homeostasis of neurons. In AD brains, autophagy defects attribute to accumulation of cellular waste and autophagic organelles in dystrophic neurites (Nixon et al., 2005). In neurons, autophagosomes often fuse with MVBs before depositing contents to the lysosome for degradation (Colacurcio et al., 2018). Disruptions in the fusion of autophagosomes to the MVB can cause MVB accumulation of amyloid precursor protein and the toxic C-terminal fragment resulting from its cleavage, along with reduced degradation of these products (González et al., 2017). As MVBs are also a prominent site of EV biogenesis (Abels and Breakefield, 2016), disruption of MVBs due to autophagy defects may affect both the biogenesis of EVs and their contents. In line with our target prediction, a study that profiled the protein cargo of CSF EVs from AD patients found several of the same affected pathways (Muraoka et al., 2020). A prominent AD-related protein in the

autophagy pathway is PTEN (Diaz Gonzalez et al., 2021), which we identified as a potential gene target of miR-454-3p (**Supplementary Tables S4, S5**). Pharmacological upregulation of PTEN has been shown to induce autophagy and increase clearance of A β in primary neuronal cells (Wani et al., 2019). Together, these data could contribute to the discovery of novel therapeutic targets for proteins and pathways regulated by miRNAs that are involved in AD.

APOE-e4 has been shown to disrupt exosome biogenesis and decreases the production of brain EVs in aged non-AD human brain and a humanized APOE-e4 mouse model (Peng et al., 2019; Ben Khedher et al., 2021). Furthermore, APOE-positive particles produced in cultured astrocytes can transport miRNAs to neurons with astrocyte-derived APOE-e4 particles carrying fewer miRNAs compared to APOE-e3 particles (Li et al., 2021). Both APOE alleles have also been shown to regulate miRNA expression (Han et al., 2013; Sharma and Eghbali, 2014). APOE-e4 decreases expression of miR-146a in the brain of both wild-type and 5xFAD AD mice compared to APOE-e3 (Teter et al., 2016). Aside from APOE-e4, miRNA expression is also regulated by both hormonal (estradiol, progesterone, and testosterone) and genetic differences between females and males (Waltering et al., 2011; Klinge, 2012; Lam et al., 2012; Sharma and Eghbali, 2014). Hormones can regulate miRNA expression directly through gene transcription *via* ligand bound nuclear hormone receptors that bind and recruit co-activators and-repressors to gene promoter elements. In rodents, 17 β -estradiol differentially regulates miRNA expression in an age-dependent manner in the hippocampus (Rao et al., 2013). Furthermore, in cultured neurons, 17 β -estradiol regulates miR-125b expression and prevents A β -induced neurotoxicity (Micheli et al., 2016). While these studies all implicate an effect of AD, APOE genotype status, and sex on EV and/or miRNAs, there is a knowledge gap in human studies. To the best of our knowledge, aside from the study herein, eight studies have investigated miRNA expression in CSF EVs from AD participants (Liu et al., 2014; Gui et al., 2015; Riancho et al., 2017; McKeever et al., 2018; Schneider et al., 2018; Jain et al., 2019; Kumar and Reddy, 2021; Tan et al., 2021), and we are the first to investigate the effects of sex and APOE-e4 status on CSF EVs and their miRNA cargo. Considering the relatively limited number of studies that have investigated sex-dependent effects on EVs and their RNA cargo, future studies that address basic questions such as the effect of sex on EV biogenesis, circulating EV concentration, and total concentration of RNA cargo are warranted.

In summary, our studies identified that AD and two of its risk factors, sex and APOE-e4 status, impact CSF EV miRNA levels. Importantly, APOE-e4 status within each sex-altered different subsets of miRNAs. Furthermore, the miRNAs differentially expressed in AD CSF EVs predicted gene targets and pathways that are known to contribute to neurodegeneration in AD. Together, these data demonstrate the need to perform large scale studies that take into consideration key biological factors in addition to the disease state in order to more precisely define biomarker profiles for the at-risk population. Our data also demonstrate that EV miRNAs could facilitate the identification of new targets for the therapeutic treatment of AD and define how EV-derived miRNAs may render females and/or APOE-e4 carriers more prone to AD.

RESOURCE IDENTIFICATION INITIATIVE

Antibody or Tool/Resource	RRID
Albumin antibody	AB_2225785
Annexin V antibody	AB_1949660
APOA1 antibody	AB_1119020
APOE antibody	AB_2904241
CD9 antibody	AB_627213
CD11b antibody	AB_2650514
CD63 bntibody	AB_2800495
CD81 bntibody	AB_2275892
Donkey anti-rabbit IgG HRP antibody	AB_10015282
Donkey anti-goat IgG HRP antibody	AB_2340390
Donkey anti-mouse IgG HRP antibody	AB_2340770
Flotillin antibody	AB_11156367
GLAST antibody	AB_2190597
NCAM-1 antibody	AB_2904242
Synaptophysin antibody	AB_2799098
TMEM119 antibody	AB_2882272
TSG101 antibody	AB_10974262
Bio-Rad ChemiDoc Touch	SCR_021693
Bio-Rad T100 PCR	SCR_021921
CytoFlex S	SCR_019627
ExpressionSuite software	SCR_021095
FCS Express software	SCR_016431
GraphPad Prism software	SCR_002798
Ingenuity Pathway analysis	SCR_008653
IZON Control Suite software	SCR_021922
IZON qNANO Gold	SCR_021923
miRBase	SCR_003152
miRDB	SCR_010848
Orbitor RS2 Microplate Robot	SCR_020721
QuantStudio™ 12K Flex real-time PCR system	SCR_021098
QuantStudio™ 12K Flex real-time PCR software	SCR_021096
TargetScan v.7.2	SCR_010845
OHSU Electron Microscopy Core	SCR_009969
OHSU Flow Cytometry Shared Resource	SCR_009974
OHSU Gene Profiling Shared Resource Core Laboratory	SCR_009975

DATA AVAILABILITY STATEMENT

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept a article that does not adhere to our open data policies.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Boards of OHSU (IRB

6845) and UCSD (IRB 80012). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

US, TM, and JS contributed to conception and design of the study. DG and JQ provided the human Alzheimer's disease and control CSF samples. US and TM performed the experiments. US, TM, and SS performed the analyses. US, TM, SS, and JS wrote and edited the manuscript. All authors read and approved the submitted version.

FUNDING

This work was supported by the Oregon Health and Science University, Center for Women's Health Circle of Giving grant (JAS, USS), and the National Institutes of Health, National Institutes on Aging, RF1 AG059392 (JAS), P30 AG024978 (JFQ), and P30 AG062429 (DRG).

ACKNOWLEDGMENTS

The authors acknowledge the support provided by the OHSU Research Cores and Shared Resources, specifically Dr. Claudia Lopez and Steven Adamou at the Multiscale Microscopy Core, Dr. Phil Streeter and Matthew Schleisman at the Flow Cytometry Shared Resource, and Dr. Chris Harrington at the Gene Profiling Shared Resource. We also thank Dr. Randy Woltjer for human brain tissue from the Oregon Brain Bank and Dr. Philip Copenhaver for generously sharing the Synaptophysin antibody.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2022.864022/full#supplementary-material>

REFERENCES

- Abels, E. R., and Breakefield, X. O. (2016). Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. *Cell Mol. Neurobiol.* 36, 301–312. doi:10.1007/s10571-016-0366-z
- Agarwal, V., Bell, G. W., Nam, J. W., and Bartel, D. P. (2015). Predicting Effective microRNA Target Sites in Mammalian mRNAs. *Elife* 4. doi:10.7554/eLife.05005
- Ainsztein, A. M., Brooks, P. J., Dugan, V. G., Ganguly, A., Guo, M., Howcroft, T. K., et al. (2015). The NIH Extracellular RNA Communication Consortium. *J. Extracellular Vesicles* 4, 27493. doi:10.3402/jev.v4.27493
- Akers, J. C., Hua, W., Li, H., Ramakrishnan, V., Yang, Z., Quan, K., et al. (2017). A Cerebrospinal Fluid microRNA Signature as Biomarker for Glioblastoma. *Oncotarget* 8, 68769–68779. doi:10.18632/oncotarget.18332
- Alexandrov, P. N., Dua, P., Hill, J. M., Bhattacharjee, S., Zhao, Y., and Lukiw, W. J. (2012). microRNA (miRNA) Speciation in Alzheimer's Disease (AD) Cerebrospinal Fluid (CSF) and Extracellular Fluid (ECF). *Int. J. Biochem. Mol. Biol.* 3, 365–373.
- Altmann, A., Tian, L., Henderson, V. W., Greicius, M. D., and Alzheimer's Disease Neuroimaging Initiative, I. (2014). Sex Modifies theAPOE-Related Risk of Developing Alzheimer Disease. *Ann. Neurol.* 75, 563–573. doi:10.1002/ana.24135

- Alzheimer's Association (2021). 2021 Alzheimer's Disease Facts and Figures. *Alzheimers Dement* 17, 327–406. doi:10.1002/alz.12328
- Anastasi, F., Masciandaro, S. M., Carratore, R. D., Dell'anno, M. T., Signore, G., Falleni, A., et al. (2021). Proteomics Profiling of Neuron-Derived Small Extracellular Vesicles from Human Plasma: Enabling Single-Subject Analysis. *Int. J. Mol. Sci.* 22. doi:10.3390/ijms22062951
- Asai, H., Ikezu, T., Tsunoda, S., Medalla, M., Luebke, J., Haydar, T., et al. (2015). Depletion of Microglia and Inhibition of Exosome Synthesis Halt Tau Propagation. *Nat. Neurosci.* 18, 1584–1593. doi:10.1038/nn.4132
- Ben Khedher, M. R., Haddad, M., Laurin, D., and Ramassamy, C. (2021). Apolipoprotein E4-Driven Effects on Inflammatory and Neurotrophic Factors in Peripheral Extracellular Vesicles from Cognitively Impaired, No Dementia Participants Who Converted to Alzheimer's Disease. *Alzheimers Dement (N Y)* 7, e12124. doi:10.1002/trc2.12124
- Bhat, R., Crowe, E. P., Bitto, A., Moh, M., Katsetos, C. D., Garcia, F. U., et al. (2012). Astrocyte Senescence as a Component of Alzheimer's Disease. *PLoS ONE* 7, e45069. doi:10.1371/journal.pone.0045069
- Böing, A. N., Van Der Pol, E., Grootemaat, A. E., Coumans, F. A., Sturk, A., and Nieuwland, R. (2014). Single-Step Isolation of Extracellular Vesicles by Size-Exclusion Chromatography. *J. Extracell. Vesicles* 3. doi:10.3402/jev.v3.23430
- Burgos, K., Malenica, I., Metpally, R., Courtright, A., Rakela, B., Beach, T., et al. (2014). Profiles of Extracellular miRNA in Cerebrospinal Fluid and Serum from Patients with Alzheimer's and Parkinson's Diseases Correlate with Disease Status and Features of Pathology. *PLoS One* 9, e94839. doi:10.1371/journal.pone.0094839
- Caraci, F., Spampinato, S. F., Morgese, M. G., Tascadda, F., Salluzzo, M. G., Giambirtone, M. C., et al. (2018). Neurobiological Links Between Depression and AD: The Role of TGF- β 1 Signaling as a New Pharmacological Target. *Pharmacol. Res.* 130, 374–384. doi:10.1016/j.phrs.2018.02.007
- Chen, Y., and Wang, X. (2020). miRDB: An Online Database for Prediction of Functional microRNA Targets. *Nucleic Acids Res.* 48, D127–D131. doi:10.1093/nar/gkz757
- Chivet, M., Hemming, F., Pernet-Gallay, K., Fraboulet, S., and Sadoul, R. (2012). Emerging Role of Neuronal Exosomes in the central Nervous System. *Front. Physiol.* 3, 145. doi:10.3389/fphys.2012.00145
- Chou, C.-H., Shrestha, S., Yang, C.-D., Chang, N.-W., Lin, Y.-L., Liao, K.-W., et al. (2018). miRTarBase Update 2018: A Resource for Experimentally Validated microRNA-Target Interactions. *Nucleic Acids Res.* 46, D296–D302. doi:10.1093/nar/gkx1067
- Cocucci, E., Racchetti, G., and Meldolesi, J. (2009). Shedding Microvesicles: Artefacts No More. *Trends Cel. Biol.* 19, 43–51. doi:10.1016/j.tcb.2008.11.003
- Cogswell, J. P., Ward, J., Taylor, I. A., Waters, M., Shi, Y., Cannon, B., et al. (2008). Identification of miRNA Changes in Alzheimer's Disease Brain and CSF Yields Putative Biomarkers and Insights into Disease Pathways. *Jad* 14, 27–41. doi:10.3233/jad-2008-14103
- Cohn, W., Melnik, M., Huang, C., Teter, B., Chandra, S., Zhu, C., et al. (2021). Multi-Omics Analysis of Microglial Extracellular Vesicles from Human Alzheimer's Disease Brain Tissue Reveals Disease-Associated Signatures. *Front. Pharmacol.* 12, 766082. doi:10.3389/fphar.2021.766082
- Colacurcio, D. J., Pensalfini, A., Jiang, Y., and Nixon, R. A. (2018). Dysfunction of Autophagy and Endosomal-Lysosomal Pathways: Roles in Pathogenesis of Down Syndrome and Alzheimer's Disease. *Free Radic. Biol. Med.* 114, 40–51. doi:10.1016/j.freeradbiomed.2017.10.001
- Colombo, M., Moita, C., Van Niel, G., Kowal, J., Vigneron, J., Benaroch, P., et al. (2013). Analysis of ESCRT Functions in Exosome Biogenesis, Composition and Secretion Highlights the Heterogeneity of Extracellular Vesicles. *J. Cel. Sci.* 126, 5553–5565. doi:10.1242/jcs.128868
- Corder, E. H., Saunders, A. M., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Small, G. W., et al. (1993). Gene Dose of Apolipoprotein E Type 4 Allele and the Risk of Alzheimer's Disease in Late Onset Families. *Science* 261, 921–923. doi:10.1126/science.8346443
- Crescitelli, R., Lässer, C., Szabó, T. G., Kittel, A., Eldh, M., Dianzani, I., et al. (2013). Distinct RNA Profiles in Subpopulations of Extracellular Vesicles: Apoptotic Bodies, Microvesicles and Exosomes. *J. Extracell. Vesicles* 2. doi:10.3402/jev.v2i0.20677
- Cressatti, M., Galindez, J. M., Juwara, L., Orlovetskies, N., Velly, A. M., Eintracht, S., et al. (2021). Characterization and Heme Oxygenase-1 Content of Extracellular Vesicles in Human Biofluids. *J. Neurochem.* 157, 2195–2209. doi:10.1111/jnc.15167
- Crews, F. T., Zou, J., and Coleman, L. G., Jr. (2021). Extracellular Microvesicles Promote Microglia-Mediated Pro-Inflammatory Responses to Ethanol. *J. Neurosci. Res.*
- Denk, J., Boelmans, K., Siegmund, C., Lassner, D., Arlt, S., and Jahn, H. (2015). MicroRNA Profiling of CSF Reveals Potential Biomarkers to Detect Alzheimer's Disease. *PLoS One* 10, e0126423. doi:10.1371/journal.pone.0126423
- Díaz González, M., Buberman, A., Morales, M., Ferrer, I., and Knafo, S. (2021). Aberrant Synaptic PTEN in Symptomatic Alzheimer's Patients May Link Synaptic Depression to Network Failure. *Front. Synaptic Neurosci.* 13, 683290. doi:10.3389/fnsyn.2021.683290
- Dickens, A. M., Tovar-Y-Romo, L. B., Yoo, S. W., Trout, A. L., Bae, M., Kanmogne, M., et al. (2017). Astrocyte-Shed Extracellular Vesicles Regulate the Peripheral Leukocyte Response to Inflammatory Brain Lesions. *Sci. Signal.* 10. doi:10.1126/scisignal.aai7696
- Efron, B. (2010). *Large-Scale Inference: Empirical Bayes Methods for Estimation, Testing and Prediction*. United Kingdom: Cambridge University Press.
- Ev-Track, Consortium., Van Deun, J., Mestdag, P., Agostinis, P., Akay, Ö., Anand, S., et al. (2017). EV-TRACK: Transparent Reporting and Centralizing Knowledge in Extracellular Vesicle Research. *Nat. Methods* 14, 228–232. doi:10.1038/nmeth.4185
- Fan, X., and Kurgan, L. (2015). Comprehensive Overview and Assessment of Computational Prediction of microRNA Targets in Animals. *Brief Bioinform* 16, 780–794. doi:10.1093/bib/bbu044
- Fleisher, A. S., Sun, S., Taylor, C., Ward, C. P., Gamst, A. C., Petersen, R. C., et al. (2008). Volumetric MRI vs Clinical Predictors of Alzheimer Disease in Mild Cognitive Impairment. *Neurology* 70, 191–199. doi:10.1212/01.wnl.0000287091.57376.65
- Folstein, M. F., Robins, L. N., and Helzer, J. E. (1983). The Mini-Mental State Examination. *Arch. Gen. Psychiatry* 40, 812. doi:10.1001/archpsyc.1983.01790060110016
- Furi, I., Momen-Heravi, F., and Szabo, G. (2017). Extracellular Vesicle Isolation: Present and Future. *Ann. Transl. Med.* 5, 263. doi:10.21037/atm.2017.03.95
- Goetzl, E. J., Elahi, F. M., Mustapic, M., Kapogiannis, D., Pryhoda, M., Gilmore, A., et al. (2019). Altered Levels of Plasma Neuron-Derived Exosomes and Their Cargo Proteins Characterize Acute and Chronic Mild Traumatic Brain Injury. *FASEB j.* 33, 5082–5088. doi:10.1096/fj.201802319r
- Goetzl, E. J., Mustapic, M., Kapogiannis, D., Eitan, E., Lobach, I. V., Goetzl, L., et al. (2016). Cargo Proteins of Plasma Astrocyte-Derived Exosomes in Alzheimer's Disease. *FASEB J.* 30, 3853–3859. doi:10.1096/fj.201600756r
- Goetzl, E. J., Peltz, C. B., Mustapic, M., Kapogiannis, D., and Yaffe, K. (2020). Neuron-Derived Plasma Exosome Proteins after Remote Traumatic Brain Injury. *J. Neurotrauma* 37, 382–388. doi:10.1089/neu.2019.6711
- González, A. E., Muñoz, V. C., Cavieles, V. A., Bustamante, H. A., Cornejo, V. H., Januário, Y. C., et al. (2017). Autophagosomes Cooperate in the Degradation of Intracellular C-Terminal Fragments of the Amyloid Precursor Protein via the MVB/lysosomal Pathway. *FASEB J.* 31, 2446–2459.
- Gouwens, L. K., Ismail, M. S., Rogers, V. A., Zeller, N. T., Garrad, E. C., Amtashar, F. S., et al. (2018). A β 42 Protofibrils Interact with and Are Trafficked through Microglial-Derived Microvesicles. *ACS Chem. Neurosci.* 9, 1416–1425. doi:10.1021/acscchemneuro.8b00029
- Gu, J., Jin, T., Li, Z., Chen, H., Xia, H., Xu, X., et al. (2021). Exosomes Expressing Neuronal Autoantigens Induced Immune Response in Antibody-Positive Autoimmune Encephalitis. *Mol. Immunol.* 131, 164–170. doi:10.1016/j.molimm.2020.12.034
- Gui, Y., Liu, H., Zhang, L., Lv, W., and Hu, X. (2015). Altered microRNA Profiles in Cerebrospinal Fluid Exosome in Parkinson Disease and Alzheimer Disease. *Oncotarget* 6, 37043–37053. doi:10.18632/oncotarget.6158
- Guo, M., Hao, Y., Feng, Y., Li, H., Mao, Y., Dong, Q., et al. (2021). Microglial Exosomes in Neurodegenerative Disease. *Front. Mol. Neurosci.* 14, 630808. doi:10.3389/fnmol.2021.630808
- Han, H., Wang, Y.-H., Qu, G.-J., Sun, T.-T., Li, F.-Q., Jiang, W., et al. (2013). Differentiated miRNA Expression and Validation of Signaling Pathways in apoE Gene Knockout Mice by Cross-Verification Microarray Platform. *Exp. Mol. Med.* 45, e13. doi:10.1038/emmm.2013.31

- Hixson, J., and Vernier, D. (1990). Restriction Isotyping of Human Apolipoprotein E by Gene Amplification and Cleavage with HhaI. *J. Lipid Res.* 31, 545–548. doi:10.1016/s0022-2275(20)43176-1
- Jain, G., Stundl, A., Rao, P., Berulava, T., Pena Centeno, T., Kaurani, L., et al. (2019). A Combined miRNA-piRNA Signature to Detect Alzheimer's Disease. *Transl Psychiatry* 9, 250. doi:10.1038/s41398-019-0579-2
- Jeppesen, D. K., Fenix, A. M., Franklin, J. L., Higginbotham, J. N., Zhang, Q., Zimmerman, L. J., et al. (2019). Reassessment of Exosome Composition. *Cell* 177, 428–445. doi:10.1016/j.cell.2019.02.029
- Karttunen, J., Heiskanen, M., Navarro-Ferrandis, V., Das Gupta, S., Lipponen, A., Puhakka, N., et al. (2019). Precipitation-Based Extracellular Vesicle Isolation from Rat Plasma Co-Precipitate Vesicle-Free microRNAs. *J. Extracellular Vesicles* 8, 1555410. doi:10.1080/20013078.2018.1555410
- Kiko, T., Nakagawa, K., Tsuduki, T., Furukawa, K., Arai, H., and Miyazawa, T. (2014). MicroRNAs in Plasma and Cerebrospinal Fluid as Potential Markers for Alzheimer's Disease. *Jad* 39, 253–259. doi:10.3233/jad-130932
- Kim, K. M., Abdelmohsen, K., Mustapic, M., Kapogiannis, D., and Gorospe, M. (2017). *RNA in Extracellular Vesicles*, 8. Wiley Interdiscip Rev RNA. doi:10.1002/wrna.1413
- Klinge, C. M. (2012). miRNAs and Estrogen Action. *Trends Endocrinol. Metab.* 23, 223–233. doi:10.1016/j.tem.2012.03.002
- Klingenberg, M., Matsuda, A., Diederichs, S., and Patel, T. (2017). Non-Coding RNA in Hepatocellular Carcinoma: Mechanisms, Biomarkers and Therapeutic Targets. *J. Hepatol.* 67, 603–618. doi:10.1016/j.jhep.2017.04.009
- Kumar, A., Kim, S., Su, Y., Sharma, M., Kumar, P., Singh, S., et al. (2021). Brain Cell-Derived Exosomes in Plasma Serve as Neurodegeneration Biomarkers in Male Cynomolgus Monkeys Self-Administering Oxycodone. *EBioMedicine* 63, 103192. doi:10.1016/j.ebiom.2020.103192
- Kumar, S., and Reddy, P. H. (2021). Elevated Levels of MicroRNA-455-3p in the Cerebrospinal Fluid of Alzheimer's Patients: A Potential Biomarker for Alzheimer's Disease. *Biochim. Biophys. Acta (Bba) - Mol. Basis Dis.* 1867, 166052. doi:10.1016/j.bbdis.2020.166052
- Lam, E. W.-F., Shah, K., and Brosens, J. J. (2012). The Diversity of Sex Steroid Action: the Role of Micro-RNAs and FOXO Transcription Factors in Cycling Endometrium and Cancer. *J. Endocrinol.* 212, 13–25. doi:10.1530/joe-10-0480
- Lässer, C. (2019). Mapping Extracellular RNA Sheds Lights on Distinct Carriers. *Cell* 177, 228–230. doi:10.1016/j.cell.2019.03.027
- Lau, P., Bossers, K., Janky, R. s., Salta, E., Frigerio, C. S., Barbash, S., et al. (2013). Alteration of the Micro RNA Network during the Progression of Alzheimer's Disease. *EMBO Mol. Med.* 5, 1613–1634. doi:10.1002/emmm.201201974
- Levy, Y., and Levy, D. (2021). A Friend and a Foe: 50 Years of the Apolipoprotein E Research Trail. *Isr. Med. Assoc. J.* 23, 665–669.
- Li, X., Zhang, J., Li, D., He, C., He, K., Xue, T., et al. (2021). Astrocytic ApoE Reprograms Neuronal Cholesterol Metabolism and Histone-Acetylation-Mediated Memory. *Neuron* 109, 957–970. doi:10.1016/j.neuron.2021.01.005
- Li, Z., Moniruzzaman, M., Dastgheyb, R. M., Yoo, S. W., Wang, M., Hao, H., et al. (2020). Astrocytes Deliver CK1 to Neurons via Extracellular Vesicles in Response to Inflammation Promoting the Translation and Amyloidogenic Processing of APP. *J. Extracell. Vesicles* 10, e12035. doi:10.1002/jev2.12035
- Limbad, C., Oron, T. R., Alimirah, F., Davalos, A. R., Tracy, T. E., Gan, L., et al. (2020). Astrocyte Senescence Promotes Glutamate Toxicity in Cortical Neurons. *PLOS ONE* 15, e0227887. doi:10.1371/journal.pone.0227887
- Liu, C.-G., Song, J., Zhang, Y.-Q., and Wang, P.-C. (2014). MicroRNA-193b Is a Regulator of Amyloid Precursor Protein in the Blood and Cerebrospinal Fluid Derived Exosomal microRNA-193b Is a Biomarker of Alzheimer's Disease. *Mol. Med. Rep.* 10, 2395–2400. doi:10.3892/mmr.2014.2484
- Liu, W., and Wang, X. (2019). Prediction of Functional microRNA Targets by Integrative Modeling of microRNA Binding and Target Expression Data. *Genome Biol.* 20, 18. doi:10.1186/s13059-019-1629-z
- Lusardi, T. A., Phillips, J. I., Wiedrick, J. T., Harrington, C. A., Lind, B., Lapidus, J. A., et al. (2017). MicroRNAs in Human Cerebrospinal Fluid as Biomarkers for Alzheimer's Disease. *J. Alzheimers Dis.* 55, 1223–1233. doi:10.3233/JAD-160835
- Lusardi, T. A., Sandau, U. S., Sakhanenko, N. A., Baker, S. C. B., Wiedrick, J. T., Lapidus, J. A., et al. (2021). Cerebrospinal Fluid MicroRNA Changes in Cognitively Normal Veterans with a History of Deployment-Associated Mild Traumatic Brain Injury. *Front. Neurosci.* 15, 720778. doi:10.3389/fnins.2021.720778
- Maddalena, A., Papassotiropoulos, A., Müller-Tillmanns, B., Jung, H. H., Hegi, T., Nitsch, R. M., et al. (2003). Biochemical Diagnosis of Alzheimer Disease by Measuring the Cerebrospinal Fluid Ratio of Phosphorylated Tau Protein to β -Amyloid Peptide42. *Arch. Neurol.* 60, 1202–1206. doi:10.1001/archneur.60.9.1202
- Mathews, P. M., and Levy, E. (2019). Exosome Production Is Key to Neuronal Endosomal Pathway Integrity in Neurodegenerative Diseases. *Front. Neurosci.* 13, 1347. doi:10.3389/fnins.2019.01347
- McKeever, P. M., Schneider, R., Taghdiri, F., Weichert, A., Multani, N., Brown, R. A., et al. (2018). MicroRNA Expression Levels Are Altered in the Cerebrospinal Fluid of Patients with Young-Onset Alzheimer's Disease. *Mol. Neurobiol.* 55, 8826–8841. doi:10.1007/s12035-018-1032-x
- Mckhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D., and Stadlan, E. M. (1984). Clinical Diagnosis of Alzheimer's Disease: Report of the NINCDS-ADRDA Work Group* under the Auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* 34, 939. doi:10.1212/wnl.34.7.939
- Mckhann, G. M., Knopman, D. S., Chertkow, H., Hyman, B. T., Jack, C. R., Jr., Kawas, C. H., et al. (2011). The Diagnosis of Dementia Due to Alzheimer's Disease: Recommendations from the National Institute on Aging-Alzheimer's Association Workgroups on Diagnostic Guidelines for Alzheimer's Disease. *Alzheimer's Dement.* 7, 263–269. doi:10.1016/j.jalz.2011.03.005
- Micheli, F., Palermo, R., Talora, C., Ferretti, E., Vacca, A., and Napolitano, M. (2016). Regulation of Proapoptotic Proteins Bak1 and P53 by miR-125b in an Experimental Model of Alzheimer's Disease: Protective Role of 17 β -Estradiol. *Neurosci. Lett.* 629, 234–240. doi:10.1016/j.neulet.2016.05.049
- Misawa, T., Tanaka, Y., Okada, R., and Takahashi, A. (2020). Biology of Extracellular Vesicles Secreted from Senescent Cells as Senescence-associated Secretory Phenotype Factors. *Geriatr. Gerontol. Int.* 20, 539–546. doi:10.1111/ggi.13928
- Mori, M. A., Ludwig, R. G., Garcia-Martin, R., Brandão, B. B., and Kahn, C. R. (2019). Extracellular miRNAs: From Biomarkers to Mediators of Physiology and Disease. *Cel. Metab.* 30, 656–673. doi:10.1016/j.cmet.2019.07.011
- Müller, M., Kuiperij, H. B., Claassen, J. A., Küsters, B., and Verbeek, M. M. (2014). MicroRNAs in Alzheimer's Disease: Differential Expression in hippocampus and Cell-free Cerebrospinal Fluid. *Neurobiol. Aging* 35, 152–158. doi:10.1016/j.neurobiolaging.2013.07.005
- Muraoka, S., Jedrychowski, M. P., Yanamandra, K., Ikezu, S., Gygi, S. P., and Ikezu, T. (2020). Proteomic Profiling of Extracellular Vesicles Derived from Cerebrospinal Fluid of Alzheimer's Disease Patients: A Pilot Study. *Cells* 9, 1959. doi:10.3390/cells9091959
- Nagaraj, S., Laskowska-Kaszub, K., Dębski, K. J., Wojsiat, J., Dąbrowski, M., Gabryelewicz, T., et al. (2017). Profile of 6 microRNA in Blood Plasma Distinguish Early Stage Alzheimer's Disease Patients from Non-Demented Subjects. *Oncotarget* 8, 16122–16143. doi:10.18632/oncotarget.15109
- Neven, K. Y., Nawrot, T. S., and Bollati, V. (2017). Extracellular Vesicles: How the External and Internal Environment Can Shape Cell-To-Cell Communication. *Curr. Envir. Health Rpt* 4, 30–37. doi:10.1007/s40572-017-0130-7
- Nikitidou, E., Khoonsari, P. E., Shevchenko, G., Ingelsson, M., Kulitima, K., and Erlandsson, A. (2017). Increased Release of Apolipoprotein E in Extracellular Vesicles Following Amyloid- β Protofibril Exposure of Neuroglial Co-Cultures. *Jad* 60, 305–321. doi:10.3233/jad-170278
- Nixon, R. A., Wegiel, J., Kumar, A., Yu, W. H., Peterhoff, C., Cataldo, A., et al. (2005). Extensive Involvement of Autophagy in Alzheimer Disease: An Immuno-Electron Microscopy Study. *J. Neuropathol. Exp. Neurol.* 64, 113–122. doi:10.1093/jnen/64.2.113
- Nogueras-Ortiz, C. J., Mahairaki, V., Delgado-Peraza, F., Das, D., Avgerinos, K., Eren, E., et al. (2020). Astrocyte- and Neuron-Derived Extracellular Vesicles from Alzheimer's Disease Patients Effect Complement-Mediated Neurotoxicity. *Cells* 9, doi:10.3390/cells9071618
- Nonaka, T., and Wong, D. T. W. (2017). Saliva-Exosomics in Cancer: Molecular Characterization of Cancer-Derived Exosomes in Saliva. *Enzymes* 42, 125–151. doi:10.1016/bs.enz.2017.08.002

- Norman, M., Ter-Ovanesyan, D., Trieu, W., Lazarovits, R., Kowal, E. J. K., Lee, J. H., et al. (2021). L1CAM Is Not Associated with Extracellular Vesicles in Human Cerebrospinal Fluid or Plasma. *Nat. Methods* 18, 631–634. doi:10.1038/s41592-021-01174-8
- Oliveira, A. C., Bovolenta, L. A., Nachtigall, P. G., Herkenhoff, M. E., Lemke, N., and Pinhal, D. (2017). Combining Results from Distinct MicroRNA Target Prediction Tools Enhances the Performance of Analyses. *Front. Genet.* 8, 59. doi:10.3389/fgene.2017.00059
- Park, J. S., Kim, S. T., Kim, S. Y., Jo, M. G., Choi, M. J., and Kim, M. O. (2019). A Novel Kit for Early Diagnosis of Alzheimer's Disease Using a Fluorescent Nanoparticle Imaging. *Sci. Rep.* 9, 13184. doi:10.1038/s41598-019-49711-y
- Pearson, A., Ajay, R., Crynen, G., Reed, J. M., Algamal, M., Mullan, M., et al. (2020). Molecular Abnormalities in Autopsied Brain Tissue from the Inferior Horn of the Lateral Ventricles of Nonagenarians and Alzheimer Disease Patients. *BMC Neurol.* 20, 317. doi:10.1186/s12883-020-01849-3
- Peng, K. Y., Pérez-González, R., Alldred, M. J., Goulbourne, C. N., Morales-Corraliza, J., Saito, M., et al. (2019). Apolipoprotein E4 Genotype Compromises Brain Exosome Production. *Brain* 142, 163–175. doi:10.1093/brain/awy289
- Pouget, J. M., Harris, W. S., Mayron, B. R., and Naughton, J. P. (1971). Abnormal Responses of the Systolic Time Intervals to Exercise in Patients with Angina Pectoris. *Circulation* 43, 289–298. doi:10.1161/01.cir.43.2.289
- Pulliam, L., Sun, B., Mustapic, M., Chawla, S., and Kapogiannis, D. (2019). Plasma Neuronal Exosomes Serve as Biomarkers of Cognitive Impairment in HIV Infection and Alzheimer's Disease. *J. Neurovirol.* 25, 702–709. doi:10.1007/s13365-018-0695-4
- Quinn, J. F., Patel, T., Wong, D., Das, S., Freedman, J. E., Laurent, L. C., et al. (2015). Extracellular RNAs: Development as Biomarkers of Human Disease. *J. Extracellular Vesicles* 4, 27495. doi:10.3402/jev.v4.27495
- Rajan, K. B., Weuve, J., Barnes, L. L., McAninch, E. A., Wilson, R. S., and Evans, D. A. (2021). Population Estimate of People with Clinical Alzheimer's Disease and Mild Cognitive Impairment in the United States (2020–2060). *Alzheimers Dement* 17, 1966–1975. doi:10.1002/alz.12362
- Rao, Y. S., Mott, N. N., Wang, Y., Chung, W. C. J., and Pak, T. R. (2013). MicroRNAs in the Aging Female Brain: A Putative Mechanism for Age-Specific Estrogen Effects. *Endocrinology* 154, 2795–2806. doi:10.1210/en.2013-1230
- Raposo, G., and Stoorvogel, W. (2013). Extracellular Vesicles: Exosomes, Microvesicles, and Friends. *J. Cel. Biol.* 200, 373–383. doi:10.1083/jcb.201211138
- Rastogi, S., Sharma, V., Bharti, P. S., Rani, K., Modi, G. P., Nikolajeff, F., et al. (2021). The Evolving Landscape of Exosomes in Neurodegenerative Diseases: Exosomes Characteristics and a Promising Role in Early Diagnosis. *Int. J. Mol. Sci.* 22. doi:10.3390/ijms22010440
- Regev, K., Paul, A., Healy, B., Von Glenn, F., Diaz-Cruz, C., Gholipour, T., et al. (2016). Comprehensive Evaluation of Serum microRNAs as Biomarkers in Multiple Sclerosis. *Neurol. Neuroimmunol. Neuroinflamm.* 3, e267. doi:10.1212/wnx.0000000000000267
- Reiman, E. M., Arboleda-Velasquez, J. F., Arboleda-Velasquez, J. F., Quiroz, Y. T., Huentelman, M. J., Beach, T. G., et al. (2020). Exceptionally Low Likelihood of Alzheimer's Dementia in APOE2 Homozygotes from a 5,000-Person Neuropathological Study. *Nat. Commun.* 11, 667. doi:10.1038/s41467-019-14279-8
- Riancho, J., Vázquez-Higuera, J. L., Pozueta, A., Lage, C., Kazimierzczak, M., Bravo, M., et al. (2017). MicroRNA Profile in Patients with Alzheimer's Disease: Analysis of miR-9-5p and miR-598 in Raw and Exosome Enriched Cerebrospinal Fluid Samples. *Jad* 57, 483–491. doi:10.3233/jad-161179
- Ruan, Z., Pathak, D., Venkatesan Kalavai, S., Yoshii-Kitahara, A., Muraoka, S., Bhatt, N., et al. (2021). Alzheimer's Disease Brain-Derived Extracellular Vesicles Spread Tau Pathology in Interneurons. *Brain* 144, 288–309. doi:10.1093/brain/awaa376
- Saeedi, S., Nagy, C., Ibrahim, P., Theroux, J. F., Wakid, M., Fiori, L. M., et al. (2021). Neuron-Derived Extracellular Vesicles Enriched from Plasma Show Altered Size and miRNA Cargo as a Function of Antidepressant Drug Response. *Mol. Psychiatry*. doi:10.1038/s41380-021-01255-2
- Saez-Atienzar, S., and Masliah, E. (2020). Cellular Senescence and Alzheimer Disease: The Egg and The Chicken Scenario. *Nat. Rev. Neurosci.* 21, 433–444. doi:10.1038/s41583-020-0325-z
- Sampedro, F., Vilaplana, E., De Leon, M. J., Alcolea, D., Pegueroles, J., Montal, V., et al. (2015). APOE-by-sex Interactions on Brain Structure and Metabolism in Healthy Elderly Controls. *Oncotarget* 6, 26663–26674. doi:10.18632/oncotarget.5185
- Sandau, U. S., Duggan, E., Shi, X., Smith, S. J., Huckans, M., Schutzer, W. E., et al. (2020a). Methamphetamine Use Alters Human Plasma Extracellular Vesicles and Their microRNA Cargo: An Exploratory Study. *J. Extracell. Vesicles* 10, e12028. doi:10.1002/jev2.12028
- Sandau, U. S., Wiedrick, J. T., Smith, S. J., McFarland, T. J., Lusardi, T. A., Lind, B., et al. (2020b). Performance of Validated MicroRNA Biomarkers for Alzheimer's Disease in Mild Cognitive Impairment. *JAD* 78, 245–263. doi:10.3233/jad-200396
- Sando, S. B., Melquist, S., Cannon, A., Hutton, M. L., Sletvold, O., Saltvedt, I., et al. (2008). APOE ε4 Lowers Age at Onset and Is a High Risk Factor for Alzheimer's Disease; A Case Control Study from Central Norway. *BMC Neurol.* 8, 9. doi:10.1186/1471-2377-8-9
- Sathe, G., Na, C. H., Renuse, S., Madugundu, A. K., Albert, M., Moghekar, A., et al. (2019). Quantitative Proteomic Profiling of Cerebrospinal Fluid to Identify Candidate Biomarkers for Alzheimer's Disease. *Proteomics Clin. Appl.* 13, e1800105. doi:10.1002/prca.201800105
- Satoh, J.-I., Kino, Y., Asahina, N., Takitani, M., Miyoshi, J., Ishida, T., et al. (2016). TMEM119 Marks a Subset of Microglia in the Human Brain. *Neuropathology* 36, 39–49. doi:10.1111/neup.12235
- Schneider, R., McKeever, P., Kim, T., Graff, C., Van Swieten, J. C., Karydas, A., et al. (2018). Downregulation of Exosomal miR-204-5p and miR-632 as a Biomarker for FTD: A GENFI Study. *J. Neurol. Neurosurg. Psychiatry* 89, 851–858. doi:10.1136/jnnp-2017-317492
- Shah, R., Murthy, V., Pacold, M., Danielson, K., Tanriverdi, K., Larson, M. G., et al. (2017a). Extracellular RNAs Are Associated with Insulin Resistance and Metabolic Phenotypes. *Diabetes Care* 40, 546–553. doi:10.2337/dc16-1354
- Shah, R., Yeri, A., Das, A., Courtright-Lim, A., Ziegler, O., Gervino, E., et al. (2017b). Small RNA-Seq during Acute Maximal Exercise Reveal RNAs Involved in Vascular Inflammation and Cardiometabolic Health: Brief Report. *Am. J. Physiology-Heart Circulatory Physiol.* 313, H1162–H1167. doi:10.1152/ajpheart.00500.2017
- Sharma, S., and Eghbali, M. (2014). Influence of Sex Differences on microRNA Gene Regulation in Disease. *Biol. Sex Dif.* 5, 3. doi:10.1186/2042-6410-5-3
- Shi, M., Bradner, J., Hancock, A. M., Chung, K. A., Quinn, J. F., Peskind, E. R., et al. (2011). Cerebrospinal Fluid Biomarkers for Parkinson Disease Diagnosis and Progression. *Ann. Neurol.* 69, 570–580. doi:10.1002/ana.22311
- Stranska, R., Gysbrechts, L., Wouters, J., Vermeersch, P., Bloch, K., Dierickx, D., et al. (2018). Comparison of Membrane Affinity-Based Method with Size-Exclusion Chromatography for Isolation of Exosome-Like Vesicles from Human Plasma. *J. Transl. Med.* 16, 1. doi:10.1186/s12967-017-1374-6
- Tan, Y. J., Wong, B. Y. X., Vaidyanathan, R., Sreejith, S., Chia, S. Y., Kandiah, N., et al. (2021). Altered Cerebrospinal Fluid Exosomal microRNA Levels in Young-Onset Alzheimer's Disease and Frontotemporal Dementia. *Adr* 5, 805–813. doi:10.3233/adr-210311
- Ter-Ovanesyan, D., Norman, M., Lazarovits, R., Trieu, W., Lee, J. H., Church, G. M., et al. (2021). Framework for Rapid Comparison of Extracellular Vesicle Isolation Methods. *Elife* 10. doi:10.7554/eLife.70725
- Teter, B., Ladu, M. J., Sullivan, P. M., Frautschy, S. A., and Cole, G. M. (2016). Apolipoprotein E Isotype-Dependent Modulation of microRNA-146a in Plasma and Brain. *Neuroreport* 27, 791–795. doi:10.1097/wnr.0000000000000608
- Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., et al. (2018). Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): A Position Statement of the International Society for Extracellular Vesicles and Update of the MISEV2014 Guidelines. *J. Extracellular Vesicles* 7, 1535750. doi:10.1080/20013078.2018.1535750
- Togashi, H., Sakisaka, T., and Takai, Y. (2009). Cell Adhesion Molecules in the Central Nervous System. *Cell Adhes. Migration* 3, 29–35. doi:10.4161/cam.3.1.6773
- Tóth, E. Á., Turiák, L., Visnovitz, T., Cserép, C., Mázló, A., Sódar, B. W., et al. (2021). Formation of a Protein corona on the Surface of Extracellular

- Vesicles in Blood Plasma. *J. Extracell Vesicles* 10, e12140. doi:10.1002/jev2.12140
- Vassileff, N., Cheng, L., and Hill, A. F. (2020). Extracellular Vesicles - Propagators of Neuropathology and Sources of Potential Biomarkers and Therapeutics for Neurodegenerative Diseases. *J. Cel. Sci.* 133. doi:10.1242/jcs.243139
- Vickers, K. C., and Remaley, A. T. (2012). Lipid-Based Carriers of microRNAs and Intercellular Communication. *Curr. Opin. Lipidol.* 23, 91–97. doi:10.1097/mol.0b013e328350a425
- Vukojevic, V., Mastrandreas, P., Arnold, A., Peter, F., Kolassa, I.-T., Wilker, S., et al. (2020). Evolutionary Conserved Role of Neural Cell Adhesion Molecule-1 in Memory. *Transl. Psychiatry* 10, 217. doi:10.1038/s41398-020-00899-y
- Waltering, K. K., Porkka, K. P., Jalava, S. E., Urbanucci, A., Kohonen, P. J., Latonen, L. M., et al. (2011). Androgen Regulation of Micro-RNAs in Prostate Cancer. *Prostate* 71, 604–614. doi:10.1002/pros.21276
- Wang, W.-X., Fardo, D. W., Jicha, G. A., and Nelson, P. T. (2017). A Customized Quantitative PCR MicroRNA Panel Provides a Technically Robust Context for Studying Neurodegenerative Disease Biomarkers and Indicates a High Correlation between Cerebrospinal Fluid and Choroid Plexus MicroRNA Expression. *Mol. Neurobiol.* 54, 8191–8202. doi:10.1007/s12035-016-0316-2
- Wani, A., Gupta, M., Ahmad, M., Shah, A. M., Ahsan, A. U., Qazi, P. H., et al. (2019). Alborixin Clears Amyloid- β by Inducing Autophagy Through PTEN-Mediated Inhibition of the AKT Pathway. *Autophagy* 15, 1810–1828. doi:10.1080/15548627.2019.1596476
- Welsh, J. A., Van Der Pol, E., Arkesteijn, G. J. A., Bremer, M., Brisson, A., Coumans, F., et al. (2020). MIFlowCyt-EV: a Framework for Standardized Reporting of Extracellular Vesicle Flow Cytometry Experiments. *J. Extracellular Vesicles* 9, 1713526. doi:10.1080/20013078.2020.1713526
- Wiedrick, J. T., Phillips, J. I., Lusardi, T. A., McFarland, T. J., Lind, B., Sandau, U. S., et al. (2019). Validation of MicroRNA Biomarkers for Alzheimer's Disease in Human Cerebrospinal Fluid. *Jad* 67, 875–891. doi:10.3233/jad-180539
- Wierenga, C. E., Dev, S. I., Shin, D. D., Clark, L. R., Bangen, K. J., Jak, A. J., et al. (2012). Effect of Mild Cognitive Impairment and APOE Genotype on Resting Cerebral Blood Flow and its Association with Cognition. *J. Cereb. Blood Flow Metab.* 32, 1589–1599. doi:10.1038/jcbfm.2012.58
- Winston, C. N., Goetzl, E. J., Akers, J. C., Carter, B. S., Rockenstein, E. M., Galasko, D., et al. (2016). Prediction of Conversion from Mild Cognitive Impairment to Dementia with Neuronally Derived Blood Exosome Protein Profile. *Alzheimer's Dement. Diagn. Assess. Dis. Monit.* 3, 63–72. doi:10.1016/j.dadm.2016.04.001
- Yao, P. J., Eren, E., Goetzl, E. J., and Kapogiannis, D. (2021). Mitochondrial Electron Transport Chain Protein Abnormalities Detected in Plasma Extracellular Vesicles in Alzheimer's Disease. *Biomedicines* 9. doi:10.3390/biomedicines9111587
- You, Y., Muraoka, S., Jedrychowski, M. P., Hu, J., Mcquade, A. K., Young-Pearse, T., et al. (2022). Human Neural Cell Type-Specific Extracellular Vesicle Proteome Defines Disease-Related Molecules Associated with Activated Astrocytes in Alzheimer's Disease Brain. *J. Extracell Vesicles* 11, e12183. doi:10.1002/jev2.12183
- You, Y., Borgmann, K., Edara, V. V., Stacy, S., Ghorpade, A., and Ikezu, T. (2020). Activated Human Astrocyte-Derived Extracellular Vesicles Modulate Neuronal Uptake, Differentiation and Firing. *J. Extracellular Vesicles* 9, 1706801. doi:10.1080/20013078.2019.1706801
- Yuan, X., and Desiderio, D. M. (2005). Proteomics Analysis of Human Cerebrospinal Fluid. *J. Chromatogr. B* 815, 179–189. doi:10.1016/j.jchromb.2004.06.044
- Yuyama, K., and Igarashi, Y. (2016). Physiological and Pathological Roles of Exosomes in the Nervous System. *Biomol. Concepts* 7, 53–68. doi:10.1515/bmc-2015-0033
- Zhang, G., and Yang, P. (2018). A Novel Cell-Cell Communication Mechanism in the Nervous System: Exosomes. *J. Neuro Res.* 96, 45–52. doi:10.1002/jnr.24113
- Zhang, H., Freitas, D., Kim, H. S., Fabijanic, K., Li, Z., Chen, H., et al. (2018). Identification of Distinct Nanoparticles and Subsets of Extracellular Vesicles by Asymmetric Flow Field-Flow Fractionation. *Nat. Cel. Biol.* 20, 332–343. doi:10.1038/s41556-018-0040-4
- Zhang, Q., Jeppesen, D. K., Higginbotham, J. N., Graves-Deal, R., Trinh, V. Q., Ramirez, M. A., et al. (2021). Supermeres Are Functional Extracellular Nanoparticles Replete with Disease Biomarkers and Therapeutic Targets. *Nat. Cel. Biol.* 23, 1240–1254. doi:10.1038/s41556-021-00805-8
- Zheng, J.-y., Sun, J., Ji, C.-m., Shen, L., Chen, Z.-j., Xie, P., et al. (2017). Selective Deletion of Apolipoprotein E in Astrocytes Ameliorates the Spatial Learning and Memory Deficits in Alzheimer's Disease (APP/PS1) Mice by Inhibiting TGF- β /Smad2/STAT3 Signaling. *Neurobiol. Aging* 54, 112–132. doi:10.1016/j.neurobiolaging.2017.03.002
- Zheng, P., Luo, Q., Wang, W., Li, J., Wang, T., Wang, P., et al. (2018). Tumor-Associated Macrophages-Derived Exosomes Promote the Migration of Gastric Cancer Cells by Transfer of Functional Apolipoprotein E. *Cell Death Dis.* 9, 434. doi:10.1038/s41419-018-0465-5
- Zhou, L., Wang, D., Qiu, X., Zhang, W., Gong, Z., Wang, Y., et al. (2020). DHZCP Modulates Microglial M1/M2 Polarization via the P38 and TLR4/NF-K β Signaling Pathways in LPS-Stimulated Microglial Cells. *Front. Pharmacol.* 11, 1126. doi:10.3389/fphar.2020.01126
- Zipkin, M. (2019). Exosome Redux. *Nat. Biotechnol.* 37, 1395–1400. doi:10.1038/s41587-019-0326-5

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Sandau, McFarland, Smith, Galasko, Quinn and Saugstad. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Extracellular Vesicles From TNF α Preconditioned MSCs: Effects on Immunomodulation and Bone Regeneration

Miya Kang¹, Chun-Chieh Huang¹, Praveen Gajendrareddy², Yu Lu¹, Sajjad Shirazi¹, Sriram Ravindran^{1*} and Lyndon F. Cooper^{1*}

OPEN ACCESS

Edited by:

Satish Balasaheb Nimse,
Hallym University, South Korea

Reviewed by:

Sudarshana Basu,
Netaji Subhas Chandra Bose Cancer
Hospital (NCRI), India
Faheem A. Sheikh,
University of Kashmir, India

*Correspondence:

Lyndon F. Cooper
coopertf@uic.edu
Sriram Ravindran
sravin1@uic.edu

Specialty section:

This article was submitted to
Cytokines and Soluble
Mediators in Immunity,
a section of the journal
Frontiers in Immunology

Received: 17 February 2022

Accepted: 07 April 2022

Published: 02 May 2022

Citation:

Kang M, Huang C-C,
Gajendrareddy P, Lu Y, Shirazi S,
Ravindran S and Cooper LF (2022)
Extracellular Vesicles From
TNF α Preconditioned MSCs:
Effects on Immunomodulation
and Bone Regeneration.
Front. Immunol. 13:878194.
doi: 10.3389/fimmu.2022.878194

¹ Department of Oral Biology, College of Dentistry, University of Illinois Chicago, Chicago, IL, United States, ² Department of Periodontics, College of Dentistry, University of Illinois Chicago, Chicago, IL, United States

Mesenchymal stem cells show remarkable versatility and respond to extracellular and micro environmental cues by altering their phenotype and behavior. In this regard, the MSC's immunomodulatory properties in tissue repair are well documented. The paracrine effects of MSCs in immunomodulation are, in part, attributable to their secreted extracellular vesicles (EVs). When MSCs migrate to the wound bed, they are exposed to a myriad of inflammatory signals. To understand their response to an inflammatory environment from an EV perspective, we sought to evaluate the effects of the inflammatory cytokine TNF α on MSC EV mediated immunomodulation. Our results indicate that while the physical characteristics of the EVs remain unchanged, the TNF α preconditioned MSC EVs possess enhanced immunomodulatory properties. *In vitro* experiments using polarized (M1 and M2) primary mouse macrophages indicated that the preconditioned MSC EVs suppressed pro-inflammatory (M1) markers such as IL-1 β and iNOS and elevated reparatory (M2) markers such as Arg1 and CD206. When evaluated *in vivo* in a rat calvarial defect model, the TNF α preconditioned MSC EVs reduced inflammation at 1-, 3- and 7-days post wounding resulting in the subsequent enhanced bone formation at 4- and 8-weeks post wounding possibly by modulation of oncostatin M (OSM) expression. An analysis of EV miRNA composition revealed significant changes to anti-inflammatory miRNAs in the preconditioned MSC EVs hinting at a possible role for EV derived miRNA in the enhanced immunomodulatory activity. Overall, these results indicate that MSC exposure to inflammatory signals influence the MSC EV's immunomodulatory function in the context of tissue repair. The specific function of TNF α preconditioned MSC EV miRNAs in immunomodulatory control of bone regeneration merits further investigation.

Keywords: mesenchymal stem cells, TNF α , extracellular vesicles, immunomodulation, bone regeneration

INTRODUCTION

Osseous repair and regeneration following surgery or trauma begins with the inflammatory response. The importance of the inflammatory response to successful wound healing is revealed generally in studies of wound healing (1) and specifically in bone regeneration by the observed limitation in healing when effectors of inflammation are eliminated (2). Examples include the reduced healing of bone fractures in TNF α receptor knock out mice and reduced bone healing when macrophages are reduced in animal models (3, 4). Implied is the role of inflammatory cytokines in the communication between cells of the immune system and the bone forming mesenchymal cells. TNF α expression, which occurs quickly and persists for several days following injury, is a central determinant of subsequent cellular healing events (5). Mechanistically, TNF α is a mediator of MSC function that can affect the MSC proliferation and phenotype (6).

It is generally acknowledged that MSCs contribute to tissue regeneration by paracrine regulation of host cells (7, 8) resulting in immunomodulation at the site of surgery or injury. The communication and interaction of MSCs with the cells of the immune system is well documented (9). Co-culture of MSCs with dendritic cells, T-cells, and NK cells resulted in reduced inflammatory cytokine expression by all cell types. MSCs are known to direct macrophage polarization toward an M2-like (regenerative) phenotype to enhance tissue repair. The paracrine immunomodulatory function of MSCs has been attributed to the secretion of anti-inflammatory proteins and cytokines including IL-10, PGE2, NO, and TGF α .

An emerging view of immunomodulation includes the bi-directional signaling of inflammatory cells and MSCs. In the process of wound healing and tissue regeneration or repair, MSCs are recruited to an inflammatory environment. Thus, the characteristic feature of the MSC during the early stages of wound healing is one that is influenced by inflammatory cytokines with TNF α representing a dominant effector of inflammatory signaling at the site of injury of surgery. Presently, the knowledge regarding both acute and chronic TNF α exposure of MSCs suggests that acute and lower concentration TNF α signaling of undifferentiated MSCs promotes osteoinductive signaling (10, 11), while chronic and higher does MSC exposure to TNF α inhibits osteoinduction and bone formation (12, 13). The ability of the MSC to control TNF α expression by immune cells such as the macrophage may represent a key aspect of its immunomodulatory role in wound healing and in bone repair.

The paracrine signaling of MSCs is multifaceted and attributed to its production and secretion of extracellular matrix proteins, growth factors and cytokines as well as extracellular vesicles (or exosomes). Several studies indicate that many functions attributed to the MSC can be recapitulated by MSC conditioned media alone, suggesting a prominent role for the MSC secretome in control of wound healing and bone repair (14). Recent investigations have highlighted a role for MSC extracellular vesicles in the paracrine regulation of wound healing and regeneration of diverse tissues including bone (15, 16). The cargo of MSC

extracellular vesicles includes protein, mRNA and microRNAs that are able to alter the function of target cells. The MSC cargo can be changed physiologically or experimentally to alter the paracrine function of the MSC. For example, overexpression of BMP2 in MSCs alters the cargo and function of secreted extracellular vesicles to enhance their osteoinductive function by directly affecting the BMP2 pathway of target cells (17).

More broadly, the concept of preconditioning of MSCs involves the exposure of MSCs to alternative culture conditions (e.g., hypoxia), to various cytokines or growth factor or through genetic modification (18). One intent of preconditioning is to increase the number of extracellular vesicles produced by the parental cell (19). Another intent is to alter the functionality of the extracellular vesicles. By example, the lineage specific differentiation of MSCs results in the production of extracellular vesicles that promote the same lineage specific differentiation of targeted MSCs (20). Other studies have demonstrated that hypoxia (21) or pro-inflammatory cytokine treatment (22) of MSCs enhanced their immunomodulatory and wound healing function. IFN γ preconditioning of MSCs also increased the anti-inflammatory protein content of MSC extracellular vesicles (23). These and other studies indicate that MSCs secrete exosomes alter immunoregulatory function during wound healing. For example, they enhance survival of allogeneic skin grafts in mice in part by targeting macrophage function (24). LPS treatment of MSCs is another preconditioning strategy in which treated-MSC extracellular vesicles promotes M2 polarization (25), and attenuated inflammation in a mouse model of myocardial infarction (26).

In the present study, the treatment of MSCs with TNF α was performed to model the acute response of MSCs to inflammation following injury or surgery and to assess the effect of TNF α preconditioning of MSCs on the MSC extracellular vesicle function in bone regeneration. Comparison of TNF α treated and control MSC extracellular vesicles was performed at the structural, miRNA and functional levels both in cell culture and a calvarial model of bone regeneration. Preconditioned extracellular vesicles did not directly enhance MSC osteoinductive gene expression or differentiation in culture. However, TNF α preconditioning of MSCs resulted in the secretion of extracellular vesicles that affected macrophage polarization in cell culture and in healing calvarial tissues. Greater bone regeneration was observed in sites treated with the TNF α preconditioned MSCs compared to controls. Suggested is a bi-directional signaling of TNF α preconditioned MSCs to the macrophage that positively influences calvarial wound healing and bone regeneration.

MATERIALS AND METHODS

Cell Culture

Human bone marrow derived mesenchymal stem cells (MSCs) were purchased from Lonza. MSCs were cultured in α MEM (Gibco) containing 20% fetal bovine serum (FBS, Gibco), 1% L-Glutamine (Gibco) and 1% antibiotic-antimycotic solution (Gibco).

For MSC osteogenic differentiation induction, 100 μ g/ml ascorbic acid (Sigma), 10mM β -glycerophosphate (Sigma) and 10mM dexamethasone (Sigma) were supplemented in the α MEM growth medium (17).

Mouse bone marrow derived macrophages (BMMs) were isolated from 8-week-old C57BL/6J mice as per previously published protocol (27). BMMs were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 20% fetal bovine serum (Gibco) and 1% antibiotic-antimycotic solution (Gibco). 20ng/ml recombinant macrophage colony-stimulating factor (M-CSF, Peprotech) was supplemented into the DMEM growth medium to induce macrophage differentiation. For M1 or M2 polarization, BMMs were treated with 100ng/ml lipopolysaccharides (LPS, Sigma) with 50ng/ml Interferon gamma (IFN γ , Peprotech) or 20ng/ml Interleukin 4 (IL-4, Peprotech) for 48 hours.

EV Isolation and Characterization

For preconditioning, MSCs were treated with 20ng/ml Tumor necrosis factor alpha (TNF α) for 72 hours. Control MSCs (no TNF α treatment) and TNF α preconditioned MSCs were washed in PBS and cultured in serum free α MEM growth medium for 24 hours. EVs were isolated from serum free culture medium according to our published and standardized protocols (20). The culture medium was harvested, cell debris were removed by centrifugation (3,000 $\times$ g, 15min) and EVs were isolated using the ExoQuick-TC reagent (System Biosciences) as per the manufacturer's recommended protocols. The isolated EVs were characterized for number and size distribution and presence of membrane markers by nano tracking analysis (NTA) and immunoblotting. For NTA, a 1/100 dilution of the EV suspension was analyzed in the Nanosight NS-300 instrument to obtain the average number of particles and the size distribution plot. Based on the NTA results, approximately equal concentration of control and TNF α EVs (1.8×10^{10} particles/ml) were used for each experiment.

For immunoblotting, exosomal proteins were isolated in RIPA buffer and 20-30 μ g of EV lysate was resolved by SDS-PAGE gel, transferred onto nitrocellulose membranes and probed with primary mouse monoclonal anti-CD63 [TS63] (1/1000, ab59479, Abcam) and mouse monoclonal anti-HSP70 [C92F3A-5] (1/200, sc-66048, Santa Cruz) antibodies and near infrared dye conjugated secondary antibodies (1/15,000, Licor) as per previously published protocols (28). The blots were imaged using a Licor Odyssey imager.

Quantitative and Qualitative Endocytosis of EVs

EVs were fluorescently labeled using the ExoGlow exosome protein labeling kit (System Biosciences) as per the manufacturer's protocols. For quantitative experiment, MSCs and BMMs were seeded onto 96 well tissue culture plates (10,000 MSCs/well, 20,000 BMMs/well) and incubated for overnight to facilitate cell attachment. The cells were then incubated with increasing numbers of fluorescently labeled EVs for 2 hours at 37°C, washed with PBS and fixed in neutral buffered 4%

paraformaldehyde (PFA). The fluorescence from the endocytosed EVs was observed and quantified by using a BioTek Synergy 2 96 well plate reader equipped with the appropriate filter sets to measure green fluorescence. The results were plotted as mean ($\pm$ SD) relative fluorescence intensity % increase (normalized to no EV group) as a function of dosage (n=6 per group).

For qualitative endocytosis experiments, 50,000 MSCs or BMMs were seeded onto cover glasses placed in 12 well or 24 well cell culture plates. Fluorescently labeled EVs (1.8×10^9 particles/well) were added and incubated for 2 hours. The cells were then washed with PBS, fixed in 4% PFA, permeabilized and counter stained using Alexa Fluor[®] 568 Phalloidin (1/2000, A12390, Invitrogen) antibody. The cover glasses were then mounted using mounting medium with DAPI (Vector Laboratories) and imaged using a Zeiss LSM 710 Meta confocal microscope.

EV Mediated MSC Osteogenic Differentiation

To examine the osteoinductive function of EVs, 50,000 MSCs were seeded onto 12 well tissue culture plates and cultured in growth medium with EVs (1.8×10^9 particles/well) for 3 days. Total RNA was isolated using RNeasy mini kit (Qiagen) as per the manufacturer's protocol. The RNA concentration was measured using NanoDrop One. After first strand cDNA synthesis, osteogenic related gene specific primers (**Table 1**) were used to direct PCR amplification and SYBR Green probe incorporation using a BioRad CFX96 thermocycler. All expression data were normalized to housekeeping genes GAPDH and fold change was calculated using $\Delta\Delta$ Ct method (n=4 per group).

For *in vitro* differentiation, MSCs were differentiated as described above using osteogenic differentiation medium. For the alkaline phosphatase (ALP) assays, MSCs (50,000 cells/well) were seeded onto 12 well tissue culture plates and EVs (1.8×10^9 particles/well) were added to the cells and cultured up to 7 days. ALP activity was quantified using Alkaline phosphatase assay kit (Abcam) by measuring p- nitrophenyl (pNP) based on the spectrophotometric absorbance at 405nm. The fold change of ALP activity at each time point was calculated with respect to relative enzymatic activity of day 0 (n=4 per group). To observe the calcium deposition, alizarin red staining was performed. MSCs (100,000 cells/well) were seeded onto 6 well tissue culture plates and EVs (3.6×10^9 particles/well) were added to the cells and incubated for 14 days. The cells were then washed with PBS, fixed with 4% PFA and stained with Alizarin red solution (Sigma).

EV Mediated BMM Polarization

The functionality of EVs on BMM polarization was assessed by qRT-PCR and MILLIPLEX multiplex assay (Millipore Sigma). Briefly, 250,000 BMMs were seeded onto 24 well tissue culture plates and incubated for overnight prior to the treatment. The cells were polarized to M1 or M2 phenotype as described in cell culture section and EVs (4.5×10^9 particles/well) were added to

TABLE 1 | Primer pairs used for qRT-PCR.

Genes	Forward (5'-3')	Reverse (5'-3')
mouse GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
mouse IL-1 β	GCAACTGTTCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
mouse iNOS	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTAC
mouse TNF α	CAGGCGGTGCCTATGTCTC	CGATCACCCGAAGTTCAGTAG
mouse CD206	CTCTGTTCACTATTGGACGC	CGGAATTTCTGGGATTCACTTC
mouse Arg1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCTATTAGGGACATC
mouse IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
mouse OSM	ATGCAGACACGGCTTCTAAGA	TTGGAGCAGCCACGATTGG
human GAPDH	CAGGGCTGCTTTTAACTCTGG	TGGGTGGAATCATATTGGAACA
human BMP2	ACTACCAGAAACGAGTGGGAA	GCATCTGTTCTCGGAAAACCT
human RUNX2	TGGTTACTGTCATGGCGGGTA	TCTCAGATCGTTGAACCTTGCTA
human OSX	CCTCTGCGGGACTCAACAAC	AGCCCATAGTGCTTGTAAGG
rat IL-1 β	CACCTCTCAAGCAGACAGCAG	GGGTTCCATGGTGAAAGTCAAC
rat TNF α	AAATGGGCTCCCTCTCATCAGTTC	TCTGCTTGGTGGTTTGCTACGAC

the cells and cultured for 48 hours. For multiplex assay, the culture medium was harvested, and cell debris were removed by centrifugation (3,000g, 15min). The protein concentration was measured using Pierce BCA protein assay kit (Thermo Fisher) and culture medium was adjusted to have equal amount of protein per well (n=4 per group). The multiplex assay was performed as per manufacture's recommended protocols and the concentration of macrophage secreted protein was quantified using Magpix system at UIC RRC Flow cytometry core. For qRT-PCR, the total RNA from BMs were isolated using RNeasy mini kit (Qiagen) as per the manufacturer's protocol and the concentration of RNA was measured by NanoDrop One. Equal amount of RNA was used to complete first strand cDNA synthesis and macrophage polarization related gene specific primers (**Table 1**) were used to performed qRT-PCR. All expression data were normalized to housekeeping genes GAPDH and fold change was calculated using $\Delta\Delta C_t$ method (n=4 per group).

Rat Calvaria Defects

To evaluate the effects of EVs on bone healing, the rat calvaria defect model was used. The rats were anesthetized intraperitoneally using Ketamine (80mg/kg)/Xylazine (10mg/kg) and a vertical incision was made in the head at the midline to expose the calvarial bone. Two 5mm calvarial defects were created bilaterally in the calvarium without dura perforation using a trephine burr. A clinical grade collagen scaffold (OraPLUG, Salvin) was placed on the wound with PBS (control), control EVs or TNF α EVs (4.5×10^9 particles/defect). The rats were sacrificed by carbon dioxide asphyxiation followed by cervical dislocation at each timepoint. For early time points, the embedded scaffolds were harvested and subjected to histology and qRT-PCR at day 1, 3 and 7 post-surgeries. Total RNA was isolated using miRNeasy mini Kit (Qiagen) as per the manufacturer's protocol and qRT-PCR was performed as described in the previous section using rat specific primers (**Table 1**). The fold change of expression level was calculated using ΔC_t method (n=4 per group). At 4- and 8-weeks post-surgery, the calvaria were harvested, fixed in neutral buffered 4% paraformaldehyde and subjected to 3D μ CT analysis using a Scanco40 μ CT scanner. The data obtained from the μ CT

scanner was quantitatively analyzed using a custom-built MATLAB Program.

Histology and Immunohistochemistry

After 3D μ CT analysis, the calvaria samples were decalcified in 10% EDTA solution. The harvested scaffolds at day 1, 3, and 7 and the decalcified calvaria at 4- and 8- weeks were then embedded in paraffin and sectioned into 5-10 μ m sections. The Hematoxylin and eosin (H&E) staining was performed as per previously published protocols (28). For immunofluorescent staining, the slides were pre-treated with 5% BSA blocking buffer for an hour at room temperature. Macrophage markers were stained with rabbit polyclonal anti-inducible nitric oxide synthase (iNOS) antibody (1/100, ab15323, Abcam), rabbit monoclonal [D4E3M] anti-arginase 1 (Arg1) antibody (1/100, 93668, Cell signaling), and mouse monoclonal [A-9] anti-oncostatin m (OSM) antibody (1/100, sc-374039, Santa Cruz). Osteomarkers were stained with mouse monoclonal [65529.111] anti-bone morphogenetic protein 2 (BMP2) antibody (1/100, ab6285, Abcam), mouse monoclonal [LFMb-31] anti-DMP-1 antibody (1/100, sc-73633, Santa Cruz), and mouse monoclonal [LFMb-25] anti-BSPII antibody (1/100, sc-73630, Santa Cruz). Sections were then stained with anti-mouse FITC and anti-rabbit TRITC secondary antibodies (1/200, Sigma) and imaged using Zeiss LSM 710 laser scanning confocal microscope. ImageJ was used to quantify the immunostained number or %area per field (n=4 per group). The positive number of iNOS and Arg 1 cells was normalized to the number of nuclei per field.

MicroRNA Sequencing

To study the microRNA (miRNA) profiles of TNF α EVs, miRNA sequencing was performed. Total RNA in EVs was extracted using miRNeasy mini Kit (Qiagen). Next-generation sequencing (NGS) was performed for miRNA expression profile. One microgram of total RNA was used for the construction of cDNA library using the TruSeq Small RNA sample prep kits (Illumina). Sequencing was performed with Illumina NovaSeq 6000 (LC Sciences). A proprietary analysis pipeline by LC Sciences, ACGT101-miR v4.2, was used for data analysis, where reads were mapped to the human genome (GRCh38) and miRBase (<https://mirbase.org>, Release 22.1).

The normalization method of the data was to divide the sequence counts of individual samples by the corresponding normalization factors, which were the median of the ratios of specific sample counts to geometric mean counts of all samples. Student's t-test (two-tailed, $n=3$) was used to analyze expression differences between TNF α EV and control EV group, and the differentially expressed miRNAs were showed in the heatmap.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using ACGT101-miR v4.2 to evaluate the functions of differentially expressed miRNA targets and gene interactions. Significance was determined by performing Fisher's exact test ($P < 0.05$).

Statistical Analysis

For experiments involving two groups, student's t-test with a confidence interval of 95% was utilized. For the experiments involving comparison of more than two groups, One-way ANOVA was performed with a confidence interval of 95%, following by pairwise comparisons using Tukey's *ad-hoc* method ($P < 0.05$).

RESULTS

Effect of TNF α Treatment on MSC EV Properties and EV Characterization

To observe if preconditioning of MSCs by treatment with TNF α affects the anti-inflammatory property of MSC EVs, MSCs were treated with varying concentrations of TNF α . The anti-inflammatory effects of the derivative EVs were analyzed by evaluating the expression levels of IL-1 β (a pro-inflammatory cytokine) and CD206 (a marker for M2 polarized macrophage) in M1 polarized BMMs. Results presented in **Figure 1A** indicate that TNF α preconditioning enhanced the anti-inflammatory

property of MSC EVs by reducing IL-1 β expression of M1 polarized BMMs at 10ng/ml and enhancing the expression of CD206 at 20ng/ml. Based on the results, 20ng/ml concentration was determined to be the ideal concentration for preconditioning and this concentration was used in subsequent experiments to generate TNF α preconditioned MSC EVs (TNF α EVs).

The properties of the TNF α EVs were analyzed in comparison to naïve MSC EVs by immunoblotting for EV specific markers CD63, HSP70 and TSG101 as well as by nano particle tracking analysis (NTA) to obtain the EV size distribution. Results presented in **Figures 1B** shows the similarity in the expression levels of the selected EV markers. NTA (**Figure 1C**) showed a minor increase in the average particle size of the TNF α EVs although this was not statistically significant. Similar polydispersity index between control and TNF α EVs was observed. The EVs from TNF α treated MSCs showed similar expression of EV markers as well as size distribution and PDI. Overall, these results indicated that treatment of MSC with TNF α did not cause global changes in structure or measured biochemistry of MSC EVs.

Effects of TNF α EVs on Osteogenic Differentiation of MSCs

MSC EVs influence osteogenic differentiation of MSCs. To evaluate if preconditioning of MSCs with TNF α further influences the osteoinductive ability of the derivative EVs, we first analyzed the ability of control and TNF α EVs to be endocytosed by MSCs. Congruent with their biophysical similarity, the results presented in **Figures 2A, B** show qualitatively and quantitatively that no significant differences were observed in the endocytic properties of the two types of EVs. Both were effectively endocytosed. When MSCs were treated with control or TNF α EVs in the presence of growth medium, both EVs triggered a positive change in the expression

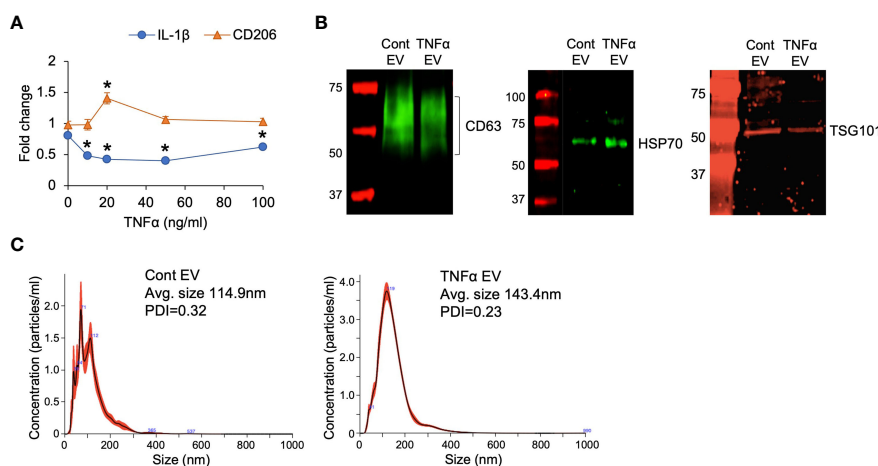


FIGURE 1 | EV characterization. **(A)** The effect of TNF α preconditioned MSC EVs on the expression of IL-1 β and CD206 in M1 polarized primary macrophages. Data is presented for two genes (IL-1 β and CD206) as representatives of M1 and M2 markers respectively. Fold change was calculated with respect to M1 macrophages in the absence of EVs. X-axis points refer to different concentrations of TNF α used for preconditioning. 20ng/ml was chosen as an ideal concentration for the subsequent experiments. *: statistical significance ($P < 0.05$) with respect to no EV control by Student's t-test. **(B)** Expression of EV markers CD63, HSP70 and TSG101 in naïve (control) and TNF α EVs. **(C)** NTA plots of control and TNF α EVs showing their size distribution.

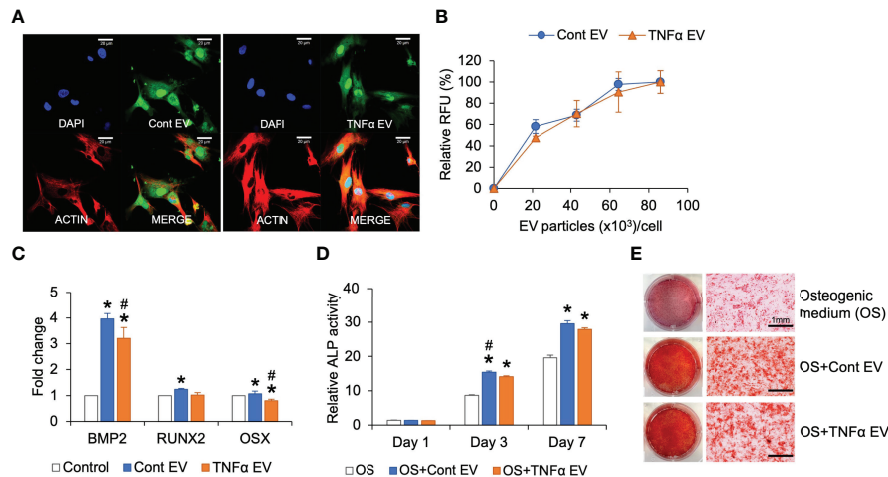


FIGURE 2 | Effect of TNF α EVs on MSC osteogenic differentiation. **(A)** Representative confocal images of fluorescently labeled control and TNF α EVs (green) endocytosed by MSCs. Scale bar=20 μ m in all images. The red staining in the images indicate actin counter stain and blue staining shows the nuclei (DAPI). **(B)** Dose-dependent and saturable endocytosis of fluorescently labeled control and TNF α EVs by MSCs. **(C)** Fold change in the expression of osteoinductive marker genes in MSCs in the presence of control and TNF α EVs (72hrs post treatment) in the presence of growth medium. Data represents fold change with respect to no EV controls. Note that TNF α preconditioning does not generate osteoinductive property in MSC EVs. **(D)** ALP activity in MSCs cultured in osteogenic differentiation medium in the presence/absence of control or TNF α EVs. Note that while EVs increase ALP activity in general, TNF α preconditioning does not increase osteoinductive potential. **(E)** Representative images of alizarin red stained MSC cultures after 14 days of culture in osteogenic medium in the presence/absence of EVs. Note that while EV presence increased calcium deposition, no change was observed between control and TNF α preconditioned groups. *: statistical significance ($P < 0.05$) with respect to untreated control, #: statistical significance ($P < 0.05$) between control and TNF α EV groups as calculated by Tukey's test post ANOVA.

level of osteogenic marker BMP2 with TNF α EVs less effective than control EVs. The changes effected by both types of EVs were statistically significant ($P < 0.05$, Tukey's test post ANOVA) with respect to untreated controls (* in **Figure 2C**). Little change was observed in the expression levels of RUNX2 and osterix (OSX) transcription factors. We also evaluated alkaline phosphatase (ALP) activity in MSCs under the influence of osteogenic differentiation medium in the presence/absence of control and TNF α EVs. Results presented in **Figure 2D** show that both EVs enhance ALP activity. However, the TNF α EVs were less effective than control EVs with the day 3 data point being statistically significant ($P < 0.05$, Tukey's test post ANOVA) and the day 7 results not statistically significant while showing a minor reduction with the TNF α EVs. Concurrently, MSCs cultured under osteogenic differentiation medium for 14 days in the presence/absence of control and TNF α EVs were stained with alizarin red to evaluate calcium deposition. While both EVs enhanced calcium deposition with respect to control, no differences were observed among the two types of EVs (**Figure 2E**). Overall, these experiments indicated that preconditioning of MSCs with TNF α did not significantly alter the osteoinductive properties of MSC EVs.

Effects of TNF α EVs on Macrophage Polarization

Results presented in **Figure 1A** showed that preconditioning MSCs with TNF α altered the immunomodulatory effects of the MSC EVs. To characterize this further, we evaluated the effects of TNF α EVs on polarized (M1 and M2) BMMs in comparison to

control EVs. As in the case of MSCs, we first evaluated the ability of control and TNF α EVs to be endocytosed by BMMs. Results presented in **Figures 3A, B** indicate that there is no significant difference between the two types of EVs with respect to endocytic properties. However, when M1 polarized macrophages were treated with control or TNF α EVs, the TNF α EVs had the anti-inflammatory effect of reducing the gene expression levels of IL-1 β , iNOS and TNF α significantly compared to control EVs (**Figure 3C**). The protein expression levels of IL-1 β , IL-12 and TNF α was also measured by multiplex ELISA for this experiment. Results presented in **Figure 3E** show that the TNF α EVs significantly reduced the expression levels of all three inflammatory cytokines with respect to controls (*) as well as control EVs (#).

BMMs were alternatively polarized to M2 phenotype and subjected to treatment with the control or TNF α EVs. Results presented in **Figure 3D** show that the TNF α EVs significantly enhanced the gene expression levels of M2 markers CD206, IL-10 and Arg1 with respect to both untreated controls (*) as well as control EVs (#). Protein expression levels were measured by multiplex ELISA and results presented in **Figure 3F** show that the anti-inflammatory cytokine IL-10 expression was significantly increased in TNF α EV group compared to control (*) and control groups (#). VEGF levels were significantly reduced with respect to control EV group although they remained at higher levels with respect to untreated controls.

Overall, the results from these studies showed that preconditioning of MSCs with TNF α did not affect the ability of the derivative EVs to be endocytosed by macrophages.

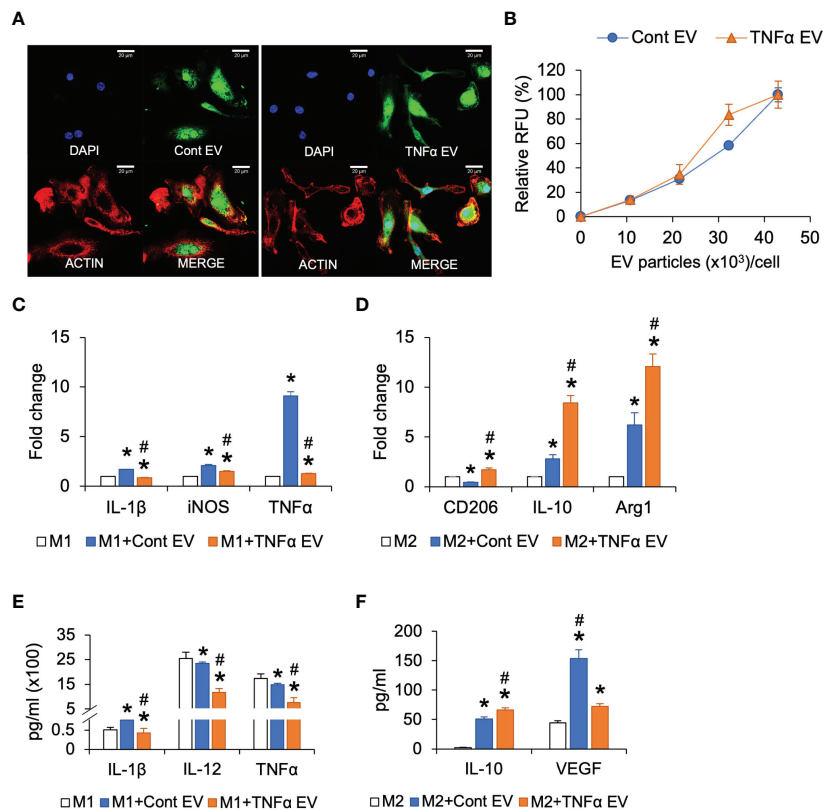


FIGURE 3 | Effect of TNF α EVs on macrophage polarization. **(A)** Representative confocal images of fluorescently labeled control and TNF α EVs (green) endocytosed by macrophages. Scale bar=20 μ m in all images. The red staining in the images indicate actin counter stain and blue staining shows the nuclei (DAPI). **(B)** Dose-dependent endocytosis of fluorescently labeled control and TNF α EVs by primary macrophages. **(C)** Fold change in gene expression of pro-inflammatory markers in M1 polarized macrophages in the presence/absence of EVs. Note that TNF α EVs significantly reduce the expression levels of IL-1 β , iNOS and TNF α indicating a reduction in M1 polarization. **(D)** Fold change in the expression levels of M2 marker genes in M2 polarized macrophages in the presence/absence of EVs. Note the significant increase in the expression levels of M2 markers CD206, IL-10 and Arg1 indicating an increase in M2 polarization. **(E)** Multiplex ELISA determined quantitative expression of pro-inflammatory cytokines in M1 polarized macrophages in the presence/absence of EVs. Note the significant reduction in cytokine expression in the presence of TNF α preconditioned MSC EVs. **(F)** Multiplex ELISA determined quantitative expression of IL-10 and VEGF in M2 polarized macrophages. *: statistical significance ($P < 0.05$) with respect to untreated control, #: represents statistical significance ($P < 0.05$) between control and TNF α EV groups as calculated by Tukey's test post ANOVA.

However, when compared to control EVs treatment of polarized macrophages, TNF α EVs treatment caused greater reduction in pro-inflammatory M1 markers and increased expression of anti-inflammatory and reparative M2 markers.

Immunomodulatory Effects of TNF α EVs *In Vivo*

Results presented in **Figure 3** indicated that TNF α preconditioning can reduce pro-inflammatory activity and enhance the anti-inflammatory/reparative activity of macrophages. We extended these *in vitro* observations by evaluating the ability of control and TNF α EVs to influence inflammation occurring *in vivo* in a rat calvarial defect model. For these experiments, we chose to evaluate the effects of the EVs at days 1, 3 and 7 post wounding qualitatively and quantitatively. The wounds were treated with collagen membranes containing the respective EVs or PBS and samples were harvested at days 1, 3 and 7 post wounding. Paraffin embedded samples were

immunoassayed for iNOS as a pro-inflammatory marker (M1-like) and Arg1 as an anti-inflammatory marker (M2-like). Results presented in **Figure 4A** indicate a reduced number of iNOS positive cells in the TNF α EV group compared to untreated (PBS) and control EV group. Quantitation of number of cells per field of view across 5 samples is presented in **Figures 4B, C**. The results validate the *in vitro* observations and show a clear (30–90%) reduction in iNOS positive cells and an increase (>200%) in Arg1 positive cells over the treatment period. The gene expression levels of IL-1 β and TNF α were also evaluated by qRT-PCR from the samples ($n=4$). Results are presented as log 10 fold change over untreated control at day 1 for both genes. Expression levels depicted in **Figures 4D, E** show a robust reduction in the expression levels of IL-1 β and TNF α in TNF α EV group compared to both untreated controls (*) as well as naïve MSC EV group (#).

Published studies have shown the relationship between inflammation and bone regeneration and the effects of the

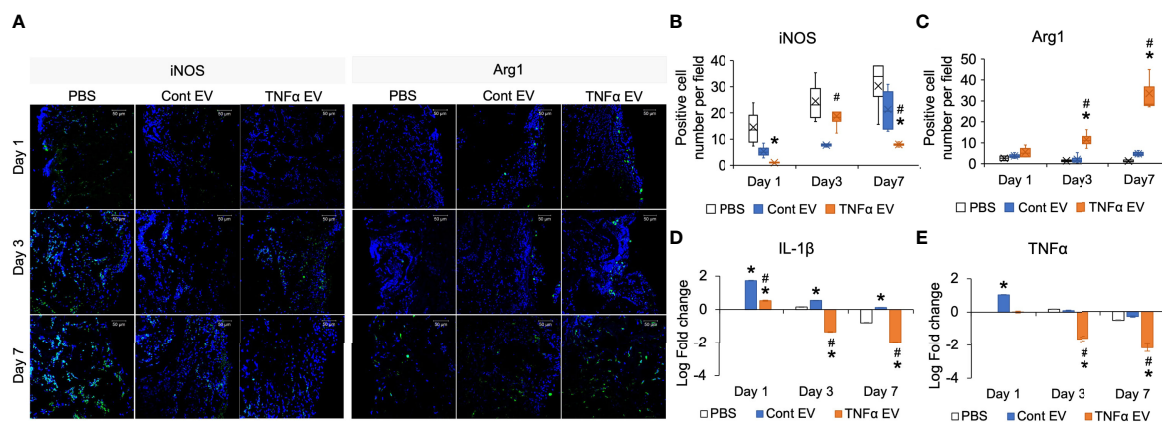


FIGURE 4 | Modulation of *in vivo* inflammatory response by TNF α EVs. **(A)** Representative confocal images showing IHC staining for pro-inflammatory (M1) marker iNOS (green) and anti-inflammatory (M2) marker Arg1 (green) in tissue sections from rat calvarial defects 1-, 3- and 7-days post wounding. Scale bar=50 μ m in all images. Note the reduced presence of iNOS positive and increased presence of Arg1 positive cells in the TNF α EV group compared to controls. **(B, C)** Image J based quantitation of number of positive cells per field of view for iNOS and Arg1 expression (n = 5). Note the significant reduction in iNOS positive cells and increase in Arg1 positive cells. **(D, E)** Gene expression of IL-1 β and TNF α respectively in tissue samples of calvarial wounds 1, 3 and 7 days post wounding. Data are represented as log 10 of fold change. Note the significant drop in the expression of these pro-inflammatory cytokines in the presence of TNF α EVs with respect to controls. *: statistical significance ($P < 0.05$) with respect to untreated control, #: statistical significance ($P < 0.05$) between control and TNF α EV groups as calculated by Tukey's test post ANOVA.

secretome of macrophages on MSC differentiation and osteoinduction (27, 28). One of the factors secreted by macrophages that positively influences bone repair is oncostatin M (OSM) (29). Therefore, to evaluate if, apart from controlling the expression of inflammatory markers, TNF α EVs also influence the expression of OSM, we quantified OSM

expression by immunoassay of days 1, 3 and 7 sections for OSM. Results presented in **Figure 5A, B** show that TNF α EVs triggered a robust increase in the expression levels of OSM *in vivo*. This was also verified in *in vitro* experiments in macrophages polarized to M0, M1 and M2 phenotypes in the presence/absence of control or TNF α EVs (**Figure 5C**).

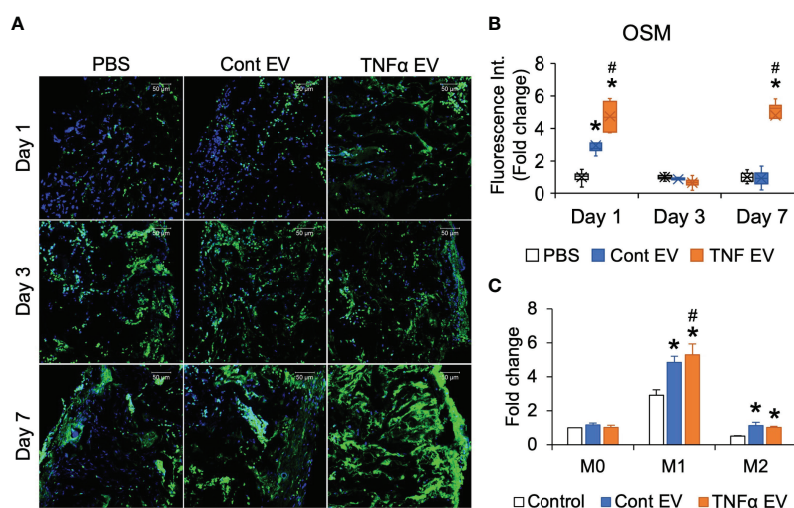


FIGURE 5 | OSM expression is influenced by TNF α EVs. **(A)** Representative confocal micrographs of 1-, 3- and 7-day calvarial sections immunoassayed for OSM (green). Scale bar=50 μ m in all images. **(B)** Image J based quantitation of fluorescence intensity in tissue sections (n = 5). Data are presented as fold change in intensity with respect to untreated (PBS) controls. Note the significant increase in OSM intensity in the TNF α EV group compared to controls. *: statistical significance ($P < 0.05$) with respect to untreated control, #: statistical significance ($P < 0.05$) between control and TNF α EV groups as calculated by Tukey's test post ANOVA. **(C)** Fold change in OSM expression in primary macrophages under naïve (M0), M1 and M2 polarization conditions in the presence/absence of control and TNF α EVs. Data are presented as fold change over M0 control (no EV) OSM expression. *: statistical significance ($P < 0.05$) with respect to no EV control in each polarization condition, #: statistical significance ($P < 0.05$) between control and TNF α EV groups as calculated by Tukey's test post ANOVA.

Overall, these *in vivo* results corroborated our *in vitro* observations and show that TNF α preconditioning enhances the positive immunomodulatory effects of MSC EVs by reducing the expression of pro-inflammatory markers and enhancing the expression of reparative anti-inflammatory markers.

Effect of TNF α EVs on Bone Repair *In Vivo*

To evaluate if the immunomodulatory effects observed at the shorter time points translated to increased bone repair at later time points, we evaluated the effects of the two types of EVs in the calvarial defect model after 4- and 8-weeks post wounding. Quantitative μ CT analysis of the calvarial samples indicated that TNF α EVs enhanced bone repair at 4 and 8 weeks (greater than 2-fold at 8 weeks; **Figures 6A, B**). Confirmatory histology of the decalcified samples revealed increased bone formation in the TNF α EV group at both time points (**Figure 6C**). The sections were also immunoassayed for the expression of BMP2, bone sialoprotein (BSP) and dentin matrix protein 1 (DMP1). Results presented in **Figure 7A** and quantified in **Figures 7B–D** indicate that while both control and TNF α EVs both triggered an increase in the expression of all three osteoinductive proteins, no significant differences were observed in expression levels between the two groups. Overall, these results indicate that the immunomodulatory effects of TNF α EVs translate to enhanced healing of calvarial wounds in wild type rats.

MiRNA Composition of TNF α EVs

In prior studies, MSC EV functionality has been directly linked to its miRNA composition (30). We sequenced both control and

TNF α EVs to identify differentially expressed miRNAs that might contribute towards the EVs altered functionality. The heat map presented in **Figure 8A** shows the differentially regulated miRNAs in the TNF α EVs compared to control EVs. The corresponding KEGG analysis highlights that the change in miRNA composition accounts for multiple pathways that involve immunomodulation, cancer biology, cell cycle and cell survival (**Figure 8B**).

DISCUSSION

Tissue engineering of bone using MSCs has its foundations based on the observations that MSCs give rise to osteoprogenitor cells (31) and their transplantation into large segmental defects supports bone regeneration (32). The mechanisms affecting MSC-mediated bone regeneration include the role of the MSC secretome in paracrine regulation of bone regeneration, particularly with respect to immunomodulatory function. Among the included secretome elements, exosomes or EVs have emerged as alone being able to reproduce many of the MSC-mediated effects in tissue regeneration (33). MSC EVs are emerging as potential regenerative tools in tissue engineering (34, 35).

MSC EVs have been used in many tissues for regeneration with varying levels of success. This generalized promotion of regeneration may reflect a more central role of the MSC in immunomodulation (9). In fact, MSCs role in immunomodulation is widely recognized and the MSC EV are

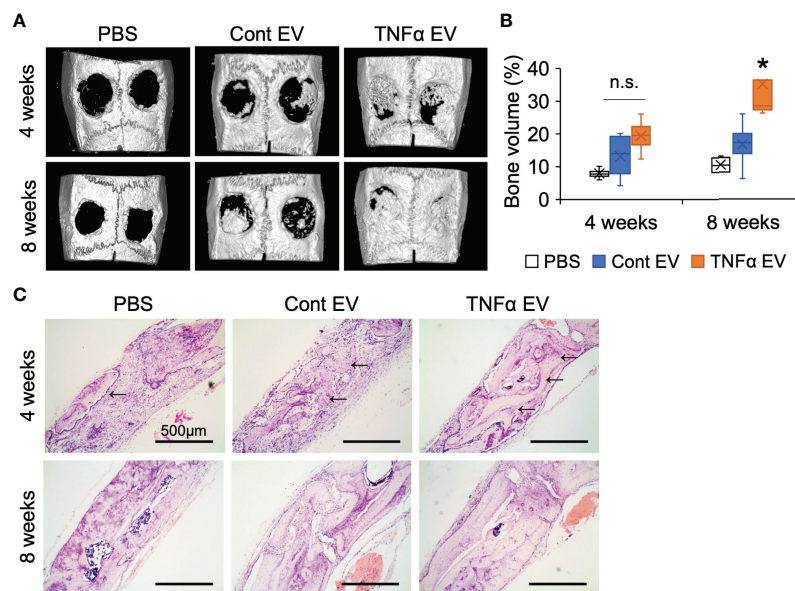


FIGURE 6 | Bone regeneration is influenced by TNF α EVs. **(A)** Representative 3D μ CT images of rat calvarial defects 4- and 8-weeks post wounding. Note the increased presence of bone in EV groups compared to control and the increased bone volume in the TNF α EV group compared to the other two. **(B)** volumetric quantitation of regenerated bone represented as percentage of BV/TV. Note the significant increase in bone volume at 8 weeks in the TNF α EV group compared to controls. n.s., Not significant; *: statistical significance ($P < 0.05$) with respect to PBS and control EV group as calculated by Tukey's test post ANOVA. **(C)** Representative micrographs of calvarial defect sections stained with H&E. Black arrows point to newly formed bone in the defect. Scale bar=500 μ m.

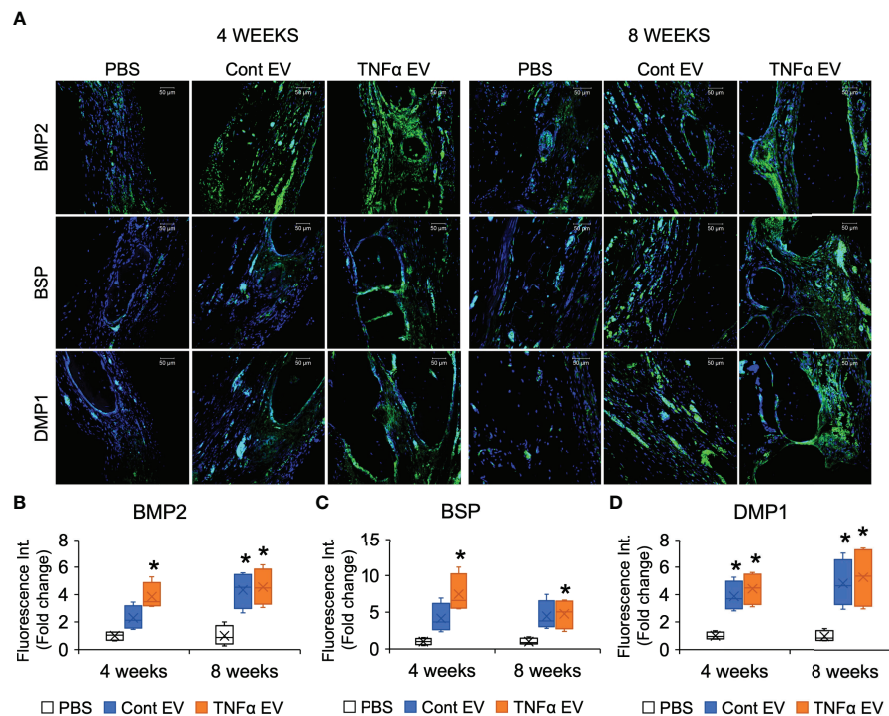


FIGURE 7 | Expression of osteogenic markers in rat calvarial defects. **(A)** Representative confocal micrographs representing the expression of osteogenic markers BMP2 (green), BSP (green) and DMP1 (green) in decalcified calvarial sections 4- and 8- weeks post wounding. Scale bar=50 μ m in all sections. **(B–D)** are Image J based quantitation of fluorescence intensity of BMP2, BSP and DMP1 in the sections (n = 5) represented as fold change with respect to untreated (PBS) control. *: statistical significance ($P < 0.05$) with respect to PBS controls calculated by Tukey's test post ANOVA. Note that while the EV treated groups showed increased expression compared to PBS control, no significant difference was observed in expression intensity between control and TNF α EVs.

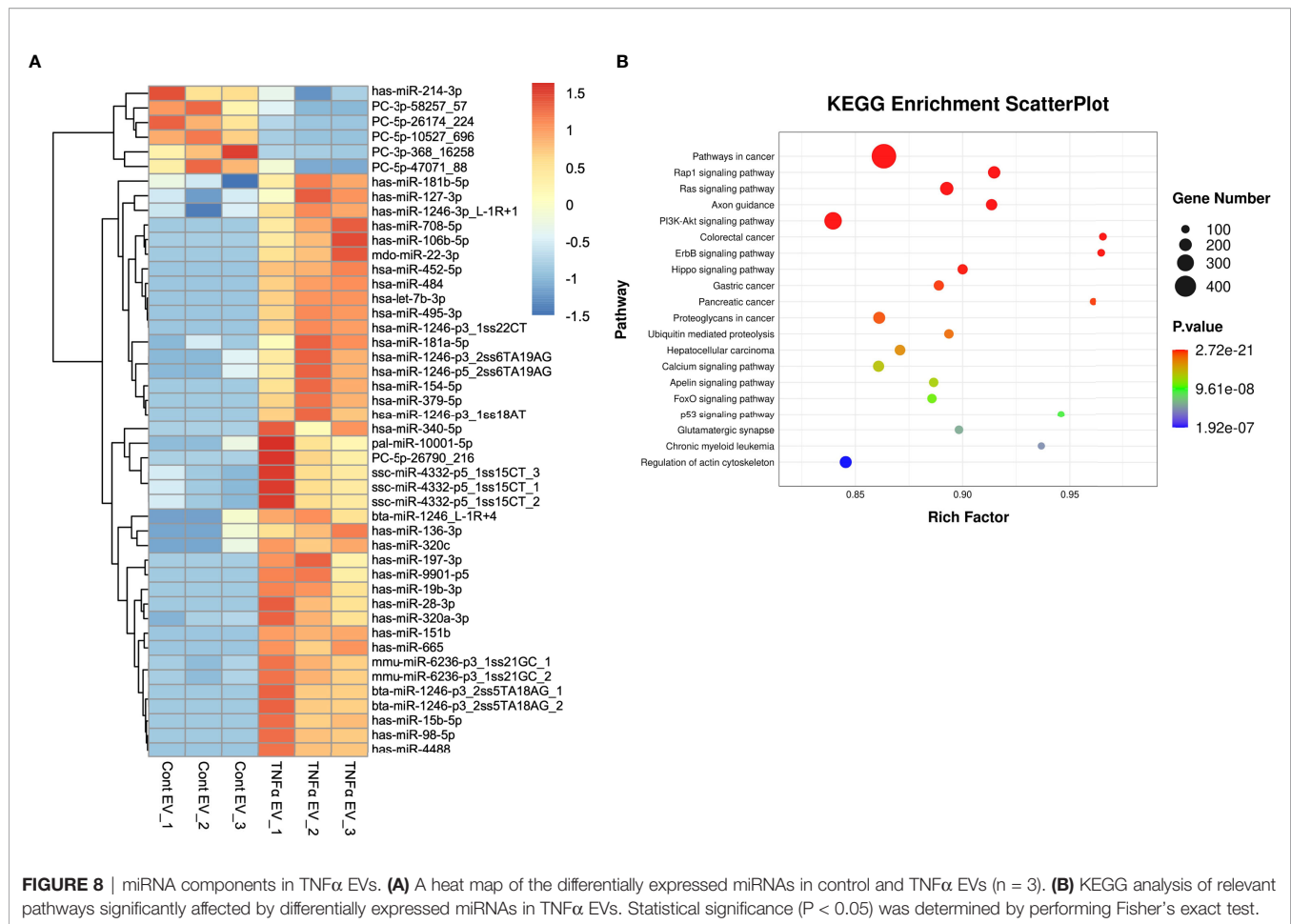
able to influence immune cell function (36). More notably, MSCs and their EVs are able to influence macrophage polarization, a phenomenon well known as a central mediator of wound healing and regeneration (37).

The process of wound healing and regeneration involves the establishment of an early tissue comprised of an immature extracellular matrix containing abundant immune cells including macrophages. These macrophages appear as pro-inflammatory (M1-like) cells that function to direct the important inflammatory steps in early wound healing. TNF α is among the pro-inflammatory cytokines expressed early in the process by macrophages. It is in this environment that MSCs are recruited to sites for wound healing and regeneration. Concomitant with MSCs populating this tissue, macrophage polarization pivots toward a regenerative (M2) phenotype. The MSC is able to affect this immunomodulatory macrophage polarization switch in cell culture (28) and we proposed that MSC exposure to TNF α supports the MSC immunomodulatory function.

The present study demonstrates that TNF α preconditioning of MSCs alters the immunomodulatory effects of MSC EVs on primary macrophages. This is consistent with other studies that have demonstrated that MSC EVs are able to influence macrophage polarization [reviewed in (37)] and that TNF α treatment of gingival MSCs resulted in greater anti-inflammatory effects and a reduction in periodontal bone loss

in the mouse model (38). Here, despite present cell culture studies that indicated TNF α EVs did not enhance osteoinduction in MSC cultures compare to control EVs, TNF α EVs treatment of calvaria defects significantly increased bone formation at 4 and 8 weeks. This increased bone formation was associated with the reduction in M1 macrophages and increase in M2-like macrophages in these treated tissues during the pro-inflammatory, formative period of 3 to 7 days during regeneration. A recent study demonstrated that the treatment of calvaria defects with M2 macrophage EVs promoted bone regeneration to greater extent than naïve and M1 macrophage EVs (28).

Preconditioning has been examined in the present study at the level of miRNA cargo of MSC EVs. Importantly, preconditioning has been shown to alter the primary metabolites and proteins associated with anti-inflammatory and immunoregulatory functions (15). Preconditioning of cultured MSCs is now recognized as one approach to improving MSC functions *in vivo*. Hypoxia is recognized as an injury-related condition that when used to precondition MSCs leads to increased engraftment, angiogenesis and regenerative function (39). This study further underscores those factors such as TNF α in wounded tissues promote expression of MSC EVs with regenerative features. MSC preconditioning is considered an important approach to improving cultured MSC activity (40).



The use of TNF α preconditioning may have selected advantages over other methods of altering MSC-derived EV function. TNF α preconditioning does not appear to affect the general biophysical properties of the EVs which may account for their similar endocytosis when compared to naïve MSC EVs. One possible advantage of preconditioned MSC EVs is that preconditioning may be part of a cell culture strategy that minimizes variation from primary native MSCs. Another advantage of preconditioning of MSCs is that it increases the consistency of EV cargo as demonstrated at the protein and metabolite level as well as the miRNA level (41). Overexpression of a single miRNA affects a single miRNA function whereas preconditioning with cytokine or morphogen more comprehensively alters the miRNA content of the MSC EVs.

The present results indicate that MSC EVs treatment of the calvaria defect promotes bone regeneration by an indirect mechanism affecting macrophage polarization and that the resulting change in macrophage polarization precedes and influences subsequent and enhanced bone repair. Macrophage OSM expression was observed in these healing tissues and is indicative of one osteoinductive signal produced by macrophages that can be increased by macrophage exposure to TNF α EVs. Preliminary studies indicated that macrophages are essential for calvaria bone regeneration within these same collagen scaffolds.

The reduction of monocytes using either clodronate liposome treatment in wild type mice or ablation of monocytes in MaFIA mice treated with AP20187 is associated with the marked reduction in calvaria bone regeneration (**Supplementary Figure 1**). This is consistent with reports of the role for osteomacs in fracture repair (42).

The control of macrophage plasticity ranging from M1 to M2 phenotypes is a critical process in the immunomodulatory control of bone regeneration (43). In the present investigation, TNF α EVs treatment of calvaria wounds was associated with a marked switch from an M1 predominant to an M2 predominant regenerative environment at the early times of healing. M1 macrophage-elaborated factors such as OSM assist in promoting osteogenesis (29, 43) and M2 macrophage-elaborated factors promote ongoing osseous regeneration (44).

When TNF α treated human gingiva derived MSC EVs were investigated, the preconditioning increased exosome number, induced M2 macrophage expression in a CD73-dependent manner and protected alveolar bone from ligature-induced periodontal bone loss. The TNF α preconditioning was associated with significant changes in the EVs miRNA cargo (38). Another study involving adipose stem cells demonstrated that exosomes isolated from cells preconditioned with TNF α promoted human primary osteoblastic cell proliferation and differentiation (45).

Our miRNA sequencing results show a significant change in the miRNA composition of the preconditioned MSC EVs. It is possible that a subset of these altered miRNA contributes to the enhanced immunomodulatory function. In preliminary analyses of some of the highly expressed miRNAs that are also differentially expressed, candidates such as miR-15b (46), miR-19b (47) and miR-22 (48, 49) stand out due to their established roles in immunomodulation. For example, miR-22 is a negative regulator of the NLRP3 inflammasome pathway (50) and has established anti-inflammatory function (50, 51). KEGG pathway analysis also indicated significant changes in pathways related to cancer, PI3K-Akt as well as the FoxO signaling pathways. Cancer related pathways are primarily immunomodulatory in nature as are the PI3K-Akt and the FoxO signaling cascades (52). These results indicate that the miRNA EV cargo shifts towards an immunomodulatory role when MSCs are preconditioned with TNF α . Ongoing studies continue to investigate more broadly the inflammation preconditioning of MSC EVs. Such studies are aimed at further defining the role of EVs and their miRNA cargo in the immunoregulatory effects of MSCs on tissue regeneration and repair.

We have observed an indirect effect of MSC EVs acting on macrophages to promote osteogenesis that may be complex in mechanism. Macrophages influence osteoblasts directly by secreted cytokines included OSM, BMP, and others. Here we demonstrate that MSC EVs treatment of calvaria resulted in the increased expression of OSM in healing tissues. Other effects cannot be excluded. For example, calvaria defect treatment with exosomes promoted neovascularization in support of osteogenesis (53). While the preconditioning of MSCs also influences cytokine expression that affects macrophage polarization, the isolation of EVs from conditioned media likely excludes cytokine effects in the present study; studies comparing EV depleted conditioned media with EVs demonstrate the effects of EVs on target cells is distinct from the EV depleted media (54). While TNF α EVs may interact with diverse cell types *in vivo*, the present cell culture studies discount a direct EV-mediated effect on osteoprogenitor cells in the calvaria.

In conclusion, the TNF α preconditioning of human MSCs results in EVs able to alter the macrophage phenotype *in vitro* and *in vivo*. While both control/naïve and TNF α EVs promote MSC osteoinduction at similar levels, the TNF α EVs increase bone regeneration in calvaria in a process that is accompanied by significant immunomodulation represented by an increase in the M2 macrophage population and suppression of M1 inflammatory cytokine production. Given the present observations, the TNF α preconditioned MSC EV's promotion of bone regeneration is indirect and dependent on their immunomodulatory function in the regeneration process.

REFERENCES

- Koh TJ, DiPietro LA. Inflammation and Wound Healing: The Role of the Macrophage. *Expert Rev Mol Med* (2011) 13:e23. doi: 10.1017/S1462399411001943
- Osta B, Benedetti G, Miossec P. Classical and Paradoxical Effects of TNF- α on Bone Homeostasis. *Front Immunol* (2014) 5:48. doi: 10.3389/fimmu.2014.00048

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/>, accession ID: PRJNA814986.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Illinois Chicago Animal Care Committee (UIC Animal Welfare Assurance No. A3460-01).

AUTHOR CONTRIBUTIONS

MK planned and executed the immunomodulatory components of this manuscript. C-CH planned and executed the bone regeneration components of this manuscript. SS performed *in vitro* experiments with respect to MSC differentiation and assisted in animal surgery. YL performed the EV characterization and some *in vitro* experiments. PG performed all the animal surgeries. SR and LC conceptualized, supervised and wrote the manuscript together. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Institute of Dental and Craniofacial Research (R01DE027404, R01DE030495).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.878194/full#supplementary-material>

Supplementary Figure 1 | Macrophage depletion leads to impair bone healing.

(A) Normalized cell number of F4/80-CD11b double positive cells by flow cytometry analysis. To deplete macrophages, clodronate liposome (Liposoma, clodronateliposomes.com) was administrated via intraperitoneal injection as per manufacture's recommended protocol up to 2 weeks. First administration was performed 3 days prior to ensure depletion of macrophages upon the surgery. The blood samples were collected retro-orbitally at day 4 and day 14 of clodronate liposome administration. *: statistical significance ($P < 0.05$) with respect to control calculated by Student's t-test ($n = 3$). **(B)** Representative 3D μ CT images of mouse calvarial defects at 8 weeks post wounding. Unilateral 3.5mm diameter defect was created using a trephine burr and recombinant BMP2 (500ng) soaked collagen scaffold was placed onto the defect. Note the impaired bone healing in clodronate group.

- Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Cruceta J, Graves BD, et al. Impaired Intramembranous Bone Formation During Bone Repair in the Absence of Tumor Necrosis Factor-Alpha Signaling. *Cells Tissues Organs* (2001) 169:285–94. doi: 10.1159/000047893
- Krum SA, Chang J, Miranda-Carboni G, Wang C-Y. Novel Functions for Nfkb: Inhibition of Bone Formation. *Nat Rev Rheumatol* (2010) 6:607–11. doi: 10.1038/nrrheum.2010.133

5. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF Receptor Superfamilies: Integrating Mammalian Biology. *Cell* (2001) 104:487–501. doi: 10.1016/s0092-8674(01)00237-9
6. Zhang E, Miramini S, Patel M, Richardson M, Ebeling P, Zhang L. Role of TNF- α in Early-Stage Fracture Healing Under Normal and Diabetic Conditions. *Comput Methods Prog BioMed* (2022) 213:106536. doi: 10.1016/j.cmpb.2021.106536
7. Phinney DG, Prockop DJ. Concise Review: Mesenchymal Stem/Multipotent Stromal Cells: The State of Transdifferentiation and Modes of Tissue Repair—Current Views. *Stem Cells* (2007) 25:2896–902. doi: 10.1634/stemcells.2007-0637
8. Samsonraj RM, Raghunath M, Nurcombe V, Hui JH, van Wijnen AJ, Cool SM. Concise Review: Multifaceted Characterization of Human Mesenchymal Stem Cells for Use in Regenerative Medicine. *Stem Cells Transl Med* (2017) 6:2173–85. doi: 10.1002/sctm.17-0129
9. Prockop DJ, Oh JY. Mesenchymal Stem/Stromal Cells (MSCs): Role as Guardians of Inflammation. *Mol Ther* (2012) 20:14–20. doi: 10.1038/mt.2011.211
10. Hess K, Ushmorov A, Fiedler J, Brenner RE, Wirth T. TNF α Promotes Osteogenic Differentiation of Human Mesenchymal Stem Cells by Triggering the NF- κ B Signaling Pathway. *Bone* (2009) 45:367–76. doi: 10.1016/j.bone.2009.04.252
11. Glass GE, Chan JK, Freidin A, Feldmann M, Horwood NJ, Nanchahal J. TNF- α Promotes Fracture Repair by Augmenting the Recruitment and Differentiation of Muscle-Derived Stromal Cells. *Proc Natl Acad Sci USA* (2011) 108:1585–90. doi: 10.1073/pnas.1018501108
12. Kotake S, Nanke Y. Effect of Tnf α on Osteoblastogenesis From Mesenchymal Stem Cells. *Biochim Biophys Acta* (2014) 18403:1209–13. doi: 10.1016/j.bbagen.2013.12.013
13. Mountziaris PM, Lehman ED, Mountziaris I, Sing DC, Kasper FK, Mikos AG. Effect of Temporally Patterned TNF- α Delivery on *In Vitro* Osteogenic Differentiation of Mesenchymal Stem Cells Cultured on Biodegradable Polymer Scaffolds. *J Biomater Sci Polym Ed* (2013) 24:1794–813. doi: 10.1080/09205063.2013.803455
14. Phinney DG, Pittenger MF. Concise Review: MSC-Derived Exosomes for Cell-Free Therapy. *Stem Cells* (2017) 35:851–8. doi: 10.1002/stem.2575
15. Zhai M, Zhu Y, Yang M, Mao C. Human Mesenchymal Stem Cell Derived Exosomes Enhance Cell-Free Bone Regeneration by Altering Their miRNAs Profiles. *Adv Sci (Weinh)* (2020) 7:2001334. doi: 10.1002/adv.202001334
16. Lyu H, Xiao Y, Guo Q, Huang Y, Luo X. The Role of Bone-Derived Exosomes in Regulating Skeletal Metabolism and Extrasosseous Diseases. *Front Cell Dev Biol* (2020) 8:89. doi: 10.3389/fcell.2020.00089
17. Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Cruceta J, Graves BD, et al. Functionally Engineered Extracellular Vesicles Improve Bone Regeneration. *Acta Biomater* (2020) 109:182–94. doi: 10.1016/j.actbio.2020.04.017
18. Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Cruceta J, Graves BD, et al. Priming Approaches to Improve the Efficacy of Mesenchymal Stromal Cell-Based Therapies. *Stem Cell Res Ther* (2019) 10:131. doi: 10.1186/s13287-019-1224-y
19. Phan J, Kumar P, Hao D, Gao K, Farmer D, Wang A. Engineering Mesenchymal Stem Cells to Improve Their Exosome Efficacy and Yield for Cell-Free Therapy. *J Extracell Vesicles* (2018) 7:1522236. doi: 10.1080/20013078.2018.1522236
20. Huang C-C, Kang M, Narayanan R, DiPietro LA, Cooper LF, Gajendradreddy P, et al. Evaluating the Endocytosis and Lineage-Specification Properties of Mesenchymal Stem Cell Derived Extracellular Vesicles for Targeted Therapeutic Applications. *Front Pharmacol* (2020) 11:163. doi: 10.3389/fphar.2020.00163
21. Liu W, Li L, Rong Y, Qian D, Chen J, Zhou Z, et al. Hypoxic Mesenchymal Stem Cell-Derived Exosomes Promote Bone Fracture Healing by the Transfer of miR-126. *Acta Biomater* (2020) 103:196–212. doi: 10.1016/j.actbio.2019.12.020
22. Kim M, Shin DI, Choi BH, Min B-H. Exosomes From IL-1 β -Primed Mesenchymal Stem Cells Inhibited IL-1 β - and TNF- α -Mediated Inflammatory Responses in Osteoarthritic SW982 Cells. *Tissue Eng Regen Med* (2021) 18:525–36. doi: 10.1007/s13770-020-00324-x
23. Magne B, Lataillade J-J, Trouillas M. Mesenchymal Stromal Cell Preconditioning: The Next Step Toward a Customized Treatment For Severe Burn. *Stem Cells Dev* (2018) 27:1385–405. doi: 10.1089/scd.2018.0094
24. Zhang B, Yin Y, Lai RC, Tan SS, Choo ABH, Lim SK. Mesenchymal Stem Cells Secrete Immunologically Active Exosomes. *Stem Cells Dev* (2014) 23:1233–44. doi: 10.1089/scd.2013.0479
25. Ti D, Hao H, Tong C, Liu J, Dong L, Zheng J, et al. LPS-Preconditioned Mesenchymal Stromal Cells Modify Macrophage Polarization for Resolution of Chronic Inflammation via Exosome-Shuttled Let-7b. *J Transl Med* (2015) 13:308. doi: 10.1186/s12967-015-0642-6
26. Xu R, Zhang F, Chai R, Zhou W, Hu M, Liu B, et al. Exosomes Derived From Pro-Inflammatory Bone Marrow-Derived Mesenchymal Stem Cells Reduce Inflammation and Myocardial Injury via Mediating Macrophage Polarization. *J Cell Mol Med* (2019) 23:7617–31. doi: 10.1111/jcmm.14635
27. Kang M, Thalji G, Huang C-C, Shirazi S, Lu Y, Ravindran S, et al. Macrophage Control of Incipient Bone Formation in Diabetic Mice. *Front Cell Dev Biol* (2020) 8:596622. doi: 10.3389/fcell.2020.596622
28. Kang M, Thalji G, Huang C-C, Shirazi S, Lu Y, Ravindran S, et al. Bone Regeneration Is Mediated by Macrophage Extracellular Vesicles. *Bone* (2020) 141:115627. doi: 10.1016/j.bone.2020.115627
29. Guihard P, Danger Y, Brounais B, David E, Brion R, Delecun J, et al. Induction of Osteogenesis in Mesenchymal Stem Cells by Activated Monocytes/Macrophages Depends on Oncostatin M Signaling. *Stem Cells* (2012) 30:762–72. doi: 10.1002/stem.1040
30. Shirazi S, Huang C-C, Kang M, Lu Y, Ravindran S, Cooper LF. The Importance of Cellular and Exosomal miRNAs in Mesenchymal Stem Cell Osteoblastic Differentiation. *Sci Rep* (2021) 11:5953. doi: 10.1038/s41598-021-85306-2
31. Prockop DJ, Kota DJ, Bazhanov N, Reger RL. Evolving Paradigms for Repair of Tissues by Adult Stem/Progenitor Cells (MSCs). *J Cell Mol Med* (2010) 14:2190–9. doi: 10.1111/j.1582-4934.2010.01151.x
32. Bruder SP, Kraus KH, Goldberg VM, Kadiyala S. The Effect of Implants Loaded With Autologous Mesenchymal Stem Cells on the Healing of Canine Segmental Bone Defects. *J Bone Joint Surg Am* (1998) 80:985–96. doi: 10.2106/00004623-199807000-00007
33. Kumar P, Kandoi S, Misra R, Vijayalakshmi S, Rajagopal K, Verma RS. The Mesenchymal Stem Cell Secretome: A New Paradigm Towards Cell-Free Therapeutic Mode in Regenerative Medicine. *Cytokine Growth Factor Rev* (2019) 46:1–9. doi: 10.1016/j.cytogfr.2019.04.002
34. Huber J, Griffin MF, Longaker MT, Quarto N. Exosomes: A Tool for Bone Tissue Engineering. *Tissue Eng Part B Rev* (2021) 28:101–13. doi: 10.1089/ten.TEB.2020.0246
35. Tan SHS, Wong JRY, Sim SJY, Tjio CKE, Wong KL, Chew JRJ, et al. Mesenchymal Stem Cell Exosomes in Bone Regenerative Strategies-A Systematic Review of Preclinical Studies. *Mater Today Bio* (2020) 7:100067. doi: 10.1016/j.mtbio.2020.100067
36. Matheakakis A, Batsali A, Papadaki HA, Pontikoglou CG. Therapeutic Implications of Mesenchymal Stromal Cells and Their Extracellular Vesicles in Autoimmune Diseases: From Biology to Clinical Applications. *Int J Mol Sci* (2021) 22:10132. doi: 10.3390/ijms221810132
37. Arabpour M, Saghaadeh A, Rezaei N. Anti-Inflammatory and M2 Macrophage Polarization-Promoting Effect of Mesenchymal Stem Cell-Derived Exosomes. *Int Immunopharmacol* (2021) 97:107823. doi: 10.1016/j.intimp.2021.107823
38. Nakao Y, Fukuda T, Zhang Q, Sanui T, Shinjo T, Kou X, et al. Exosomes From TNF- α -Treated Human Gingiva-Derived MSCs Enhance M2 Macrophage Polarization and Inhibit Periodontal Bone Loss. *Acta Biomater* (2021) 122:306–24. doi: 10.1016/j.actbio.2020.12.046
39. Crisostomo PR, Wang Y, Markel TA, Wang M, Lahm T, Meldrum DR. Human Mesenchymal Stem Cells Stimulated by TNF- α , LPS, or Hypoxia Produce Growth Factors by an NF Kappa B- But Not JNK-Dependent Mechanism. *Am J Physiol Cell Physiol* (2008) 294:C675–682. doi: 10.1152/ajpcell.00437.2007
40. Hu C, Li L. Preconditioning Influences Mesenchymal Stem Cell Properties *In Vitro* and *In Vivo*. *J Cell Mol Med* (2018) 22:1428–42. doi: 10.1111/jcmm.13492
41. Nakao Y, Fukuda T, Zhang Q, Sanui T, Shinjo T, Kou X, et al. Primed Mesenchymal Stem Cells Package Exosomes With Metabolites Associated With Immunomodulation. *Biochem Biophys Res Commun* (2019) 512:729–35. doi: 10.1016/j.bbrc.2019.03.119

42. Wu AC, Raggatt LJ, Alexander KA, Pettit AR. Unraveling Macrophage Contributions to Bone Repair. *BoneKEy Rep* (2013) 2:1–7. doi: 10.1038/bonekey.2013.107
43. Schlundt C, Fischer H, Bucher CH, Rendenbach C, Duda GN, Schmidt-Bleek K. The Multifaceted Roles of Macrophages in Bone Regeneration: A Story of Polarization, Activation and Time. *Acta Biomater* (2021) 133:46–57. doi: 10.1016/j.actbio.2021.04.052
44. Katagiri W, Takeuchi R, Saito N, Suda D, Kobayashi T. Migration and Phenotype Switching of Macrophages at Early-Phase of Bone-Formation by Secretomes From Bone Marrow Derived Mesenchymal Stem Cells Using Rat Calvaria Bone Defect Model. *J Dent Sci* (2022) 17:421–9. doi: 10.1016/j.jds.2021.08.012
45. Lu Z, Chen Y, Dunstan C, Roohani-Esfahani S, Zreiqat H. Priming Adipose Stem Cells With Tumor Necrosis Factor-Alpha Preconditioning Potentiates Their Exosome Efficacy for Bone Regeneration. *Tissue Eng Part A* (2017) 23:1212–20. doi: 10.1089/ten.tea.2016.0548
46. Zhao T, Jin Q, Kong L, Zhang D, Teng Y, Lin L, et al. microRNA-15b-5p Shuttled by Mesenchymal Stem Cell-Derived Extracellular Vesicles Protects Podocytes From Diabetic Nephropathy via Downregulation of VEGF/PDK4 Axis. *J Bioenerg Biomembr* (2022) 54:17–30. doi: 10.1007/s10863-021-09919-y
47. Zhao T, Jin Q, Kong L, Zhang D, Teng Y, Lin L, et al. miR-19b Downregulates Intestinal SOCS3 to Reduce Intestinal Inflammation in Crohn's Disease. *Sci Rep* (2015) 5:10397. doi: 10.1038/srep10397
48. Youn GS, Park JK, Lee CY, Jang JH, Yun SH, Kwon HY, et al. MicroRNA-22 Negatively Regulates LPS-Induced Inflammatory Responses by Targeting HDAC6 in Macrophages. *BMB Rep* (2020) 53(141):223–8. doi: 10.5483/BMBRep.2020.53.4.209
49. Li S, Liang X, Ma L, Shen L, Li T, Zheng L, et al. MiR-22 Sustains NLRP3 Expression and Attenuates H. pylori-Induced gastric Carcinogenesis. *Oncogene* (2018) 37(7):884–96. doi: 10.1038/onc.2017.381
50. Boxberger N, Hecker M, Zettl UK. Dysregulation of Inflammasome Priming and Activation by MicroRNAs in Human Immune-Mediated Diseases. *J Immunol* (2019) 202:2177–87. doi: 10.4049/jimmunol.1801416
51. Tezcan G, Martynova EV, Gilazieva ZE, McIntyre A, Rizvanov AA, Khaiboullina SF. MicroRNA Post-Transcriptional Regulation of the NLRP3 Inflammasome in Immunopathologies. *Front Pharmacol* (2019) 10:451. doi: 10.3389/fphar.2019.00451
52. Kraehenbuehl L, Weng C-H, Eghbali S, Wolchok JD, Merghoub T. Enhancing Immunotherapy in Cancer by Targeting Emerging Immunomodulatory Pathways. *Nat Rev Clin Oncol* (2022) 19:2177–87. doi: 10.1038/s41571-021-00552-7
53. Liang B, Liang J-M, Ding J-N, Xu J, Xu J-G, Chai Y-M. Dimethylxaloylglycine-Stimulated Human Bone Marrow Mesenchymal Stem Cell-Derived Exosomes Enhance Bone Regeneration Through Angiogenesis by Targeting the AKT/mTOR Pathway. *Stem Cell Res Ther* (2019) 10:335. doi: 10.1186/s13287-019-1410-y
54. Mathew B, Ravindran S, Liu X, Torres L, Chennakesavalu M, Huang C-C, et al. Mesenchymal Stem Cell-Derived Extracellular Vesicles and Retinal Ischemia-Reperfusion. *Biomaterials* (2019) 197:146–60. doi: 10.1016/j.biomaterials.2019.01.016

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Kang, Huang, Gajendrareddy, Lu, Shirazi, Ravindran and Cooper. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Exosome: The “Off-the-Shelf” Cellular Nanocomponent as a Potential Pathogenic Agent, a Disease Biomarker, and Neurotherapeutics

Satyajit Ghosh and Surajit Ghosh*

Department of Bioscience and Bioengineering, Indian Institute of Technology Jodhpur, Karwar, India

OPEN ACCESS

Edited by:

Gal Bitan,
University of California, Los Angeles,
United States

Reviewed by:

Livia Civitelli,
University of Oxford, United Kingdom
Eric Daniel Hamlett,
Medical University of South Carolina,
United States
Lynn Pulliam,
University of California, San Francisco,
United States

*Correspondence:

Surajit Ghosh
sghosh@iitj.ac.in

Specialty section:

This article was submitted to
Experimental Pharmacology and Drug
Discovery,
a section of the journal
Frontiers in Pharmacology

Received: 17 February 2022

Accepted: 19 April 2022

Published: 24 May 2022

Citation:

Ghosh S and Ghosh S (2022)
Exosome: The “Off-the-Shelf” Cellular
Nanocomponent as a Potential
Pathogenic Agent, a Disease
Biomarker, and Neurotherapeutics.
Front. Pharmacol. 13:878058.
doi: 10.3389/fphar.2022.878058

Exosomes are nanosized “off-the-shelf” lipid vesicles released by almost all cell types and play a significant role in cell–cell communication. Exosomes have already been proven to carry cell-specific cargos of proteins, lipids, miRNA, and noncoding RNA (ribonucleic acid). These vesicles can be selectively taken up by the neighboring cell and can regulate cellular functions. Herein, we have discussed three different roles of exosomes in neuroscience. First, we have discussed how exosomes play the role of a pathogenic agent as a part of cell–cell communication and transmit pathogens such as amyloid-beta ($A\beta$), further helping in the propagation of neurodegenerative and other neurological diseases. In the next section, the review talks about the role of exosomes in biomarker discovery in neurological disorders. Toward the end, we have reviewed how exosomes can be harnessed and engineered for therapeutic purposes in different brain diseases. This review is based on the current knowledge generated in this field and our comprehension of this domain.

Keywords: exosome, disease biomarker, cell-cell communication, neurotherapeutics, exosome engineering, pathogenic agent

1 INTRODUCTION

Exosomes, saucer-shaped vesicles of approximately 30–100 nm diameter (Théry et al., 2002b), are one of the different types of “extracellular vesicle” (EV) that are delimited by a lipid bilayer and are naturally released from the cell and play major roles in cell–cell communications. These vesicles are of endosomal origin, cannot replicate, i.e., do not contain a functional nucleus, and float at a density of 1.13–1.19 g ml⁻¹ in sucrose gradients (Théry et al., 2018). The process of exosome release can be divided into three steps: exosome biogenesis, intracellular movement of multivesicular bodies (MVBs), and MVB fusion with the plasma membrane. In the first step of exosome biogenesis (Figure 1), early endosomes are formed by inward invagination of the plasma membrane or from the trans-Golgi network. These early endosomes mature to form late endosomes. Invagination of the endosomal membrane into the lumen leads to the formation of intraluminal vesicles (ILVs), which finally leads to the generation of MVBs. Lastly, the generated MVBs will fuse with the plasma membrane or alternatively with lysosomes or autophagosomes. The former results in the release of the exosome, and the latter results in the degradation of MVBs. Several molecules are involved in this complicated process, and the details are summarized elsewhere (Teng and Fussenegger, 2021).

Exosomes are not merely lipid vesicles; they also contain membrane-associated proteins, transmembrane proteins, mRNA, noncoding RNAs, and other cell-specific cargos. Exosomes are equipped with endosomal sorting complex required for transport (ESCRT), Alix, TSG101,

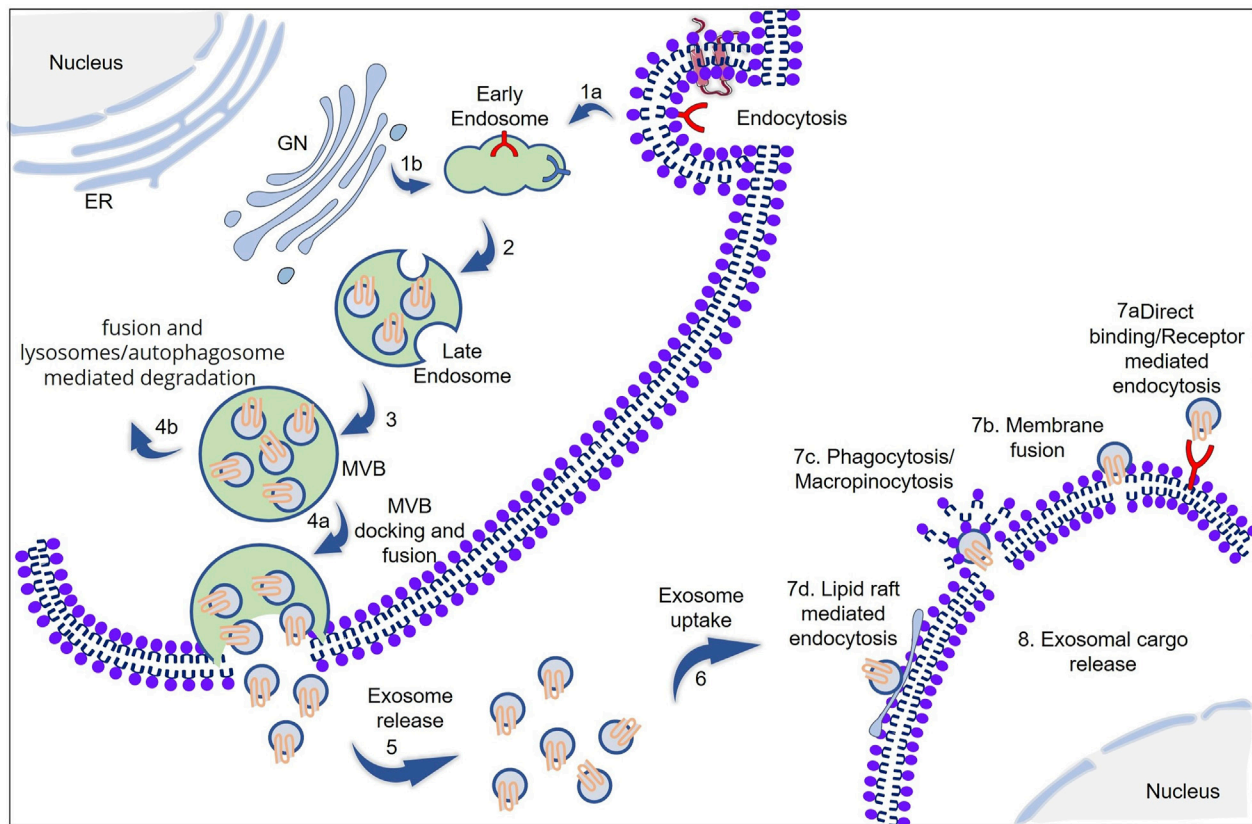
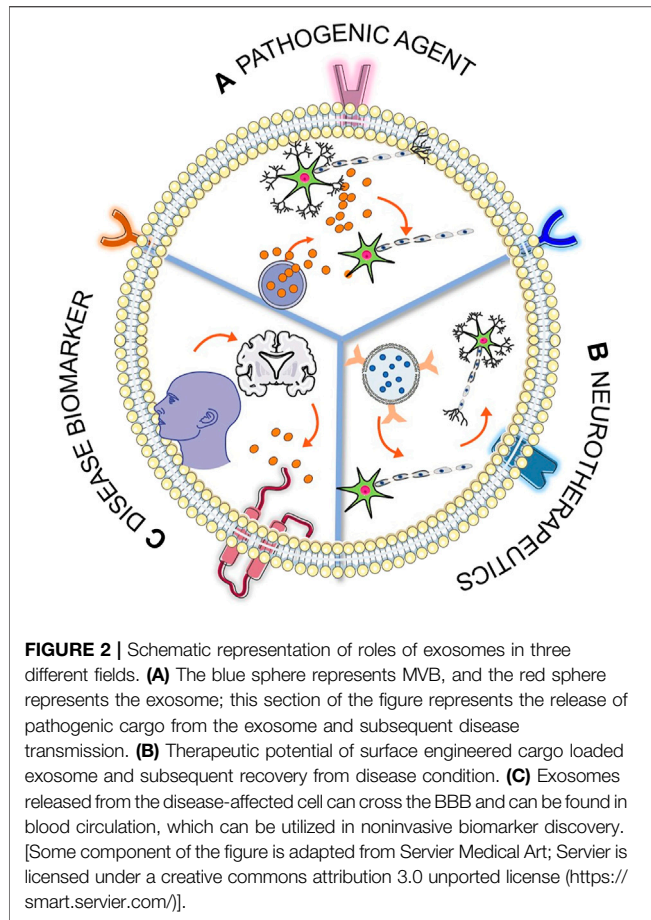


FIGURE 1 | Schematic representation of exosome biogenesis, cargo packing, and cellular uptake. **(1a,b)** Early endosomes are formed from the inward budding of the plasma membrane or Golgi network (GN). **(2,3)** Early endosomes mature to form late endosomes and subsequently to multivesicular bodies (MVB). **(4a)** MVB docking into the plasma membrane via SNAREs and SNAP23, and subsequent fusion with the plasma membrane results in exosome release **(5)**. Alternatively, MVBs can fuse with lysosome and subsequent degradation **(4b)**. **(6)** The released exosome will be taken up by the neighboring cell. The cellular uptake of exosomes can be **(7a)** direct binding and receptor-mediated endocytosis, **(7b)** membrane fusion, **(7c)** phagocytosis/micropinocytosis, or **(7d)** lipid raft-mediated endocytosis. The ESCRT machinery plays a key role in protein sorting, particularly for ubiquitinated cargos. During the process of exosome biogenesis, various proteins, including RNA-binding proteins (RBPs), are selectively sequestered into exosomes; these RBPs help in RNA cargo packing into the exosome. (ER: endoplasmic reticulum; GN: Golgi network; MVB: multivesicular bodies; ESCRT: endosomal sorting complex required for transport).

HSC70, CD63, CD81, and HSP90 β protein that help in the formation and release of exosomes. CD63 and CD81 (Theos et al., 2006; Stuffers et al., 2009) are tetraspanin family proteins that are thought to help in exosome formation and release by an ESCRT-independent mechanism. These proteins are enriched in exosomes compared to the cell lysate, and these are termed “exosomal marker proteins,” which can be identified using Western blot with appropriate antibodies (Thompson et al., 2016; Doyle and Wang, 2019). ExoCarta database hosts about 41,860 proteins, >7540 RNA, and 1,116 lipid molecules from more than 286 exosomal studies; this can help us to have an idea about the diversity of exosomal content from different cell types, current cell state (e.g., transformed, differentiated, stimulated, and stressed), and culture conditions (Keerthikumar et al., 2016). A different method of isolation of exosomes from condition media has been introduced, from the most commonly used methods ultra-centrifugation, sucrose gradient centrifugation, different kit-based to microfluidics-based methods (**Supplementary Table S1**) (Théry et al., 2006; Wu et al., 2017). The choice of exosome isolation

method greatly impacts the exosome quality and quantity (Patel et al., 2019; Brennan et al., 2020). The isolated exosomes can be characterized using nanoparticle tracking analysis (NTA) for size distribution and surface charge, transmission electron microscopy for size distribution and morphology (TEM), atomic force microscopy (AFM) for size distribution and surface morphology, and Western blotting to check the presence and absence of protein markers (Wu et al., 2015; Chopra et al., 2019). Experimental evidence and live-cell imaging (Sung et al., 2020) have already shown that exosomes are secreted by all cell types (Peters et al., 1989; Raposo et al., 1996; Théry et al., 2002a; Morelli et al., 2004) and brain cells like neurons, astrocytes, microglia, and oligodendrocytes are not the exception (Poticchio et al., 2005; Fauré et al., 2006; Krämer-Albers et al., 2007; Taylor et al., 2007). In the late 1980s, when exosome was first discovered, it was thought to be cellular waste resulting from cell damage or by-products of cell homeostasis and had no significant impact on neighboring cells (Johnstone et al., 1987). But currently, it is crystal clear that exosomes and their cargo can play a major role in cellular processes like in



immune response (Greening et al., 2015), signal transduction (Gangoda et al., 2015), antigen presentation (Mittelbrunn et al., 2011) as well as in disease state like chronic inflammation (Lässer et al., 2016), cardiovascular and renal diseases (Gonzalez-Calero et al., 2014), neurodegenerative diseases (Howitt and Hill, 2016), lipid metabolic diseases (Record et al., 2014), traumatic brain injury (Zhang et al., 2021), mental disorder (Saeedi et al., 2019), and tumors (Salem et al., 2016). It is now a well-established fact that exosomes can be found in almost all biological fluids like blood (Hornung et al., 2020), urine (Street et al., 2017), saliva (Machida et al., 2015), breast milk (Qin et al., 2016), cerebrospinal fluid (Yagi et al., 2017), semen (Madison et al., 2015), and amniotic fluid (Keller et al., 2007). Exosomes isolated from these fluids will reflect the cellular origin and its physiological state as a “fingerprint” or “signature” of the donor cell. From this, it is very clear that exosomes can be a potential target for biomarker discovery and early detection of many diseases. Apart from the role of exosomes in signal transduction and biomarker discovery, they can also be harnessed to be used as therapeutics in many diseases (Cooper et al., 2014; Zhuang et al., 2011). The ability to cross blood–brain barrier (BBB), non-immunogenicity, the option of surface engineering, and selective cargo packaging make exosomes emerge as a blockbuster therapeutic option in many diseases (Ghosh

et al., 2020; Zhan et al., 2020; Mishra et al., 2021). The probable roles that can be played by exosomes are schematically summarized in Figure 2.

2 EXOSOME AS A PATHOGENIC AGENT IN NEUROLOGICAL DISEASES

The cells in the central nervous system (CNS) communicate between themselves by intercellular and extracellular interactions. The former is mediated by ions and can be transduced and sensed by the cell through ion channels and neurotransmitter receptors present in neurons and glial cells (Yamazaki et al., 2007; Debanne and Rama, 2011). The latter could consist of either wiring transmission, which is primarily dependent on synapses or volume transmission, mediated by exosome for major vesicular carrier or by exocytosis of neurotransmitters (Trueta and De-Miguel, 2012; Borroto-Escuela et al., 2015). The secretion of exosomes from CNS cells was first demonstrated in cultured embryonic cortical neurons, and it can be released either presynaptically at the neuromuscular junction or postsynaptically by cortical neurons upon activation of synaptic NMDA receptors, which will then bind presynaptically to hippocampal neurons (Fauré et al., 2006; Zhang and Yang, 2018). Experimental evidence has also shown that neuron-derived exosomes are 50 times more abundant in soma and dendrites than axons in both peripheral nervous system (PNS) and CNS (Von Bartheld and Altick, 2011). In the brain, the exosomes act as local or distant messengers and communicators and can play a significant role in neural homeostasis, modulation of synaptic plasticity, synaptic transduction, modifying the cell surface properties of target cells, auto-protective mechanism for neurons, sequestering “toxic” (pathogenic) proteins, and promoting regeneration and neuroprotection both in the CNS and the PNS (Korkut et al., 2013; Lopez-Verrilli et al., 2013; Chivet et al., 2014; Kalani et al., 2014; Hornung et al., 2020). In addition to interneuronal communication, exosomes from neuronal culture when added to astrocyte culture have shown to have an effect in extracellular glutamate levels and modulation of synaptic activation (Morel et al., 2013). In the opposite case, when glial cell-derived exosomes are added to the neuronal culture, they significantly increase the firing rate of neurons and has a neuroprotective role under oxidative stress and starvation conditions (Smalheiser, 2007; Morel et al., 2013; Fröhlich et al., 2014). Apart from normal brain function, it is already a proven fact that exosomes have a role in disease progression and can act as a pathogen delivery agent (Hornung et al., 2020; Zhang et al., 2021). The different roles of exosomes in disease are as follows and are schematically represented in Figure 3.

2.1 Alzheimer’s Disease

Extracellular deposition of polymerized amyloid- β (A β) protein, also called plaques, and intracellular filamentous inclusions of hyperphosphorylated tau protein, known as neurofibrillary tangles, are the two major neuropathology involved in Alzheimer’s disease (AD) (Yamaguchi et al., 1989; Lee et al.,

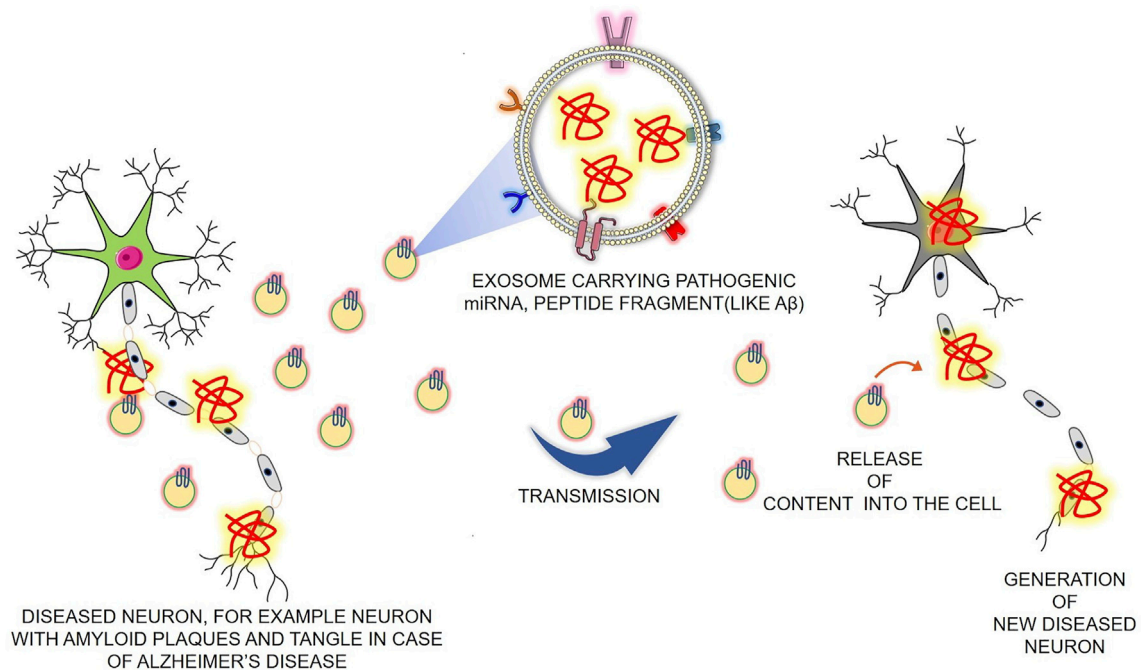


FIGURE 3 | Schematic representation of the role of exosomes as the pathogenic carrier in neurodegenerative disease. Pathogenic cargo-loaded exosomes are released from the diseased cell, and the cells that take up the exosomes get a similar kind of disease. This picture represents a similar phenomenon, taking an example of how exosomes can carry A β pathogenic peptides in their lumen and cause the propagation of Alzheimer's disease. (A β : amyloid-beta) (Some component of the figure is adapted from Servier Medical Art; Servier is licensed under a creative commons attribution 3.0 unported license (<https://smart.servier.com/>)).

1991). Several lines of evidence pointed toward the fact that the exosomes have a role in amyloid pathology in AD. Scientists have shown that in HeLa and N2a cells, after beta-cleavage of amyloid precursor protein (APP) in early endosomes, a minute fraction of A β peptides can be secreted from the cells in association with exosomes; not only that but also exosomal proteins have also been found to accumulate in the plaques of AD patients' brains (Rajendran et al., 2006). Intraperitoneal injection of GW4869, the moiety responsible for inhibition of neutral sphingomyelinase 2 (nSMase2) in the 5XFAD mouse, shows reduced levels of brain and serum exosomes, brain ceramide, and also reduces A β 1–42 plaque load. This result suggests that exosomes are involved in the generation of A β plaques (Dinkins et al., 2014). In recent years, it has been experimentally proven that exosomes isolated from AD brains contain elevated levels of amyloid-beta oligomers; these exosomes can act as vehicles for the neuron-to-neuron transfer of such toxic species. Inhibition of the formation, secretion, or uptake of exosomes has been found to reduce both the spread of oligomers and the related toxicity (Sinha et al., 2018). Recent experimental evidence indicates that soluble pre-fibrillar A β species are more toxic than insoluble fibrils (Ladiwala et al., 2012). Scientists have shown that the exosomes of microglial origin are strikingly high in AD patients and in subjects with mild cognitive impairment and are toxic for cultured neurons. Studies have also found that the neurotoxicity of these exosomes is due to the capability of exosome lipids to promote the formation of soluble A β

species and from the trafficking of neurotoxic A β *via* exosomes after A β got internalized into microglia (Joshi et al., 2014). Apart from A β , exosomes are also involved in tauopathy. A recent study shows much of the tau phosphorylated at Thr-181 is secreted by M1C cells and occurs *via* exosomal release (Saman et al., 2012). A group of scientists developed an adeno-associated virus-based model exhibiting rapid tau propagation from the entorhinal cortex to the dentate gyrus and has shown that microglia spread tau *via* exosomes secretion. Inhibiting exosome synthesis significantly reduces tau propagation *in-vitro* and *in-vivo* (Asai et al., 2015). Another group of scientists has discovered gambinol, an inhibitor of the neutral sphingomyelinase 2 (nSMase2) enzyme, and shown that gambinol works in a dose-dependent manner and suppresses extracellular vesicle (EV) production, which in turn reduce tau seed propagation (Bilousova et al., 2018). Apolipoprotein E (apoE) and bridging integrator-1 (Bin1), the genetic risk factors for late-onset AD (LOAD), are involved in exosome biogenesis and cargo sorting (Cohn et al., 2021). Experiments have shown that overexpression of BIN1 in PS19 mice promotes the release of Tau *via* extracellular vesicles (Crotti et al., 2019). On the other hand, the apolipoprotein E4 genotype is involved in the downregulation of exosome biosynthesis and release; this will lead to decreased elimination of materials from the endo-lysosomal system. The failure of the endo-lysosomal system will contribute to amyloidogenic amyloid- β precursor protein processing, compromise trophic signaling and synaptic

function, and interfere with a neuron's ability to degrade material, all of which will result in neuronal vulnerability and a higher risk of AD development (Peng et al., 2019).

2.2 Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disease (Lebouvier et al., 2009), which is characterized by the degenerative death of dopaminergic (DA) neurons in the substantia nigra, a significant decrease in striatal DA content, and the appearance of Lewy bodies due to the accumulation and aggregation of α -synuclein (α -syn) in the cytoplasm of residual nigrostriatal neurons (Spillantini et al., 1998). Experimental evidence has shown that α -syn is packaged into exosomes *via* the endosome pathway, and it can fuse with the plasma membrane for secretion as exosomal cargo with the assistance of VPS4 and SUMO proteins (Cabin et al., 2002; Lee et al., 2005). Recent studies suggest that exosomes provide favorable conditions for α -syn aggregate formation; this, in turn, promotes the propagation of PD pathology (Grey et al., 2015). Scientists have already discovered the presence of oligomeric α -syn in the exosome, which is readily taken up by the neighboring cell and is more toxic as compared to free α -syn (Danzer et al., 2012). In an experiment with an SH-SY5Y cell line, overexpressing α -syn has shown to have α -syn in isolated exosomes, and this can get transferred to normal SH-SY5Y cells (Alvarez-Erviti et al., 2011a). Later, it was also shown that the exosome-packed α -syn could promote the cell death of recipient neuronal cells. These experiments provide support to the hypothesis of exosome-mediated α -syn propagation between neurons and facilitate PD progression (Emmanouilidou et al., 2010). Recent research by a group of scientists suggested that the presence of α -syn oligomers in CD11b + exosomes of microglia origin can induce α -syn aggregation within neurons (Guo et al., 2020). Overall, it is very clear that exosomes can act as a pathogenic agent in PD propagation.

2.3 Frontotemporal Dementia and Amyotrophic Lateral Sclerosis

FTD involves progressive deficits in behavior, executive function, or language (Bang et al., 2015). Transactive response DNA-binding protein (TDP-43), its aggregation, and cytoplasmic translocation are thought to represent significant steps in the pathogenesis of FTD or ALS (Hu and Grossman, 2009). ALS is a distinct neurodegenerative disease affecting motor neurons in the brain and spinal cord; SOD1 was the first gene discovered to cause familial ALS and was the most studied cause of ALS (Sheng et al., 2012). FTD and ALS appear to be on a spectrum, and some patients display mixed phenotypes of both diseases (Kawakami et al., 2019). The TDP-43 gene is involved in the pathogenesis of both the disease, but SOD1 is only related to ALS but not FTD (Hornung et al., 2020; Jo et al., 2020). Using mouse motor neuron-like NSC-34 cells overexpressing human wild-type or mutant SOD1, scientists have shown that exosomes derived from NSC-34 cells contain SOD1; this gave the evidence of secretion and cell-to-cell transmission of SOD1 (Gomes et al.,

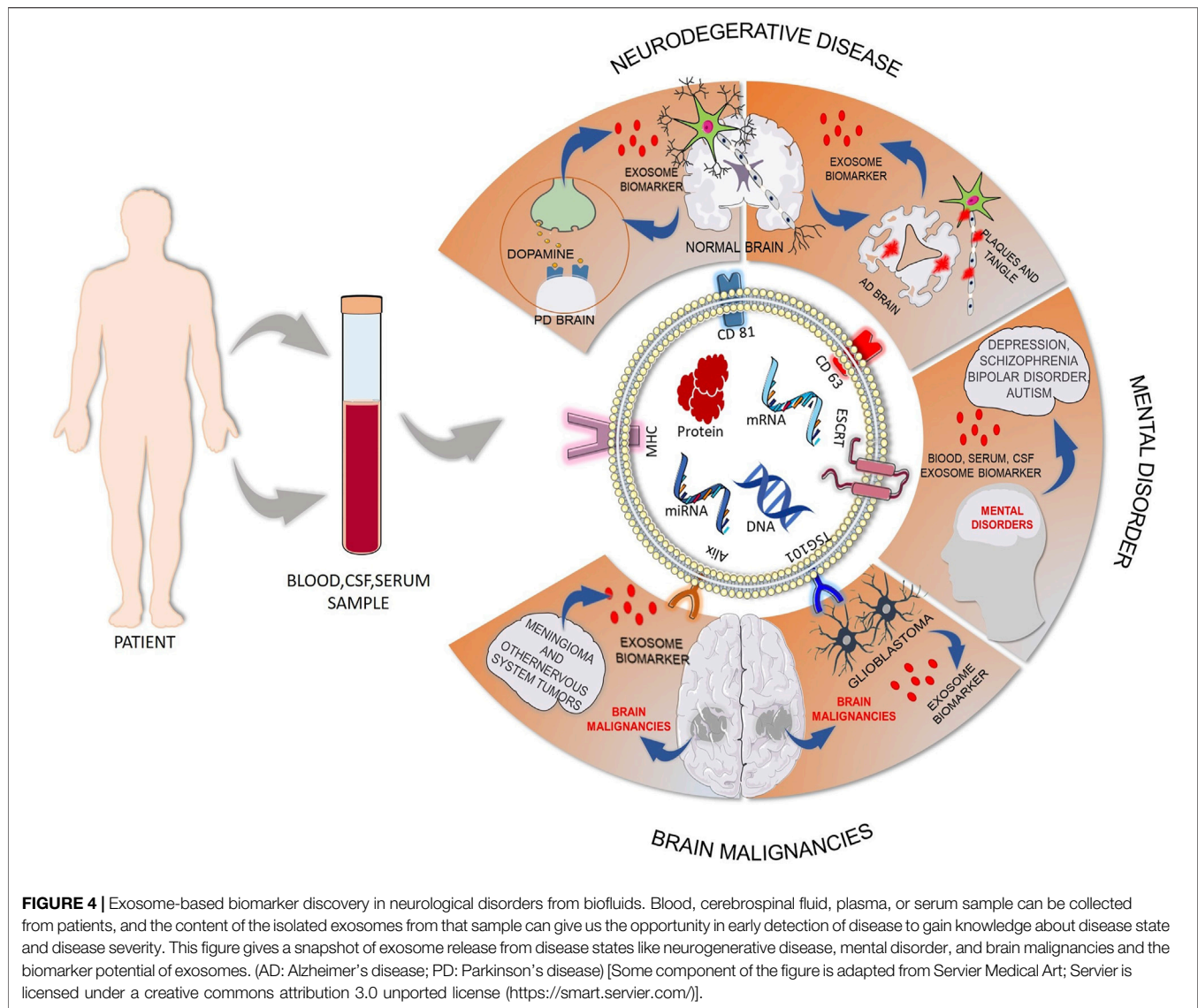
2007). In the similar way, TDP-43 can also get transmitted from cell to cell (Nonaka et al., 2013; Iguchi et al., 2016).

2.4 Traumatic Brain Injury

TBI occurs due to the sudden external force in the brain that leads to temporary or permanent neurological deficits. TBI pathogenesis is a complex process due to primary and secondary injuries. The primary deficit occurs immediately, and the secondary injury can occur from minutes to days after the primary impact and consists of a molecular, chemical, and inflammatory cascade responsible for further cerebral damage. The injury involves depolarization of the neurons and release of excitatory neurotransmitters such as glutamate and aspartate that lead to increased intracellular calcium levels, which in turn activates caspases and free radicals that result in the degradation of cells either directly or indirectly through an apoptotic process. These cell deaths result in an inflammatory response that further damages neuronal cells and the blood-brain barrier (BBB) and promotes cerebral edema. The secondary injury phase is followed by the recovery period that involves reorganization at an anatomical, molecular, and functional level. The brain parenchyma, cerebrospinal fluid, and blood make up the volume of the intracranial compartment. An increase in intracranial volume *via* mass effect from blood, both cytotoxic and vasogenic edema, and venous congestion is also a hallmark of TBI. This would lead to pathological brain compression and, finally, death (Galgano et al., 2017). Exosomes are actively participating in traumatic brain injury pathogenesis; in a case study involving military personnel with mild TBIs (mTBI) and chronic symptoms, it is found that there is a higher level of tau, amyloid-beta 42, and IL-10 in neuron-derived exosomes (NDEs) (Gill et al., 2018). A group of scientists postulated that exosomes could mediate the induction of chronic traumatic encephalopathy (CTE) from mTBI. They have hypothesized a pathway to show how exosomes can mediate pathogenesis from normal to mild deterioration after one mTBI to advanced CTE pathology after the repeated occurrence of mTBIs. According to their hypothesis, initial mTBI leads to the production of NDEs that contains pathogenic complexes of PRPC-Abo-Fyn, SNGY3 + P-tau, and IL-6-sIL-6R; this leads to damage in the donor neurons and other neurons that receive the neurotoxic NDEs. Apart from the neurons, microglia (MG) and astrocytes (AG) will also produce microglia-derived exosomes (MDEs) and astrocytes-derived exosomes (ADEs), respectively, carrying elevated levels of APP, BACE-1, and IL-6, which will further cause neurotoxic damage to neurons. With subsequent episodes of mTBI, this series of processes will increase and cause neuronal apoptosis, which will subsequently lead to the induction of CTE (Goetzl et al., 2019). From these studies, it is believable that the NDE from TBI patients contains neurotoxic cargo, and this NDE causes neuronal damage in proximal or distal cells that receive it.

2.5 Glioblastoma

Apart from its role as a mediator of neurodegenerative disease, exosomes also play a significant role as a pathogenic agent in brain malignancies. The most frequent intrinsic tumors of the central nervous system are glioma. It encompasses two principal



subgroups: (World Health Organisation) WHO grade I or “nondiffuse gliomas,” showing a more circumscribed growth pattern, and WHO grades II–IV or “diffusely infiltrating gliomas,” arising from glial cells or glial precursors (Wesseling and Capper, 2018). Scientifically accepted hallmark of cancers includes sustaining proliferative signaling, resisting cell death, evading growth suppression, activating invasion and metastasis, enabling replicative immortality, and inducing angiogenesis (Hanahan and Weinberg, 2011). Exosomes play a significant role as a pathogenic agent in all the six hallmark scenarios (Choi et al., 2018; Oushy et al., 2018; Bian et al., 2019; Hallal et al., 2019; Gao et al., 2020; Lucero et al., 2020). Apart from these roles, studies have shown that glioblastoma-derived exosomes can promote the immunosuppressive properties of microglia when they are taken up by tumor-associated microglia (Abels et al., 2019). Recent studies have shown that exosomes have active participation in the acquisition of resistance to therapy in glioblastomas (Yekula et al., 2021). From the aforementioned

evidence, it is clear that exosomes play a major role as a pathogenic agent in maintaining the tumor microenvironment and further metastasis of tumors.

3 EXOSOME IN BIOMARKER DISCOVERY FOR NEUROLOGICAL DISEASES

The blood–brain barrier (BBB) is a complex physical barrier between the brain and the peripheral circulation that regulates the influx and efflux of molecules to the brain to preserve CNS homeostasis and maintains the stable local ionic microenvironment necessary for neuronal function (Armulik et al., 2010; Kheirandish-Gozal et al., 2017). This barrier makes it difficult for biomolecules to pass from the brain side to the peripheral circulation and remains the main obstacle in the discovery of biomarkers from peripheral blood or serum for brain-related diseases. After the discovery that exosomes can

cross BBB and the exosomal content remains active, the interest in exosome-based biomarker discovery in neurological disorder has increased (Saeedi et al., 2019). Exosomal content can help us gain insights into early disease detection, disease state, and disease severity (Figure 4). The ability to compare the biomarkers in exosomes originating from different cell types gives an added advantage to biomarker analysis in CNS-derived blood exosomes as compared to CSF (Hornung et al., 2020). Different biofluids and their exosomal content for biomarker discovery in neurological diseases are listed in **Supplementary Table S2**.

4 EXOSOME AS NEUROTHERAPEUTICS

In the previous section, we briefly introduced the role of exosomes as a biomarker and pathogenic agent. In this section, we will be focusing on the recent progress surrounding exosomal surface engineering and engineering exosome for packaging cargo of interest. We will also discuss how exosome engineering can increase the value of exosomes as therapeutics in different neurological disorders.

4.1 Engineered Exosome

The main issue with exosome therapeutics is it does not have targeting ability. There are two types of exosome engineering: surface engineering and packaging of cargo of interest. The former endows the exosomes with targetability, and the latter makes the exosomes a better delivery agent and increases therapeutic value.

4.1.1 Methods of Exosome Engineering

Methods of exosome engineering involve two strategies: i) surface engineering strategy (Richardson and Ejima, 2019) and ii) exosome packaging strategy (Donoso-Quezada et al., 2020).

4.1.1.1 Surface Engineering Strategy

Currently available methods for exosome surface functionalization can be classified into two main approaches: 1) genetic engineering and 2) chemical modification. The former method is effective for displaying genetically engineered proteins on the surface of exosomes, but it is only limited to genetically encodable peptides and proteins. But the latter chemical modification method can be used to functionalize exosomes with a wide range of molecules by utilizing noncovalent or covalent interactions. This method remains challenging because of the membrane complexity and because of the various issues with the purification steps necessary to separate the unreacted chemicals from the exosomes (Richardson and Ejima, 2019).

(1) Genetic engineering-based surface engineering includes designing plasmids, transfecting cells with the designed plasmid, and exosomes isolation which itself is a challenging and expensive task. In many works, lactadherin that localizes to exosomes *via* binding of its C1C2 domain to exosome lipids has been utilized for the generation of chimeric protein and exosome functionalization (Delcayre et al., 2005). The protein of interest is cloned to the C1C2 domain of the lactadherin, which results in chimeric proteins being trafficked to the exosomes, and the N-terminal region was displayed outward on the exosome

surface. Different scientific groups have utilized lactadherin to display GLuc reporter protein, carcinoembryonic antigen, and HER2 and anti-HER2 antibodies on the exosome surface (Hartman et al., 2011; Takahashi et al., 2013; Wang et al., 2018b). In another set, scientists have utilized exosomal membrane protein, Lamp2b, and fused targeting peptides, e.g., RVG and RGD peptides to the N terminus of the protein for exosome surface functionalization and targeted delivery to neurons (Kumar et al., 2007) and breast cancer cell (Tian et al., 2014). Yim et al. fused CIBN to the N-terminus of EGFP tagged CD9 (CIBN-EGFP-CD9) for the vector preparation and fused the cargo proteins with CRY2 (cargo protein-CRY2). This system helped to immobilize proteins to the inner surface of exosomes and loaded cargo proteins into the newly generated exosomes (Yim et al., 2016a). Lai et al. (2014) have generated a lentivirus vector encoding the transmembrane domain of PDGFR, BAP domain, and GLuc, and this construct generates surface-engineered exosomes with Gluc and BAP domain which gives an opportunity for *in vivo* multimodal imaging to monitor tissue distribution, blood levels, and clearance dynamics of the EV. Though the genetic engineering-based methods have advantages, this method still has risks of the engineered biomolecules appearing on the internal exosome surface rather than the desired external surface.

(2) Chemical modification involves covalent or noncovalent interaction for exosome surface functionalization. Alkyl chains can be utilized to anchor molecules into the lipid bilayer membranes of exosomes through hydrophobic interactions. Using this strategy, PEG-lipid conjugates were inserted into exosomal membranes to increase blood circulation time (Kooijmans et al., 2016). Wan et al. (2018) utilized lipids to modify cell membranes for the formation of exosome-mimetic vesicles. They conjugated nucleolin-targeting aptamer AS1411 to cholesterol-PEG and generated surface-functionalized exosomes for *in vivo* anticancer drug delivery. Functionalized exosomes can also be generated by fusing liposomes, consisting of DOPS and PEG-DSPE with exosomes *via* freeze-thaw cycles. This method also justifies the efficient packing of liposomal cargo into the newly generated liposome fused exosome (Sato et al., 2016). Wan et al. (2017) have demonstrated the hybridization-mediated assembly of DNA on the exosomal surface for the generation of targeted exosomes. Apart from noncovalent modifications, exosomes can be covalently modified; these modifications are less prone to dissociate away from the exosomes, unlike noncovalent modifications. Smyth et al. (2014) modified the exosomes with alkyne-containing 4-pentynoic acid used carbodiimide coupling onto amines in the exosomal membrane. Then, they used these functionalized exosomes to conjugated azide-tagged fluorophores *via* azide-alkyne Huisgen cycloaddition or click chemistry. Wang et al. (2015) used metabolic engineering to introduce azide groups on the surface of exosomes; they have also used azide-alkyne Huisgen cycloaddition reaction to covalently introduce small molecules and proteins onto the exosomal surface. Many other works also used similar chemical-based approaches for surface engineering of exosomes (Qi et al., 2016; Kumar et al., 2018; Tian et al., 2018).

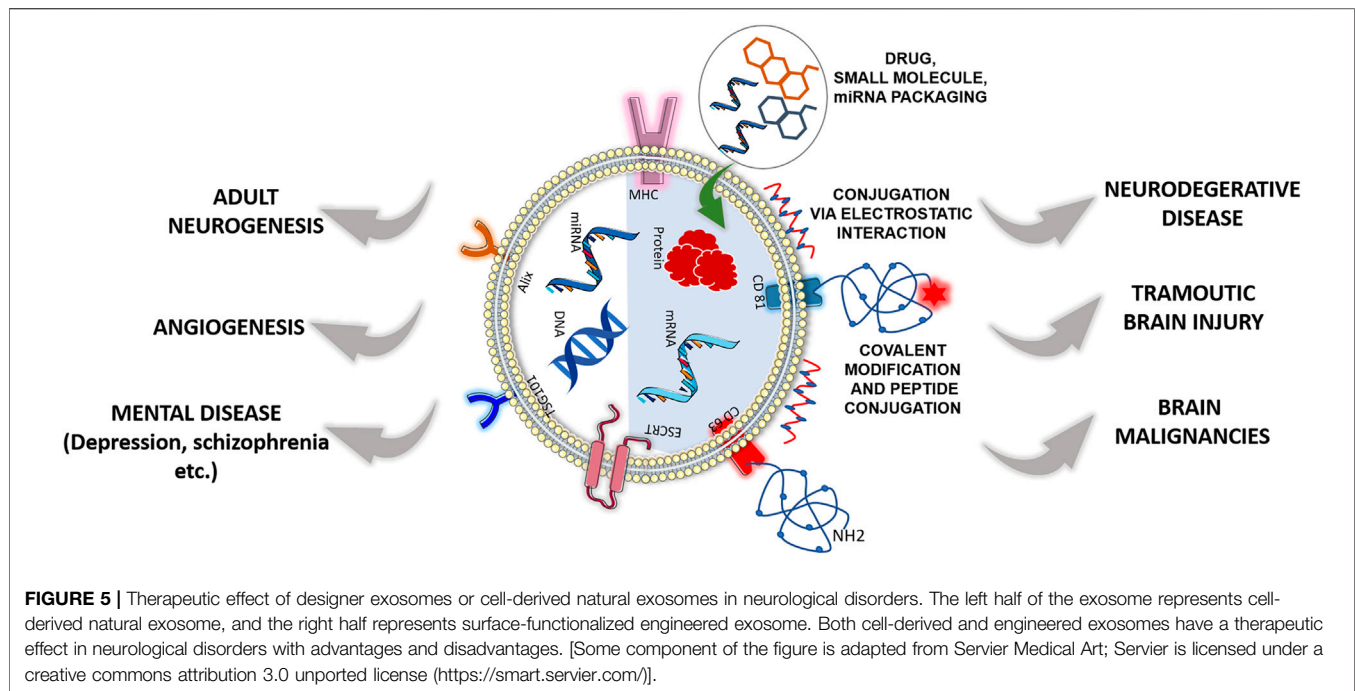
4.1.1.2 Exosome Packaging Strategy

Several studies have explored the natural properties of exosomes as nanocarriers, and in recent years, numerous techniques have been developed to improve the immunogenicity, drug loading efficiency, or targeting ability of exosomes. In this section, we will discuss the state-of-the-art packaging strategies to load a cargo of interest into the exosome. Packaging strategies include

- (1) **Passive loading of hydrophobic compounds:** The lipidic nature of the exosome membrane enables several hydrophobic compounds to be passively loaded into the exosomes by co-incubation. Using this strategy, curcumin (Sun et al., 2010), doxorubicin, and paclitaxel (Yang et al., 2015) have been successfully incorporated into different cell-derived exosomes. Apart from therapeutic molecules, large protein, e.g., the tetrameric protein catalase, has efficiently been loaded into Raw 264.7-derived exosomes by simple diffusion (Haney et al., 2015).
- (2) **Physical methods for molecule loading:** The previously discussed passive loading method is not efficient for packaging hydrophobic molecules like DNA or RNA. In these scenarios, physical methods like electroporation, sonication, and extrusion-based methods are used. Momen-Heravi et al. (2014) loaded miRNA-155 into the exosomes using electroporation; they concluded that higher voltages (between 0.14 and 0.2 kV) and a total exosomal protein concentration between 500 and 1,000 µg/ml resulted in better loading yields. Wahlgren et al. (2012) used a similar method to load mitogen-activated protein kinase-1 siRNA (MAPK1-siRNA), and they have found that the optimum electroporation voltage was between 0.150 and 0.200 kV and the exosomal protein concentration was between 250 L and 1,000 µg/ml. Larger nucleic acids, like double-stranded DNA, have also been successfully packaged into exosomes by electroporation; it was found that the loading efficiency of dsDNA significantly decreases for sizes above 750 bp (Lamichhane et al., 2015). By using membrane-permeabilizing agents, the issue of aggregation and fusion of exosomes after electroporation can be resolved (Hood et al., 2014). Another physical method is sonication, and it is reported that sonication can successfully incorporate doxorubicin and paclitaxel into exosomes with more efficiency than other physical methods (Kim et al., 2016). Though this technique is more efficient as it is the most damaging technique for exosomal membrane, sonication is very rarely used for exosomal cargo packaging. Another less explored method for exosome cargo loading is cell extrusion, where cells are extruded through 100–400 nm pore size membrane filters to break up the cell and then cells reform the cell membrane to generate exosome-mimics. Using this method, exosome-mimics have been generated from MCF10A cells and loaded with siRNA by electroporation (Yang et al., 2016). Similarly, catalase was loaded into Raw 264.7-derived exosomes by extruding the catalase mixture with exosomes (Haney et al., 2015).
- (3) **Hydrophobic modification of nucleic acids:** To avoid the problem of aggregation, vesicle fusion, and variations of surface zeta potential associated with electroporation-based siRNA, miRNA cargo loading, hydrophobic modification of nucleic acids has evolved as a strategy to pack cargo into the exosomes. Didiot et al. have modified the siRNA by adding a cholesterol moiety conjugated to the 3' end of the passenger strand and successfully loaded the modified cargo into U87 glioblastoma cell-derived exosomes (Didiot et al., 2016).
- (4) **Labeling of target proteins for loading into exosomes:** This method gives the opportunity to utilize the protein that plays a major role in exosomal cargo packaging; one such protein is ESCRT which specifically shortens the ubiquitinated proteins in the exosomes. In many studies, scientists are leveling cargo protein with a peptide that can selectively interact with ESCRT, which increases the probability of cargo protein getting packed into the exosomes (Villarroya-Beltri et al., 2014). Cheng and Schorey (2016) fused ubiquitin to the C-terminal region of enhanced green fluorescent protein (EGFP), tumor antigenic protein nHer2, and *Mycobacterium tuberculosis* proteins Ag58B and ESAT6; ubiquitin labeling increases the loading of all the protein into the exosomes. Other than ESCRT, late-domains (L-domains), which recognizes the WW tag in the protein of interest, also give similar opportunity in cargo loading (Sterzenbach et al., 2017).
- (5) **Light-induced exosome loading:** optically reversible protein–protein interaction (EXPLORE) can be used to load proteins into exosomes. This process involves endogenous biogenesis processes and the delivery of cargo proteins into the cytosol by light-mediated signal (Yim et al., 2016b). Scientists have explored exosomal CD9-CIBN-CRY2-based systems to pack many cargos into the exosomes (Yim et al., 2016a; Huang et al., 2019).

4.2 Applications of Cell-Derived and Surface-Engineered Novel Cargo-Loaded Exosome as Neurotherapeutics

Natural exosomes have various potentials; their clinical application is associated with some inherent limitations of targetability, immunogenicity, and less efficient cargo delivery. Recently, to overcome these limitations, exosome engineering and the development of designer exosomes are coming into the picture. In this section, we will first discuss the role of natural exosomes as neurotherapeutics, and toward the end, we will discuss the role of surface engineered novel cargo-loaded exosomes as neurotherapeutics (Figure 5). Mesenchymal stem cell (MSC) is already extensively studied for regenerative medicine, cell therapy, and tissue engineering (Ankrum and Karp, 2010). Accordingly, research based on exosomes derived from MSCs (MSC-exosomes) has great value as this has the



advantage of exosomes and also the characteristics of MSCs (Ghosh et al., 2020). Numerous studies have demonstrated the therapeutic value of MSC-exosomes in tumors, neurodegenerative diseases, cardiovascular and cerebrovascular diseases, wound repair, etc (Cui et al., 2018; Zhao et al., 2018; Moon et al., 2019; Rao et al., 2019; Riazifar et al., 2019). MSC-derived exosomes can exert their therapeutic effect by removing or inhibiting pathological processes or by promoting regenerative mechanisms. In the former case, it is known to reduce amyloid-beta ($A\beta$) aggregate in AD, rescue dopaminergic neurons from 6-OHDA-induced apoptosis in PD, reduce demyelination in multiple sclerosis (MS), and inhibit apoptosis, inflammation, and promotes angiogenesis in TBI and stroke (Jarmalavičiūtė et al., 2015; Huang et al., 2017; Yang et al., 2017b; Ding et al., 2018; Elia et al., 2019). In the latter case of regeneration, MSC-derived exosomes exert their effect by neuroprotection, neurogenesis, neuromodulation, and angiogenesis in many disease conditions like AD, TBI, and stroke (Doeppner et al., 2015; Zhang et al., 2015; Elia et al., 2019; Otero-Ortega et al., 2020). Other than the aforementioned process, MSC-exosomes show their effect in reducing oxidative stress, restoring the integrity of the BBB, inhibiting tumor growth, and improving behavioral and biochemical deficits in mental disorders like schizophrenia (Jarmalavičiūtė et al., 2015; Chen et al., 2016; Li et al., 2019; Tsivion-Visbord et al., 2020; Luo et al., 2021). Apart from MSC-exosomes, other cell-derived exosomes are explored for a similar application. Sharma et al. (2019) have shown exosomes released by neural cultures can rescue deficits in neuronal proliferation, differentiation, synaptogenesis, and synchronized firing in MECP2-knockdown human primary neural cultures, a model for Rett syndrome. Chen et al. (2020b) have shown exosomes derived from astrocytes promoted the recovery of damaged

neurons by downregulation of the apoptosis rate and upregulating mitochondrial function. Lopez-Verrilli et al. (2013) have shown Schwann cells (SC)-derived exosomes increase axonal regeneration *in-vitro* and increase regeneration after sciatic nerve injury *in vivo*. Webb et al. (2018) demonstrated that human neural stem cell-derived EVs improve behavior and mobility by removing intracranial hemorrhage, reducing the volume of the cerebral lesions and brain swelling, which eventually leads to recovery from ischemic stroke in a pig model. From the previous discussion, it is clear that natural exosomes have a potential therapeutic effect; many attempts have been made to improve the therapeutic potential and load cargo of interest. Alvarez-Erviti et al. (2011b) have generated engineered dendritic cells that express Lamp2b fused to the neuron-specific RVG peptide and isolated exosomes from the engineered cell. They have packed the exosomes with exogenous siRNA by electroporation and demonstrated the targeted delivery of cargo specifically to neurons, microglia, and oligodendrocytes in the brain, resulting in a specific gene knockdown and subsequent therapeutic effect in AD (Alvarez-Erviti et al., 2011b). Using a similar approach, Yang et al. (2017a) have successfully delivered miR-124 to the infarct site, which leads to amplification of adult neurogenesis in ischemia. Tian et al. (2018) have developed c (RGDyK)-conjugated curcumin-loaded exosomes (cRGD-Exo), which can target the lesion region and show strong suppression of the inflammatory response and cellular apoptosis in the lesion region of the ischemic brain after intravenous administration. A group of scientists developed superparamagnetic iron oxide nanoparticles (SPIONs) and curcumin (Cur)-loaded exosomes. By click chemistry, they have conjugated the exosome membrane with neuropilin-1-targeted peptide (RGERPPR, RGE); these

engineered exosomes have the ability to target glioma cells and also have imaging and therapeutic functions (Jia et al., 2018). Ye et al. (2018) have developed methotrexate (MTX)-loaded EVs functionalized with therapeutic [Lys-Leu-Ala (KLA)] and targeted [low-density lipoprotein (LDL)] peptides which show targetability toward brain tumors and show therapeutic effects. Wang and Han (2019) have modified the exosomes and loaded a plasmid expressing B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X-protein (Bax) short hairpin RNA (shRNA); these exosomes show therapeutic effect in apoptosis and neural functions after TBI. Zhang et al. (2019) have developed c (RGDyK) peptide conjugated, cholesterol-modified miR-210 engineered exosomes, which show upregulated expressions of integrin β 3, vascular endothelial growth factor (VEGF), and CD34 and subsequent angiogenesis after middle cerebral artery occlusion (MCAO). In addition to different scientific groups, many companies are developing exosome-based neurotherapeutics; one such company is Evox Therapeutics. Evox Therapeutics uses a biotechnological-based approach to generate drug-loaded brain and central nervous system targeted engineered exosomes (<https://www.evoxtherapeutics.com/>). Aruna Bio is working on pharmaceutical exosomes for drug delivery to the brain and neurons (<https://www.arunabio.com/>). Some major disadvantages of cell therapy (induced pluripotent stem cell, iPSC), such as necrosis or abnormal cell differentiation, tumorigenesis, immune rejection caused by cell transplantation, and microvascular embolism, can be overcome by exosome-based therapies (Ghosh et al., 2020). The main advantages of exosome therapies are as followed. First, exosomes mediate stem cell paracrine action, participate in cell–cell communication and are already proven as the main mechanism of disease treatment mediated by cell-based therapies. Second, exosomes can be engineered and can be packed with a cargo of interest like existing, newly developed compositions, and can work as drug delivery vehicles. Third, in some cases, exosomes have autonomous targeting capabilities which make exosome-specific tissue or cell-targeted drug carriers (Mathieu et al., 2019; Wei et al., 2021). From the aforementioned studies, we can have an idea that exosome-based neurotherapeutics have made huge progress in recent years and with the development of new technologies, more progress will follow in upcoming days and can be an alternative to cell-based therapies, like iPSC therapies.

5 DISCUSSION AND FUTURE PERSPECTIVE

Exosomes are a rising star and a complete package in the era of advanced medical science due to their multiple roles in cell–cell communication, biomarker discovery, disease progression, and therapeutics. The following are some of the benefits that exosomes have: 1) they can pass the blood–brain barrier, and are less invasive, non-tumorigenic, and non-immunogenic, 2) their shelf life and half-life in

patients are longer, allowing for long-term storage without loss of function, and 3) they do not reproduce or cause a microvascular embolism (Ghosh et al., 2020). These advantages make the exosomes a superior tool for biomarker discovery and therapeutic development. Apart from advantages, the main challenges for bringing exosomes into the clinical practice include the following: First, the urgent need for standard, efficient, and sensitive methods with a low biofluid volume requirement and high purity and yield for classification and extraction of exosomes from different body fluids and cells. Second, the identification of specific subtypes of EVs is urgently needed, as different vesicles may exert various biological effects. Current methods of exosome isolation are too diverse to confirm the purity of the product. Therefore, it is necessary to standardize the protocols and identification methods when attempting to use exosomes widely in clinical testing. Additionally, more reliable biomarkers should be confirmed. Although many molecules carried by exosomes have been documented to serve as potential biomarkers, few of them are qualified for application. Documented biomarkers need to be validated on a larger scale to create a standard hallmark for diseases. Third, for exosome-based therapeutics development, the targetability of exosomes needs to be checked, as different culture conditions change exosomal cargo. A standardized protocol needs to be developed for large production of exosomes from the cell; cell banks need to be developed. A specific purification method and sensitive method for specific exosomal marker identification need to be developed to avoid ambiguity. Last but not least, the biological safety, targeted efficacy, and adverse effects of exosomes must be confirmed before clinical use. In recent years, to endow the exosomes with targetability and to make exosomes more potent delivery and therapeutic agents, exosome engineering is coming into the picture, which will resolve many issues that cell-derived exosomes have. In conclusion, if the abovementioned lags are resolved and guidelines prescribed by the International Society for Extracellular Vesicles (Théry et al., 2018) are followed, then exosomes can be in the spotlight of clinical practice as biomarkers and therapeutics in the near future.

AUTHOR CONTRIBUTIONS

SaG and SuG discussed this area for exploration of the role of exosomes as pathogenic agents, disease biomarkers, and neurotherapeutics and jointly wrote the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2022.878058/full#supplementary-material>

REFERENCES

- Abels, E. R., Maas, S. L. N., Nieland, L., Wei, Z., Cheah, P. S., Tai, E., et al. (2019). Glioblastoma-associated Microglia Reprogramming Is Mediated by Functional Transfer of Extracellular miR-21. *Cell Rep.* 28 (12), 3105–e7. doi:10.1016/j.celrep.2019.08.036
- Akers, J. C., Ramakrishnan, V., Kim, R., Skog, J., Nakano, I., Pingle, S., et al. (2013). MiR-21 in the Extracellular Vesicles (EVs) of Cerebrospinal Fluid (CSF): a Platform for Glioblastoma Biomarker Development. *PLoS One* 8 (10), e78115. doi:10.1371/journal.pone.0078115
- Alvarez-Erviti, L., Seow, Y., Schapira, A. H., Gardiner, C., Sargent, I. L., Wood, M. J., et al. (2011a). Lysosomal Dysfunction Increases Exosome-Mediated Alpha-Synuclein Release and Transmission. *Neurobiol. Dis.* 42 (3), 360–367. doi:10.1016/j.nbd.2011.01.029
- Alvarez-Erviti, L., Seow, Y., Yin, H., Betts, C., Lakhal, S., and Wood, M. J. (2011b). Delivery of siRNA to the Mouse Brain by Systemic Injection of Targeted Exosomes. *Nat. Biotechnol.* 29 (4), 341–345. doi:10.1038/nbt.1807
- Ankrum, J., and Karp, J. M. (2010). Mesenchymal Stem Cell Therapy: Two Steps Forward, One Step Back. *Trends Mol. Med.* 16 (5), 203–209. doi:10.1016/j.molmed.2010.02.005
- Armulik, A., Genové, G., Mäe, M., Nisancioglu, M. H., Wallgard, E., Niaudet, C., et al. (2010). Pericytes Regulate the Blood-Brain Barrier. *Nature* 468 (7323), 557–561. doi:10.1038/nature09522
- Asai, H., Ikezu, S., Tsunoda, S., Medalla, M., Luecke, J., Haydar, T., et al. (2015). Depletion of Microglia and Inhibition of Exosome Synthesis Halt Tau Propagation. *Nat. Neurosci.* 18 (11), 1584–1593. doi:10.1038/nn.4132
- Banack, S. A., Dunlop, R. A., and Cox, P. A. (2020). An miRNA Fingerprint Using Neural-Enriched Extracellular Vesicles from Blood Plasma: towards a Biomarker for Amyotrophic Lateral Sclerosis/motor Neuron Disease. *Open Biol.* 10 (6), 200116. doi:10.1098/rsob.200116
- Bang, J., Spina, S., and Miller, B. L. (2015). Frontotemporal Dementia. *Lancet* 386 (10004), 1672–1682. doi:10.1016/S0140-6736(15)00461-4
- Bian, E. B., Chen, E. F., Xu, Y. D., Yang, Z. H., Tang, F., Ma, C. C., et al. (2019). Exosomal lncRNA-ATB A-ctivates A-strocytes that P-romote G-lioma C-ell I-nvasion. *Int. J. Oncol.* 54 (2), 713–721. doi:10.3892/ijo.2018.4644
- Bilousova, T., Elias, C., Miyoshi, E., Alam, M. P., Zhu, C., Campagna, J., et al. (2018). Suppression of Tau Propagation Using an Inhibitor that Targets the DK-Switch of nSmase2. *Biochem. Biophys. Res. Commun.* 499 (4), 751–757. doi:10.1016/j.bbrc.2018.03.209
- Borrotto-Escuela, D. O., Agnati, L. F., Bechter, K., Jansson, A., Tarakanov, A. O., and Fuxe, K. (2015). The Role of Transmitter Diffusion and Flow versus Extracellular Vesicles in Volume Transmission in the Brain Neural-Glial Networks. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370 (1672), 20140183. doi:10.1098/rstb.2014.0183
- Brennan, K., Martin, K., FitzGerald, S. P., O'Sullivan, J., Wu, Y., Blanco, A., et al. (2020). A Comparison of Methods for the Isolation and Separation of Extracellular Vesicles from Protein and Lipid Particles in Human Serum. *Sci. Rep.* 10 (1), 1–13. doi:10.1038/s41598-020-57497-7
- Cabin, D. E., Shimazu, K., Murphy, D., Cole, N. B., Gottschalk, W., McIlwain, K. L., et al. (2002). Synaptic Vesicle Depletion Correlates with Attenuated Synaptic Responses to Prolonged Repetitive Stimulation in Mice Lacking Alpha-Synuclein. *J. Neurosci.* 22 (20), 8797–8807. doi:10.1523/JNEUROSCI.22-20-08797.2002
- Cao, X. Y., Lu, J. M., Zhao, Z. Q., Li, M. C., Lu, T., An, X. S., et al. (2017). MicroRNA Biomarkers of Parkinson's Disease in Serum Exosome-like Microvesicles. *Neurosci. Lett.* 644, 94–99. doi:10.1016/j.neulet.2017.02.045
- Ceylan, D., Tufekci, K. U., Keskinoglu, P., Genc, S., and Özerdem, A. (2020). Circulating Exosomal microRNAs in Bipolar Disorder. *J. Affect. Disord.* 262, 99–107. doi:10.1016/j.jad.2019.10.038
- Cha, D. J., Mengel, D., Mustapic, M., Liu, W., Selkoe, D. J., Kapogiannis, D., et al. (2019). miR-212 and miR-132 Are Downregulated in Neurally Derived Plasma Exosomes of Alzheimer's Patients. *Front. Neurosci.* 13, 1208. doi:10.3389/fnins.2019.01208
- Chanteloup, G., Cordonnier, M., Moreno-Ramos, T., Pytel, V., Matias-Guiu, J., Gobbo, J., et al. (2019). Exosomal HSP70 for Monitoring of Frontotemporal Dementia and Alzheimer's Disease: Clinical and FDG-PET Correlation. *J. Alzheimers Dis.* 71 (4), 1263–1269. doi:10.3233/JAD-190545
- Chen, C., Skog, J., Hsu, C. H., Lessard, R. T., Balaj, L., Wurdinger, T., et al. (2010). Microfluidic Isolation and Transcriptome Analysis of Serum Microvesicles. *Lab. Chip* 10 (4), 505–511. doi:10.1039/B916199F
- Chen, K. H., Chen, C. H., Wallace, C. G., Yuen, C. M., Kao, G. S., Chen, Y. L., et al. (2016). Intravenous Administration of Xenogenic Adipose-Derived Mesenchymal Stem Cells (ADMSC) and ADMSC-Derived Exosomes Markedly Reduced Brain Infarct Volume and Preserved Neurological Function in Rat after Acute Ischemic Stroke. *Oncotarget* 7 (46), 74537–74556. doi:10.18632/oncotarget.12902
- Chen, P. C., Wu, D., Hu, C. J., Chen, H. Y., Hsieh, Y. C., and Huang, C. C. (2020a). Exosomal TAR DNA-Binding Protein-43 and Neurofilaments in Plasma of Amyotrophic Lateral Sclerosis Patients: A Longitudinal Follow-Up Study. *J. Neurol. Sci.* 418, 117070. doi:10.1016/j.jns.2020.117070
- Chen, W., Zheng, P., Hong, T., Wang, Y., Liu, N., He, B., et al. (2020b). Astrocytes-derived Exosomes Induce Neuronal Recovery after Traumatic Brain Injury via Delivering Gap Junction Alpha 1-20 K. *J. Tissue Eng. Regen. Med.* 14 (3), 412–423. doi:10.1002/term.3002
- Cheng, Y., and Schorey, J. S. (2016). Targeting Soluble Proteins to Exosomes Using a Ubiquitin Tag. *Biotechnol. Bioeng.* 113 (6), 1315–1324. doi:10.1002/bit.25884
- Cheng, L., Doecke, J. D., Sharples, R. A., Villemagne, V. L., Fowler, C. J., Rembach, A., et al. (2015). Prognostic Serum miRNA Biomarkers Associated with Alzheimer's Disease Shows Concordance with Neuropsychological and Neuroimaging Assessment. *Mol. Psychiatry* 20 (10), 1188–1196. doi:10.1038/mp.2014.127
- Chivet, M., Javellet, C., Laulagnier, K., Blot, B., Hemming, F. J., and Sadoul, R. (2014). Exosomes Secreted by Cortical Neurons upon Glutamatergic Synapse Activation Specifically Interact with Neurons. *J. Extracell. Vesicles* 3 (1), 24722. doi:10.3402/jev.v3.24722
- Choi, D., Montermini, L., Kim, D. K., Meehan, B., Roth, F. P., and Rak, J. (2018). The Impact of Oncogenic EGFRvIII on the Proteome of Extracellular Vesicles Released from Glioblastoma Cells. *Mol. Cell Proteomics* 17 (10), 1948–1964. doi:10.1074/mcp.RA118.000644
- Chopra, N., Dutt Arya, B., Jain, N., Yadav, P., Wajid, S., Singh, S. P., et al. (2019). Biophysical Characterization and Drug Delivery Potential of Exosomes from Human Wharton's Jelly-Derived Mesenchymal Stem Cells. *ACS omega* 4 (8), 13143–13152. doi:10.1021/acsomega.9b01180
- Cohn, S. W., Melnik, M., Huang, C., Teter, B., Chandra, S., McIntire, L. B., et al. (2021). Multi-omics Analysis of Microglial Extracellular Vesicles from Human Alzheimer's Disease Brain Reveals Disease-Associated Signatures. *Front. Pharmacol.* 12, 3078. doi:10.3389/fphar.2021.766082
- Cooper, J. M., Wiklander, P. B., Nordin, J. Z., Al-Shawi, R., Wood, M. J., Vithlani, M., et al. (2014). Systemic Exosomal siRNA Delivery Reduced Alpha-Synuclein Aggregates in Brains of Transgenic Mice. *Mov. Disord.* 29 (12), 1476–1485. doi:10.1002/mds.25978
- Crotti, A., Sait, H. R., McAvoy, K. M., Estrada, K., Ergun, A., Szak, S., et al. (2019). B1N1 Favors the Spreading of Tau via Extracellular Vesicles. *Sci. Rep.* 9 (1), 1–20. doi:10.1038/s41598-019-45676-0
- Cui, G. H., Wu, J., Mou, F. F., Xie, W. H., Wang, F. B., Wang, Q. L., et al. (2018). Exosomes Derived from Hypoxia-Preconditioned Mesenchymal Stromal Cells Ameliorate Cognitive Decline by Rescuing Synaptic Dysfunction and Regulating Inflammatory Responses in APP/PS1 Mice. *FASEB J.* 32 (2), 654–668. doi:10.1096/fj.201706000R
- Cumba Garcia, L. M., Peterson, T. E., Cepeda, M. A., Johnson, A. J., and Parney, I. F. (2019). Isolation and Analysis of Plasma-Derived Exosomes in Patients with Glioma. *Front. Oncol.* 9, 651. doi:10.3389/fonc.2019.00651
- Danzer, K. M., Kranich, L. R., Ruf, W. P., Cagsal-Getkin, O., Winslow, A. R., Zhu, L., et al. (2012). Exosomal Cell-To-Cell Transmission of Alpha Synuclein Oligomers. *Mol. Neurodegener.* 7 (1), 42–48. doi:10.1186/1750-1326-7-42
- Debanne, D., and Rama, S. (2011). Astrocytes Shape Axonal Signaling. *Sci. Signal.* 4 (162), pe11. doi:10.1126/scisignal.2001884
- Delcayre, A., Estelles, A., Sperinde, J., Roulon, T., Paz, P., Aguilar, B., et al. (2005). Exosome Display Technology: Applications to the Development of New Diagnostics and Therapeutics. *Blood Cells Mol. Dis.* 35 (2), 158–168. doi:10.1016/j.bcmd.2005.07.003
- Denk, J., Oberhauser, F., Kornhuber, J., Wiltfang, J., Fassbender, K., Schroeter, M. L., et al. (2018). Specific Serum and CSF microRNA Profiles Distinguish Sporadic Behavioural Variant of Frontotemporal Dementia Compared with

- Alzheimer Patients and Cognitively Healthy Controls. *PLoS one* 13 (5), e0197329. doi:10.1371/journal.pone.0197329
- Didiot, M. C., Hall, L. M., Coles, A. H., Haraszti, R. A., Godinho, B. M., Chase, K., et al. (2016). Exosome-mediated Delivery of Hydrophobically Modified siRNA for Huntingtin mRNA Silencing. *Mol. Ther.* 24 (10), 1836–1847. doi:10.1038/mt.2016.126
- Ding, M., Shen, Y., Wang, P., Xie, Z., Xu, S., Zhu, Z., et al. (2018). Exosomes Isolated from Human Umbilical Cord Mesenchymal Stem Cells Alleviate Neuroinflammation and Reduce Amyloid-Beta Deposition by Modulating Microglial Activation in Alzheimer's Disease. *Neurochem. Res.* 43 (11), 2165–2177. doi:10.1007/s11064-018-2641-5
- Dinkins, M. B., Dasgupta, S., Wang, G., Zhu, G., and Bieberich, E. (2014). Exosome Reduction *In Vivo* Is Associated with Lower Amyloid Plaque Load in the 5XFAD Mouse Model of Alzheimer's Disease. *Neurobiol. Aging* 35 (8), 1792–1800. doi:10.1016/j.neurobiolaging.2014.02.012
- Doepfner, T. R., Herz, J., Görgens, A., Schlechter, J., Ludwig, A. K., Radtke, S., et al. (2015). Extracellular Vesicles Improve Post-Stroke Neuroregeneration and Prevent Postischemic Immunosuppression. *Stem Cells Transl. Med.* 4 (10), 1131–1143. doi:10.5966/sctm.2015-0078
- Dong, L., Zieren, R. C., Horie, K., Kim, C. J., Mallick, E., Jing, Y., et al. (2020). Comprehensive Evaluation of Methods for Small Extracellular Vesicles Separation from Human Plasma, Urine and Cell Culture Medium. *J. Extracell. Vesicles* 10 (2), e12044. doi:10.1002/jev2.12044
- Dong, Z., Gu, H., Guo, Q., Liang, S., Xue, J., Yao, F., et al. (2021). Profiling of Serum Exosome miRNA Reveals the Potential of a miRNA Panel as Diagnostic Biomarker for Alzheimer's Disease. *Mol. Neurobiol.*, 1–11. doi:10.1007/s12035-021-02323-y
- Donoso-Quezada, J., Ayala-Mar, S., and González-Valdez, J. (2020). State-of-the-art Exosome Loading and Functionalization Techniques for Enhanced Therapeutics: a Review. *Crit. Rev. Biotechnol.* 40 (6), 804–820. doi:10.1080/07388551.2020.1785385
- Doyle, L. M., and Wang, M. Z. (2019). Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. *Cells* 8 (7), 727. doi:10.3390/cells8070727
- Du, Y., Yu, Y., Hu, Y., Li, X. W., Wei, Z. X., Pan, R. Y., et al. (2019). Genome-wide, Integrative Analysis Implicates Exosome-Derived microRNA Dysregulation in Schizophrenia. *Schizophr. Bull.* 45 (6), 1257–1266. doi:10.1093/schbul/sby191
- Elia, C. A., Tamborini, M., Rasile, M., Desiato, G., Marchetti, S., Swuec, P., et al. (2019). Intracerebral Injection of Extracellular Vesicles from Mesenchymal Stem Cells Exerts Reduced A β Plaque Burden in Early Stages of a Preclinical Model of Alzheimer's Disease. *Cells* 8 (9), 1059. doi:10.3390/cells8091059
- Emmanouilidou, E., Melachroinou, K., Roumeliotis, T., Garbis, S. D., Ntzouni, M., Margaritis, L. H., et al. (2010). Cell-produced Alpha-Synuclein Is Secreted in a Calcium-dependent Manner by Exosomes and Impacts Neuronal Survival. *J. Neurosci.* 30 (20), 6838–6851. doi:10.1523/JNEUROSCI.5699-09.2010
- Fauré, J., Lachenal, G., Court, M., Hirrlinger, J., Chatellard-Causse, C., Blot, B., et al. (2006). Exosomes Are Released by Cultured Cortical Neurons. *Mol. Cell. Neurosci.* 31 (4), 642–648. doi:10.1016/j.mcn.2005.12.003
- Feneberg, E., Steinacker, P., Lehnert, S., Schneider, A., Walther, P., Thal, D. R., et al. (2014). Limited Role of Free TDP-43 as a Diagnostic Tool in Neurodegenerative Diseases. *Amyotroph. Lateral Scler. Front. Degener.* 15 (5-6), 351–356. doi:10.3109/21678421.2014.905606
- Fiandaca, M. S., Kapogiannis, D., Mapstone, M., Boxer, A., Eitan, E., Schwartz, J. B., et al. (2015). Identification of Preclinical Alzheimer's Disease by a Profile of Pathogenic Proteins in Neurally Derived Blood Exosomes: A Case-Control Study. *Alzheimers Dement.* 11 (6), 600–e1. doi:10.1016/j.jalz.2014.06.008
- Fröhlich, D., Kuo, W. P., Frühbeis, C., Sun, J.-J., Zehendner, C. M., Luhmann, H. J., et al. (2014). Multifaceted Effects of Oligodendroglial Exosomes on Neurons: Impact on Neuronal Firing Rate, Signal Transduction and Gene Regulation. *Phil. Trans. R. Soc. B* 369 (1652), 20130510. doi:10.1098/rstb.2013.0510
- Galgano, M., Toshkezi, G., Qiu, X., Russell, T., Chin, L., and Zhao, L. R. (2017). Traumatic Brain Injury: Current Treatment Strategies and Future Endeavors. *Cell Transpl.* 26 (7), 1118–1130. doi:10.1177/0963689717714102
- Gangoda, L., Boukouris, S., Liem, M., Kalra, H., and Mathivanan, S. (2015). Extracellular Vesicles Including Exosomes Are Mediators of Signal Transduction: Are They Protective or Pathogenic? *Proteomics* 15 (2-3), 260–271. doi:10.1002/pmic.201400234
- Gao, X., Zhang, Z., Mashimo, T., Shen, B., Nyagilo, J., Wang, H., et al. (2020). Gliomas Interact with Non-glioma Brain Cells via Extracellular Vesicles. *Cell Rep.* 30 (8), 2489–e5. doi:10.1016/j.celrep.2020.01.089
- Gelle, T., Samey, R. A., Plansont, B., Bessette, B., Jauberteau-Marchan, M. O., Lalloué, F., et al. (2021). BDNF and Pro-BDNF in Serum and Exosomes in Major Depression: Evolution after Antidepressant Treatment. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 109, 110229. doi:10.1016/j.pnpbp.2020.110229
- Ghosh, S., Garg, S., and Ghosh, S. (2020). Cell-derived Exosome Therapy: a Novel Approach to Treat Post-traumatic Brain Injury Mediated Neural Injury. *ACS Chem. Neurosci.* 11 (14), 2045–2047. doi:10.1021/acscchemneuro.0c00368
- Gill, J., Mustapic, M., Diaz-Arrastia, R., Lange, R., Gulyani, S., Diehl, T., et al. (2018). Higher Exosomal Tau, Amyloid-Beta 42 and IL-10 Are Associated with Mild TBIs and Chronic Symptoms in Military Personnel. *Brain Inj.* 32 (11), 1277–1284. doi:10.1080/02699052.2018.1471738
- Goetzl, E. J., Boxer, A., Schwartz, J. B., Abner, E. L., Petersen, R. C., Miller, B. L., et al. (2015). Altered Lysosomal Proteins in Neural-Derived Plasma Exosomes in Preclinical Alzheimer Disease. *Neurology* 85 (1), 40–47. doi:10.1212/WNL.0000000000001702
- Goetzl, E. J., Ledreux, A., Granholm, A. C., Elahi, F. M., Goetzl, L., Hiramoto, J., et al. (2019). Neuron-Derived Exosome Proteins May Contribute to Progression from Repetitive Mild Traumatic Brain Injuries to Chronic Traumatic Encephalopathy. *Front. Neurosci.* 13, 452. doi:10.3389/fnins.2019.00452
- Goetzl, E. J., Mustapic, M., Kapogiannis, D., Eitan, E., Lobach, I. V., Goetzl, L., et al. (2016). Cargo Proteins of Plasma Astrocyte-Derived Exosomes in Alzheimer's Disease. *FASEB J.* 30 (11), 3853–3859. doi:10.1096/fj.201600756R
- Gomes, C., Keller, S., Altevogt, P., and Costa, J. (2007). Evidence for Secretion of Cu,Zn Superoxide Dismutase via Exosomes from a Cell Model of Amyotrophic Lateral Sclerosis. *Neurosci. Lett.* 428 (1), 43–46. doi:10.1016/j.neulet.2007.09.024
- Gonzalez-Calero, L., Martin-Lorenzo, M., and Alvarez-Llamas, G. (2014). Exosomes: a Potential Key Target in Cardio-Renal Syndrome. *Front. Immunol.* 5, 465. doi:10.3389/fimmu.2014.00465
- Greening, D. W., Gopal, S. K., Xu, R., Simpson, R. J., and Chen, W. (2015). Exosomes and Their Roles in Immune Regulation and Cancer. *Semin. Cell Dev. Biol.* 40, 72–81. doi:10.1016/j.semcdb.2015.02.009
- Grey, M., Dunning, C. J., Gaspar, R., Grey, C., Brundin, P., Sparr, E., et al. (2015). Acceleration of α -synuclein Aggregation by Exosomes. *J. Biol. Chem.* 290 (5), 2969–2982. doi:10.1074/jbc.M114.585703
- Guedes, V. A., Kenney, K., Shahim, P., Qu, B. X., Lai, C., Devoto, C., et al. CENC Multisite Observational Study Investigators (2020). Exosomal Neurofilament Light: A Prognostic Biomarker for Remote Symptoms after Mild Traumatic Brain Injury? *Neurology* 94 (23), e2412–e2423. doi:10.1212/WNL.0000000000009577
- Guo, M., Wang, J., Zhao, Y., Feng, Y., Han, S., Dong, Q., et al. (2020). Microglial Exosomes Facilitate α -synuclein Transmission in Parkinson's Disease. *Brain* 143 (5), 1476–1497. doi:10.1093/brain/awaa090
- Hallal, S., Mallawaarachy, D. M., Wei, H., Ebrahimkhani, S., Stringer, B. W., Day, B. W., et al. (2019). Extracellular Vesicles Released by Glioblastoma Cells Stimulate Normal Astrocytes to Acquire a Tumor-Supportive Phenotype via P53 and MYC Signaling Pathways. *Mol. Neurobiol.* 56 (6), 4566–4581. doi:10.1007/s12035-018-1385-1
- Han, Z., Peng, C., Yi, J., Zhang, D., Xiang, X., Peng, X., et al. (2021). Highly Efficient Exosome Purification from Human Plasma by Tangential Flow Filtration Based Microfluidic Chip. *Sensors Actuators B Chem.* 333, 129563. doi:10.1016/j.snb.2021.129563
- Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of Cancer: the Next Generation. *cell* 144 (5), 646–674. doi:10.1016/j.cell.2011.02.013
- Haney, M. J., Klyachko, N. L., Zhao, Y., Gupta, R., Plotnikova, E. G., He, Z., et al. (2015). Exosomes as Drug Delivery Vehicles for Parkinson's Disease Therapy. *J. Control. Release* 207, 18–30. doi:10.1016/j.jconrel.2015.03.033
- Hartman, Z. C., Wei, J., Glass, O. K., Guo, H., Lei, G., Yang, X. Y., et al. (2011). Increasing Vaccine Potency through Exosome Antigen Targeting. *Vaccine* 29 (50), 9361–9367. doi:10.1016/j.vaccine.2011.09.133
- Hayashi, N., Doi, H., Kurata, Y., Kagawa, H., Atobe, Y., Funakoshi, K., et al. (2020). Proteomic Analysis of Exosome-Enriched Fractions Derived from

- Cerebrospinal Fluid of Amyotrophic Lateral Sclerosis Patients. *Neurosci. Res.* 160, 43–49. doi:10.1016/j.neures.2019.10.010
- Hermann, S., Buschmann, D., Kirchner, B., Borrmann, M., Brandes, F., Kotschote, S., et al. (2019). Transcriptomic Profiling of Cell-free and Vesicular microRNAs from Matched Arterial and Venous Sera. *J. Extracell. Vesicles* 8 (1), 1670935. doi:10.1080/20013078.2019.1670935
- Hisey, C. L., Dorayappan, K. D. P., Cohn, D. E., Selvendiran, K., and Hansford, D. J. (2018). Microfluidic Affinity Separation Chip for Selective Capture and Release of Label-free Ovarian Cancer Exosomes. *Lab. Chip* 18 (20), 3144–3153. doi:10.1039/C8LC00834E
- Holcar, M., Kanduđer, M., and Lenassi, M. (2021). Blood Nanoparticles - Influence on Extracellular Vesicle Isolation and Characterization. *Front. Pharmacol.* 12. doi:10.3389/fphar.2021.773844
- Hood, J. L., Scott, M. J., and Wickline, S. A. (2014). Maximizing Exosome Colloidal Stability Following Electroporation. *Anal. Biochem.* 448, 41–49. doi:10.1016/j.ab.2013.12.001
- Hornung, S., Dutta, S., and Bitan, G. (2020). CNS-derived Blood Exosomes as a Promising Source of Biomarkers: Opportunities and Challenges. *Front. Mol. Neurosci.* 13, 38. doi:10.3389/fnmol.2020.00038
- Howitt, J., and Hill, A. F. (2016). Exosomes in the Pathology of Neurodegenerative Diseases. *J. Biol. Chem.* 291 (52), 26589–26597. doi:10.1074/jbc.R116.757955
- Hu, W. T., and Grossman, M. (2009). TDP-43 and Frontotemporal Dementia. *Curr. Neurol. Neurosci. Rep.* 9 (5), 353–358. doi:10.1007/s11910-009-0052-3
- Huang, J. H., Yin, X. M., Xu, Y., Xu, C. C., Lin, X., Ye, F. B., et al. (2017). Systemic Administration of Exosomes Released from Mesenchymal Stromal Cells Attenuates Apoptosis, Inflammation, and Promotes Angiogenesis after Spinal Cord Injury in Rats. *J. Neurotrauma* 34 (24), 3388–3396. doi:10.1089/neu.2017.5063
- Huang, K., Fang, C., Yi, K., Liu, X., Qi, H., Tan, Y., et al. (2018). The Role of PTRF/Cavin1 as a Biomarker in Both Glioma and Serum Exosomes. *Theranostics* 8 (6), 1540–1557. doi:10.7150/thno.22952
- Huang, L., Gu, N., Zhang, X.-E., and Wang, D.-B. (2019). Light-Inducible Exosome-Based Vehicle for Endogenous RNA Loading and Delivery to Leukemia Cells. *Adv. Funct. Mat.* 29 (9), 1807189. doi:10.1002/adfm.201807189
- Iguchi, Y., Eid, L., Parent, M., Soucy, G., Bareil, C., Riku, Y., et al. (2016). Exosome Secretion Is a Key Pathway for Clearance of Pathological TDP-43. *Brain* 139 (12), 3187–3201. doi:10.1093/brain/aww237
- Jarmalavičiūtė, A., Tunaitis, V., Pivoraitė, U., Venalis, A., and Pivoriūnas, A. (2015). Exosomes from Dental Pulp Stem Cells Rescue Human Dopaminergic Neurons from 6-Hydroxy-Dopamine-Induced Apoptosis. *Cytotherapy* 17 (7), 932–939. doi:10.1016/j.jcyt.2014.07.013
- Jia, G., Han, Y., An, Y., Ding, Y., He, C., Wang, X., et al. (2018). NRP-1 Targeted and Cargo-Loaded Exosomes Facilitate Simultaneous Imaging and Therapy of Glioma *In Vitro* and *In Vivo*. *Biomaterials* 178, 302–316. doi:10.1016/j.biomaterials.2018.06.029
- Jia, L., Qiu, Q., Zhang, H., Chu, L., Du, Y., Zhang, J., et al. (2019). Concordance between the Assessment of Aβ₄₂, T-Tau, and P-T181-Tau in Peripheral Blood Neuronal-Derived Exosomes and Cerebrospinal Fluid. *Alzheimers Dement.* 15 (8), 1071–1080. doi:10.1016/j.jalz.2019.05.002
- Jia, L., Zhu, M., Kong, C., Pang, Y., Zhang, H., Qiu, Q., et al. (2021). Blood Neuro-Exosomal Synaptic Proteins Predict Alzheimer's Disease at the Asymptomatic Stage. *Alzheimers Dement.* 17 (1), 49–60. doi:10.1002/alz.12166
- Jiang, Y., Qian, J., Yang, J., Yan, X., Xue, X., and Chang, Q. (2018). Advances in Exosome-Related Biomarkers for Glioblastoma: Basic Research and Clinical Application. *Glioma* 1 (5), 159. doi:10.4103/glioma.glioma_35_1
- Jo, M., Lee, S., Jeon, Y. M., Kim, S., Kwon, Y., and Kim, H. J. (2020). The Role of TDP-43 Propagation in Neurodegenerative Diseases: Integrating Insights from Clinical and Experimental Studies. *Exp. Mol. Med.* 52 (10), 1652–1662. doi:10.1038/s12276-020-00513-7
- Johnstone, R. M., Adam, M., Hammond, J. R., Orr, L., and Turbide, C. (1987). Vesicle Formation during Reticulocyte Maturation. Association of Plasma Membrane Activities with Released Vesicles (Exosomes). *J. Biol. Chem.* 262 (19), 9412–9420. doi:10.1016/S0021-9258(18)48095-7
- Joshi, P., Turola, E., Ruiz, A., Bergami, A., Libera, D. D., Benussi, L., et al. (2014). Microglia Convert Aggregated Amyloid-β into Neurotoxic Forms through the Shedding of Microvesicles. *Cell Death Differ.* 21 (4), 582–593. doi:10.1038/cdd.2013.180
- Kalani, A., Tyagi, A., and Tyagi, N. (2014). Exosomes: Mediators of Neurodegeneration, Neuroprotection and Therapeutics. *Mol. Neurobiol.* 49 (1), 590–600. doi:10.1007/s12035-013-8544-1
- Kawakami, I., Arai, T., and Hasegawa, M. (2019). The Basis of Clinicopathological Heterogeneity in TDP-43 Proteinopathy. *Acta Neuropathol.* 138 (5), 751–770. doi:10.1007/s00401-019-02077-x
- Keerthikumar, S., Chisanga, D., Ariyaratne, D., Al Saffar, H., Anand, S., Zhao, K., et al. (2016). ExoCarta: a Web-Based Compendium of Exosomal Cargo. *J. Mol. Biol.* 428 (4), 688–692. doi:10.1016/j.jmb.2015.09.019
- Keller, S., Rupp, C., Stoeck, A., Runz, S., Fogel, M., Lugert, S., et al. (2007). CD24 Is a Marker of Exosomes Secreted into Urine and Amniotic Fluid. *Kidney Int.* 72 (9), 1095–1102. doi:10.1038/sj.ki.5002486
- Kheirandish-Gozal, L., Khalyfa, A., and Gozal, D. (2017). Exosomes, Blood-Brain Barrier, and Cognitive Dysfunction in Pediatric Sleep Apnea. *Sleep. Biol. Rhythms* 15 (4), 261–267. doi:10.1007/s41105-017-0108-8
- Kim, M. S., Haney, M. J., Zhao, Y., Mahajan, V., Deygen, I., Klyachko, N. L., et al. (2016). Development of Exosome-Encapsulated Paclitaxel to Overcome MDR in Cancer Cells. *Nanomedicine* 12 (3), 655–664. doi:10.1016/j.nano.2015.10.012
- Kooijmans, S. A. A., Fliervoet, L. A. L., Van Der Meel, R., Fens, M. H. A. M., Heijnen, H. F. G., van Bergen En Henegouwen, P. M. P., et al. (2016). PEGylated and Targeted Extracellular Vesicles Display Enhanced Cell Specificity and Circulation Time. *J. Control. Release* 224, 77–85. doi:10.1016/j.jconrel.2016.01.009
- Korkut, C., Li, Y., Koles, K., Brewer, C., Ashley, J., Yoshihara, M., et al. (2013). Regulation of Postsynaptic Retrograde Signaling by Presynaptic Exosome Release. *Neuron* 77 (6), 1039–1046. doi:10.1016/j.neuron.2013.01.013
- Krämer-Albers, E. M., Bretz, N., Tenzer, S., Winterstein, C., Möbius, W., Berger, H., et al. (2007). Oligodendrocytes Secrete Exosomes Containing Major Myelin and Stress-Protective Proteins: Trophic Support for Axons? *Proteomics Clin. Appl.* 1 (11), 1446–1461. doi:10.1002/prca.200700522
- Kumar, P., Wu, H., McBride, J. L., Jung, K. E., Kim, M. H., Davidson, B. L., et al. (2007). Transvascular Delivery of Small Interfering RNA to the Central Nervous System. *Nature* 448 (7149), 39–43. doi:10.1038/nature05901
- Kumar, S., Michael, I. J., Park, J., Granick, S., and Cho, Y. K. (2018). Cloaked Exosomes: Biocompatible, Durable, and Degradable Encapsulation. *Small* 14 (34), e1802052. doi:10.1002/smll.201802052
- Ladivala, A. R., Litt, J., Kane, R. S., Aucoin, D. S., Smith, S. O., Ranjan, S., et al. (2012). Conformational Differences between Two Amyloid β Oligomers of Similar Size and Dissimilar Toxicity. *J. Biol. Chem.* 287 (29), 24765–24773. doi:10.1074/jbc.M111.329763
- Lai, C. P., Mardini, O., Ericsson, M., Prabhakar, S., Maguire, C., Chen, J. W., et al. (2014). Dynamic Biodistribution of Extracellular Vesicles *In Vivo* Using a Multimodal Imaging Reporter. *ACS Nano* 8 (1), 483–494. doi:10.1021/nn404945r
- Lamichhane, T. N., Raiker, R. S., and Jay, S. M. (2015). Exogenous DNA Loading into Extracellular Vesicles *via* Electroporation Is Size-dependent and Enables Limited Gene Delivery. *Mol. Pharm.* 12 (10), 3650–3657. doi:10.1021/acs.molpharmaceut.5b00364
- Lässer, C., O'Neil, S. E., Shelke, G. V., Sihlbom, C., Hansson, S. F., Gho, Y. S., et al. (2016). Exosomes in the Nose Induce Immune Cell Trafficking and Harbour an Altered Protein Cargo in Chronic Airway Inflammation. *J. Transl. Med.* 14 (1), 1–14. doi:10.1186/s12967-016-0927-4
- Lebouvier, T., Chaumette, T., Paillusson, S., Duyckaerts, C., Bruley des Varannes, S., Neunlist, M., et al. (2009). The Second Brain and Parkinson's Disease. *Eur. J. Neurosci.* 30 (5), 735–741. doi:10.1111/j.1460-9568.2009.06873.x
- Lee, H. J., Patel, S., and Lee, S. J. (2005). Intravascular Localization and Exocytosis of Alpha-Synuclein and its Aggregates. *J. Neurosci.* 25 (25), 6016–6024. doi:10.1523/JNEUROSCI.0692-05.2005
- Lee, V. M., Balin, B. J., Otvos, L., and Trojanowski, J. Q. (1991). A68: a Major Subunit of Paired Helical Filaments and Derivatized Forms of Normal Tau. *Science* 251 (4994), 675–678. doi:10.1126/science.1899488
- Li, C., Fei, K., Tian, F., Gao, C., and Yang, S. (2019). Adipose-derived Mesenchymal Stem Cells Attenuate Ischemic Brain Injuries in Rats by Modulating miR-21-3p/MAT2B Signaling Transduction. *Croat. Med. J.* 60 (5), 439–448. doi:10.3325/cmj.2019.60.439
- Li, M., Huang, L., Chen, J., Ni, F., Zhang, Y., and Liu, F. (2021). Isolation of Exosome Nanoparticles from Human Cerebrospinal Fluid for Proteomic Analysis. *ACS Appl. Nano Mat.* 4 (4), 3351–3359. doi:10.1021/acsnm.0c02622

- Liu, C. G., Song, J., Zhang, Y. Q., and Wang, P. C. (2014). MicroRNA-193b Is a Regulator of Amyloid Precursor Protein in the Blood and Cerebrospinal Fluid Derived Exosomal microRNA-193b Is a Biomarker of Alzheimer's Disease. *Mol. Med. Rep.* 10 (5), 2395–2400. doi:10.3892/mmr.2014.2484
- Lopez-Verrilli, M. A., Picou, F., and Court, F. A. (2013). Schwann Cell-Derived Exosomes Enhance Axonal Regeneration in the Peripheral Nervous System. *Glia* 61 (11), 1795–1806. doi:10.1002/glia.22558
- Lucero, R., Zappulli, V., Sammarco, A., Murillo, O. D., Cheah, P. S., Srinivasan, S., et al. (2020). Glioma-derived miRNA-Containing Extracellular Vesicles Induce Angiogenesis by Reprogramming Brain Endothelial Cells. *Cell Rep.* 30 (7), 2065–e4. doi:10.1016/j.celrep.2020.01.073
- Luo, Q., Xian, P., Wang, T., Wu, S., Sun, T., Wang, W., et al. (2021). Antioxidant Activity of Mesenchymal Stem Cell-Derived Extracellular Vesicles Restores Hippocampal Neurons Following Seizure Damage. *Theranostics* 11 (12), 5986–6005. doi:10.7150/thno.58632
- Machida, T., Tomofuji, T., Ekuni, D., Maruyama, T., Yoneda, T., Kawabata, Y., et al. (2015). MicroRNAs in Salivary Exosome as Potential Biomarkers of Aging. *Int. J. Mol. Sci.* 16 (9), 21294–21309. doi:10.3390/ijms160921294
- Madison, M. N., Jones, P. H., and Okeoma, C. M. (2015). Exosomes in Human Semen Restrict HIV-1 Transmission by Vaginal Cells and Block Intravaginal Replication of LP-BM5 Murine AIDS Virus Complex. *Virology* 482, 189–201. doi:10.1016/j.virol.2015.03.040
- Mathieu, M., Martin-Jaular, L., Lavie, G., and Théry, C. (2019). Specificities of Secretion and Uptake of Exosomes and Other Extracellular Vesicles for Cell-To-Cell Communication. *Nat. Cell Biol.* 21 (1), 9–17. doi:10.1038/s41556-018-0250-9
- McKeever, P. M., Schneider, R., Taghdiri, F., Weichert, A., Multani, N., Brown, R. A., et al. (2018). MicroRNA Expression Levels Are Altered in the Cerebrospinal Fluid of Patients with Young-Onset Alzheimer's Disease. *Mol. Neurobiol.* 55 (12), 8826–8841. doi:10.1007/s12035-018-1032-x
- Mishra, A., Singh, P., Qayoom, I., Prasad, A., and Kumar, A. (2021). Current Strategies in Tailoring Methods for Engineered Exosomes and Future Avenues in Biomedical Applications. *J. Mat. Chem. B* 9 (32), 6281–6309. doi:10.1039/D1TB01088C
- Mittelbrunn, M., Gutiérrez-Vázquez, C., Villarroja-Beltri, C., González, S., Sánchez-Cabo, F., González, M. Á., et al. (2011). Unidirectional Transfer of microRNA-Loaded Exosomes from T Cells to Antigen-Presenting Cells. *Nat. Commun.* 2 (1), 1–10. doi:10.1038/ncomms1285
- Momen-Heravi, F., Bala, S., Bukong, T., and Szabo, G. (2014). Exosome-mediated Delivery of Functionally Active miRNA-155 Inhibitor to Macrophages. *Nanomedicine* 10 (7), 1517–1527. doi:10.1016/j.nano.2014.03.014
- Mondello, S., Guedes, V. A., Lai, C., Czeiter, E., Amrein, K., Kobeissy, F., et al. (2020). Circulating Brain Injury Exosomal Proteins Following Moderate-To-Severe Traumatic Brain Injury: Temporal Profile, Outcome Prediction and Therapy Implications. *Cells* 9 (4), 977. doi:10.3390/cells9040977
- Moon, G. J., Sung, J. H., Kim, D. H., Kim, E. H., Cho, Y. H., Son, J. P., et al. (2019). Application of Mesenchymal Stem Cell-Derived Extracellular Vesicles for Stroke: Biodistribution and microRNA Study. *Transl. Stroke Res.* 10 (5), 509–521. doi:10.1007/s12975-018-0668-1
- Morel, L., Regan, M., Higashimori, H., Ng, S. K., Esau, C., Vidensky, S., et al. (2013). Neuronal Exosomal miRNA-dependent Translational Regulation of Astroglial Glutamate Transporter GLT1. *J. Biol. Chem.* 288 (10), 7105–7116. doi:10.1074/jbc.M112.410944
- Morelli, A. E., Larregina, A. T., Shufesky, W. J., Sullivan, M. L., Stolz, D. B., Papworth, G. D., et al. (2004). Endocytosis, Intracellular Sorting, and Processing of Exosomes by Dendritic Cells. *Blood* 104 (10), 3257–3266. doi:10.1182/blood-2004-03-0824
- Muraoka, S., Jedrychowski, M. P., Tatebe, H., DeLeo, A. M., Ikezu, S., Tokuda, T., et al. (2019). Proteomic Profiling of Extracellular Vesicles Isolated from Cerebrospinal Fluid of Former National Football League Players at Risk for Chronic Traumatic Encephalopathy. *Front. Neurosci.* 13, 1059. doi:10.3389/fnins.2019.01059
- Muraoka, S., Jedrychowski, M. P., Yanamandra, K., Ikezu, S., Gygi, S. P., and Ikezu, T. (2020). Proteomic Profiling of Extracellular Vesicles Derived from Cerebrospinal Fluid of Alzheimer's Disease Patients: A Pilot Study. *Cells* 9 (9), 1959. doi:10.3390/cells9091959
- Nasca, C., Dobbin, J., Bigio, B., Watson, K., de Angelis, P., Kautz, M., et al. (2021). Insulin Receptor Substrate in Brain-Enriched Exosomes in Subjects with Major Depression: on the Path of Creation of Biosignatures of Central Insulin Resistance. *Mol. Psychiatry* 26 (9), 5140–5149. doi:10.1038/s41380-020-0804-7
- Neerukonda, S. N., Egan, N. A., Patria, J., Assakhi, I., Tavarides-Hontz, P., Modla, S., et al. (2020). A Comparison of Exosome Purification Methods Using Serum of Marek's Disease Virus (MDV)-vaccinated and -Tumor-Bearing Chickens. *Heliyon* 6 (12), e05669. doi:10.1016/j.heliyon.2020.e05669
- Nonaka, T., Masuda-Suzukake, M., Arai, T., Hasegawa, Y., Akatsu, H., Obi, T., et al. (2013). Prion-like Properties of Pathological TDP-43 Aggregates from Diseased Brains. *Cell Rep.* 4 (1), 124–134. doi:10.1016/j.celrep.2013.06.007
- Otake, K., Kamiguchi, H., and Hirozane, Y. (2019). Identification of Biomarkers for Amyotrophic Lateral Sclerosis by Comprehensive Analysis of Exosomal mRNAs in Human Cerebrospinal Fluid. *BMC Med. Genomics* 12 (1), 7–11. doi:10.1186/s12920-019-0473-z
- Otero-Ortega, L., Laso-García, F., Frutos, M. C. G., Diekhof, L., Martínez-Arroyo, A., Alonso-López, E., et al. (2020). Low Dose of Extracellular Vesicles Identified that Promote Recovery after Ischemic Stroke. *Stem Cell Res. Ther.* 11 (1), 70–15. doi:10.1186/s13287-020-01601-1
- Oushy, S., Hellwinkel, J. E., Wang, M., Nguyen, G. J., Gunaydin, D., Harland, T. A., et al. (2018). Glioblastoma Multiforme-Derived Extracellular Vesicles Drive Normal Astrocytes towards a Tumour-Enhancing Phenotype. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 373 (1737), 20160477. doi:10.1098/rstb.2016.0477
- Patel, G. K., Khan, M. A., Zubair, H., Srivastava, S. K., Khushman, M., Singh, S., et al. (2019). Comparative Analysis of Exosome Isolation Methods Using Culture Supernatant for Optimum Yield, Purity and Downstream Applications. *Sci. Rep.* 9 (1), 1–10. doi:10.1038/s41598-019-41800-2
- Peng, K. Y., Pérez-González, R., Alldred, M. J., Goulbourne, C. N., Morales-Corraliza, J., Saito, M., et al. (2019). Apolipoprotein E4 Genotype Compromises Brain Exosome Production. *Brain* 142 (1), 163–175. doi:10.1093/brain/awy289
- Peters, P. J., Geuze, H. J., Van der Donk, H. A., Slot, J. W., Griffith, J. M., Stam, N. J., et al. (1989). Molecules Relevant for T Cell-Target Cell Interaction Are Present in Cytolytic Granules of Human T Lymphocytes. *Eur. J. Immunol.* 19 (8), 1469–1475. doi:10.1002/eji.1830190819
- Potolichio, I., Carven, G. J., Xu, X., Stipp, C., Riese, R. J., Stern, L. J., et al. (2005). Proteomic Analysis of Microglia-Derived Exosomes: Metabolic Role of the Aminopeptidase CD13 in Neuropeptide Catabolism. *J. Immunol.* 175 (4), 2237–2243. doi:10.4049/jimmunol.175.4.2237
- Qi, H., Liu, C., Long, L., Ren, Y., Zhang, S., Chang, X., et al. (2016). Blood Exosomes Endowed with Magnetic and Targeting Properties for Cancer Therapy. *ACS Nano* 10 (3), 3323–3333. doi:10.1021/acsnano.5b06939
- Qin, W., Tsukasaki, Y., Dasgupta, S., Mukhopadhyay, N., Ikebe, M., and Sauter, E. R. (2016). Exosomes in Human Breast Milk Promote EMT. *Clin. Cancer Res.* 22 (17), 4517–4524. doi:10.1158/1078-0432.CCR-16-0135
- Rajendran, L., Honsho, M., Zahn, T. R., Keller, P., Geiger, K. D., Verkade, P., et al. (2006). Alzheimer's Disease Beta-Amyloid Peptides Are Released in Association with Exosomes. *Proc. Natl. Acad. Sci. U. S. A.* 103 (30), 11172–11177. doi:10.1073/pnas.0603838103
- Rao, F., Zhang, D., Fang, T., Lu, C., Wang, B., Ding, X., et al. (2019). Exosomes from Human Gingiva-Derived Mesenchymal Stem Cells Combined with Biodegradable Chitin Conduits Promote Rat Sciatic Nerve Regeneration. *Stem Cells Int.* 2019, 2546367. doi:10.1155/2019/2546367
- Raposo, G., Nijman, H. W., Stoorvogel, W., Liejendekker, R., Harding, C. V., Melief, C. J., et al. (1996). B Lymphocytes Secrete Antigen-Presenting Vesicles. *J. Exp. Med.* 183 (3), 1161–1172. doi:10.1084/jem.183.3.1161
- Record, M., Poirot, M., and Silvente-Poirot, S. (2014). Emerging Concepts on the Role of Exosomes in Lipid Metabolic Diseases. *Biochimie* 96, 67–74. doi:10.1016/j.biochi.2013.06.016
- Rekker, K., Saare, M., Roost, A. M., Kubo, A. L., Zarovni, N., Chiesi, A., et al. (2014). Comparison of Serum Exosome Isolation Methods for microRNA Profiling. *Clin. Biochem.* 47 (1–2), 135–138. doi:10.1016/j.clinbiochem.2013.10.020
- Riazifar, M., Mohammadi, M. R., Pone, E. J., Yeri, A., Lässer, C., Segaliny, A. I., et al. (2019). Stem Cell-Derived Exosomes as Nanotherapeutics for Autoimmune and Neurodegenerative Disorders. *ACS Nano* 13 (6), 6670–6688. doi:10.1021/acsnano.9b01004
- Richardson, J. J., and Ejima, H. (2019). Surface Engineering of Extracellular Vesicles through Chemical and Biological Strategies. *Chem. Mat.* 31 (7), 2191–2201. doi:10.1021/acs.chemmater.9b00050

- Saeedi, S., Israel, S., Nagy, C., and Turecki, G. (2019). The Emerging Role of Exosomes in Mental Disorders. *Transl. Psychiatry* 9 (1), 1–11. doi:10.1038/s41398-019-0459-9
- Salem, K. Z., Moschetta, M., Sacco, A., Imberti, L., Rossi, G., Ghobrial, I. M., et al. (2016). Exosomes in Tumor Angiogenesis. *Methods Mol. Biol.* 1464, 25–34. doi:10.1007/978-1-4939-3999-2_3
- Saman, S., Kim, W., Raya, M., Visnick, Y., Miro, S., Saman, S., et al. (2012). Exosome-associated Tau Is Secreted in Tauopathy Models and Is Selectively Phosphorylated in Cerebrospinal Fluid in Early Alzheimer Disease. *J. Biol. Chem.* 287 (6), 3842–3849. doi:10.1074/jbc.M111.277061
- Santangelo, A., Imbrucè, P., Gardenghi, B., Belli, L., Agushi, R., Tamanini, A., et al. (2018). A microRNA Signature from Serum Exosomes of Patients with Glioma as Complementary Diagnostic Biomarker. *J. Neurooncol.* 136 (1), 51–62. doi:10.1007/s11060-017-2639-x
- Sardar Sinha, M., Ansell-Schultz, A., Civitelli, L., Hildesjö, C., Larsson, M., Lannfelt, L., et al. (2018). Alzheimer's Disease Pathology Propagation by Exosomes Containing Toxic Amyloid-Beta Oligomers. *Acta Neuropathol.* 136 (1), 41–56. doi:10.1007/s00401-018-1868-1
- Sato, Y. T., Umezaki, K., Sawada, S., Mukai, S. A., Sasaki, Y., Harada, N., et al. (2016). Engineering Hybrid Exosomes by Membrane Fusion with Liposomes. *Sci. Rep.* 6 (1), 1–11. doi:10.1038/srep21933
- Shao, H., Chung, J., Balaj, L., Charest, A., Bigner, D. D., Carter, B. S., et al. (2012). Protein Typing of Circulating Microvesicles Allows Real-Time Monitoring of Glioblastoma Therapy. *Nat. Med.* 18 (12), 1–11. doi:10.1038/nm.2994
- Sharma, P., Mesci, P., Carromeu, C., McClatchy, D. R., Schiapparelli, L., Yates, J. R., et al. (2019). Exosomes Regulate Neurogenesis and Circuit Assembly. *Proc. Natl. Acad. Sci. U. S. A.* 116 (32), 16086–16094. doi:10.1073/pnas.1902513116
- Sheng, Y., Chattopadhyay, M., Whitelegge, J., and Valentine, J. S. (2012). SOD1 Aggregation and ALS: Role of Metallation States and Disulfide Status. *Curr. Top. Med. Chem.* 12 (22), 2560–2572. doi:10.2174/1568026611212220010
- Shi, M., Kovac, A., Korff, A., Cook, T. J., Ginghina, C., Bullock, K. M., et al. (2016). CNS Tau Efflux via Exosomes Is Likely Increased in Parkinson's Disease but Not in Alzheimer's Disease. *Alzheimers Dement.* 12 (11), 1125–1131. doi:10.1016/j.jalz.2016.04.003
- Shi, M., Liu, C., Cook, T. J., Bullock, K. M., Zhao, Y., Ginghina, C., et al. (2014). Plasma Exosomal α -synuclein Is Likely CNS-Derived and Increased in Parkinson's Disease. *Acta Neuropathol.* 128 (5), 639–650. doi:10.1007/s00401-014-1314-y
- Shtam, T., Evtushenko, V., Samsonov, R., Zabrodskaya, Y., Kamyshinsky, R., Zabagina, L., et al. (2020). Evaluation of Immune and Chemical Precipitation Methods for Plasma Exosome Isolation. *Plos one* 15 (11), e0242732. doi:10.1371/journal.pone.0242732
- Smalheiser, N. R. (2007). Exosomal Transfer of Proteins and RNAs at Synapses in the Nervous System. *Biol. Direct* 2 (1), 35–15. doi:10.1186/1745-6150-2-35
- Smyth, T., Petrova, K., Payton, N. M., Persaud, I., Redzic, J. S., Graner, M. W., et al. (2014). Surface Functionalization of Exosomes Using Click Chemistry. *Bioconjug. Chem.* 25 (10), 1777–1784. doi:10.1021/bc500291r
- Soares Martins, T., Catita, J., Martins Rosa, I., A B da Cruz E Silva, O., and Henriques, A. G. (2018). Exosome Isolation from Distinct Biofluids Using Precipitation and Column-Based Approaches. *PloS one* 13 (6), e0198820. doi:10.1371/journal.pone.0198820
- Spillantini, M. G., Crowther, R. A., Jakes, R., Cairns, N. J., Lantos, P. L., and Goedert, M. (1998). Filamentous Alpha-Synuclein Inclusions Link Multiple System Atrophy with Parkinson's Disease and Dementia with Lewy Bodies. *Neurosci. Lett.* 251 (3), 205–208. doi:10.1016/S0304-3940(98)00504-7
- Sterzenbach, U., Putz, U., Low, L. H., Silke, J., Tan, S. S., and Howitt, J. (2017). Engineered Exosomes as Vehicles for Biologically Active Proteins. *Mol. Ther.* 25 (6), 1269–1278. doi:10.1016/j.ymthe.2017.03.030
- Street, J. M., Barran, P. E., Mackay, C. L., Weidt, S., Balmforth, C., Walsh, T. S., et al. (2012). Identification and Proteomic Profiling of Exosomes in Human Cerebrospinal Fluid. *J. Transl. Med.* 10 (1), 5–7. doi:10.1186/1479-5876-10-5
- Street, J. M., Koritzinsky, E. H., Glispie, D. M., Star, R. A., and Yuen, P. S. (2017). Urine Exosomes: an Emerging Trove of Biomarkers. *Adv. Clin. Chem.* 78, 103–122. doi:10.1016/bs.acc.2016.07.003
- Stuffers, S., Sem Wegner, C., Stenmark, H., and Brech, A. (2009). Multivesicular Endosome Biogenesis in the Absence of ESCRTs. *Traffic* 10 (7), 925–937. doi:10.1111/j.1600-0854.2009.00920.x
- Sun, D., Zhuang, X., Xiang, X., Liu, Y., Zhang, S., Liu, C., et al. (2010). A Novel Nanoparticle Drug Delivery System: the Anti-inflammatory Activity of Curcumin Is Enhanced when Encapsulated in Exosomes. *Mol. Ther.* 18 (9), 1606–1614. doi:10.1038/mt.2010.105
- Sung, B. H., Von Lersner, A., Guerrero, J., Krystofiak, E. S., Inman, D., Pelletier, R., et al. (2020). A Live Cell Reporter of Exosome Secretion and Uptake Reveals Pathfinding Behavior of Migrating Cells. *Nat. Commun.* 11 (1), 1–15. doi:10.1038/s41467-020-15747-2
- Takahashi, Y., Nishikawa, M., Shinotsuka, H., Matsui, Y., Ohara, S., Imai, T., et al. (2013). Visualization and *In Vivo* Tracking of the Exosomes of Murine Melanoma B16-BL6 Cells in Mice after Intravenous Injection. *J. Biotechnol.* 165 (2), 77–84. doi:10.1016/j.jbiotec.2013.03.013
- Tan, Y. J., Wong, B. Y. X., Vaidyanathan, R., Sreejith, S., Chia, S. Y., Kandiah, N., et al. (2021). Altered Cerebrospinal Fluid Exosomal microRNA Levels in Young-Onset Alzheimer's Disease and Frontotemporal Dementia. *J. Alzheimers Dis. Rep.* 5, 805–813. doi:10.3233/ADR-210311
- Taylor, A. R., Robinson, M. B., Gifondorwa, D. J., Tytell, M., and Milligan, C. E. (2007). Regulation of Heat Shock Protein 70 Release in Astrocytes: Role of Signaling Kinases. *Dev. Neurobiol.* 67 (13), 1815–1829. doi:10.1002/dneu.20559
- Teng, F., and Fussenegger, M. (2021). Shedding Light on Extracellular Vesicle Biogenesis and Bioengineering. *Adv. Sci.* 8 (1), 2003505. doi:10.1002/adv.202003505
- Theos, A. C., Truschel, S. T., Tenza, D., Hurbain, I., Harper, D. C., Berson, J. F., et al. (2006). A Lumenal Domain-dependent Pathway for Sorting to Intraluminal Vesicles of Multivesicular Endosomes Involved in Organelle Morphogenesis. *Dev. Cell* 10 (3), 343–354. doi:10.1016/j.devcel.2006.01.012
- Théry, C., Amigorena, S., Raposo, G., and Clayton, A. (2006). Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids. *Curr. Protoc. Cell Biol.* Chapter 3 (1), Unit–22. doi:10.1002/0471143030.cb0322s30
- Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., et al. (2018). Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): a Position Statement of the International Society for Extracellular Vesicles and Update of the MISEV2014 Guidelines. *J. Extracell. Vesicles.* 7 (1), 1535750. doi:10.1080/20013078.2018.1535750
- Théry, C., Duban, L., Segura, E., Véron, P., Lantz, O., and Amigorena, S. (2002a). Indirect Activation of Naïve CD4+ T Cells by Dendritic Cell-Derived Exosomes. *Nat. Immunol.* 3 (12), 1156–1162. doi:10.1038/ni854
- Théry, C., Zitvogel, L., and Amigorena, S. (2002b). Exosomes: Composition, Biogenesis and Function. *Nat. Rev. Immunol.* 2 (8), 569–579. doi:10.1038/nri855
- Thompson, A. G., Gray, E., Heman-Ackah, S. M., Mäger, I., Talbot, K., Andaloussi, S. E., et al. (2016). Extracellular Vesicles in Neurodegenerative Disease - Pathogenesis to Biomarkers. *Nat. Rev. Neurol.* 12 (6), 346–357. doi:10.1038/nrneurol.2016.68
- Tian, T., Zhang, H. X., He, C. P., Fan, S., Zhu, Y. L., Qi, C., et al. (2018). Surface Functionalized Exosomes as Targeted Drug Delivery Vehicles for Cerebral Ischemia Therapy. *Biomaterials* 150, 137–149. doi:10.1016/j.biomaterials.2017.10.012
- Tian, Y., Li, S., Song, J., Ji, T., Zhu, M., Anderson, G. J., et al. (2014). A Doxorubicin Delivery Platform Using Engineered Natural Membrane Vesicle Exosomes for Targeted Tumor Therapy. *Biomaterials* 35 (7), 2383–2390. doi:10.1016/j.biomaterials.2013.11.083
- Trueta, C., and De-Miguel, F. F. (2012). Extrasynaptic Exocytosis and its Mechanisms: a Source of Molecules Mediating Volume Transmission in the Nervous System. *Front. Physiol.* 3, 319. doi:10.3389/fphys.2012.00319
- Tsvion-Visbord, H., Perets, N., Sofer, T., Bikovski, L., Goldshmit, Y., Ruban, A., et al. (2020). Mesenchymal Stem Cells Derived Extracellular Vesicles Improve Behavioral and Biochemical Deficits in a Phencyclidine Model of Schizophrenia. *Transl. Psychiatry* 10 (1), 305–310. doi:10.1038/s41398-020-00988-y
- Villarroya-Beltri, C., Baixauli, F., Gutiérrez-Vázquez, C., Sánchez-Madrid, F., and Mittelbrunn, M. (2014). Sorting it Out: Regulation of Exosome Loading. *Seminars Cancer Biol.* 28, 3–13. doi:10.1016/j.semcancer.2014.04.009
- Von Bartheld, C. S., and Altick, A. L. (2011). Multivesicular Bodies in Neurons: Distribution, Protein Content, and Trafficking Functions. *Prog. Neurobiol.* 93 (3), 313–340. doi:10.1016/j.pneurobio.2011.01.003
- Wahlgren, J., De L Karlson, T., Brisslert, M., Vaziri Sani, F., Teleme, E., Sunnerhagen, P., et al. (2012). Plasma Exosomes Can Deliver Exogenous Short Interfering RNA to Monocytes and Lymphocytes. *Nucleic Acids Res.* 40 (17), e130. doi:10.1093/nar/gks463

- Wan, S., Zhang, L., Wang, S., Liu, Y., Wu, C., Cui, C., et al. (2017). Molecular Recognition-Based DNA Nanoassemblies on the Surfaces of Nanosized Exosomes. *J. Am. Chem. Soc.* 139 (15), 5289–5292. doi:10.1021/jacs.7b00319
- Wan, Y., Wang, L., Zhu, C., Zheng, Q., Wang, G., Tong, J., et al. (2018). Aptamer-conjugated Extracellular Nanovesicles for Targeted Drug Delivery. *Cancer Res.* 78 (3), 798–808. doi:10.1158/0008-5472.CAN-17-2880
- Wang, B., and Han, S. (2019). Modified Exosomes Reduce Apoptosis and Ameliorate Neural Deficits Induced by Traumatic Brain Injury. *Asaio J.* 65 (3), 285–292. doi:10.1097/MAT.0000000000000810
- Wang, G., Wen, Y., Faletti, O. D., Zhao, Q., Liu, J., Zhang, G., et al. (2020). A Panel of Exosome-Derived Mirnas of Cerebrospinal Fluid for the Diagnosis of Moyamoya Disease. *Front. Neurosci.* 14, 548278. doi:10.3389/fnins.2020.548278
- Wang, H., Atik, A., Stewart, T., Ginghina, C., Aro, P., Kerr, K. F., et al. (2018a). Plasma α -synuclein and Cognitive Impairment in the Parkinson's Associated Risk Syndrome: A Pilot Study. *Neurobiol. Dis.* 116, 53–59. doi:10.1016/j.nbd.2018.04.015
- Wang, J. H., Forterre, A. V., Zhao, J., Frimannsson, D. O., Delcayre, A., Antes, T. J., et al. (2018b). Anti-HER2 scFv-Directed Extracellular Vesicle-Mediated mRNA-Based Gene Delivery Inhibits Growth of HER2-Positive Human Breast Tumor Xenografts by Prodrug Activation. *Mol. Cancer Ther.* 17 (5), 1133–1142. doi:10.1158/1535-7163.MCT-17-0827
- Wang, M., Altinoglu, S., Takeda, Y. S., and Xu, Q. (2015). Integrating Protein Engineering and Bioorthogonal Click Conjugation for Extracellular Vesicle Modulation and Intracellular Delivery. *PLoS One* 10 (11), e0141860. doi:10.1371/journal.pone.0141860
- Wang, S., Liu, Z., Ye, T., Mabrouk, O. S., Maltbie, T., Aasly, J., et al. (2017). Elevated LRRK2 Autophosphorylation in Brain-Derived and Peripheral Exosomes in LRRK2 Mutation Carriers. *Acta Neuropathol. Commun.* 5 (1), 86–13. doi:10.1186/s40478-017-0492-y
- Webb, R. L., Kaiser, E. E., Jurgielewicz, B. J., Spellacy, S., Scoville, S. L., Thompson, T. A., et al. (2018). Human Neural Stem Cell Extracellular Vesicles Improve Recovery in a Porcine Model of Ischemic Stroke. *Stroke* 49 (5), 1248–1256. doi:10.1161/STROKEAHA.117.020353
- Wei, R., Zhao, L., Kong, G., Liu, X., Zhu, S., Zhang, S., et al. (2020). Combination of Size-Exclusion Chromatography and Ultracentrifugation Improves the Proteomic Profiling of Plasma-Derived Small Extracellular Vesicles. *Biol. Proced. Online* 22 (1), 12–11. doi:10.1186/s12575-020-00125-5
- Wei, W., Ao, Q., Wang, X., Cao, Y., Liu, Y., Zheng, S. G., et al. (2021). Mesenchymal Stem Cell-Derived Exosomes: A Promising Biological Tool in Nanomedicine. *Front. Pharmacol.* 11. doi:10.3389/fphar.2020.590470
- Welton, J. L., Loveless, S., Stone, T., von Ruhland, C., Robertson, N. P., and Clayton, A. (2017). Cerebrospinal Fluid Extracellular Vesicle Enrichment for Protein Biomarker Discovery in Neurological Disease; Multiple Sclerosis. *J. Extracell. vesicles* 6 (1), 1369805. doi:10.1080/20013078.2017.1369805
- Wesseling, P., and Capper, D. (2018). WHO 2016 Classification of Gliomas. *Neuropathol. Appl. Neurobiol.* 44 (2), 139–150. doi:10.1111/nan.12432
- Winston, C. N., Goetzl, E. J., Akers, J. C., Carter, B. S., Rockenstein, E. M., Galasko, D., et al. (2016). Prediction of Conversion from Mild Cognitive Impairment to Dementia with Neuronally Derived Blood Exosome Protein Profile. *Alzheimers Dement. (Amst)* 3, 63–72. doi:10.1016/j.dadm.2016.04.001
- Winston, C. N., Romero, H. K., Ellisman, M., Nauss, S., Julovich, D. A., Conger, T., et al. (2019). Assessing Neuronal and Astrocyte Derived Exosomes from Individuals with Mild Traumatic Brain Injury for Markers of Neurodegeneration and Cytotoxic Activity. *Front. Neurosci.* 13, 1005. doi:10.3389/fnins.2019.01005
- Wu, M., Ouyang, Y., Wang, Z., Zhang, R., Huang, P. H., Chen, C., et al. (2017). Isolation of Exosomes from Whole Blood by Integrating Acoustics and Microfluidics. *Proc. Natl. Acad. Sci. U. S. A.* 114 (40), 10584–10589. doi:10.1073/pnas.1709210114
- Wu, Y., Deng, W., and Klinke, D. J., II (2015). Exosomes: Improved Methods to Characterize Their Morphology, RNA Content, and Surface Protein Biomarkers. *Analyst* 140 (19), 6631–6642. doi:10.1039/C5AN00688K
- Xu, Q., Zhao, Y., Zhou, X., Luan, J., Cui, Y., and Han, J. (2018). Comparison of the Extraction and Determination of Serum Exosome and miRNA in Serum and the Detection of miR-27a-3p in Serum Exosome of ALS Patients. *Intractable Rare Dis. Res.* 7 (1), 13–18. doi:10.5582/irdr.2017.01091
- Yagi, Y., Ohkubo, T., Kawaji, H., Machida, A., Miyata, H., Goda, S., et al. (2017). Next-generation Sequencing-Based Small RNA Profiling of Cerebrospinal Fluid Exosomes. *Neurosci. Lett.* 636, 48–57. doi:10.1016/j.neulet.2016.10.042
- Yamaguchi, H., Hirai, S., Shoji, M., Harigaya, Y., Okamoto, Y., and Nakazato, Y. (1989). Alzheimer Type Dementia: Diffuse Type of Senile Plaques Demonstrated by Beta Protein Immunostaining. *Prog. Clin. Biol. Res.* 317, 467–474. doi:10.1007/BF00687420
- Yamazaki, Y., Hozumi, Y., Kaneko, K., Sugihara, T., Fujii, S., Goto, K., et al. (2007). Modulatory Effects of Oligodendrocytes on the Conduction Velocity of Action Potentials along Axons in the Alveus of the Rat Hippocampal CA1 Region. *Neuron Glia Biol.* 3 (4), 325–334. doi:10.1017/S1740925X08000070
- Yang, J., Zhang, X., Chen, X., Wang, L., and Yang, G. (2017a). Exosome Mediated Delivery of miR-124 Promotes Neurogenesis after Ischemia. *Mol. Ther. Nucleic Acids* 7, 278–287. doi:10.1016/j.omtn.2017.04.010
- Yang, T., Martin, P., Fogarty, B., Brown, A., Schurman, K., Phipps, R., et al. (2015). Exosome Delivered Anticancer Drugs across the Blood-Brain Barrier for Brain Cancer Therapy in *Danio rerio*. *Pharm. Res.* 32 (6), 2003–2014. doi:10.1007/s11095-014-1593-y
- Yang, T. T., Liu, C. G., Gao, S. C., Zhang, Y., and Wang, P. C. (2018). The Serum Exosome Derived MicroRNA-135a, -193b, and -384 Were Potential Alzheimer's Disease Biomarkers. *Biomed. Environ. Sci.* 31 (2), 87–96. doi:10.3967/bes2018.011
- Yang, Y., Ye, Y., Su, X., He, J., Bai, W., and He, X. (2017b). MSCs-Derived Exosomes and Neuroinflammation, Neurogenesis and Therapy of Traumatic Brain Injury. *Front. Cell. Neurosci.* 11, 55. doi:10.3389/fncel.2017.00055
- Yang, Z., Xie, J., Zhu, J., Kang, C., Chiang, C., Wang, X., et al. (2016). Functional Exosome-Mimic for Delivery of siRNA to Cancer: *In Vitro* and *In Vivo* Evaluation. *J. Control. Release* 243, 160–171. doi:10.1016/j.jconrel.2016.10.008
- Ye, Z., Zhang, T., He, W., Jin, H., Liu, C., Yang, Z., et al. (2018). Methotrexate-loaded Extracellular Vesicles Functionalized with Therapeutic and Targeted Peptides for the Treatment of Glioblastoma Multiforme. *ACS Appl. Mat. Interfaces* 10 (15), 12341–12350. doi:10.1021/acsami.7b18135
- Yekula, A., Taylor, A., Beecroft, A., Kang, K. M., Small, J. L., Muralidharan, K., et al. (2021). The Role of Extracellular Vesicles in Acquisition of Resistance to Therapy in Glioblastomas. *Cdr* 4 (1), 1–16. doi:10.20517/cdr.2020.61
- Yim, N., Ryu, S. W., Choi, K., Lee, K. R., Lee, S., Choi, H., et al. (2016a). Exosome Engineering for Efficient Intracellular Delivery of Soluble Proteins Using Optically Reversible Protein-Protein Interaction Module. *Nat. Commun.* 7 (1), 12277–12279. doi:10.1038/ncomms12277
- Yim, N., Ryu, S.-W., Choi, K., Lee, K. R., Lee, S., Choi, H., et al. (2016b). “Abstract 2167: Efficient and Rapid Cellular Delivery of Bioactive Proteins Using EXPLOR: Exosomes Engineered for Protein Loading via Optically Reversible Protein-Protein Interaction,” in Proceedings of the 107th Annual Meeting of the American Association for Cancer Research. doi:10.1158/1538-7445.AM2016-2167
- Yoo, C. E., Kim, G., Kim, M., Park, D., Kang, H. J., Lee, M., et al. (2012). A Direct Extraction Method for microRNAs from Exosomes Captured by Immunoaffinity Beads. *Anal. Biochem.* 431 (2), 96–98. doi:10.1016/j.ab.2012.09.008
- Zhan, Q., Yi, K., Qi, H., Li, S., Li, X., Wang, Q., et al. (2020). Engineering Blood Exosomes for Tumor-Targeting Efficient Gene/chemo Combination Therapy. *Theranostics* 10 (17), 7889–7905. doi:10.7150/thno.45028
- Zhang, G., and Yang, P. (2018). A Novel Cell-Cell Communication Mechanism in the Nervous System: Exosomes. *J. Neurosci. Res.* 96 (1), 45–52. doi:10.1002/jnr.24113
- Zhang, H., Wu, J., Wu, J., Fan, Q., Zhou, J., Wu, J., et al. (2019). Exosome-mediated Targeted Delivery of miR-210 for Angiogenic Therapy after Cerebral Ischemia in Mice. *J. Nanobiotechnology* 17 (1), 29–13. doi:10.1186/s12951-019-0461-7
- Zhang, Y., Chopp, M., Meng, Y., Katakowski, M., Xin, H., Mahmood, A., et al. (2015). Effect of Exosomes Derived from Multipotent Mesenchymal Stromal Cells on Functional Recovery and Neurovascular Plasticity in Rats after Traumatic Brain Injury. *J. Neurosurg.* 122 (4), 856–867. doi:10.3171/2014.11.JNS14770
- Zhang, Y., Zhang, Y., Chopp, M., Pang, H., Zhang, Z. G., Mahmood, A., et al. (2021). MiR-17-92 Cluster-Enriched Exosomes Derived from Human Bone Marrow Mesenchymal Stromal Cells Improve Tissue and Functional Recovery in Rats after Traumatic Brain Injury. *J. Neurotrauma* 38 (11), 1535–1550. doi:10.1089/neu.2020.7575
- Zhao, X., Wu, X., Qian, M., Song, Y., Wu, D., and Zhang, W. (2018). Knockdown of TGF- β 1 Expression in Human Umbilical Cord Mesenchymal Stem Cells Reverts Their Exosome-Mediated EMT Promoting Effect on Lung Cancer Cells. *Cancer Lett.* 428, 34–44. doi:10.1016/j.canlet.2018.04.026
- Zhao, Z. H., Chen, Z. T., Zhou, R. L., Zhang, X., Ye, Q. Y., and Wang, Y. Z. (2018). Increased DJ-1 and α -Synuclein in Plasma Neural-Derived Exosomes as Potential Markers for Parkinson's Disease. *Front. Aging Neurosci.* 10, 438. doi:10.3389/fnagi.2018.00438

- Zhuang, X., Xiang, X., Grizzle, W., Sun, D., Zhang, S., Axtell, R. C., et al. (2011). Treatment of Brain Inflammatory Diseases by Delivering Exosome Encapsulated Anti-inflammatory Drugs from the Nasal Region to the Brain. *Mol. Ther.* 19 (10), 1769–1779. doi:10.1038/mt.2011.164
- Zou, J., Guo, L., Wei, B., Yu, A., Yu, B., and Xu, A. (2020). Long Noncoding RNA POU3F3 and α -Synuclein in Plasma L1CAM Exosomes Combined with β -Glucocerebrosidase Activity: Potential Predictors of Parkinson's Disease. *Neurotherapeutics* 17 (3), 1104–1119. doi:10.1007/s13311-020-00842-5

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Ghosh and Ghosh. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Potentiality of Exosomal Proteins as Novel Cancer Biomarkers for Liquid Biopsy

Chunmiao Hu^{1,2}, Wei Jiang², Mingjin Lv², Shuhao Fan¹, Yujia Lu¹, Qingjun Wu^{2*} and Jiang Pi^{1*}

¹ Institute of Laboratory Medicine, Guangdong Provincial Key Laboratory of Medical Molecular Diagnostics, School of Medical Technology, The First Dongguan Affiliated Hospital, Guangdong Medical University, Dongguan, China, ² Department of Cellular Biology, Dakewe, Shenzhen, China

OPEN ACCESS

Edited by:

Suman Dutta,
University of California, Los Angeles,
United States

Reviewed by:

Suchandrima Ghosh,
Institute of Post Graduate Medical
Education and Research (IPGIMER),
India

Somnath Paul,
University of Texas MD Anderson
Cancer Center, United States

*Correspondence:

Qingjun Wu
wu_qingjun@dakewe.com
Jiang Pi
jiangpi@gdmu.edu.cn

Specialty section:

This article was submitted to
Cytokines and Soluble
Mediators in Immunity,
a section of the journal
Frontiers in Immunology

Received: 09 October 2021

Accepted: 22 April 2022

Published: 09 June 2022

Citation:

Hu C, Jiang W, Lv M, Fan S, Lu Y,
Wu Q and Pi J (2022) Potentiality of
Exosomal Proteins as Novel Cancer
Biomarkers for Liquid Biopsy.
Front. Immunol. 13:792046.
doi: 10.3389/fimmu.2022.792046

Liquid biopsy has been rapidly developed in recent years due to its advantages of non-invasiveness and real-time sampling in cancer prognosis and diagnosis. Exosomes are nanosized extracellular vesicles secreted by all types of cells and abundantly distributed in all types of body fluid, carrying diverse cargos including proteins, DNA, and RNA, which transmit regulatory signals to recipient cells. Among the cargos, exosomal proteins have always been used as immunoaffinity binding targets for exosome isolation. Increasing evidence about the function of tumor-derived exosomes and their proteins is found to be massively associated with tumor initiation, progression, and metastasis in recent years. Therefore, exosomal proteins and some nucleic acids, such as miRNA, can be used not only as targets for exosome isolation but also as potential diagnostic markers in cancer research, especially for liquid biopsy. This review will discuss the existing protein-based methods for exosome isolation and characterization that are more appropriate for clinical use based on current knowledge of the exosomal biogenesis and function. Additionally, the recent studies for the use of exosomal proteins as cancer biomarkers are also discussed and summarized, which might contribute to the development of exosomal proteins as novel diagnostic tools for liquid biopsy.

Keywords: exosomal proteins, biomarkers, cancer diagnosis, liquid biopsy, exosome

INTRODUCTION

Tissue biopsy is widely applied as the gold standard for clinical cancer diagnosis. Nonetheless, tissue sampling becomes a substantial challenge once the tumor is adjacent to major blood vessels, which would make the surgical sampling procedures extremely invasive and painful. In addition, there are also some circumstances in which the small dissected tissues of patients may not be sufficient to represent the pathological profile of the primary tumor (1). Due to the above limitations, liquid biopsy has emerged as the most capable substitute for tissue biopsy due to the advantage of the much more easily accessible samples, such as urine, blood, and cerebrospinal fluid. Multiple sources of tumor-derived substances including circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and exosomes can be detected from these body fluid samples and can be quickly analyzed (2). Hence, reliable real-time information can be acquired that can help in making a cancer prognosis and monitoring the physiological state of patients with repeated non-invasive sampling (3).

Masked by billions of host cells and vast quantities of free DNA released from normal cells, CTCs and ctDNA are scarcely dispersed in blood. Therefore, it is essential to isolate and enrich CTCs and ctDNA with elaborate techniques that possess high selectivity and sensitivity to carry out clinical analysis (4, 5). However, the enrichment of CTCs remains a big challenge for the application of CTCs, which is restricted by limited cancer surface markers and hindered by a rather limited number of CTCs themselves in the body fluids of cancer patients (6). CtDNA is generally thought to be secreted by necrotic and apoptotic cells. Although its abundance is higher than that of CTCs (7), ctDNA fragments have a very short half-life, even less than 1 h, and are rapidly cleared off (8). Based on these difficulties, the development of exosomes for liquid biopsy has gained increasing attention.

One of the greatest strengths of exosome-based liquid biopsy over CTCs (0–1,000 cells per 7.5 ml of blood) (9) and ctDNA is the larger distributed quantities of exosomes in the body fluids (up to 10^{11} exosomes per ml in the blood) (10). It is not an exaggeration that up to 10% of the circulating exosomes in a cancer patient would be tumor-derived exosomes at the late tumor stage (10, 11). Circulating RNA including mRNA and miRNA is also suggested to be a significant functional mediator in some cancers (8), but the worse stability in the plasma and other intricate causes hinder the clinical use of cell-free RNA (12). On the contrary, another advantage of exosomes as a promising diagnostic marker is that the cargos inside exosomes are well-protected by their lipid bilayer, which makes the intact tumor-derived entities such as RNA, DNA, and proteins carrying the comprehensive oncogenic information to be obtained and identified right after exosome isolation (13). An example of that is the family of phosphorylated proteins, which are usually degraded by the free circulating phosphatase, making them very difficult to be detected in the body fluid. However, these phosphorylated proteins can be well isolated from exosomes and stored stably in the exosomal form for as long as 5 years at -80°C (14). The fact that different cargos are sorted into exosomes as a protective vesicle makes multicomponent analysis feasible, which is conducive to a full-scale knowledge of how exosomes function and what the consequence is. Therefore, the sum of tumor-derived exosomes can be representative of the tumor heterogeneity (15). Moreover, combined research data have shown and confirmed that tumor-derived exosomes and their encapsulated proteins play important roles in cancer progression, which indicates the enormous potentiality for tumor-derived exosomal proteins as novel cancer biomarkers in liquid biopsy (16).

Nucleic acids in exosomes have been intensively investigated as cancer biomarkers. Nonetheless, increasing evidence suggests that proteins of tumor-derived exosomes circulating in the body fluids can also be the precise representative of relevant tumors in distant tissues. Here in this review, the potential of exosomal proteins as liquid biopsy biomarkers in future clinical research has been discussed. The biogenesis and function of exosomes, along with the latest isolation and characterization methods, have been discussed. The application of different exosomal

proteins as tumor liquid biopsy targets in different research studies and the future development of exosomal proteins as powerful diagnostic targets in hospitals have been elaborated on in this review.

BIOLOGY AND FUNCTION OF EXOSOMES

Biogenesis and Composition of Exosomes

Extracellular vesicles (EVs) are particles naturally secreted by cells. They are delimited by a lipid bilayer but do not have a functional nucleus for self-replication (17). According to the process of formation, EVs are classified into two categories: ectosomes and exosomes. Ectosomes are formed by direct exocytosis of the plasma membrane (PM) (18). By contrast, exosomes are the product of consecutive internalizations of PM and are finally released by fusion with PM. Consecutive PM internalizations include the first inward budding of PM to form early endosomes, which will then mature into late endosomes. Second, invagination of the membrane in late endosomes gives rise to several intraluminal vesicles (ILVs) containing various constituents such as DNA, RNA, enzymes, and proteins. These late endosome-containing ILVs are also recognized as multi-vesicular body (MVB). Finally, MVB will fuse with PM, and ILVs will be released as exosomes into the extracellular milieu with PM-derived membrane (19, 20) (**Figure 1**). The generation of exosomes can be both ESCRT (endosomal sorting complexes required for transport)-dependent and ESCRT-independent (21).

Exosomes can be secreted by all types of cells including eukaryotic and prokaryotic cells (14, 20). Negatively stained exosomes display classic cup-shape and lipid bilayer structure under electron microscopy (EM), with a generic size range of 40–160 nm (~ 100 nm on average) in diameter and a density range of 1.15–1.19 g/ml (22). Diverse contents including nucleic acid, protein, lipid, and metabolite can be sorted and packaged into exosomes, which reflect the biological properties of parental cells in different scenarios.

Similar to cell purification and isolation, proteins are widely used as surface and internal markers for the isolation, characterization, and investigation of exosomes. According to the statistical study of data from ExoCarta, a database of exosome cargos, there are approximately 10,000 different exosomal proteins that have been characterized, and the number is believed to be growing over time (23). The proteins wrapped up in exosomes can be divided into three major groups according to their functions: biogenesis relevant (tetraspanins and ESCRT machinery), transport and secretion relevant (heat shock proteins and membrane transport proteins and cytoskeletal proteins), and cell of origin relevant (major histocompatibility complex (MHC) molecules and cytokines and other proteins) (10). CD9, CD63, CD81, and CD82 are the main tetraspanins that are associated with cargo selection machinery and biogenesis of exosomes, and CD63 is the protein marker mostly utilized for immunocapture of exosomes. Representative ESCRT machinery proteins are TSG101 and Alix. Heat shock protein, Rab GTPase, annexin, flotillin, syntaxin, actin, and tubulin are the common

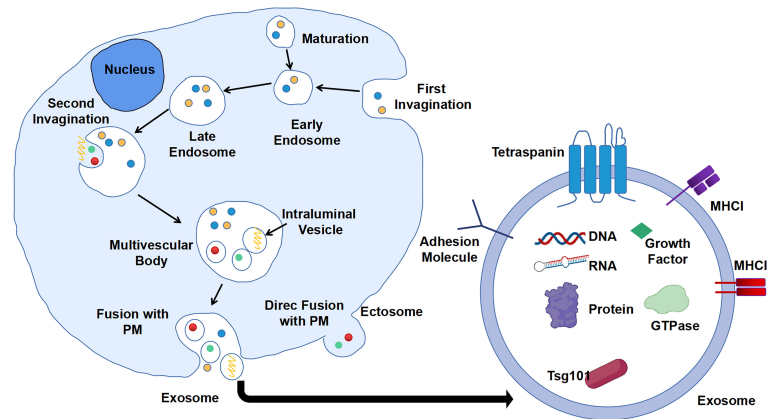


FIGURE 1 | Biogenesis and composition of exosomes. First invagination of plasma membrane (PM) forms the early endosomes that contain multiple constituents, which will then mature into late endosomes. Second, invagination of membrane of late endosomes generates multi-vesicular body (MVB) containing intraluminal vesicles (ILVs). ILVs are secreted into extracellular milieu as exosomes with cargos including DNA, RNA, and proteins.

exosome transport-related proteins. However, the role of heat shock proteins in the biogenesis of exosomes needs further investigation (24). Some specific exosomes also contain MHC molecules of antigen-presenting cells (APCs) and cytokines like interleukins that are accommodated with immune responses (19).

Exosomal nucleic acids are comprised of DNA, mRNA, miRNA, and lncRNA. As research moves further, the level of miRNA in exosomes was found to be more condensed over other species of nucleic acids from parental cells (25). Compared to other nucleic acids, miRNA is the most widely studied component in exosomes and is found to play a significant role in exosome-mediated cell-cell communication (26). Additionally, differential expression levels of miRNA are vastly discovered in cancer cells by various clinical studies, indicating that miRNA has great potential for cancer diagnosis (27). Nonetheless, there are still difficulties to integrate exosomal miRNA into cancer diagnosis and therapy (28).

In fact, the history of exosome research is relatively short, which is only several decades. The initial discovery of the existence of exosomes was in 1946 (29). But until 1987, Johnstone et al. provided pivotal evidence for the selective release of vesicles that are approximately 50 nm (in diameter) as reticulocytes maturing to erythrocytes. These vesicles were first termed exosomes by the same group (30). Later, a growing body of research data demonstrated in detail that exosomes played important roles in cell-cell communication in both healthy and disease conditions (26, 26, 31, 32), which therefore attracted increasing attention from the scientific field to explore the potential of exosomes as the diagnostic analyte and drug delivery carrier ever since.

Function of Exosomes

The biogenesis and content of exosomes have determined their destiny and mission as intercellular messengers. When parental cells produce and secrete exosomes, those exosomes carrying

proteins, lipids, metabolites, and nucleotides can transfer *via* the extracellular matrix (ECM) and circulation system and to any site of our body. Recipient cells with different varieties can internalize those exosomes by different patterns, such as clathrin-dependent endocytosis (33) or direct fusion with PM (34). Once uptaken, exosomes will deliver their cargos into cells for the regulation of multiple cellular activities, such as cellular development, immune response, and disease condition of recipient cells (19). For example, exosomal DNA can promote the cGAS-STING signaling pathway and pro-inflammatory response (35). Additionally, exosomal miRNA can also show regulatory functions during pregnancy (36). APCs like macrophages or dendritic cells can produce exosomes that carry MHC molecules with antigen peptides. Afterward, T cells are primed and activated when they uptake those exosomes *via* T-cell receptor (TCR)-MHC-peptide recognition (37).

Tumor-Derived Exosomes and Their Role in Cancer

It is now widely accepted that exosomes also participate in sophisticated cellular interactions between tumor cells and the tumor microenvironment (TME) in every step of cancer development. First, tumor-derived exosomal miRNA modifies gene expression in epithelial cells or fibroblasts, promoting their malignant transformation (19). Meanwhile, other soluble growth factors are also delivered by exosomes to those tumor-associated cells, activating different signaling events like PI3K/AKT pathway (38) or Akt and ERK pathways (39) and leading to the proliferation of recipient cells. Second, tumor-derived exosomes can promote epithelial-to-mesenchymal transition (EMT) and tumor metastasis by activating the resting cancer cells to aggressively metastasize *via* multiple inducible signaling molecules like Notch1 and HIF1 α (40, 41). During TME, hypoxic conditions promote tumor cells to express much more exosomal proteins than those outside the niche, which promotes ECM remodeling and angiogenesis (42, 43). In the meantime,

tumor-derived exosomes can assist tumor cells to escape from the surveillance of the immune system and develop the capability of chemoresistance, which further promote tumor progression. Some studies have revealed that tumor-derived exosomes can inhibit the cytotoxicity of natural killer cells and cytotoxic T lymphocytes, both of which are indispensable players of the immune mechanism against tumor progression (44, 45). Macrophages and dendritic cells are the pivotal mediators of innate and adaptive immunity, and they are not spared. Tumor-derived exosomes have shown to induce the polarization of immature macrophages into M2 macrophages and display anti-inflammatory activities favoring the ongoing tumor progression (46). Evidence has also shown that maturation of dendritic cells can be suppressed by tumor-derived exosomes, which affects the antigen priming and the activation of antitumor T cells eventually (47). Intrinsically chemoresistant cancer-associated fibroblasts (CAFs) can generate chemoresistant exosomes by sorting relevant molecules into exosomes. Recipient cells including tumor cells in TME will acquire the ability of chemoresistance after the uptake of these chemoresistant exosomes (48). Monitoring exchange of exosomes *in vivo* remains a difficult subject to investigate, which if resolved may shed more light on their tumor-associated function.

ISOLATION AND CHARACTERIZATION METHODS

The characteristics of exosomes are exploited as powerful targets in liquid biopsy and therapy. Nevertheless, the biggest challenge faced for their further application in clinical research and liquid biopsy is the difficulty in obtaining a purified population of exosomes from different sources of samples and accurately characterizing them as authentic exosomes. Although traditional methods like ultracentrifugation remain the gold standard, their unsuitability becomes increasingly obvious due to the requirement of expensive equipment, prolonged working hours, and a large amount of labor (49, 50). On the contrary, new methods like microfluidics take advantage of different traits of exosomes and create opportunities for the transition of exosome research from bench to bedside. The isolation method based on size exclusion or hydrophobic interaction-related mechanism can separate total exosomes from fluid samples. However, the results and yield of purified exosomes among different isolation methods are distinct from one another (51). In addition, there are specific methods that can select subpopulations of exosomes by surface protein markers. In this section, methods based on exosomal proteins will be briefly discussed in terms of suitability for clinical application.

Isolation Methods

Polymer Co-Precipitation

Polychylene glycol (PEG) is cheap and easy to obtain, and the whole purification process does not require complex equipment, except for low-speed centrifugation. Therefore, many

commercial kits have emerged based on the above strengths of PEG co-precipitation, like ExoQuick, Exo-spin, and Total Exosome Isolation. Commercial kits not only save time but also provide relatively standard experimental procedures to eliminate human errors, which therefore are very favorable to clinical labs. Samples of purified exosomes are often contaminated by junk components during isolation (52). When dealing with a serum that contains plenty of lipid proteins and albumin, it is recommended to remove such interference before the use of kits if high purity is required.

Affinity-Based Isolation

The membrane of exosomes is enriched with phosphatidylserines, which can bind to a type I transmembrane protein called Tim-4. The binding specificity was developed as a novel method by Nakai et al. to separate phosphatidylserine-rich exosomes (53). Compared to PEG co-precipitation, separation by this method results in fewer contaminants. However, the yield of exosomes might also include other components such as microvesicles that also contain phosphatidylserine. Interestingly, there are two commercial kits that are likely associated with affinity membrane, including exoEasy from QIAGEN and Capturem from TaKaRa.

Ultrafiltration

Based on the size and molecular weight, ultrafiltration is used for exosomes enrichment. However, before loading the sample onto an ultrafiltration column, it is usually recommended to perform sequential centrifugation steps to totally remove cells and debris, followed by a one-time filtration step through a 0.45- μ m filter to remove large apoptotic bodies (54). Likewise, ultrafiltration is easy to conduct, and it saves both time and labor. But exosomes can remain retained in the filtration membrane during centrifugation, which not only hinders effective separation but also leads to a big loss of yield with insufficient elution (55). Moreover, when it comes to viscous samples like a serum, ultrafiltration shows lesser efficiency. Therefore, the method should be chosen according to the sample of interest to ensure a better separation of exosomes.

Size Exclusion Chromatography

Size exclusion chromatography (SEC) is another popular exosome purification method based on the size property of exosomes. It can separate exosomes efficiently from impurities (proteins, lipids, etc.) with well-maintained biological activity. Nonetheless, traditional SEC requires a very long time to run with the existence of contamination in the products (56, 57). But there is a commercial automated instrument from iZON that can be taken into consideration for clinical uses, which is claimed to optimize the running time from hours to minutes.

Immunoaffinity Capture

Methods of immunoaffinity isolation usually contain two components, streptavidin-coated magnetic beads and biotinylated purified monoclonal antibodies, which can be combined *via* a streptavidin-biotin bond. Antibodies with high affinity will catch corresponding target exosomal proteins to make sure that the exosomes are captured and gathered by a

magnet (58). Tetraspanins including CD9, CD63, and CD81 are generally used as targets because these proteins are recognized as ubiquitous markers expressed among exosomes regardless of origin (19). Methods of immunoaffinity do not involve complicated procedures, while the field of monoclonal antibody production is mature enough to guarantee the quality of antibodies. Therefore, the immunoaffinity capture method is appropriate for clinical applications. However, it is worth to be considered that exosomes isolated by the immunoaffinity method cannot get rid of magnetic bead-bound antibodies, which may influence the bioactivity of exosomes and the experimental results of downstream assays. Surprisingly, a new branch of aptamer-based technique emerged recently to show strong potential to resolve the disadvantage of antibody-based technique. The main advantage of the development of aptamers by exosome researchers is that aptamers are easier to synthesize and produce than antibodies (3). Notably, a group successfully separated exosomes with CD63 aptamer and then discharged bound exosomes by adding complementary nucleotide strands to separate exosomes from aptamer. This method could largely maintain the integrity and activity of exosomes, which further reinforces the power of the aptamer-based isolation technique for exosome research (59). **Figure 2** provides an illustrative procedure of representative methods, and **Table 1** provides the comparisons among the mentioned isolation methods with their characteristics.

To achieve a higher yield and purity of exosomes from clinical samples like body fluids, it is recommended to combine different isolation methods along with the pretreatment of clinical samples as mentioned above. In a methodology study of

exosome isolation, Lobb et al. showed that ultrafiltration competed over ultracentrifugation, and coupling ultrafiltration with SEC resulted in a high yield of exosomes with high purity (60). They pointed out that the yield of exosomes differed among the application of different isolation methods, which suggested that the size and species of samples need to be taken into account before choosing a compatible isolation method. Another study used ultrafiltration in a company with size exclusion liquid chromatography, which proved that the combination of different methods could also preserve the bioactivity of purified exosomes (61). Scientific and clinical research on exosomes has attracted much attention so far, which impelled the development and revolution of the exosome isolation methodology. Nonetheless, existing methods are still mostly working with tissue cultures, and the results sometimes are not compatible with physical conditions (62). Additionally, the experimental results obtained on similar samples are controversial due to the different amounts of samples and different protocols that are used by different groups (63). Therefore, it is urgent to develop standardized methods to better assist the research of exosomes as well as to facilitate the translation of exosome-based techniques for clinical application in liquid biopsy.

Characterization Method

Microscopy and Nanoparticle Tracking

It is recommended and encouraged to characterize exosomes by two indispensable and complementary techniques, which usually are EM and nanoparticle tracking analysis (NTA) (19). The former provides the image of exosome morphology, and the latter gives information on the size distribution and

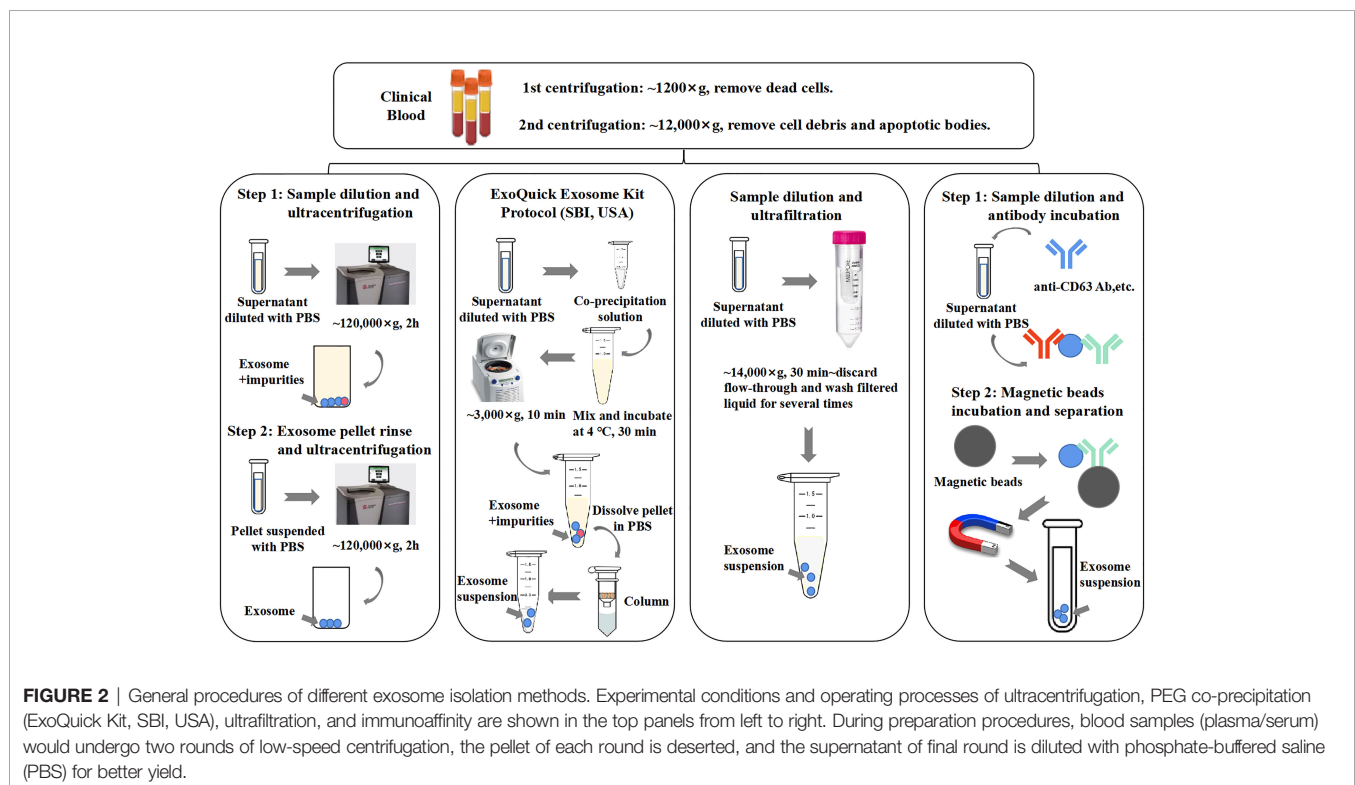


TABLE 1 | Comparison of different exosome isolation techniques.

Isolation technique	Underlying mechanism	Working scalability	Sample purity	Advantage	Disadvantage	Reference
Polymer co-precipitation	Hydrophobicity of protein and lipid	Small	Low	Cheap, fast, easy	Contaminated by co-precipitated particles	(52)
Affinity-based isolation	Affinity	Small	High	Fast, easy	Expensive, contaminated by microvesicles	(53)
Ultrafiltration	Molecular weight	Medium	Medium to high	Fast, easy	Plugged up easily by vesicles	(54, 55)
Size exclusion chromatography	Size and molecular weight	Flexible	High	Cheap, easy, reproducible	Time-consuming	(56, 57)
Immunoaffinity capture	Immunoaffinity-antibody	Small	High	Fast, easy	Expensive, contaminated by magnetic beads	(58)
Ultracentrifugation	Immunoaffinity-aptamer	Small	High	Cheap, fast, easy	Low recovery	(3, 59)
	Differential density	Flexible	Medium	Well-established and commonly used	Time-consuming, ultraspeed centrifuge required	(49, 50)

concentration of exosomes (17). It is necessary to obtain a clear and high-resolution image of exosomes to ensure that the intact structure of exosomes is maintained, which requires hours of microscopy operation and observation (64). Cellular experiments like the uptake of exosomes in the absence of cell-cell contact are sometimes used to demonstrate the bioactivity of purified exosomes. NTA detects the Brownian movement of total nanoparticles of a sample but is unable to distinguish the phenotype or the origin of exosomes (65).

Bicinchoninic Acid Assay, Western Blotting, and ELISA

Bicinchoninic acid assay (BCA) is one of the most widely used methods for exosome protein quantification. By combining the examined amount of protein with the concentration of exosome particles, general knowledge of the quantity and purity of the exosome sample can be obtained. Nevertheless, the contaminant proteins can influence the authentic result. Western blotting and ELISA are the most used methods for the characterization of the exosomal composition, and the presence of certain proteins can be confirmed; however, the results are also affected by contaminant proteins, as they cannot separate exosomal proteins from non-exosomal proteins (66). It is suggested by MISEV that at least three positive and one negative protein marker of exosomes should be conducted in Western blotting for exosome characterization (17). For samples like serum or plasma, the most recommended negative markers for exosome characterization are apolipoproteins A1/2 and B as well as albumin (17, 67). However, there are also some contradictory studies indicating the presence of apolipoproteins A and albumin in exosomes (68, 69). Therefore, cytochrome C, a protein of mitochondria, and other endoplasmic reticulum (ER) and Golgi proteins such as calnexin, BIP, GRP94, and GM130 are suggested as negative markers for exosomes instead (17). It is sometimes necessary to remove disturbing proteins to get a cleaner background and a better readout.

Flow Cytometric Analysis

Regular flow cytometric devices detect and analyze targets at the cellular level, and thus it is a great challenge to apply them at the

nanosized level. To be analyzed by a flow cytometric machine, exosomes should be immobilized on microbeads first and then bound with fluorescent antibodies, which make the fluorescence detectable as a positive signal. The good news is that the recent development of flow cytometric instruments reduces the detection limit to 100 nm (9), which strongly favors future exosome analysis in clinical situations. A major strength of flow cytometry is that it can recognize and select specific subpopulations of exosomes by specific protein markers. Meanwhile, the number of those fluorescence-bearing exosomes can be calculated (70).

Microfluidics

As increasing evidence has revealed the significant role of exosomes in cancer progression, some research groups have coined microfluidics for clinical isolation and analysis of exosomes. Usually, microfluidics is a combined system of isolation, characterization, and analysis modules, enabling a complete flow of diagnosis based on exosomes. Microfluidics has the same principles as conventional isolation methods such as immunoaffinity capture and fluorescent analysis. But compared to conventional methods, microfluidics requires much less time and amount of sample, which makes it more clinically favorable. One of the representative examples of microfluidics is developed by the combination with flow cytometry, whose sensitivity reaches 10,000 exosomes per ml of serum (9). We can recognize the microfluidics platform as a scaled-down version of common methods, but with higher sensitivity and throughput, which therefore maintains the great potential for liquid biopsy (72). Nonetheless, the method of microfluidics should be further standardized before being widely applied. A brief comparison of different methods of exosome characterization is summarized in **Table 2**.

APPLICATION OF EXOSOMAL PROTEINS IN LIQUID BIOPSY

The field of exosome research has put a huge effort into exploring exosomal miRNA as an attractive biomarker for cancer diagnosis because of the discovered correlation between the increased level of miRNA and the manifestation of cancer. However, it has been

TABLE 2 | Comparison of different exosome characterization techniques.

Characterization method	Exosome property	Advantage	Disadvantage	Reference
Physical characterization				
Electronic microscopy	Morphology, size distribution	Necessary process for nanosized exosome morphological feature characterization	Time-consuming, affected by human factors like visual sense	(64) (17)
Nanoparticle tracking analysis	Size distribution, concentration in solution	Fast and easy, in combination with microscopy for exosome physical property characterization	Unable to determine the phenotype of exosomes	(17) (65)
Biochemistry characterization				
Western blotting	Presence and level of protein	Classic and standardized method for protein analysis	Low sensitivity, long preparation procedure, unable to exclude contaminants from exosomes	(71) (66)
ELISA	Presence and level of protein	Classic and standardized method for protein analysis	Expensive, unable to exclude contaminants from exosomes	(66)
Flow cytometry	Protein specificity and concentration in solution	Able to identify subpopulation of exosomes with specific protein markers	Resolution limit restricts the sensitivity, low amount of protein reduces the fluorescence signal for detection	(70)

neglected that exosomal proteins can also serve as diagnostic biomarkers of cancer due to the following advantages: 1) protein constituents from tumor-derived exosomes reflect the proteomic profile of their parent tumor cells. Thus, circulating exosomes in the body fluids can provide comprehensive information about the distal primary tumor by the exosomal proteins (62). 2) Tumor-secreted proteins are greatly diluted by background substances in the circulating blood, making them very difficult to be detected. Enrichment of exosomes from blood can make it much easier to detect the proteins wrapped up in exosomes (13). 3) Many proteins secreted by tumor cells are unstable and vulnerable due to the presence of free protease in the body fluids. But these proteins can be well protected within tumor-derived exosomes, enabling in-depth analyses of relevant tumors (14). 4) Different miRNAs can lead to a similar consequence of gene expression sometimes (26), while the phenotypes of proteomic profiles can be more straightforward and representative of parent cells. Nevertheless, with increasing research on exosomal proteins for biomarker application, new challenges arise. For instance, the inconsistency among different cohorts of study is found even in the analysis of the same exosomal protein. Although there is still a long way to go, the development and application of exosomal protein as a powerful diagnostic tool for cancer therapy possess a bright future with tremendous potential. In this section, exosomal proteins that have been identified as tumor-associated markers by clinical research will be summarized and discussed. *In vitro* studies with cell culture or mouse models will not be covered because more attention has been paid to clinical studies, which present more relevant results to authentic physical situations. Exosomal proteins discussed here are summarized in **Tables 3–6**.

Tetraspanin and Surface Protein

Tetraspanins such as CD9, CD63, and CD81 are considered the major components of exosomal transmembrane proteins, which participate in the biogenesis and cargo sorting of exosomes (108). These three tetraspanins are also used most frequently as targets conjugated with the immunoaffinity capture method. Nevertheless, it has been noted that CD63 was less presented

in all of the tested samples (109–111), which queries the widely accepted concept that CD63 is ubiquitously existed on the surface of exosomes and urges us to explore new exosomal signature proteins. Tumor-derived exosomes are known to play important roles in the process of tumor generation, progression, and EMT, as exosomal tetraspanins can interact with receptors like integrins on recipient cells for the enhanced uptake of tumor-derived exosomes. Hence, exosomes with increased expression of tetraspanins have been widely identified in the body fluids of cancer patients by different research groups. The correlation of tetraspanins with tumors also makes these exosomal surface proteins strong biomarkers for various cancers (112). An earlier proteomics study suggested that the expression of exosomal CD9 significantly varied among cancer tissues and relevant normal tissues (113). Recently, these proteins, including CD9, have been increasingly demonstrated to be closely relevant to cancer-derived exosomes by different groups, thus possessing tremendous potential as cancer biomarkers. An assay called ExoScreen was developed by the group of Yoshioka et al. (76) to screen exosomes with surface expressions of CD63 and CD9 from only 5 μ l of serum from colorectal cancer patients without any sample preparation procedures such as dilution or purification. This assay combined two groups of antibodies conjugated with a donor photosensitizer bead and an acceptor photosensitizer bead. It was also found that the level of CD9 and another surface molecule CD147 was significantly increased in the serum of colorectal cancer patients (114). Notably, the higher level of CD147/CD9 in serum exosomes could also be detected at an early stage of cancer, with the area under the curve (AUC) of 0.820 among healthy donors versus patients. The regular reference tumor-associated antigens carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) were within the normal value range (termed non-cancer) with the AUC of 0.669 and 0.622, respectively, indicating the great potential of exosomal proteins as cancer biomarkers with higher specificity and sensitivity than a regular signature. In another smaller cohort of studies about prostate cancer, Krishn et al. also confirmed that the CD9 level increased in plasma exosomes from patients

TABLE 3 | Exosomal tetraspanin and surface markers.

Type of cancer	Protein marker	Number of patients/controls	Source of exosome/amount of Sample	Isolation technique	Diagnostic accuracy of proposed marker	Reference
Lung cancer	CD91	105/73	Serum/50 μ l	MSIA (immunoaffinity capture)	AUC = 0.724, sensitivity = 60.0%, specificity = 89.0%. Control group includes 54 healthy individuals and 19 interstitial pneumonia patients.	(73)
	TSPAN8, CD151	336/126	Plasma/10 μ l	EV array (immunoaffinity capture)	AUC _{TSPAN8} = 0.60, AUC _{CD151} = 0.68. Patient group is composed of individuals with three types of lung cancer. Control group is composed of non-cancer patients having symptoms of cancer.	(74)
Breast cancer	CD82	80/80	Serum/500 μ l	ExoQuick Kit (SBI)	Significantly increased in 30 representative samples. Control group is composed of patients with benign breast disease.	(75)
Colorectal cancer	CD9, CD147	194/191	Serum/5 μ l	ExoScreen (immunoaffinity capture)	AUC _{CD9/CD147} = 0.820. Double-positive exosomes were used as diagnostic markers.	(76)
Pancreatic cancer	TSPAN8	131/79	Serum/1~1.5 ml	Sucrose gradient ultracentrifugation	Positive in 90% of patient samples. Control group include patients with chronic pancreatitis benign pancreatic tumor and non-pancreatic tumor and healthy volunteers.	(77)
Prostate cancer	CD9, CD81	70/14	Plasma/over 3 ml	Ultracentrifugation	Significantly increased (CD9) in five representative patient samples. Significantly decreased (CD81) in four representative patient samples.	(78)
	CD9	6/10	Plasma/2,500 μ g of protein	Ultracentrifugation/ EV assay	Significantly increased in six representative samples. Control group is composed of patients with benign prostate hyperplasia.	(79)
	P-glycoprotein	4/6	Serum/1 ml	Differential centrifugation	Increased in 4 docetaxel-resistant prostate cancer compared to 6 treatment-naïve patients.	(80)
Melanoma	CD63	90/58	Plasma/ unspecified	Ultracentrifugation	Sensitivity = 96.5%, specificity = 43%. AUC unspecified.	(81)
Oral squamous cell carcinoma	CD63	10	Plasma/1 ml	Ultracentrifugation	Significantly decreased among 10 representative samples. Comparison was made between patients before and after surgery.	(82)

EV, extracellular vesicle; AUC, area under the curve.

compared to that of the healthy control group. Along with the upregulated CD9 levels on exosomes, the authors also found that α v β 3 integrin was also expressed in plasma exosomes of cancer patients, but the expression of another classic tetraspanin CD81 decreased somehow in cancer patients. However, a similar change was not found in the RNA level of CD9 and CD81 between healthy and disease conditions, suggesting that there should be an intriguingly complicated regulatory network involved in the process. Additionally, exosomal proteins should be more straightforward and convincing than exosomal RNA to reflect the cellular activity in a sense (78). Moreover, a higher expression of exosomal CD9 in plasma from prostate cancer patients had also been revealed in another study by differential centrifugation. However, these results came from a small-scale study with only six recruited patients and thus should be verified by identical experiments with a larger cohort. Nevertheless, they investigated the role of CD9 in prostate cancer and found that exosomal CD9 could promote cancer cell proliferation (79). An additional study on bladder cancer also reported an elevated level of CD9-positive exosomes in urinary samples (105). It looks like CD9 can promote tumor growth in different types of cancers, but an *in vivo* experiment has shown a contradictory result that the knockout of CD9 in hepatocellular carcinoma can facilitate cancer development instead. Additionally, it was also reported that there was no difference between the number of exosomes released by hepatocellular carcinoma cells before and after the

overexpression of CD9 and CD81 (115). Although the results obtained from cell lines do not always display exactly the same in clinical scenarios, the complicated roles of CD9 discussed here should remind researchers that solid conclusions require more reliable comparative discoveries, especially regarding exosomes that are naturally heterogeneous.

Meanwhile, an in-house study of melanoma reported that there was a higher level of CD63⁺ exosomes in cancer plasma, which was correlative with the level of caveolin-1, a component of caveolae (81). Another pilot study with oral squamous cell carcinoma patients showed that the level of CD63⁺ exosomes decreased immediately after resection surgery, suggesting that the tumor was responsible for the high level of CD63⁺ exosomes in the circulation (82). In addition, to be potential candidates for cancer biomarkers, a recent study using immunohistochemistry (IHC) showed that tumor exosomal CD9 and CD63 might also act as potential prognostic monitors due to the increased expression in rectal tumor tissue after chemoradiation treatment (116). Although it is helpful to build our understanding of pathology by tissue biopsy, the result should be further verified with body fluid samples since liquid biopsy application of exosomes holds great promise in non-invasive personal precision medicine. So far, very few studies have investigated three tetraspanins (CD63, CD9, and CD81) together in clinical settings; therefore, it is still unclear whether the level of CD9, CD63, or CD81 correlates with different types of cancers.

TABLE 4 | Exosomal transport protein, heat shock protein, and adhesion protein.

Type of cancer	Protein marker	Number of patients/controls	Source of exosome/amount of sample	Isolation technique	Diagnostic accuracy of proposed marker	Reference
Lung cancer	HSP70	18/19	Plasma/10 ml blood	Ultracentrifugation	AUC = 0.8968 for distinguishing metastatic stage of cancer (including lung cancer and breast cancer). Comparison was made between patients with metastatic cancer and non-metastatic cancer.	(83)
	Fibronectin	21/41	Serum/ unspecified	Sucrose gradient ultracentrifugation	AUC = 0.844.	(84)
Gastric cancer	PSMA3/PSMA6	24/13	Serum/2 ml	exoEasy Maxi Kit (QIAGEN)	Significantly increased in six patients with late-stage gastric cancer.	(85)
Colorectal cancer	HSP90	18/18	Serum/250 μ l	Total Exosome Isolation Kit (Invitrogen)	Significantly decreased in 18 representative samples.	(86)
Prostate cancer	α v β 3	70/14	Plasma/over 3 ml	Ultracentrifugation	Significantly increased in representative samples.	(78)
	ACTN4	20/8	Serum/ unspecified	Sucrose gradient ultracentrifugation	Significantly increased in castration-resistant prostate cancer compared to prostate cancer patients receiving primary androgen deprivation therapy.	(87)
Pancreatic cancer	ZIP4	24/78	Serum/500 μ l	ExoQuick Kit (SBI)	AUC = 0.89 for distinguishing malignant cancer from benign pancreatic disease. AUC = 0.8931 for distinguishing malignant cancer group from normal group. [Number of controls include benign pancreatic disease (32) and normal subjects (46)]	(88)
Endometrial cancer	ANXA2	41/20	Plasma/500 μ l	ExoGAG	AUC = 0.748.	(89)
Gynecologic cancer	HSP22	30	Serum/1 ml	Ultracentrifugation	Comparison was made among patients with ovarian cancer, patients with endometrial cancer and patients with endometriosis. Although no significant result was concluded.	(90)

If no specified AUC, sensitivity, or specificity is claimed in the reference, no precise numerical data for diagnostic accuracy are included in this table.
AUC, area under the curve.

Some other surface molecules expressed on exosomes were also reported as strong cancer biomarkers. In a large cohort study of lung cancer patients, an EV array was built that contained 49 exosomal and tumor proteins to collect the heterogeneous populations of exosomes from 472 isolated plasma samples. The expressions of two tetraspanins CD151 and TSPAN8 were found to be significantly elevated in exosomes from cancer patients, which could distinguish cancer from healthy control with AUC of 0.68 and 0.60, respectively, independently of disease stage and histological subtype. Following that, the combination of 8 proteins along with CD151 and TSPAN8 was determined to have the best separation outcome to distinguish cancer sample from healthy control with the largest AUC of 0.74, which suggested that a group of protein markers may have higher sensitivity than an individual marker in diagnosis (74). Another proteomic analysis presented similar data to show that the expression of exosomal TSPAN8 was associated with pancreatic cancer as well (77). A study on breast cancer demonstrated that the level of CD82 in serum-derived exosomes was significantly higher in a malignant group than in the healthy control group. The results also suggested that the level of CD82 was positively correlated with the malignancy of breast cancer and thus could sensitively serve as a breast cancer marker (75). There were also some other reports showing the increased level of exosomal CD91 and P-glycoprotein in the clinical case of lung cancer and prostate cancer, respectively (73, 80), indicating their potential for cancer analysis. Interestingly, the AUC of CD91 combined with CEA in

distinguishing lung cancer reached 0.882 (73, 80), which substantiated CD91 as a very good candidate for potential lung cancer diagnosis.

There is a great chance that the surface molecules discussed above will be applied widely in future clinical research on cancer diagnosis and liquid biopsy, while most of these encouraging findings were involved with two correlated proteins, like CD9 and CD147 or CD63 and caveolin-1, because it may not be enough to get a whole picture of a tumor merely by a single protein marker. By combining various biomarkers in one panel, the signature of cancer can be identified and analyzed more reliably, accurately, and efficiently. Though promising, the results shown here came from different groups by using different purification processes without any normalization. Future studies with standardized methods are necessary to exclude the contingencies of experiments that might induce the methods of exosome enrichment.

Transport Protein, Heat Shock Protein, and Adhesion Protein

TSG101, Alix, and heat shock proteins take part in the intracellular transport of exosomes, which therefore shows important implications in cancer (117–119). Elmira et al. reported in a recent study with over 400 samples that the level of exosomal TSG101 was significantly upregulated in colorectal tumor tissue (120). ANXA2, a member of the annexin family involved in the endocytosis and exocytosis processes, was identified to have an increasing level in plasma exosomes of endometrial cancer (EC) patients. The AUC of

TABLE 5 | Tumor-associated exosomal protein.

Type of cancer	Protein marker	Number of patients/control	Source of exosome/amount of sample	Isolation technique	Diagnostic accuracy of proposed marker	Reference
Glioblastoma	EGFR	24/8	Plasma/ unspecified	Differential centrifugation	$AUC_{EGFR} = 0.78$, sensitivity = 64.0%, specificity = 88.0%. $AUC_{EGFR_{VIII}} = 0.88$, sensitivity = 68.0%, specificity = 100.0%.	(91)
Lung cancer	EGFR	9	Plasma/ 100 μ l	Immunoaffinity capture	Positive in five representative samples.	(92)
Hepatocellular carcinoma	LG3BP, PIGR	29/32	Serum/1 ml	Ultracentrifugation	$AUC_{LG3BP} = 0.904$. Sensitivity = 96.6%, specificity = 71.8%. $AUC_{PIGR} = 0.837$. Sensitivity = 82.8%, specificity = 71.8%.	(93)
Colorectal cancer	Glypican-1	102/80	Plasma/ 10 ml blood	ExoCap™ kit (JSR)/ immunoaffinity capture	Significantly decreased in cancer patients after surgery treatment compared to patients before surgery treatment. Significantly increased in patients compared to healthy control.	(94)
	Glypican-1	85	Plasma/ 20 ml blood	ExoCap™ kit (JSR)/ immunoaffinity capture	Significantly decreased in cancer patients after surgery treatment compared to patients before surgery treatment. Significantly increased in late stage of cancer.	(95)
Pancreatic cancer	c-Met	30/40	Serum/250 μ l	Total Exosome Isolation Kit (Invitrogen)	Sensitivity = 70%, specificity = 85%. Diagnostic odds ratio = 13:2. $AUC_{diagnostic}$ not provided. $AUC_{prognostic} = 0.779$. Control group include non-malignant subjects, serous cystadenoma subjects, and chronic pancreatitis subjects.	(96)
	Glypican-1	62/20	Serum/250 μ l	Sucrose gradient ultracentrifugation	$AUC = 1.0$. Sensitivity = 100.0%, specificity = 100.0%.	(97)
	Glypican-1	27/16	Plasma/ 1~1.5 ml	Ultracentrifugation	$AUC = 0.59$. Sensitivity = 74.0%, specificity = 44.0%. Control group is composed of patients with benign pancreatic disease.	(98)
	Glypican-1	24/26	Serum/2 ml	Sucrose gradient ultracentrifugation	$AUC = 0.885$.	(99)
Pancreatic ductal adenocarcinoma	Glypican-1	22/28	Serum/250 μ l	Total Exosome Isolation Kit (Invitrogen)	$AUC = 0.78$ for GPC1 ⁺ exosomes in portal and peripheral blood. Sensitivity = 64.0%, specificity = 90.0%.	(100)
Ovarian cancer	CD24, EpCAM, CA-125	15/5	Plasma/20 μ l	ExoSearch Chip/ immunoaffinity capture	$AUC_{CD24} = 0.9067$. $AUC_{EpCAM} = 1.000$. $AUC_{CA-125} = 1.000$	(101)
Melanoma	PD-L1	44/11	Plasma/ 250 μ l	TEI kit (Invitrogen)/ ultracentrifugation	$AUC = 0.9184$. Sensitivity = 80.00%, specificity = 89.47%.	(102)
	Caveolin-1	90/58	Plasma/ unspecified	Ultracentrifugation	Sensitivity = 69%, specificity = 96.3%.	(81)
Head and neck squamous cell carcinomas	PD-L1	40	Plasma/1 ml	Size exclusion chromatography/ Immunoaffinity capture	Significantly increased in cancer patients with active disease and late stage (UICC stage III/IV) cancer.	(103)

If no specified AUC, sensitivity, or specificity is claimed in the reference, no precise numerical data for diagnostic accuracy are included in this table.
AUC, area under the curve.

ANXA2 as a biomarker for EC is 0.74, which therefore can serve as a relatively good marker suggested by the authors (89). HSP70 is actively released as exosome surface protein under the stimulation of high levels of IL-10 and IFN- γ in the serum of cancer patients, suggesting the role of exosomal HSP70 in tumor immunity and the potential of HSP70 as a cancer biomarker (121). Moreover, it was found that the number of HSP70⁺ exosomes in the plasma samples from metastatic lung and breast cancer patients was significantly higher than that of healthy volunteers, in which the level of HSP70⁺ exosomes was barely detectable (83). The same group further compared the performance of exosomal HSP70 vs. CTCs in their pilot attempt under clinical conditions and found that exosomal HSP70 could act as a discriminative marker between patients with non-metastatic lung cancer and metastatic lung cancer, possessing better AUC of 0.8968 than that of CTC detection ($AUC = 0.7857$).

Under stress, the induced form of HSP70 is expressed exclusively by cancer cells, which is called HSP72. One of the conclusive reviews of HSP70 discussed that tumor-derived HSP72-containing exosomes promoted the immunosuppressive function of tumor-associated suppressor cells, indicating the diagnostic value of HSP72 as the target of cancer (121, 122). Other than HSP70, an integrin-mediated pathway-related heat shock protein HSP90 was also reported to be strongly downregulated in the serum of colorectal cancer patients compared to the healthy control group (86). Moreover, one of small heat shock proteins (sHSP), named HSP22, was found at a high level in exosomes from gynecologic cancer patients, which was further suggested to be correlated with host cytotoxic immune response (90).

Subunits PSMA3 and PSMA6, belonging to the 26S proteasome complex that is responsible for the functional

TABLE 6 | Tumor-associated exosomal protein in urine and ascites.

Type of Cancer	Protein Marker	Number of patients/controls	Source of exosome/amount of sample	Isolation technique	Diagnostic accuracy of proposed marker	Reference
Prostate cancer	TMEM256	16/15	Urine/50~150 ml	Ultracentrifugation	AUC = 0.87. Sensitivity = 94.0%, specificity = 100.0%.	(104)
Bladder cancer	TACSTD2	28/12	Urine/12.5 ml	Ultracentrifugation	AUC = 0.741. Control group is composed of 12 hernia patients. A higher AUC = 0.80 of TACSTD2 was obtained in a larger cohort of 221 samples with ELISA.	(105)
Pancreatic cancer	CD133	19	Ascites/unspecified	exoEasy Maxi Kit (QIAGEN)	The intensity of high-density glycosylation of CD133 significantly correlated with survival days of pancreatic patients. Non-malignant ascites from alcoholic and hepatitis C-related cirrhotic patients were considered as control.	(106)
Endometriosis	ANXA2	22/6	Peritoneal fluid/1 ml	Exo-spin Kit (Cell Guidance)	Specifically existed in endometriosis patients regardless of disease stage.	(18)
Renal disease	Polycystin-1	6	Urine/50 ml	Ultracentrifugation	Significantly increased in urinary exosomes. Comparison was made between urinary samples and kidney tissue samples.	(107)

If no specified AUC, sensitivity, or specificity is claimed in the reference, no precise numerical data for diagnostic accuracy are included in this table. AUC, area under the curve.

modification and the degradation of cellular proteins, were shown to be enriched in serum-derived exosomes of gastric cancer patients (85). In addition, exosomal zinc transporter ZIP4 and integrin $\alpha\beta 3$ were also reported to be significantly related to tumor growth of pancreatic and prostate cancers, respectively (78, 88). In the latter study, to determine the origin of exosomes, C4-2B- $\beta 3$ -GFP cells were injected into NOD mice to develop prostate cancer, and the authors successfully isolated plasma exosomes with GFP-tagged $\alpha\beta 3$ integrin in the circulation, indicating the direct relevance between tissue-derived exosomes and circulating exosomes (78). Moreover, exosomal fibronectin was also confirmed by proteomic analysis on non-small cell lung cancer-derived exosomes to display great diagnostic potential in the clinical cohort (84). The actin cross-linking protein actinin-4, which could facilitate cancer metastasis, was also identified as a signature protein of metastatic prostate cancer with a high expression level (87).

Tumor-Specific Protein

Tumor-associated proteins such as CA19-9, CEA, and prostate-specific antigen (PSA) have been used as indicators in cancer diagnosis for a long time. Among those significant biomarkers, PD-L1 has become the focus of immunotherapy development. PD-L1, existing on the surface of various cell types including epithelial cells and tumor cells, can bind with PD-1 on the surface of T cells to act as the key immune checkpoint mediator in healthy conditions; however, it can also suppress the immune response of cytotoxic T cells in cancer. Overexpression of PD-L1 has been reported to be indicative of the status of tumors by using methods like IHC (123). Compared to tissue biopsy, liquid biopsy offers a safer way of sampling, which can be conducted at any time to acquire accurate information about the ongoing illness in patients. Due to that, research starts to focus on the investigation of the value of exosomal PD-L1 in liquid biopsy. A mechanism study has demonstrated that metastatic melanoma cells managed to escape from immune supervision by producing

exosomes that carry PD-L1 to facilitate tumor growth as well as suppress CD8⁺ T cells. Additionally, the authors also found that compared to that in healthy donors, the level of PD-L1 in plasma exosomes was significantly higher in melanoma patients (102). Similarly, in patients with head and neck cancer, the expression of PD-L1 on the membrane of plasma exosomes was discovered to be associated with the progression of the disease, whereas soluble PD-L1 was not correlated with any of the results found with exosomal surface PD-L1 (103). This odd observation may suggest that the exosomal surface proteins exhibit the same expression pattern as parental cells. Nevertheless, a study on pancreatic cancer revealed controversial results showing that the level of PD-L1 on circulating exosomes was not correlated with cancer. On the contrary, another molecule c-Met displayed a strong correlation with cancerous parameters (96). Thus, based on these favorable findings, more future work should be done to provide convincing proof that exosomal PD-L1 can be developed as an excellent cancer biomarker.

Glypican-1 (GPC-1), a surface-bound proteoglycan that regulates signaling pathways mediated by TGF- β , Wnt, and other growth factors, is overexpressed in a variety of cancer tissues to promote cancer progression, especially pancreatic cancer (124, 125). Melo et al. purified serum exosomes from patients with pancreatic ductal adenocarcinoma (PDAC) and found that all exosome samples of PDAC patients (n = 190) were marked with high expression of GPC-1, suggesting a strong correlation between cancer and serum-derived exosomes. More than that, the authors analyzed the primary tumor tissue of PDAC patients and found the mutant transcript of Kras, an oncogenic gene that is frequently seen in PDAC. They confirmed the presence of similar Kras mutation in GPC-1⁺ serum exosomes of PDAC patients. These results indicated that a part of circulating exosomes was derived from relevant tumors, which underscored the importance of exosomal GPC-1 as a pancreatic cancer biomarker (97). A similar study with PDAC further emphasized the potential of exosomal GPC-1 by showing that the level of GPC-1 was elevated in plasma exosomes of PDAC

patients, which was decreased accordingly after the resection surgery of PDAC patients (98). However, in many studies with PDAC, the investigation of exosomal GPC-1 was usually combined with other surface markers, which were claimed to possess higher sensitivity and specificity for cancer detection. Buscail et al. reported that the combination with CA19-9 and CD63⁺ GPC-1⁺ circulating exosomes could define PDAC much better with an accuracy reaching 84% (100). Xiao et al. also addressed that a sensitive and reproducible detection panel consisting of exosomal GPC-1 and CD82 and serum CA19-9 could efficiently distinguish PDAC patients from healthy people (99). Furthermore, a multiparametric exosome profiling was also developed and conducted by screening exosomes from patient plasma with a combination of ten surface markers including four biomarkers from a major group of cancers, three putative PDAC markers including GPC-1, and three pan-exosome markers (126). Exosomal GPC-1 has also been demonstrated to be increased in colorectal cancer (94). The percentage of GPC-1⁺ exosomes among total exosome numbers was significantly higher in patients with more serious disease conditions (95). This percentage was also significantly increased in relapsed patients, indicating GPC-1 as a biomarker for diagnosis and poor prognosis (95). Collectively, these research data obtained with clinical samples greatly support the value of exosomal GPC-1 as a potential diagnostic marker for cancer screening.

CD24 is a type of cell adhesion molecule participating in cell recognition, activation, signal transduction, proliferation, etc., which has been found to be abnormally expressed in various cancers. Notably, Zhao et al. developed a multiplexed detection chip combined with protein markers CA-125, EpCAM, and CD24, which indicated a three-fold increase of exosomal CD24 in the plasma of ovarian cancer patients. The AUC values of CA-125, EpCAM, and CD24 were 1.0, 1.0, and 0.91, respectively, all indicating the great value of diagnosis (101). But Soltész et al. found that in the same type of cancer, the expression of CD24 RNA was not detectable in all plasma or exosome samples of the patients, though there is a significant alteration in the tissue of the same ovarian cancer patients (127). Thus the bias existing between exosomal protein and RNA remains to be solved, and it also signifies the importance of exosomal protein in liquid biopsy. Epidermal growth factor receptor (EGFR), another protein specifically overexpressed in tumors, is closely associated with cancer progression. Yamashita et al. showed that the expression of EGFR on plasma exosomes was significantly higher in lung cancer patients compared to healthy controls, whereas the level of soluble EGFR in plasma showed no significant difference between healthy and disease conditions (92). In another study, a microfluidic chip combined with a nuclear magnetic resonance detection system was developed by Shao et al. to analyze the expressions of EGFR and its variant EGFRvIII on plasma exosomes of glioblastoma patients (91). They identified that the levels of EGFR and EGFRvIII in exosomes were significantly higher in patients compared to healthy individuals.

Survivin-2B, a pro-apoptotic protein mainly found in primary tumors rather than high-grade tumors, could be further developed as a diagnostic marker for breast cancer (128).

Caveolin-1, referred to in the above section as exosomal CD63, was reported to be increased in plasma-derived exosomes of melanoma patients, with even higher sensitivity than CD63 as a putative melanoma biomarker (81). Galectin-3-binding protein (LG3BP), a protein promoting tumor growth, was found in the serum-derived exosomes of hepatocellular carcinoma (HCC; liver cancer) patients with remarkably increased expression compared to that of the healthy control group. It was worth noting that although exosomal miRNAs in liver cancer were widely studied, there was a paucity of studies with exosomal protein biomarkers, which urged researchers to pay more attention to potential protein biomarkers for liver cancer. The diagnostic AUC of LG3BP was 0.904, which was much higher than that of a non-specific tumor marker alpha-fetoprotein (AUC = 0.802). However, another tumor marker PIGR was surprisingly found to be elevated in serum-derived exosomes with the diagnostic AUC of 0.837, higher than that of alpha-fetoprotein (93). Thus, LG3BP and PIGR may work as novel biomarkers for liver cancer together. Common cancer like lung cancer and breast cancer are both discussed in this review regarding different cancer-associated exosomal protein markers. However, rather than exosomal protein, there are much more research data in the clinical field of HCC that showed nucleic acids in exosomes including miRNA and lncRNA can act as novel biomarkers (129).

Exosomal proteins discussed in the above section almost came from the study with either plasma or serum. Other body fluids like urine or ascites, which are less viscous than plasma or serum, are much easier to deal with. Exosomes from these sources also have been revealed as potential tools for diagnosis (31). For example, a proteome profile study of urine-derived exosomes identified proteins from renal tubule epithelial cells. Polycystin-1, specifically identified in urine-derived exosomes, was associated with multiple renal diseases (107). The same group further developed an efficient protocol for urinary exosome isolation and storage (130). Different from plasma or serum-derived exosomes, urine-derived exosomes are sometimes more relevant to the urinary system, which suggests that they might possess greater potential as early liquid biopsy biomarkers for renal-related diseases. In urological cancers like prostate cancer or bladder cancer, urine-derived exosomes still display great value as diagnosis signature. One of the comprehensive proteomic studies of prostate cancer described in a review (104) suggested that TMEM256 displayed excellent accuracy for cancer diagnosis with a high AUC of 0.94 in clinical urinary samples. Nevertheless, in the discussion of another review, though another group proposed TMEM256 as a potential biomarker for prostate cancer, two other groups could not detect the presence of TMEM256 in two sets of clinical urinary samples. Moreover, the protein profiles obtained by these two groups in the urinary sample of prostate cancer were also distinct, possibly due to the different isolation and quantification methods or a different control group (131).

Moreover, there was also a study by the group of Chen et al. about a potential biomarker TACSTD2 for bladder cancer, which was significantly increased in urinary exosomes of patients

compared to individuals with hernia (105). The great potential of TACSTD2 as a diagnostic biomarker for bladder cancer was further supported by the validation of another cohort of study (131) and by the fact that it was exclusively made by cancer cells (132). Ascite-derived exosomes are also found as a strong diagnostic tool for their expression of highly glycosylated CD133 in pancreatic cancer (106). Meanwhile, the group of Nazri et al. discovered the connection between peritoneal fluid-derived exosomes and endometriosis by systemic liquid chromatography with tandem mass spectrometry and identified five associated exosomal proteins including AXNA2 to be potential diagnostic biomarkers for endometriosis (18). The discussion about tumor-associated exosomal proteins as potential cancer biomarkers should also remind researchers of the fact that the clinical data are still limited. For example, when investigating prostate cancer, exosomal proteins including CD9, CD81, P-glycoprotein, and ACTN4 together can act as a detection panel with high specificity. CD9, CD63, or glypican-1 can be taken into consideration as generic tumor-associated markers of exosomes produced by a major group of cancers. In the future, more comprehensive research should be conducted in representative clinical cohorts to further substantiate the existing data.

CONCLUSION AND PERSPECTIVE

With the increasing knowledge of exosomal functions in cell–cell communication, the application of exosomal proteins as a potential tool for cancer diagnosis and liquid biopsy has become more and more attractive. Under normal circumstances, tissue biopsy or pulmonary lavage is carried out to examine the state of lung cancer, which undoubtedly causes damage to the body. Notably, in a proteomic study of non-small cell lung cancer, Li et al. demonstrated that AQP-2 (kidney tissue-related)-positive urinary exosomes expressed a high level of LRG1 protein. Similarly, LRG1 was also found highly expressed in the lung tissue of cancer patients (133). The study has presented us with a clue to the pulmonary origin of exosomes dispersed in urine and a special perspective on diagnostic utilization of urinary exosomes for lung cancer. Moreover, the discovered correlations between urine-derived exosomes and distant cancerous lung tissue encourage researchers to look deeper into the origin, transport, circulation, and uptake of exosomes.

Compared to the detection of tumors by classic antigens, the combination of exosomal protein markers offers better diagnostic results (76). Although increasing research data have suggested the importance of exosomal proteins, there are also some inconsistent and paradoxical results that we have discussed in this review among different studies, which urge a more precise investigation of exosomes. The most important reason for this result is that no standardized methodology of isolation and characterization has been established in the whole field of exosome research. Additionally, exosomes are heterogeneous, which makes the creation of standardized methods very

necessary to promote the development of the clinical application of exosomal proteins. In a recent comparative study on the tetraspanin profile of exosomes, the heterogeneity of exosomes was underlined to show a strong impact on the diagnostic sensitivity of exosomal surface markers. Good consistency exists in the tetraspanin profile of exosomes from the same source regardless of isolation methods. But the results confirmed that tetraspanin profiles of exosomes from a different source are distinct (134). Additionally, other exosomal cargos can also differ among different sources. From the long-term point of view, the development of a microfluidics system is more likely to be rapidly upgraded because it represents the cutting-edge technology that can satisfy the needs of exosome investigation. Microfluidics can integrate separate isolation procedures and characterization procedures into a single platform that skips the conservation of exosomes. Even more encouraging, it can analyze multiple samples that have a small volume to detect exosomes with high throughput and high precision (135). **Figure 3** shows a microfluidic system conceived by our group that delivers a general workflow or construction that can isolate and characterize tumor-derived exosomes in clinical samples.

In a recent study, Zhang et al. presented a microfluidic system in which exosomes are captured by an anti-CD63 aptamer and diagnosed by an anti-EpCAM aptamer (136). To detect those captured exosomes, a hybridization chain reaction (HCR) is applied. In this way, the signal of EpCAM aptamer on the surface of CD63⁺ exosomes is amplified in a linear form that can be detected with high sensitivity. The detection limit is confirmed as 0.5 exosomes/ μ l, and Zhang et al. also compared the performance of the system to that of NTA, which showed that the system could isolate cancer-derived exosomes and spare non-cancerous exosomes at the same time. Notably, a similar microfluidic aptasensor (aptamer-combined sensor) platform was constructed by Wang et al. to detect tumor-derived exosomes (137). The principle of HCR was also used, but in a different way to accurately amplify the signal of EpCAM⁺ exosomes by the formation of a multidirectional reaction. Surprisingly, the detection limit reached 285 exosomes/ μ l, significantly higher than that of other systems. The huge difference that existed between the two systems suggested that the mechanism applied for the amplification and detection of signals in an aptasensor system had a great impact on the sensitivity. However, a remaining issue of these two studies is that the exosomes they used for testing are enriched either by ultracentrifugation of supernatant of cell culture or by isolation of human serum with commercial isolation kit. Therefore, the detection sensitivity and limit of these two systems for exosomal EpCAM diagnosis of more complicated clinical samples without exosome enrichment remain unknown. Moreover, in these two studies, the use of a single biomarker CD63 for exosome capture resulted in the omission of exosomes deprived of surface CD63. Similarly, use of single biomarker EpCAM resulted in the omission of tumorous exosomes lack of EpCAM.

While these issues are taken into consideration in our proposed model (**Figure 3**), the combination of multiple

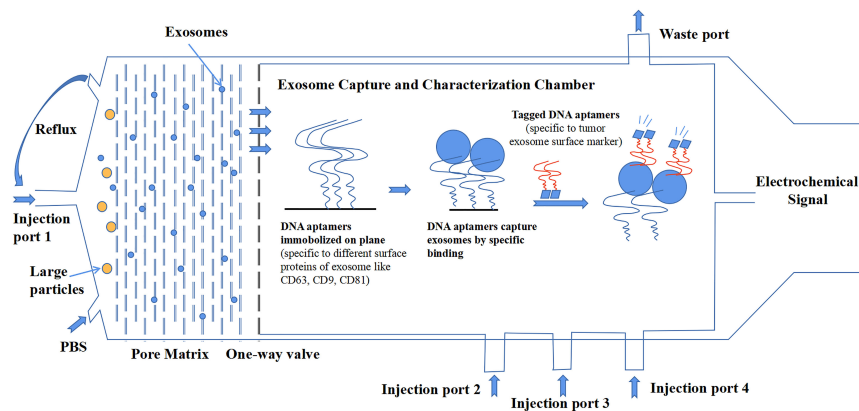


FIGURE 3 | A design of microfluidics system for clinical exosome isolation and characterization with high efficiency and high sensitivity. The first module of the system is a pore matrix (pore size ~200 nm). When microliters of plasma/serum is injected through the injection port 1, pressure will be added to the module, which makes particles inside the sample move toward the pore matrix. Nanoparticles including exosomes will move through pore matrix, while large particles will be left in the chamber. Then, phosphate-buffered saline (PBS) solution is injected into the chamber to create reflux to wash the pore matrix to ensure all exosomes move across the matrix and reach the one-way valve. In the second module, exosomes were captured in a characterization chamber. Enough quantities of DNA aptamers that specifically recognize exosome surface proteins like CD63, CD81, and CD9 are immobilized on the plane. Exosomes were captured while flowing across the plane, and buffer was injected through port 2. Impurities were discharged through the waste port. After that, tagged DNA aptamers that specifically recognize tumor-specific antigens were injected through port 3, and tumor-derived exosomes were bound by those aptamers. Finally, the chemiluminescence reagent was injected through port 4 and reacted with the tagged aptamers. The chemical signal was captured and exported eventually.

exosomal surface markers in this model can promote the probability to capture almost all exosomes in samples; however, the actual performance of the device still relies on the specificity of current exosomal markers and the identification of novel exosomal markers. Moreover, the sensitivity for detecting or capturing tumor-derived exosomes by the model also relies on the binding affinity of tumor-associated aptamers to exosomes, as well as the technique applied. The exclusion of non-cancerous exosomes would be favored by the technical improvement of detection strategy and the discovery of some novel tumor markers with high specificity, which would result in the enrichment of cancerous exosomes. For clinical use of exosomes, the methods should be handy, highly efficient, and supersensitive. The methods should also be compatible with different clinical samples. When dealing with blood, coagulation will cause the release of platelet-derived exosomes in the serum; thus it is better to choose plasma for exosome isolation (138). However, coagulation sometimes happens with cancer progression; thus, it is extremely necessary to distinguish tumor-derived exosomes from immune cell-derived exosomes in the blood sample, which further emphasizes the need for the discovery of tumor-specific exosomal markers. Additionally, the development of cancer-stage-specific exosomal markers is also very attractive to the current precision medicine theory.

It is worth mentioning that the standardization of exosome isolation methods will favor future clinical utilization of exosomes for immunotherapy at the same time. Safety is the most important issue to be concerned about when talking about therapy, which holds back many cell-therapy strategies. On the contrary, the high purity of exosome products will be a strong candidate for therapeutic treatment. For example, mesenchymal

stem cell (MSC)-derived exosomes have already been proved to mediate immune response in multiple murine models with acute graft-versus-host disease (aGVHD) due to their anti-inflammatory or immunomodulatory effects. MSC-derived exosomes can promote the expansion of regulatory T cells, which suppress the inflammatory response and can also induce the production of anti-inflammatory cytokines like IL-10 while inhibiting the production of inflammatory cytokines like TNF- α (139, 140). In a typical clinical treatment against aGVHD, the response of patients to MSC-derived exosomes administration was found to be positive, and the clinical GVHD symptom was significantly improved, suggesting the great potential of MSC-derived exosomes for clinical therapies (141). Beyond that, the function of MSC-derived exosomes has also been recently introduced into the treatment of COVID-19 for the first time. Patients infected with COVID-19 received 5 days of MSC-derived exosome infusion. At the endpoint, data showed that the percentage of CD3⁺, CD4⁺, and CD8⁺ T cells increased in patients, implying the exciting outcome of immunomodulatory effects of MSC-derived exosomes (142).

In summary, it is believed that with the advancement of isolation technique and characterization strategy, the clinical application of exosomal proteins would finally act as a powerful diagnostic target in liquid biopsy as well as effective immunotherapy.

AUTHOR CONTRIBUTIONS

CH and WJ drafted the manuscript. ML, SF, and YL helped to revise the manuscript. JP and QW were responsible for leading

this work and revising the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This study was supported by the Open Research Fund of Songshan Lake Materials Laboratory (2021SLABFN10), the Natural Science Foundation of Guangdong Province

(2022A1515011223), Project of Educational Commission of Guangdong Province of China (2021KTSCX038), and Funds for PhD researchers of Guangdong Medical University in 2021 (4SG21236G and 4SG22067G).

ACKNOWLEDGMENTS

We apologize to those authors whose deserving research was not cited in this manuscript.

REFERENCES

- Oshi M, Murthy V, Takahashi H, Huyser M, Okano M, Tokumaru Y, et al. Urine as a Source of Liquid Biopsy for Cancer. *Cancers (Basel)* (2021) 13 (11):2652. doi: 10.3390/cancers13112652
- Watanabe K, Nakamura Y, Low SK. Clinical Implementation and Current Advancement of Blood Liquid Biopsy in Cancer. *J Hum Genet* (2021) 66 (9):909–26. doi: 10.1038/s10038-021-00939-5
- Wu L, Wang Y, Zhu L, Liu Y, Wang T, Liu D, et al. Aptamer-Based Liquid Biopsy. *ACS Appl Bio Materials* (2020) 3(5):2743–64. doi: 10.1021/acsabm.9b01194
- Gormally E, Hainaut P, Caboux E, Airoldi L, Autrup H, Malaveille C, et al. Amount of DNA in Plasma and Cancer Risk: A Prospective Study. *Int J Cancer* (2004) 111(5):746–9. doi: 10.1002/ijc.20327
- Sharma S, Zhuang R, Long M, Pavlovic M, Kang Y, Ilyas A, et al. Circulating Tumor Cell Isolation, Culture, and Downstream Molecular Analysis. *Biotechnol Adv* (2018) 36(4):1063–78. doi: 10.1016/j.biotechadv.2018.03.007
- Wu CP, Wu P, Zhao HF, Liu WL, Li WP. Clinical Applications of and Challenges in Single-Cell Analysis of Circulating Tumor Cells. *DNA Cell Biol* (2018) 37(2):78–89. doi: 10.1089/dna.2017.3981
- Diehl F, Li M, Dressman D, He Y, Shen D, Szabo S, et al. Detection and Quantification of Mutations in the Plasma of Patients With Colorectal Tumors. *Proc Natl Acad Sci USA* (2005) 102(45):16368–73. doi: 10.1073/pnas.0507904102
- Schwarzenbach H, Hoon DS, Pantel K. Cell-Free Nucleic Acids as Biomarkers in Cancer Patients. *Nat Rev Cancer* (2011) 11(6):426–37. doi: 10.1038/nrc3066
- Ko J, Carpenter E, Issadore D. Detection and Isolation of Circulating Exosomes and Microvesicles for Cancer Monitoring and Diagnostics Using Micro-/Nano-Based Devices. *Analyst* (2016) 141(2):450–60. doi: 10.1039/c5an01610j
- Valencia K, Montuenga LM. Exosomes in Liquid Biopsy: The Nanometric World in the Pursuit of Precision Oncology. *Cancers (Basel)* (2021) 13 (9):2147. doi: 10.3390/cancers13092147
- Vaidyanathan R, Soon RH, Zhang P, Jiang K, Lim CT. Cancer Diagnosis: From Tumor to Liquid Biopsy and Beyond. *Lab Chip* (2018) 19(1):11–34. doi: 10.1039/c8lc00684a
- Lombardi CP, Bossola M, Princi P, Boscherini M, La Torre G, Raffaelli M, et al. Circulating Thyroglobulin mRNA Does Not Predict Early and Midterm Recurrences in Patients Undergoing Thyroidectomy for Cancer. *Am J Surg* (2008) 196(3):326–32. doi: 10.1016/j.amjsurg.2007.09.047
- Zhou B, Xu K, Zheng X, Chen T, Wang J, Song Y, et al. Application of Exosomes as Liquid Biopsy in Clinical Diagnosis. *Signal Transduct Target Ther* (2020) 5(1):144. doi: 10.1038/s41392-020-00258-9
- Li W, Li C, Zhou T, Liu X, Liu X, Li X, et al. Role of Exosomal Proteins in Cancer Diagnosis. *Mol Cancer* (2017) 16(1):145. doi: 10.1186/s12943-017-0706-8
- Fitts CA, Ji N, Li Y, Tan C. Exploiting Exosomes in Cancer Liquid Biopsies and Drug Delivery. *Adv Healthc Mater* (2019) 8(6):e1801268. doi: 10.1002/adhm.201801268
- Liu Y, Shi K, Chen Y, Wu X, Chen Z, Cao K, et al. Exosomes and Their Role in Cancer Progression. *Front Oncol* (2021) 11:639159. doi: 10.3389/fonc.2021.639159
- Thery C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsohaina R, et al. Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): A Position Statement of the International Society for Extracellular Vesicles and Update of the MISEV2014 Guidelines. *J Extracell Vesicles* (2018) 7(1):1535750. doi: 10.1080/20013078.2018.1535750
- Nazri HM, Imran M, Fischer R, Heilig R, Manek S, Dragovic RA, et al. Characterization of Exosomes in Peritoneal Fluid of Endometriosis Patients. *Fertil Steril* (2020) 113(2):364–73 e2. doi: 10.1016/j.fertnstert.2019.09.032
- Kalluri R, LeBleu VS. The Biology, Function, and Biomedical Applications of Exosomes. *Science* (2020) 367(6478):eaa6977. doi: 10.1126/science.aaa6977
- van Niel G, D'Angelo G, Raposo G. Shedding Light on the Cell Biology of Extracellular Vesicles. *Nat Rev Mol Cell Biol* (2018) 19(4):213–28. doi: 10.1038/nrm.2017.125
- Gurunathan S, Kang MH, Qasim M, Khan K, Kim JH. Biogenesis, Membrane Trafficking, Functions, and Next Generation Nanotherapeutics Medicine of Extracellular Vesicles. *Int J Nanomedicine* (2021) 16:3357–83. doi: 10.2147/IJN.S310357
- Thery C, Amigorena S, Raposo G, Clayton A. Isolation and Characterization of Exosomes From Cell Culture Supernatants and Biological Fluids. *Curr Protoc Cell Biol* (2006) 93:3.22.1–29. doi: 10.1002/0471143030.cb0322s30
- Keerthikumar S, Chisanga D, Ariyaratne D, Al Saffar H, Anand S, Zhao K, et al. ExoCarta: A Web-Based Compendium of Exosomal Cargo. *J Mol Biol* (2016) 428(4):688–92. doi: 10.1016/j.jmb.2015.09.019
- Reddy VS, Madala SK, Trinath J, Reddy GB. Extracellular Small Heat Shock Proteins: Exosomal Biogenesis and Function. *Cell Stress Chaperones* (2018) 23(3):441–54. doi: 10.1007/s12192-017-0856-z
- Goldie BJ, Dun MD, Lin M, Smith ND, Verrills NM, Dayas CV, et al. Activity-Associated miRNA Are Packaged in Map1b-Enriched Exosomes Released From Depolarized Neurons. *Nucleic Acids Res* (2014) 42(14):9195–208. doi: 10.1093/nar/gku594
- Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-Mediated Transfer of mRNAs and microRNAs Is a Novel Mechanism of Genetic Exchange Between Cells. *Nat Cell Biol* (2007) 9(6):654–9. doi: 10.1038/ncb1596
- Salehi M, Sharifi M. Exosomal miRNAs as Novel Cancer Biomarkers: Challenges and Opportunities. *J Cell Physiol* (2018) 233(9):6370–80. doi: 10.1002/jcp.26481
- Chatterjee N, Rana S, Espinosa-Diez C, Anand S. MicroRNAs in Cancer: Challenges and Opportunities in Early Detection, Disease Monitoring, and Therapeutic Agents. *Curr Pathobiol Rep* (2017) 5(1):35–42. doi: 10.1007/s40139-017-0123-0
- Chargaff E, West R. The Biological Significance of the Thromboplastic Protein of Blood. *J Biol Chem* (1946) 166(1):189–97. doi: 10.1016/S0021-9258(17)34997-9
- Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle Formation During Reticulocyte Maturation. Association of Plasma Membrane Activities With Released Vesicles (Exosomes). *J Biol Chem* (1987) 262(19):9412–20. doi: 10.1016/S0021-9258(18)48095-7
- Keller S, Ridinger J, Rupp AK, Janssen JW, Altevogt P. Body Fluid Derived Exosomes as a Novel Template for Clinical Diagnostics. *J Transl Med* (2011) 9:86. doi: 10.1186/1479-5876-9-86
- Wei Y, Lai X, Yu S, Chen S, Ma Y, Zhang Y, et al. Exosomal miR-221/222 Enhances Tamoxifen Resistance in Recipient ER-Positive Breast Cancer

- Cells. *Breast Cancer Res Treat* (2014) 147(2):423–31. doi: 10.1007/s10549-014-3037-0
33. Tian T, Zhu YL, Zhou YY, Liang GF, Wang YY, Hu FH, et al. Exosome Uptake Through Clathrin-Mediated Endocytosis and Macropinocytosis and Mediating miR-21 Delivery. *J Biol Chem* (2014) 289(32):22258–67. doi: 10.1074/jbc.M114.588046
 34. Parolini I, Federici C, Raggi C, Lugini L, Palleschi S, De Milito A, et al. Microenvironmental pH Is a Key Factor for Exosome Traffic in Tumor Cells. *J Biol Chem* (2009) 284(49):34211–22. doi: 10.1074/jbc.M109.041152
 35. Kitai Y, Kawasaki T, Sueyoshi T, Kobiyama K, Ishii KJ, Zou J, et al. DNA-Containing Exosomes Derived From Cancer Cells Treated With Topotecan Activate a STING-Dependent Pathway and Reinforce Antitumor Immunity. *J Immunol* (2017) 198(4):1649–59. doi: 10.4049/jimmunol.1601694
 36. Sheller-Miller S, Trivedi J, Yellon SM, Menon R. Exosomes Cause Preterm Birth in Mice: Evidence for Paracrine Signaling in Pregnancy. *Sci Rep* (2019) 9(1):608. doi: 10.1038/s41598-018-37002-x
 37. Vincent-Schneider H, Stumptner-Cuvellette P, Lankar D, Pain S, Raposo G, Benaroch P, et al. Exosomes Bearing HLA-DR1 Molecules Need Dendritic Cells to Efficiently Stimulate Specific T Cells. *Int Immunol* (2002) 14(7):713–22. doi: 10.1093/intimm/dfx048
 38. Qu JL, Qu XJ, Zhao MF, Teng YE, Zhang Y, Hou KZ, et al. Gastric Cancer Exosomes Promote Tumour Cell Proliferation Through PI3K/Akt and MAPK/ERK Activation. *Dig Liver Dis* (2009) 41(12):875–80. doi: 10.1016/j.dld.2009.04.006
 39. Yang L, Wu XH, Wang D, Luo CL, Chen LX. Bladder Cancer Cell-Derived Exosomes Inhibit Tumor Cell Apoptosis and Induce Cell Proliferation *In Vitro*. *Mol Med Rep* (2013) 8(4):1272–8. doi: 10.3892/mmr.2013.1634
 40. Aga M, Bentz GL, Raffa S, Torrisi MR, Kondo S, Wakisaka N, et al. Exosomal HIF1 α Supports Invasive Potential of Nasopharyngeal Carcinoma-Associated LMP1-Positive Exosomes. *Oncogene* (2014) 33(37):4613–22. doi: 10.1038/ncr.2014.66
 41. Yoshizaki T, Kondo S, Wakisaka N, Muroso S, Endo K, Sugimoto H, et al. Pathogenic Role of Epstein-Barr Virus Latent Membrane Protein-1 in the Development of Nasopharyngeal Carcinoma. *Cancer Lett* (2013) 337(1):1–7. doi: 10.1016/j.canlet.2013.05.018
 42. Fiaschi T, Giannoni E, Taddei ML, Cirri P, Marini A, Pintus G, et al. Carbonic Anhydrase IX From Cancer-Associated Fibroblasts Drives Epithelial-Mesenchymal Transition in Prostate Carcinoma Cells. *Cell Cycle* (2013) 12(11):1791–801. doi: 10.4161/cc.24902
 43. Yue S, Mu W, Erb U, Zoller M. The Tetraspanins CD151 and Tspan8 Are Essential Exosome Components for the Crosstalk Between Cancer Initiating Cells and Their Surrounding. *Oncotarget* (2015) 6(4):2366–84. doi: 10.18632/oncotarget.2958
 44. Berchem G, Noman MZ, Bosseler M, Paggetti J, Baconnais S, Le Cam E, et al. Hypoxic Tumor-Derived Microvesicles Negatively Regulate NK Cell Function by a Mechanism Involving TGF- β and Mir23a Transfer. *Oncoimmunology* (2016) 5(4):e1062968. doi: 10.1080/2162402X.2015.1062968
 45. Taylor DD, Gercel-Taylor C. Exosomes/microvesicles: Mediators of Cancer-Associated Immunosuppressive Microenvironments. *Semin Immunopathol* (2011) 33(5):441–54. doi: 10.1007/s00281-010-0234-8
 46. Ying X, Wu Q, Wu X, Zhu Q, Wang X, Jiang L, et al. Epithelial Ovarian Cancer-Secreted Exosomal miR-222-3p Induces Polarization of Tumor-Associated Macrophages. *Oncotarget* (2016) 7(28):43076–87. doi: 10.18632/oncotarget.9246
 47. Ding G, Zhou L, Qian Y, Fu M, Chen J, Chen J, et al. Pancreatic Cancer-Derived Exosomes Transfer miRNAs to Dendritic Cells and Inhibit RFXAP Expression via miR-212-3p. *Oncotarget* (2015) 6(30):29877–88. doi: 10.18632/oncotarget.4924
 48. Richards KE, Zeleniak AE, Fishel ML, Wu J, Littlepage LE, Hill R. Cancer-Associated Fibroblast Exosomes Regulate Survival and Proliferation of Pancreatic Cancer Cells. *Oncogene* (2017) 36(13):1770–8. doi: 10.1038/ncr.2016.353
 49. Momen-Heravi F, Balaj L, Alian S, Trachtenberg AJ, Hochberg FH, Skog J, et al. Impact of Biofluid Viscosity on Size and Sedimentation Efficiency of the Isolated Microvesicles. *Front Physiol* (2012) 3:162. doi: 10.3389/fphys.2012.00162
 50. Witwer KW, Buzas EI, Bemis LT, Bora A, Lasser C, Lotvall J, et al. Standardization of Sample Collection, Isolation and Analysis Methods in Extracellular Vesicle Research. *J Extracell Vesicles* (2013) 2(1):20360. doi: 10.3402/jev.v2i0.20360
 51. Jung HH, Kim JY, Lim JE, Im YH. Cytokine Profiling in Serum-Derived Exosomes Isolated by Different Methods. *Sci Rep* (2020) 10(1):14069. doi: 10.1038/s41598-020-70584-z
 52. Taylor DD, Shah S. Methods of Isolating Extracellular Vesicles Impact Down-Stream Analyses of Their Cargoes. *Methods* (2015) 87:3–10. doi: 10.1016/j.ymeth.2015.02.019
 53. Nakai W, Yoshida T, Diez D, Miyatake Y, Nishibu T, Imawaka N, et al. A Novel Affinity-Based Method for the Isolation of Highly Purified Extracellular Vesicles. *Sci Rep* (2016) 6:33935. doi: 10.1038/srep33935
 54. Ning B, Huang Z, Youngquist BM, Scott JW, Niu A, Bojanowski CM, et al. Liposome-Mediated Detection of SARS-CoV-2 RNA-Positive Extracellular Vesicles in Plasma. *Nat Nanotechnol* (2021) 16(9):1039–44. doi: 10.1038/s41565-021-00939-8
 55. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in Exosome Isolation Techniques. *Theranostics* (2017) 7(3):789–804. doi: 10.7150/thno.18133
 56. Baranyai T, Herczeg K, Onodi Z, Voszka I, Módos K, Marton N, et al. Isolation of Exosomes From Blood Plasma: Qualitative and Quantitative Comparison of Ultracentrifugation and Size Exclusion Chromatography Methods. *PLoS One* (2015) 10(12):e0145686. doi: 10.1371/journal.pone.0145686
 57. Hong P, Koza S, Bouvier ES. Size-Exclusion Chromatography for the Analysis of Protein Biotherapeutics and Their Aggregates. *J Liq Chromatogr Relat Technol* (2012) 35(20):2923–50. doi: 10.1080/10826076.2012.743724
 58. Clayton A, Court J, Navabi H, Adams M, Mason MD, Hobot JA, et al. Analysis of Antigen Presenting Cell Derived Exosomes, Based on Immuno-Magnetic Isolation and Flow Cytometry. *J Immunol Methods* (2001) 247(1–2):163–74. doi: 10.1016/S0022-1759(00)00321-5
 59. Zhang K, Yue Y, Wu S, Liu W, Shi J, Zhang Z. Rapid Capture and Nondestructive Release of Extracellular Vesicles Using Aptamer-Based Magnetic Isolation. *ACS Sensors* (2019) 4(5):1245–51. doi: 10.1021/acsensors.9b00060
 60. Lobb RJ, Becker M, Wen SW, Wong CS, Wiegman AP, Leimgruber A, et al. Optimized Exosome Isolation Protocol for Cell Culture Supernatant and Human Plasma. *J Extracell Vesicles* (2015) 4:27031. doi: 10.3402/jev.v4.27031
 61. Nordin JZ, Lee Y, Vader P, Mager I, Johansson HJ, Heusermann W, et al. Ultrafiltration With Size-Exclusion Liquid Chromatography for High Yield Isolation of Extracellular Vesicles Preserving Intact Biophysical and Functional Properties. *Nanomedicine* (2015) 11(4):879–83. doi: 10.1016/j.nano.2015.01.003
 62. Shurtleff MJ, Temoche-Diaz MM, Schekman R. Extracellular Vesicles and Cancer: Caveat Lector. *Annu Rev Cancer Biol* (2018) 2(1):395–411. doi: 10.1146/annurev-cancerbio-030617-050519
 63. Jeppesen DK, Fenix AM, Franklin JL, Higginbotham JN, Zhang Q, Zimmerman LJ, et al. Reassessment of Exosome Composition. *Cell* (2019) 177(2):428–445 e18. doi: 10.1016/j.cell.2019.02.029
 64. van der Pol E, Hoekstra AG, Sturk A, Otto C, van Leeuwen TG, Nieuwland R. Optical and Non-Optical Methods for Detection and Characterization of Microparticles and Exosomes. *J Thromb Haemost* (2010) 8(12):2596–607. doi: 10.1111/j.1538-7836.2010.04074.x
 65. Dragovic RA, Gardiner C, Brooks AS, Tannetta DS, Ferguson DJ, Hole P, et al. Sizing and Phenotyping of Cellular Vesicles Using Nanoparticle Tracking Analysis. *Nanomedicine* (2011) 7(6):780–8. doi: 10.1016/j.nano.2011.04.003
 66. Coumans FAW, Gool EL, Nieuwland R. Bulk Immunoassays for Analysis of Extracellular Vesicles. *Platelets* (2017) 28(3):242–8. doi: 10.1080/09537104.2016.1265926
 67. Karimi N, Cvjetkovic A, Jang SC, Crescitelli R, Hosseini Pour Feizi MA, Nieuwland R, et al. Detailed Analysis of the Plasma Extracellular Vesicle Proteome After Separation From Lipoproteins. *Cell Mol Life Sci* (2018) 75(15):2873–86. doi: 10.1007/s00018-018-2773-4
 68. Feng Y, Zhong X, Tang T-T, Wang C, Wang L-T, Li Z-L, et al. Rab27a Dependent Exosome Releasing Participated in Albumin Handling as a Coordinated Approach to Lysosome in Kidney Disease. *Cell Death Dis* (2020) 11(7):513. doi: 10.1038/s41419-020-2709-4

69. Li C, Zhou Y, Liu J, Su X, Qin H, Huang S, et al. Potential Markers From Serum-Purified Exosomes for Detecting Oral Squamous Cell Carcinoma Metastasis. *Cancer Epidemiol Biomarkers Prev* (2019) 28(10):1668–81. doi: 10.1158/1055-9965.Epi-18-1122
70. Lacroix R, Robert S, Poncelet P, Dignat-George F. Overcoming Limitations of Microparticle Measurement by Flow Cytometry. *Semin Thromb Hemost* (2010) 36(8):807–18. doi: 10.1055/s-0030-1267034
71. Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, et al. Proteomic Comparison Defines Novel Markers to Characterize Heterogeneous Populations of Extracellular Vesicle Subtypes. *Proc Natl Acad Sci USA* (2016) 113(8):E968–77. doi: 10.1073/pnas.1521230113
72. Contreras-Naranjo JC, Wu HJ, Ugaz VM. Microfluidics for Exosome Isolation and Analysis: Enabling Liquid Biopsy for Personalized Medicine. *Lab Chip* (2017) 17(21):3558–77. doi: 10.1039/c7lc00592j
73. Ueda K, Ishikawa N, Tatsuguchi A, Saichi N, Fujii R, Nakagawa H. Antibody-Coupled Monolithic Silica Microtips for Highthroughput Molecular Profiling of Circulating Exosomes. *Sci Rep* (2014) 4, 6232. doi: 10.1038/srep06232
74. Sandfeld-Paulsen B, Jakobsen KR, Baek R, Folkersen BH, Rasmussen TR, Meldgaard P, et al. Exosomal Proteins as Diagnostic Biomarkers in Lung Cancer. *J Thorac Oncol* (2016) 11(10):1701–10. doi: 10.1016/j.jtho.2016.05.034
75. Wang X, Zhong W, Bu J, Li Y, Li R, Nie R, et al. Exosomal Protein CD82 as a Diagnostic Biomarker for Precision Medicine for Breast Cancer. *Mol Carcinog* (2019) 58(5):674–85. doi: 10.1002/mc.22960
76. Yoshioka Y, Kosaka N, Konishi Y, Ohta H, Okamoto H, Sonoda H, et al. Ultra-Sensitive Liquid Biopsy of Circulating Extracellular Vesicles Using ExoScreen. *Nat Commun* (2014) 5, 3591. doi: 10.1038/ncomms4591
77. Madhavan B, Yue S, Galli U, Rana S, Gross W, Muller M, et al. Combined Evaluation of a Panel of Protein and miRNA Serum-Exosome Biomarkers for Pancreatic Cancer Diagnosis Increases Sensitivity and Specificity. *Int J Cancer* (2015) 136(11):2616–27. doi: 10.1002/ijc.29324
78. Krishn SR, Singh A, Bowler N, Duffy AN, Friedman A, Fedele C, et al. Prostate Cancer Sheds the Alpha5beta3 Integrin *In Vivo* Through Exosomes. *Matrix Biol* (2019) 77:41–57. doi: 10.1016/j.matbio.2018.08.004
79. Soekmadji C, Riches JD, Russell PJ, Ruelcke JE, McPherson S, Wang C, et al. Modulation of Paracrine Signaling by CD9 Positive Small Extracellular Vesicles Mediates Cellular Growth of Androgen Deprived Prostate Cancer. *Oncotarget* (2017) 8(32):52237–55. doi: 10.18632/oncotarget.11111
80. Kato T, Mizutani K, Kameyama K, Kawakami K, Fujita Y, Nakane K, et al. Serum Exosomal P-Glycoprotein Is a Potential Marker to Diagnose Docetaxel Resistance and Select a Taxoid for Patients With Prostate Cancer. *Urol Oncol* (2015) 33(9):385 e15–20. doi: 10.1016/j.urolonc.2015.04.019
81. Logozzi M, De Milito A, Lugini L, Borghi M, Calabro L, Spada M, et al. High Levels of Exosomes Expressing CD63 and Caveolin-1 in Plasma of Melanoma Patients. *PLoS One* (2009) 4(4):e5219. doi: 10.1371/journal.pone.0005219
82. Rodriguez Zorrilla S, Perez-Sayans M, Fais S, Logozzi M, Gallas Torreira M, Garcia Garcia A. A Pilot Clinical Study on the Prognostic Relevance of Plasmatic Exosomes Levels in Oral Squamous Cell Carcinoma Patients. *Cancers (Basel)* (2019) 11(3):429. doi: 10.3390/cancers11030429
83. Chanteloup G, Cordonnier M, Isambert N, Bertaute A, Hervieu A, Hennequin A, et al. Monitoring HSP70 Exosomes in Cancer Patients' Follow Up: A Clinical Prospective Pilot Study. *J Extracell Vesicles* (2020) 9 (1):1766192. doi: 10.1080/20013078.2020.1766192
84. An T, Qin S, Sun D, Huang Y, Hu Y, Li S, et al. Unique Protein Profiles of Extracellular Vesicles as Diagnostic Biomarkers for Early and Advanced Non-Small Cell Lung Cancer. *Proteomics* (2019) 19(12):e1800160. doi: 10.1002/pmic.201800160
85. Ding XQ, Wang ZY, Xia D, Wang RX, Pan XR, Tong JH. Proteomic Profiling of Serum Exosomes From Patients With Metastatic Gastric Cancer. *Front Oncol* (2020) 10:1113. doi: 10.3389/fonc.2020.01113
86. Chen Y, Xie Y, Xu L, Zhan S, Xiao Y, Gao Y, et al. Protein Content and Functional Characteristics of Serum-Purified Exosomes From Patients With Colorectal Cancer Revealed by Quantitative Proteomics. *Int J Cancer* (2017) 140(4):900–13. doi: 10.1002/ijc.30496
87. Ishizuya Y, Uemura M, Narumi R, Tomiyama E, Koh Y, Matsushita M, et al. The Role of Actinin-4 (ACTN4) in Exosomes as a Potential Novel Therapeutic Target in Castration-Resistant Prostate Cancer. *Biochem Biophys Res Commun* (2020) 523(3):588–94. doi: 10.1016/j.bbrc.2019.12.084
88. Jin H, Liu P, Wu Y, Meng X, Wu M, Han J, et al. Exosomal Zinc Transporter ZIP4 Promotes Cancer Growth and Is a Novel Diagnostic Biomarker for Pancreatic Cancer. *Cancer Sci* (2018) 109(9):2946–56. doi: 10.1111/cas.13737
89. Herrero C, Abal M, Muinelo-Romay L. Circulating Extracellular Vesicles in Gynecological Tumors: Realities and Challenges. *Front Oncol* (2020) 10:565666. doi: 10.3389/fonc.2020.565666
90. Wyciszkievicz A, Kalinowska-Lyszczarz A, Nowakowski B, Kaźmierczak K, Osztynowicz K, Michalak S. Expression of Small Heat Shock Proteins in Exosomes From Patients With Gynecologic Cancers. *Sci Rep* (2019) 9(1), 9817. doi: 10.1038/s41598-019-46221-9
91. Shao H, Chung J, Balaj L, Charest A, Bigner DD, Carter BS, et al. Protein Typing of Circulating Microvesicles Allows Real-Time Monitoring of Glioblastoma Therapy. *Nat Med* (2012) 18(12):1835–40. doi: 10.1038/nm.2994
92. Yamashita T, Kamada H, Kanasaki S, Maeda Y, Nagano K, Abe Y, et al. Epidermal Growth Factor Receptor Localized to Exosome Membranes as a Possible Biomarker for Lung Cancer Diagnosis. *Pharmazie* (2013) 68 (12):969–73. doi: 10.1691/ph.2013.3599
93. Arbelaz A, Azkargorta M, Krawczyk M, Santos-Laso A, Lapitz A, Perugorria MJ, et al. Serum Extracellular Vesicles Contain Protein Biomarkers for Primary Sclerosing Cholangitis and Cholangiocarcinoma. *Hepatology* (2017) 66(4):1125–43. doi: 10.1002/hep.29291
94. Li J, Chen Y, Guo X, Zhou L, Jia Z, Peng Z, et al. GPC1 Exosome and its Regulatory miRNAs Are Specific Markers for the Detection and Target Therapy of Colorectal Cancer. *J Cell Mol Med* (2017) 21(5):838–47. doi: 10.1111/jcmm.12941
95. Li J, Li B, Ren C, Chen Y, Guo X, Zhou L, et al. The Clinical Significance of Circulating GPC1 Positive Exosomes and Its Regulatory miRNAs in Colon Cancer Patients. *Oncotarget* (2017) 8(60):101189–202. doi: 10.18632/oncotarget.20516
96. Lux A, Kahlert C, Grutzmann R, Pilarsky C. C-Met and PD-L1 on Circulating Exosomes as Diagnostic and Prognostic Markers for Pancreatic Cancer. *Int J Mol Sci* (2019) 20(13):3305. doi: 10.3390/ijms20133305
97. Melo SA, Luecke LB, Kahlert C, Fernandez AF, Gammon ST, Kaye J, et al. Glypican-1 Identifies Cancer Exosomes and Detects Early Pancreatic Cancer. *Nature* (2015) 523(7559):177–82. doi: 10.1038/nature14581
98. Frampton AE, Prado MM, Lopez-Jimenez E, Fajardo-Puerta AB, Jawad ZAR, Lawton P, et al. Glypican-1 Is Enriched in Circulating-Exosomes in Pancreatic Cancer and Correlates With Tumor Burden. *Oncotarget* (2018) 9 (27):19006–13. doi: 10.18632/oncotarget.24873
99. Xiao D, Dong Z, Zhen L, Xia G, Huang X, Wang T, et al. Combined Exosomal GPC1, CD82, and Serum CA19-9 as Multiplex Targets: A Specific, Sensitive, and Reproducible Detection Panel for the Diagnosis of Pancreatic Cancer. *Mol Cancer Res* (2020) 18(2):300–10. doi: 10.1158/1541-7786.MCR-19-0588
100. Buscail E, Chauvet A, Quincy P, Degrandi O, Buscail C, Lamrissi I, et al. CD63-GPC1-Positive Exosomes Coupled With CA19-9 Offer Good Diagnostic Potential for Resectable Pancreatic Ductal Adenocarcinoma. *Transl Oncol* (2019) 12(11):1395–403. doi: 10.1016/j.tranon.2019.07.009
101. Zhao Z, Yang Y, Zeng Y, He M. A Microfluidic ExoSearch Chip for Multiplexed Exosome Detection Towards Blood-Based Ovarian Cancer Diagnosis. *Lab Chip* (2016) 16(3):489–96. doi: 10.1039/c5lc01117e
102. Chen G, Huang AC, Zhang W, Zhang G, Wu M, Xu W, et al. Exosomal PD-L1 Contributes to Immunosuppression and Is Associated With Anti-PD-1 Response. *Nature* (2018) 560(7718):382–6. doi: 10.1038/s41586-018-0392-8
103. Theodoraki MN, Yerneni SS, Hoffmann TK, Gooding WE, Whiteside TL. Clinical Significance of PD-L1(+) Exosomes in Plasma of Head and Neck Cancer Patients. *Clin Cancer Res* (2018) 24(4):896–905. doi: 10.1158/1078-0432.CCR-17-2664
104. Erozceni LA, Böttger F, Bijnsdorp IV, Jimenez CR. Urinary Exosomal Proteins as (Pan-)Cancer Biomarkers: Insights From the Proteome. *FEBS Lett* (2019) 593(13):1580–97. doi: 10.1002/1873-3468.13487
105. Chen CL, Lai YF, Tang P, Chien KY, Yu JS, Tsai CH, et al. Comparative and Targeted Proteomic Analyses of Urinary Microparticles From Bladder

- Cancer and Hernia Patients. *J Proteome Res* (2012) 11(12):5611–29. doi: 10.1021/pr3008732
106. Sakaue T, Koga H, Iwamoto H, Nakamura T, Ikezono Y, Abe M, et al. Glycosylation of Ascites-Derived Exosomal CD133: A Potential Prognostic Biomarker in Patients With Advanced Pancreatic Cancer. *Med Mol Morphol* (2019) 52(4):198–208. doi: 10.1007/s00795-019-00218-5
 107. Pisitkun T, Shen RF, Knepper MA. Identification and Proteomic Profiling of Exosomes in Human Urine. *Proc Natl Acad Sci USA* (2004) 101(36):13368–73. doi: 10.1073/pnas.0403453101
 108. Jalalian SH, Ramezani M, Jalalian SA, Abnous K, Taghdisi SM. Exosomes, New Biomarkers in Early Cancer Detection. *Anal Biochem* (2019) 571:1–13. doi: 10.1016/j.ab.2019.02.013
 109. He M, Crow J, Roth M, Zeng Y, Godwin AK. Integrated Immunoisolation and Protein Analysis of Circulating Exosomes Using Microfluidic Technology. *Lab Chip* (2014) 14(19):3773–80. doi: 10.1039/c4lc00662c
 110. Jakobsen KR, Paulsen BS, Baek R, Varming K, Sorensen BS, Jorgensen MM. Exosomal Proteins as Potential Diagnostic Markers in Advanced Non-Small Cell Lung Carcinoma. *J Extracell Vesicles* (2015) 4:26659. doi: 10.3402/jev.v4.26659
 111. Jorgensen M, Baek R, Pedersen S, Sondergaard EK, Kristensen SR, Varming K. Extracellular Vesicle (EV) Array: Microarray Capturing of Exosomes and Other Extracellular Vesicles for Multiplexed Phenotyping. *J Extracell Vesicles* (2013) 2:20920. doi: 10.3402/jev.v2i0.20920
 112. Malla RR, Pandrangi S, Kumari S, Gavara MM, Badana AK. Exosomal Tetraspanins as Regulators of Cancer Progression and Metastasis and Novel Diagnostic Markers. *Asia-Pacific J Clin Oncol* (2018) 14(6):383–91. doi: 10.1111/ajco.12869
 113. Uhlen M, Bjorling E, Agaton C, Szgyarto CA, Amini B, Andersen E, et al. A Human Protein Atlas for Normal and Cancer Tissues Based on Antibody Proteomics. *Mol Cell Proteomics* (2005) 4(12):1920–32. doi: 10.1074/mcp.M500279-MCP200
 114. Yurchenko V, Constant S, Bukrinsky M. Dealing With the Family: CD147 Interactions With Cyclophilins. *Immunology* (2006) 117(3):301–9. doi: 10.1111/j.1365-2567.2005.02316.x
 115. Li Y, Yu S, Li L, Chen J, Quan M, Li Q, et al. KLF4-Mediated Upregulation of CD9 and CD81 Suppresses Hepatocellular Carcinoma Development via JNK Signaling. *Cell Death Dis* (2020) 11(4):299. doi: 10.1038/s41419-020-2479-z
 116. Khushman M, Prodduturvar P, Mneimneh W, Zotto VD, Akbar S, Grimm L, et al. The Impact of Neoadjuvant Concurrent Chemoradiation on Exosomal Markers (CD63 and CD9) Expression and Their Prognostic Significance in Patients With Rectal Adenocarcinoma. *Oncotarget* (2021) 12(15):1490–8. doi: 10.18632/oncotarget.28025
 117. Oh KB, Stanton MJ, West WW, Todd GL, Wagner KU. Tsg101 is Upregulated in a Subset of Invasive Human Breast Cancers and Its Targeted Overexpression in Transgenic Mice Reveals Weak Oncogenic Properties for Mammary Cancer Initiation. *Oncogene* (2007) 26(40):5950–9. doi: 10.1038/sj.onc.1210401
 118. Yang J, Zhang Y, Gao X, Yuan Y, Zhao J, Zhou S, et al. Plasma-Derived Exosomal ALIX as a Novel Biomarker for Diagnosis and Classification of Pancreatic Cancer. *Front Oncol* (2021) 11:628346. doi: 10.3389/fonc.2021.628346
 119. Albakova Z, Siam MKS, Sacitharan PK, Ziganshin RH, Ryazantsev DY, Sapozhnikov AM. Extracellular Heat Shock Proteins and Cancer: New Perspectives. *Trans Oncol* (2021) 14(2):100995. doi: 10.1016/j.tranon.2020.100995
 120. Gheyntanchi E, Saeednejad Zanjani L, Ghods R, Abolhasani M, Shahin M, Vafaei S, et al. High Expression of Tumor Susceptibility Gene 101 (TSG101) Is Associated With More Aggressive Behavior in Colorectal Carcinoma. *J Cancer Res Clin Oncol* (2021) 147(6):1631–46. doi: 10.1007/s00432-021-03561-2
 121. Albakova Z, Armeev GA, Kanevskiy LM, Kovalenko EI, Sapozhnikov AM. HSP70 Multi-Functionality in Cancer. *Cells* (2020) 9(3):587. doi: 10.3390/cells9030587
 122. Bausero MA, Gastpar R, Multhoff G, Asea A. Alternative Mechanism by Which IFN- γ Enhances Tumor Recognition: Active Release of Heat Shock Protein 72. *J Immunol* (2005) 175(5):2900–12. doi: 10.4049/jimmunol.175.5.2900
 123. Jiang C, Cao S, Li N, Jiang L, Sun T. PD-1 and PD-L1 Correlated Gene Expression Profiles and Their Association With Clinical Outcomes of Breast Cancer. *Cancer Cell Int* (2019) 19:233. doi: 10.1186/s12935-019-0955-2
 124. Li N, Gao W, Zhang Y-F, Ho M. Glypicans as Cancer Therapeutic Targets. *Trends Cancer* (2018) 4(11):741–54. doi: 10.1016/j.trecan.2018.09.004
 125. Lund ME, Campbell DH, Walsh BJ. The Role of Glypican-1 in the Tumour Microenvironment. *Adv Exp Med Biol* (2020) 1245:163–76. doi: 10.1007/978-3-030-40146-7_8
 126. Yang KS, Im H, Hong S, Pergolini I, Del Castillo AF, Wang R, et al. Multiparametric Plasma EV Profiling Facilitates Diagnosis of Pancreatic Malignancy. *Sci Transl Med* (2017) 9(391):eaal3226. doi: 10.1126/scitranslmed.aal3226
 127. Soltesz B, Lukacs J, Szilagyi E, Marton E, Szilagyi Bonizs M, Penyige A, et al. Expression of CD24 in Plasma, Exosome and Ovarian Tissue Samples of Serous Ovarian Cancer Patients. *J Biotechnol* (2019) 298:16–20. doi: 10.1016/j.jbiotec.2019.03.018
 128. Khan S, Bennit HF, Turay D, Perez M, Mirshahidi S, Yuan Y, et al. Early Diagnostic Value of Survivin and Its Alternative Splice Variants in Breast Cancer. *BMC Cancer* (2014) 14:176. doi: 10.1186/1471-2407-14-176
 129. Wang H, Lu Z, Zhao X. Tumorigenesis, Diagnosis, and Therapeutic Potential of Exosomes in Liver Cancer. *J Hematol Oncol* (2019) 12(1):133. doi: 10.1186/s13045-019-0806-6
 130. Zhou H, Yuen PS, Pisitkun T, Gonzales PA, Yasuda H, Dear JW, et al. Collection, Storage, Preservation, and Normalization of Human Urinary Exosomes for Biomarker Discovery. *Kidney Int* (2006) 69(8):1471–6. doi: 10.1038/sj.ki.5000273
 131. Wang Y-T, Shi T, Srivastava S, Kagan J, Liu T, Rodland KD. Proteomic Analysis of Exosomes for Discovery of Protein Biomarkers for Prostate and Bladder Cancer. *Cancers* (2020) 12(9), 2335. doi: 10.3390/cancers12092335
 132. Georgantzoglou N, Pergaris A, Masaoutis C, Theocharis S. Extracellular Vesicles as Biomarkers Carriers in Bladder Cancer: Diagnosis, Surveillance, and Treatment. *Int J Mol Sci* (2021) 22(5), 2744. doi: 10.3390/ijms22052744
 133. Li Y, Zhang Y, Qiu F, Qiu Z. Proteomic Identification of Exosomal LRG1: A Potential Urinary Biomarker for Detecting NSCLC. *Electrophoresis* (2011) 32(15):1976–83. doi: 10.1002/elps.201000598
 134. Mizenko RR, Brostoff T, Rojalin T, Koster HJ, Swindell HS, Leiserowitz GS, et al. Tetraspanins Are Unevenly Distributed Across Single Extracellular Vesicles and Bias Sensitivity to Multiplexed Cancer Biomarkers. *J Nanobiotech* (2021) 19(1):250. doi: 10.1186/s12951-021-00987-1
 135. Lin S, Yu Z, Chen D, Wang Z, Miao J, Li Q, et al. Progress in Microfluidics-Based Exosome Separation and Detection Technologies for Diagnostic Applications. *Small* (2020) 16(9):e1903916. doi: 10.1002/smll.201903916
 136. Zhang W, Tian Z, Yang S, Rich J, Zhao S, Klingeborn M, et al. Electrochemical Micro-Aptasensors for Exosome Detection Based on Hybridization Chain Reaction Amplification. *Microsystems Nanoeng* (2021) 7(1):63. doi: 10.1038/s41378-021-00293-8
 137. Wang L, Zeng L, Wang Y, Chen T, Chen W, Chen G, et al. Electrochemical Aptasensor Based on Multidirectional Hybridization Chain Reaction for Detection of Tumorous Exosomes. *Sensors Actuators B: Chem* (2021) 332:129471. doi: 10.1016/j.snb.2021.129471
 138. Palviainen M, Saraswat M, Varga Z, Kitka D, Neuvonen M, Puhka M, et al. Extracellular Vesicles From Human Plasma and Serum Are Carriers of Extravesicular Cargo-Implications for Biomarker Discovery. *PLoS One* (2020) 15(8):e0236439. doi: 10.1371/journal.pone.0236439
 139. Dal Collo G, Adamo A, Gatti A, Tamellini E, Bazzoni R, Takam Kamga P, et al. Functional Dosing of Mesenchymal Stromal Cell-Derived Extracellular Vesicles for the Prevention of Acute Graft-Versus-Host-Disease. *Stem Cells* (2020) 38(5):698–711. doi: 10.1002/stem.3160
 140. Lai P, Chen X, Guo L, Wang Y, Liu X, Liu Y, et al. A Potent Immunomodulatory Role of Exosomes Derived From Mesenchymal Stromal Cells in Preventing cGVHD. *J Hematol Oncol* (2018) 11(1):135. doi: 10.1186/s13045-018-0680-7
 141. Kordelas L, Rebmann V, Ludwig AK, Radtke S, Ruesing J, Doeppner TR, et al. MSC-Derived Exosomes: A Novel Tool to Treat Therapy-Refractory Graft-Versus-Host Disease. *Leukemia* (2014) 28(4):970–3. doi: 10.1038/leu.2014.41
 142. Sengupta V, Sengupta S, Lazo A, Woods P, Nolan A, Bremer N. Exosomes Derived From Bone Marrow Mesenchymal Stem Cells as Treatment for

Severe COVID-19. *Stem Cells Dev* (2020) 29(12):747–54. doi: 10.1089/scd.2020.0080

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in

this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Hu, Jiang, Lv, Fan, Lu, Wu and Pi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



OPEN ACCESS

EDITED BY

Kendall Van Keuren-Jensen,
Translational Genomics Research
Institute (TGen), United States

REVIEWED BY

Jodi L. McGill,
Iowa State University, United States
Zhenlong Liu,
McGill University, Canada

*CORRESPONDENCE

Tiziana Corsello
ticorsel@utmb.edu
Antonella Casola
ancasola@utmb.edu

SPECIALTY SECTION

This article was submitted to
Cytokines and Soluble
Mediators in Immunity,
a section of the journal
Frontiers in Immunology

RECEIVED 28 February 2022

ACCEPTED 28 July 2022

PUBLISHED 12 August 2022

CITATION

Corsello T, Qu Y, Ivanciuc T,
Garofalo RP and Casola A (2022)
Antiviral activity of extracellular
vesicles derived from respiratory
syncytial virus-infected airway
epithelial cells.
Front. Immunol. 13:886701.
doi: 10.3389/fimmu.2022.886701

COPYRIGHT

© 2022 Corsello, Qu, Ivanciuc, Garofalo
and Casola. This is an open-access
article distributed under the terms of
the [Creative Commons Attribution
License \(CC BY\)](#). The use, distribution
or reproduction in other forums is
permitted, provided the original author
(s) and the copyright owner(s) are
credited and that the original
publication in this journal is cited, in
accordance with accepted academic
practice. No use, distribution or
reproduction is permitted which does
not comply with these terms.

Antiviral activity of extracellular vesicles derived from respiratory syncytial virus-infected airway epithelial cells

Tiziana Corsello^{1*}, Yue Qu¹, Teodora Ivanciuc¹,
Roberto P. Garofalo^{1,2} and Antonella Casola^{1,2*}

¹Department of Pediatrics, The University of Texas Medical Branch at Galveston (UTMB), Galveston, TX, United States, ²Department of Microbiology and Immunology, The University of Texas Medical Branch at Galveston (UTMB), Galveston, TX, United States

Respiratory syncytial virus (RSV) is a major cause of acute lower respiratory tract infections in children and elderly. No vaccine or effective treatment is currently available for RSV. Extracellular vesicles (EVs) are microvesicles known to carry biologically active molecules, including RNA, DNA and proteins (i.e. cargo). Viral infections can induce profound changes in EV cargo, and the cargo can modulate cellular responses of recipient cells. We have recently shown that EVs isolated from RSV-infected cells were able to activate innate immune response by inducing cytokine and chemokine release from human monocytes and airway epithelial cells, however, we did not investigate the potential antiviral contribution of EVs to a subsequent infection. The objective of this study was to assess the presence of innate immune mediators, including type I and III interferons (IFNs) in EVs released from airway epithelial cells infected with RSV, and their potential role in modulating viral replication in recipient cells. EV-derived from cells infected with RSV were associated with significant amounts of cytokine and chemokines, as well as IFN- β and - λ , compared to EVs isolated from mock-infected cells. Cells treated with RSV-EVs showed significantly lower levels of viral replication compared to untreated or mock-EV-treated RSV infected cells. Cellular pretreatment with Cerdulatinib, an IFN receptor signaling inhibitor, inhibited the antiviral activity of RSV-EVs in recipient airway epithelial cells. Furthermore, treatment of A549 cells with RSV-EVs induced the expression of IFN-dependent antiviral genes, supporting the idea that RSV-EVs exerts their antiviral activity through an interferon-dependent mechanism. Finally, we determined the concentrations of soluble and EV-associated IFN- β and IFN- λ in five nasopharyngeal secretions (NPS) of children with viral infections. There were significant levels of IFN- λ in NPS and NPS-derived EVs, while IFN- β was not detected in either of the two types of samples. EVs released from RSV-infected cells could represent a potential therapeutic approach for modulating RSV replication in the airways.

KEYWORDS

extracellular vesicles, viral infection, airways, RSV, epithelial cells

Introduction

Respiratory syncytial virus (RSV) is a negative-sense single-stranded RNA virus belonging to the *Pneumoviridae* family (1). It is the single most important virus causing acute lower respiratory tract infections in children and a major cause of severe respiratory morbidity and mortality in the elderly (2). About 45% of hospital admissions and in-hospital deaths due to RSV occurred in children younger than 6 months (3) and 1.5 million RSV episodes in older adults in industrialized countries in 2015 (4). In addition to acute morbidity, RSV infection has been linked to both the development and the severity of asthma. No vaccine or effective treatment is currently available for RSV (5).

Extracellular vesicles (EVs) are a type of secretory vehicle released from cells and isolated from various bio-fluids as bronchial lavage, breast milk, blood, and saliva (6–10). EVs are characterized by diameter (size), and specific EV markers, including CD63, CD9, ALIX, and TSG101 (11). EVs contain nucleic acids, lipids, and proteins, known as the EV cargo, and have been shown to transfer their biologically active cargo between neighboring cells and to distant sites, therefore participating in cell-to-cell communication, inflammation, and disease pathogenesis (12, 13). Previous studies in selected viral infections have suggested that EVs can play an important role in viral pathogenesis, by contributing to viral replication and spread, as well as to modulation of virus-host cellular interactions (14–16). Conversely, other groups have shown a protective role of these vesicles, conferring host cell resistance to viral infections, as in the case of Dengue virus in an *in vitro* model of infection (17). Although it is known that infections can alter the molecular cargo associated with EVs (15), their role in viral replication and pathogenesis remains largely unexplored, particularly in the context of single stranded RNA viruses.

We have previously described that RSV infection of A549 cells is associated with changes in EV cargo, which are not a simple reflection of changes occurring within infected cells. Importantly, we showed that EVs isolated from RSV-infected cells can activate innate immune responses by inducing release of cytokine and chemokine from EV-exposed (i.e. recipient) monocytes and airway epithelial cells (18). In the present study, we investigated whether EVs released from airway epithelial cells infected with RSV would carry a specific cargo of cytokines, chemokines and IFNs and tested whether RSV-EVs affected viral replication of exposed/recipient cells that were subsequently infected. We found that EV-derived from cells infected with RSV were associated with significant levels of cytokine and chemokines, as well as IFN- β and - λ , compared to EVs isolated from uninfected cells. Moreover, recipient epithelial cells treated with RSV-EVs showed significantly lower levels of viral replication compared to untreated or mock-EV-treated RSV infected cells. Pretreatment of recipient cells with

Cerdulatinib, an IFN receptor signaling inhibitor, inhibited the antiviral activity of RSV-EVs. Furthermore, treatment of A549 cells with RSV-EVs induced the expression of IFN-dependent antiviral genes, suggesting that RSV-EVs exert their antiviral activity *via* an interferon-dependent mechanism. Finally, we determined the concentrations of soluble and EV-associated IFN- β and IFN- λ in five nasopharyngeal secretions (NPS) of children with viral infections and found significant levels of IFN- λ , but not IFN- β in NPS and NPS-derived EVs.

Materials and methods

Cell cultures and RSV infection

Human airway epithelial cell line A549 (human alveolar type II cell line -American Type Culture Collection, USA) were cultured and maintained in F12K culture media, supplemented with 10% FBS (HyClone, GE Healthcare USA), 100 U/mL penicillin G, 100 μ g/mL streptomycin and 2 mM glutamine. RSV stocks and viral pools were prepared as previously described (18, 19). Primary small airway epithelial (SAE) cells (Lonza Inc., San Diego, CA, USA), derived from the terminal bronchioli of cadaveric donors, were grown in culture medium containing 7.5 mg/mL bovine pituitary extract (BPE), 0.5 mg/mL hydrocortisone, 0.5 μ g/mL hEGF, 0.5 mg/mL epinephrine, 10 mg/mL transferrin, 5 mg/mL insulin, 0.1 μ g/mL retinoic acid, 0.5 μ g/mL triiodothyronine, 50 mg/mL gentamicin, and 50 mg/mL bovine serum albumin. SAE cells were switched to basal media (no supplemented added) several hours prior to RSV infection. When A549 cells were used to isolate EVs, they were changed to exo-free FBS medium, 4 hours before and throughout the length of the experiment. At 90 to 95% confluence, cell monolayers were infected with RSV at multiplicity of infection (MOI) of 1. An equivalent amount of 30% sucrose solution was added to uninfected A549 or SAE cells as a control (mock cells). The culture media, from both mock and RSV-infected cells, were collected after 24 hours p.i. and processed for the next analyses. Viral titers were measured by plaque assay in HEp2 cells as described in (20).

Extracellular vesicles isolation and purification

Culture media collected from 2×10^7 mock-infected or RSV-infected cells (24 hours) and nasopharyngeal secretions (NPS) samples of patients were subjected to debris removal by centrifugation at 3,000 g for 15 min at 4°C. The clear media and NPS samples were subjected to further cleaning by filtration through 0.22 μ m sterile filter to remove any remaining debris. The filtered media was transferred to Amicon® Ultra-15

centrifugation filters (Millipore, Billerica, MA, USA) and centrifuged at 2500 g for 35 min. Exoquick-TC (System Biosciences, USA) reagent was added to the filtered media or NPS, mixed thoroughly, and incubated overnight at 4°C to precipitate EVs. Next morning the mixture was subjected to centrifugation at 1,500 g for 30 min, the EV pellets were washed and resuspended in filtered PBS. To remove contaminating viral particles, EVs were subjected to CD63 immuno-purification using CD63 exosome isolation reagents (System Biosciences, USA), following manufacturer's instructions. The purified EVs were eluted from the bound CD63 beads in an average of 300 µl and used for experimental procedures. Protein concentration was determined using a protein assay kit from Bio-Rad, USA. Purified EVs from cells were screened for presence of replicating virus, to avoid using contaminated preparations. This screening was done by plaque assay, inoculating a fraction of the EV pool onto HEp2 cells.

Extracellular vesicles characterization

EV size distribution and number of particles were analyzed using the NanoSight™ LM10-HS10 system (Malvern Instruments, UK). NanoSight™ tracking analysis (NTA) software was used to produce the mean and median vesicle size together with the vesicle concentration (in millions). Samples were measured 3 times to ensure reproducibility. The instrument was rinsed between samples using filtered water. EV markers were analyzed by Western Blot assays. EV samples were lysed in a buffer (50mM Tris NaCl, 0.5% Triton, 300 mM NaCl) supplemented with a protease and phosphatase inhibitor cocktail. Equal amount of protein, 15 µg in total, were processed as described previously (18). The primary antibodies for Western blot were rabbit anti-human CD63 (1:1000; System Biosciences), anti-human Alix (1:500; Santa Cruz), anti-human apoB (1:500; Novus Biologicals) and mouse anti-human GM130 cis -Golgi (1:800; Santa Cruz).

Cytokine and chemokine analysis

Intact and lysed (pretreated with Triton 1%) EV samples were used to measure the levels of cytokine/chemokines and IFNs. EV fractions were quantified using the NanoSight instrument and normalized to the same particle number prior to IFN and cytokine assays. Cytokines and chemokines were measured using the Bio-Plex Cytokine Human Multi-Plex panel (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Immunoreactive IFN-β and IFN-λ 2/3 were measured using commercial enzyme-linked immunosorbent assays (ELISAs), following the manufacturer's protocol (PBL Biomedical Laboratories, Piscataway, NJ, USA).

EV treatments of cells

A549 cells were placed in 24-well plates and grown overnight. The next day the cell media was removed, cells were washed with PBS, and fresh media containing equal amounts of mock- or RSV- EVs (15 µg/well) was added. Mock- or RSV- EVs were isolated and purified using a two-step EV purification method, Exoquick-TC followed by CD63 immuno-EV purification. The cells were allowed to incubate in the presence of EVs for 24 hours. Negative control wells were included that consisted of mock or RSV-infected cells not treated with EVs. For inhibition of IFN signaling, cells were pretreated with 5 µM of Cerdulatinib (Selleckchem, TX) 1 h prior to and throughout the EV treatment. At the end of the EV treatment, media were removed, and cells were infected with RSV for 24h. After infection, media were collected from each well and stored at -80°C for further analyses.

Reverse transcription - qPCR

RNA was extracted from A549 cells using an Aurum Total RNA Mini Kit (BioRad, Hercules, CA, USA) according to the manufacturer instructions. RNA samples were quantified using a DS-11 Spectrophotometer (DeNovix Inc., Wilmington, DE, USA). Synthesis of cDNA was performed with 1 µg of total RNA in a 20 µL reaction using iScript Reverse Transcription Supermix reagent according to the manufacturer's instructions (BioRad, Hercules, CA, USA). PCR amplification was done using 1 µL of cDNA in a total volume of 25 µL using a SYBR Green Fast qPCR mix (ABclonal, Woburn, MA, USA). 18S RNA was used as a housekeeping gene for normalization. PCR assays were run in the BioRad CFX Connect Real-Time System. Triplicate CT values were analyzed in Microsoft Excel using the comparative CT ($\Delta\Delta CT$) method. The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalizing the endogenous reference (18S) sample. Primer sequences of Mx1, DDX58 and ISG15 genes are available upon request.

Western blot

Total cell lysates were prepared with RIPA buffer (Cell Signaling, 9806) and protein concentration was determined with Pierce BCA Protein Assay Kit (ThermoFisher, 23225). Equal amounts (15 µg) of proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane. Nonspecific binding was blocked by immersing the membrane in Tris-buffered saline-Tween (TBST) blocking solution containing 5% skim milk powder. After blocking, the membranes were incubated with the primary antibody overnight at 4°C, followed by the appropriate secondary antibody diluted in TBST for 1h at

room temperature. Proteins were detected using enhanced chemiluminescence (ECL). The primary antibody used for phospho-STAT1 was from Cell Signaling, cat#9167S. β -Actin was used as loading control protein to normalize the target proteins expressions from whole cell extracts (Sigma-Aldrich, cat#A1978).

Nasopharyngeal secretions samples collection

NPS were collected as part of an ongoing IRB-approved study on the pathogenesis of lower respiratory tract infections in children up to 2 years of age. After written informed consent was provided by the parent or legal guardian, NPS samples were collected from patients at the time of the visit in the ER, or within 24 hours after hospital admission. Control NPS were children admitted to the Pediatric Intensive Care Unit following surgery for conditions unrelated to airways disease and negative for viral infections. Samples were immediately transported to the lab in ice and tested for respiratory viruses, using the multiplex RT-PCR-based Luminex xTAG Respiratory Viral Panel (RVP, Luminex Molecular Diagnostics) to detect simultaneously 19 viral targets. An aliquot of NPS was used for direct analysis of IFN- β or IFN- λ 2/3 and another aliquot for the isolation of EVs as described in the previous section.

Statistical analysis

A two-tailed Student's *t* test using a 95% confidence level was performed in all experiments. Significance is indicated as a *p* value of <0.05 (*). Fold change of RT-PCR experiments was calculated by 2- $\Delta\Delta$ Ct method and represent mean $\pm$ SEM using GraphPad Prism v4 (GraphPad Software).

Results

Cytokines and interferons in EVs derived from airway epithelial cells

Studies over the last few years have identified EVs as a non-canonical mechanism by which cytokines can be secreted into the extracellular space and modulate functions of neighboring and distant cells. They can be membrane-associated or encapsulated within the EVs, reviewed in (21), although the cytokine packaging mechanisms into EVs is not fully known. To determine whether immune mediators such as cytokines, chemokines and IFNs were associated with EVs released from airway epithelial cells infected with RSV, we first isolated and purified EVs from A549 cells using a two-step EV purification method, Exoquick-TC followed by CD63 immuno-EV

purification as previously published from our laboratory (18). We used a precipitation reagent-based EV enrichment, followed by CD63 antibody based immuno-magnetic isolation, which results in EV preparations mostly devoid of RSV particles (18). Equal numbers of intact EVs derived from mock (mock-EVs) or RSV-infected cells (RSV-EVs), or EVs lysed with 1% Triton x solution (22), (mock-EVs Triton or RSV-EVs Triton), were used to measure surface-associated and encapsulated cytokines and chemokines by Bio-Plex Cytokine Multi-Plex array. Concentrations of IFN- β and IFN- λ in intact or lysed EVs were measured by ELISA. IL-1 β , IL-1ra, IL-15, IL-17, TNF- α , MIP-1 β and PDGF-bb were significantly increased in both RSV-EVs and RSV-EVs Triton purified from A549 cells, compared to mock-EVs or mock-EVs Triton. Significantly higher concentrations of IL-6, IL-8, IL-9 and RANTES were detected in RSV-EVs compared to mock-EVs, while MIP-1 α concentration was significantly increased in RSV-EVs Triton compared to mock-EVs Triton (Figure 1).

As type I and III IFNs secreted from infected cells represent the main host defense system against viral infections (23, 24), we also assessed IFN- β and IFN- λ levels in EVs isolated from A549 cells. We detected IFN- β and IFN- λ in intact and Triton lysed RSV-EVs, but not in mock-EVs (Figure 2A), with a trend of higher levels detected in Triton lysed EVs. We then confirmed the presence of IFNs in EVs released from normal human SAE cells, which showed results similar to A549 cells with the exception of levels of IFN- β associated with Triton lysed RSV-EVs, which was lower than the one present in intact EVs (Figure 2B).

Antiviral activity of RSV-EVs on exposed cells occurs via an IFN-mediated mechanism

To investigate whether EVs could exert an antiviral effect on recipient cells, A549 cells were pre-treated with 15 μ g of mock or RSV-EVs for 24 hours, were then infected with RSV for 24 hours and harvested to collect cell supernatants to measure viral titers. In our previous study, we observed a functional effect in A549 cells treated with EVs (10 μ g/well). This time, we increased and selected 15 μ g/well EV-dose for a stronger anti-viral effect of EVs than the previous dose. We observed a significant decrease in RSV replication in cells treated with RSV-EV, compared to untreated and mock-EV treated infected cells (Figure 3A), indicating that RSV-EV cellular exposure confers protection against a subsequent infection. To determine whether the observed antiviral effect of RSV-EVs treatment was due to the presence of IFNs in RSV-EVs, recipient cells were treated with Cerdulatinib, an IFN receptor signaling inhibitor, prior to RSV-EV addition and subsequent infection. Cerdulatinib pretreatment resulted in loss of RSV-EV antiviral activity, with increased viral replication in recipient cells treated with the

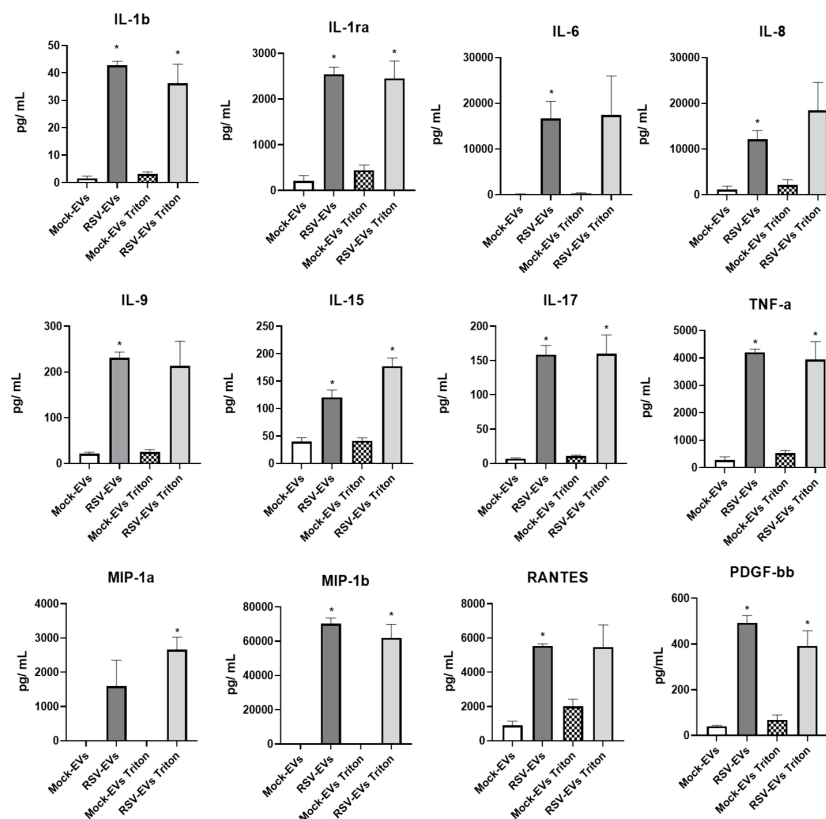


FIGURE 1

Cytokine concentrations associated with EVs released from mock- and RSV-infected A549 cells. Cytokines were measured using a human multi-plex panel array as intact or Triton lysed EVs. Data are presented as mean $\pm$ SEM. * indicates a statistical difference comparing RSV- EVs or RSV-Triton versus Mock-EVs or Mock-Triton, respectively (*p value < 0.05). Data represents the average of three independent experiments.

inhibitor, compared to recipient cells treated with RSV-EVs only, indicating that IFNs carried by RSV-EVs are biologically active (Figure 3B).

To confirm that RSV-EV exposure was associated with the induction of an antiviral gene response in recipient cells, A549 cells were treated with 15 μ g of mock- or RSV-EVs for 24 hours and harvested to collect total RNA. Levels of the antiviral gene Mx1, DDX58 and ISG15 mRNA were assessed by RT-qPCR. We found that cell exposure to RSV-EVs induced a significant increase of Mx1, DDX58 and ISG15 gene expression, compared to basal levels in untreated (mock) cells (Figure 4A). Surprisingly, exposure to mock-EVs resulted in a significant inhibition of the basal levels of these antiviral genes. Engagement of both type I and III IFN receptors leads to activation of STAT1 and 2 proteins, through their tyrosine phosphorylation, which together with IRF9 form the ISGF3 complex necessary for induction of antiviral genes. To confirm activation of this pathway following RSV-EV treatment, A549 cells were treated with 15 μ g of mock- or RSV-EVs for 24 hours and harvested to prepare total cell lysates. Levels of tyrosine phosphorylated STAT1 (pSTAT1) were then assessed by Western blot assay.

Only stimulation of cells with RSV-EV led to activation of STAT1, with no phosphorylation induced by mock-EV (Figure 4B). Collectively, these results support the concept that RSV-EVs can exert an antiviral activity in exposed cells through an interferon-dependent mechanism.

IFN content of EVs isolated from NPS of children with viral infections

We collected NPS from five children < 2 years of age who were admitted to the hospital for a lower respiratory tract infection or from two children negative for viral infections and who were admitted to the Pediatric Intensive Care Unit following surgery for conditions unrelated to airways disease (control sample). Presence of respiratory viruses was confirmed by the Luminex xTAG Respiratory Viral panel. Children were positive for RSV, rhinovirus (RV) and SARS-CoV-2. We isolated EVs from the NPS using the two-step purification protocol and we confirmed by immunoblot that they expressed the marker CD63 and Alix, and were negative for the Apolipoprotein B

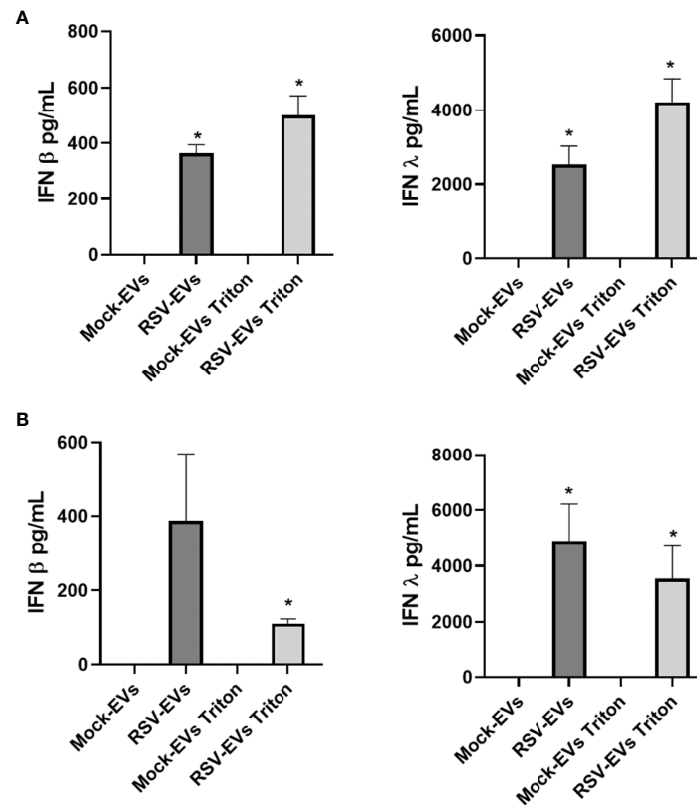


FIGURE 2

IFN-β and IFN-λ concentrations associated with EVs released from mock- and RSV-infected A549 (A) and SAE cells (B). IFN-β and IFN-λ were measured by ELISA as intact or Triton lysed EVs. Data are presented as mean ± SEM. * indicates a statistical difference comparing RSV- EVs or RSV-EVs Triton versus Mock-EVs or Mock-EVs Triton, respectively (**p* value < 0.05). Data represents the average of three independent experiments.

(ApoB) and cis-Golgi matrix protein GM130 (Figure 5A). EV size and particle number of NPS-derived EVs were measured by Nanosight instrument (Figure 5B). The average size of NPS-derived EVs of virus positive patients was 170 nm, and the average number of particles was 1.95×10^9 particles/mL, while the average size of EV from control patients was 145 nm, with an average number of particles of 2.1×10^8 particles/mL.

Next, we determined the concentrations of IFN-β and IFN-λ in NPS (Figure 5C) and NPS-derived EVs (Figure 5D) of these seven children. While IFN-β levels were below the limit of detection in both NPS and NPS-derived EVs of all children, IFN-λ was detected in five of the seven NPS samples (patients #3 and #4 had values below the limit of detection of the assay), with values ranging from 300 to 5,160 pg/mL. In addition, significant levels of IFN-λ were found to be associated with five NPS-derived EVs (upper values, 1693 pg/mL) from infected patients, excepted for the two patients with no viral infections (patients #6 and #7). Slightly higher concentrations of IFN-λ were detected in lysed (Triton-treated) EVs compared to intact EVs derived

from those viral infected NPS samples with detectable IFN-λ, while we found IFN-λ only in the Triton lysed but not intact EVs isolated from the two infected NPS sample. EVs isolated from the airway secretions of two “control” infants negative for viral infections showed no detectable levels of IFN-λ in intact or Triton lysed EVs. Overall, our results suggest that IFN-λ is associated with EVs isolated from airway secretions during episodes of viral respiratory infections.

Discussion

Each year in the United States, RSV leads to approximately 58,000 and 177,000 hospitalizations of children younger than 5 years old and adults aged 65 years or older, respectively (25). No effective drug or vaccine is currently available for RSV (5). There is a huge demand to develop therapeutic approaches and vaccines to treat respiratory viral infections such as RSV. The aims of this study were to 1) evaluate the innate immune

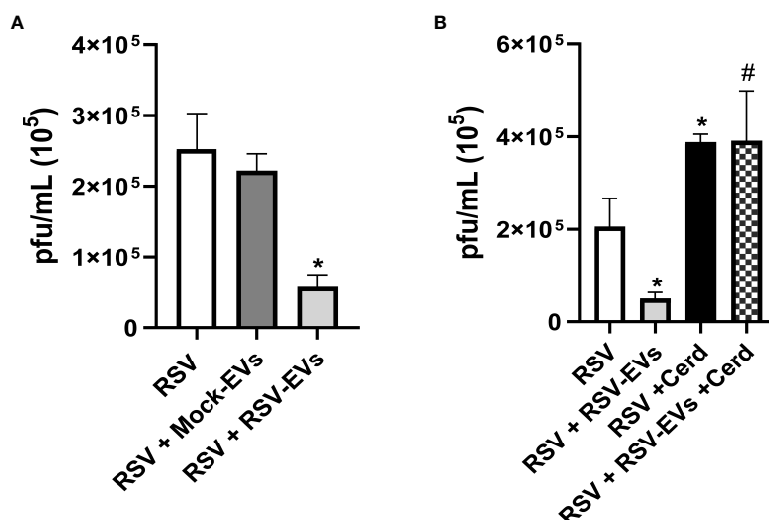


FIGURE 3

Effect of EV treatment on RSV replication. (A) A549 cells were infected with RSV alone (RSV) or treated with Mock-EVs (RSV+Mock-EVs) or RSV-EVs (RSV+RSV-EVs) (15 μ g) for 24 hours and then infected with RSV (MOI of 1). (B) A549 cells were infected with RSV alone (RSV), pretreated with RSV-EVs (15 μ g) 24 hours prior to RSV infection (RSV+RSV-EVs), infected with RSV and treated with Cerdulatinib (5 μ M) 1h prior to infection (RSV-Cerd), or were pretreated with RSV-EVs for 24 hrs, treated with Cerd 1h prior to infection and then infected with RSV at a MOI of 1 (RSV +RSV-EVs+Cerd). Supernatants of infected cells were collected at 24 hours post-infection and viral titers were determined by plaque assay. Data are expressed as mean $\pm$ SEM. * indicates a statistical difference compared to RSV alone, while # indicates a difference between RSV-EV treated and RSV-EV treated plus Cerdulatinib groups (* or # for $p \leq 0.05$). Data represents the mean average of three independent experiments.

mediator cargo of EVs derived from airway epithelial cells with RSV infection, and 2) investigate the potential antiviral activity of EVs isolated from infected airway epithelial cells.

In our previous published work, we isolated, purified, and characterized EVs from airway epithelial cells. We found significantly higher levels of cellular RNA species, named small non-coding RNAs, in EVs than in infected cells. EVs isolated from infected RSV cells carried viral RNAs as well as selected viral proteins, although they were not able to transmit infection to uninfected cells. These previous results underlined the changes of EV-cargo associated with RSV infection (18). In the current study, we show for the first time that EV-derived from cells infected with RSV carry a significant amount of cytokine, chemokines and IFN cargo, compared to EVs isolated from mock-infected cells. In the past few years, there has been a growing literature indicating that EVs can function as alternative carriers for the delivery of cytokines and chemokines, specifically these mediators can be packaged into microvesicles released from cells or can be secreted in membrane-bound form through vesicles-like exosomes, reviewed in (21). In a comprehensive study of cytokine association with EVs, Fitzgerald and colleagues have shown that that cytokine encapsulation into EVs can be found *in vitro*, *ex vivo* and *in vivo* biological experimental models, and that encapsulation in EVs is not associated with a specific cytokine but rather with the specific system and stimulus investigated (22). The same authors

also provided evidence that the EV-associated cytokines were biologically active, by using reporter cell lines that needed specific cytokines to proliferate (22). EV-associated cytokines have been shown to possess a wide range of functions in multiple biological processes. For instance, in HIV-infected individuals, plasma-derived exosomes were highly enriched in a variety of cytokines, and exposure to these exosomes resulted in the induction of CD38 expression on naive and memory CD4+ and CD8+ T cells, a mechanism that could contribute to HIV propagation *via* bystander cell activation (26). Obregon *et al.* showed that EVs derived from lipopolysaccharide (LPS)-activated dendritic cells are important carriers of tumor necrosis factor (TNF)- α , which is important for the induction of proinflammatory mediators from epithelial cells upon internalization (27). In the cancer literature, transforming growth factor β (TGF- β) associated with tumor-derived EVs has been shown to promote tumor progression by stimulating the migration of cancer cells, by inhibiting T-cell responses, and by inducing differentiation of fibroblasts into myofibroblasts that support tumor growth, vascularization, and metastasis, reviewed in (21).

In this study, we found that both IFN- β and IFN- λ were present in significant concentrations in EVs released from RSV-infected cells, while no measurable levels of IFNs were found in EVs originated from uninfected A549 and SAE cells (Figure 2). Treatment with Triton resulted in some increase in the detected

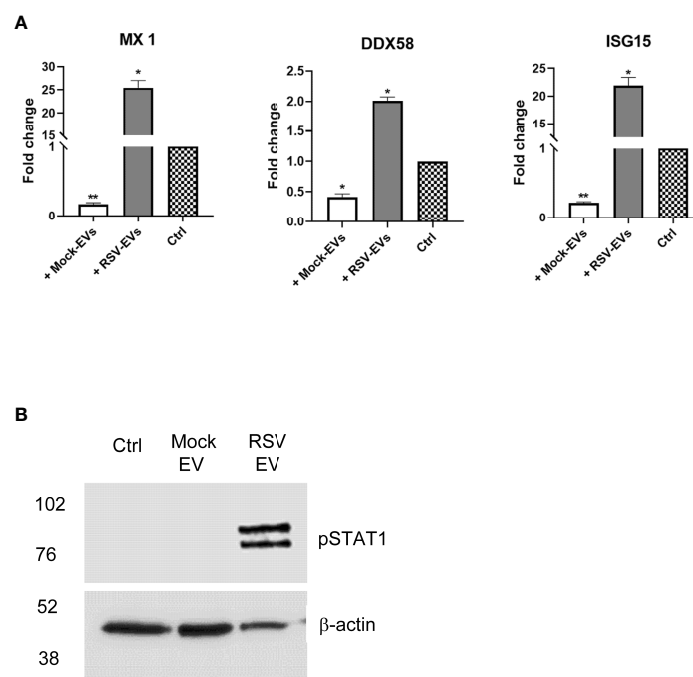


FIGURE 4

A549 cells were treated with Mock- or RSV- EVs (15 μ g) for 24 hours and harvested to either extract total RNA or to prepare total cell lysates. A549 control cells with no EVs treatment are represented by checkered bar plot (A). RNA extracted from A549 mock cells with or without EV treatment was subjected to RT-qPCR to measure the expression of antiviral genes MX1, DDX58 and ISG15. Fold changes in antiviral gene expressions were determined by $2^{-\Delta\Delta CT}$ method and represent mean $\pm$ SEM normalized to 18S. Cell treated with RSV- or Mock- EVs versus mock cells (* p value < 0.05; ** p value < 0.01). Data represents the average of three independent experiments. (B). Total cell lysates were subjected to western blot analysis using an antibody anti-phospho-STAT1 (pSTAT1). Membrane was stripped and re-probed with anti- β -actin for loading control. Western blot figure is representative of two independent experiments.

levels of IFNs in RSV-infected A549, but not in SAE cells (with even less IFN- β in Triton-treated compared to untreated ones). We do not know at this point the reason for this finding, although we can speculate that treatment of EVs with Triton, which is a milder detergent compared to other chemicals, may not lead to a full lysis of SAE-derived EVs, may interfere with the measurement of IFNs, or affect some of the experimental steps that precede the measurement of these mediators. Nonetheless, future studies will address the question regarding the relative distribution of cytokines and IFNs within the epithelial cell EVs, in particular those mediators that are surface-bound or encapsulated (22). Both type I and III IFNs are expressed in a variety of epithelial cells and released in response to viral infections, to induce an antiviral state in host cells (23, 28–30). IFN- λ was also present in EVs derived from NPS samples of children with viral respiratory infections, while there was no detectable IFN- β either in the NPS-EVs or in the originating samples. We recognize that our data are representative of a small group of young patients or controls purposely selected based on different viral pathogens that were associated with episodes of lower respiratory tract infections. Thus, this study was not

designed to address statistical differences in EV-associated IFNs levels between viral pathogens or other clinical parameters, rather to extend our *in vitro* observation to human-derived samples of airway EVs. Although larger clinical studies that will include a diverse spectrum of viral respiratory pathogens will be necessary to confirm our findings, recent elegant studies using nasal cell organoids have shown that RSV is indeed a strong inducer of IFN- λ (31). The biological significance of our discovery was supported by: 1) evidence that exposure of recipient cells to RSV-EVs was associated with significantly reduced replication of a subsequent RSV infection; 2) pre-treatment with an IFN receptor signaling inhibitor abolished this protective effect; and 3) treatment of recipient cells with RSV-EVs induced activation of STAT1 protein and a significant increase of IFN-inducible antiviral genes Mx1, DDX58 and ISG15, which have been shown to control RSV replication (32). These data altogether support the idea that IFNs carried by epithelial RSV-EVs function as messengers to help blocking viral replication in neighbor cells.

Although initial studies have shown that EVs may promote pathogen transmission and spreading of viral infections (HCV/

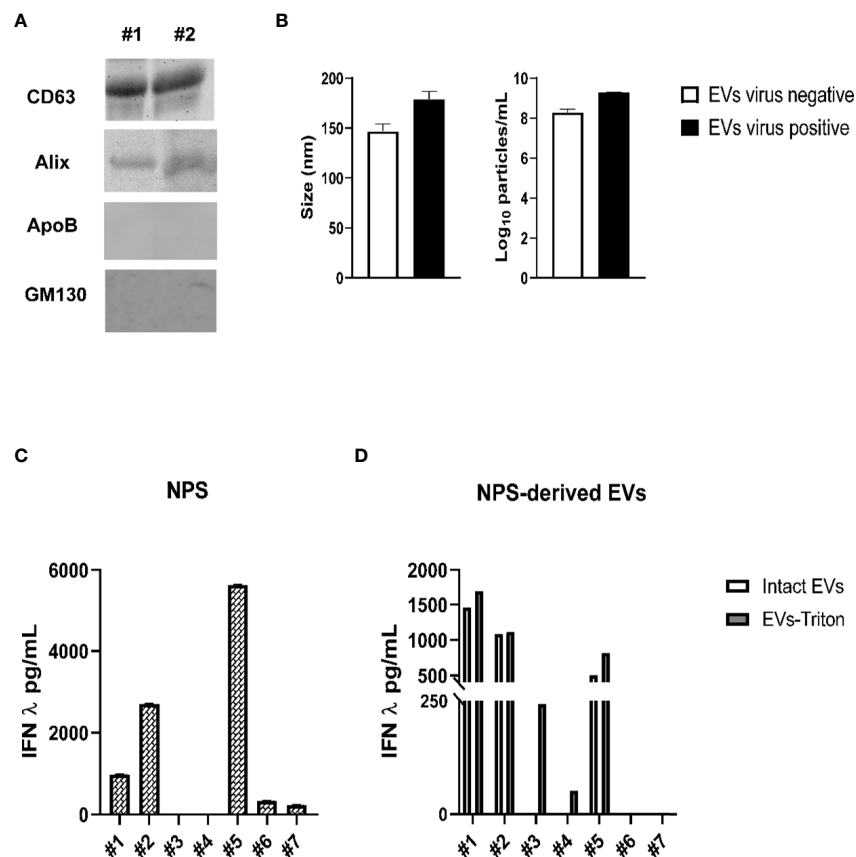


FIGURE 5

Characterization of NPS-derived EVs from children with and without viral infections. (A) Western blot analysis of equal amounts of purified EVs (5 µg) for CD63, Alix, ApoB and GM130. #1 and 2 indicates EV samples isolated from two representative patients. (B) Absolute size (left graph) and concentration (right graph) determined by Nanosight analysis of EVs isolated from virus negative (open bars) or virus positive (black bars) NPS. IFN-λ concentrations were measured by ELISA in NPS samples (C) or NPS-derived EVs (D) of children with or without viral lower respiratory tract infections. Patients #1 and #2 = RSV; Patient #3 = SARS-CoV-2; Patients #4 and #5 = Rhinovirus (RV), Patients #6 and #7 = Virus-negative.

HIV) (33–35), other studies demonstrate on the contrary that EVs have a protective role by limiting viral replication, as it has been shown for Dengue virus (17), Rift Valley Fever virus (36) and influenza virus in a rodent model of infection (37). Our study reports for the first time a significant association of cytokines, chemokines and IFNs with EVs released from airway epithelial cells following RSV infection. Furthermore, our finding of type III IFN associated with EVs isolated from respiratory secretions of children infected with respiratory viruses support the concept that the packaging of innate immune mediators in EVs could be indeed an important mechanism to modulate innate and antiviral responses both close and far from the site of initial viral entry or infection. These mediators can be concentrated within EVs and exert their activity at the surface of other cells that might not otherwise be targeted by cytokines in soluble, circulating form (22). Also, the lipid bilayer structure of EVs has been

shown to protect the antiviral molecules from extracellular degradation during the cell-to-cell communication *via* EVs and facilitate cytokine delivery and targeting to distant cells (38). Better understanding of the cargo and antiviral and immunomodulatory properties of EVs released from airway epithelial cells following respiratory virus infection could provide insight into the regulation of viral-induced responses and be the basis for potential EV-mediated antiviral strategies to treat/prevent infections.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by IRB 03-117 UTMB (The University of Texas Medical Branch). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

Author contributions

TC designed and performed the experiments, analyzed the data, wrote the paper; YQ and TI contributed data to the viral titers or interferons/cytokines studies; TC, AC and RG acquired the listed funding, conceived, designed the experiments, and finalized the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by NIH grant AI062885, AI142570 and AI125434. TC was partially supported by the Jeane B. Kempner Postdoctoral Scholar Award from UTMB and Data

Acquisition Grant from Institute for Human Infections and Immunity (IHII), UTMB.

Acknowledgments

The authors would like to thank Drs. Leticia Castillo for providing samples of bronchial aspirates and Cynthia Tribble for assistance with submission of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

- Rima B, Collins P, Easton A, Fouchier R, Kurath G, Lamb RA, et al. Ictv virus taxonomy profile: Pneumoviridae. *J Gen Virol* (2017) 98(12):2912–3. doi: 10.1099/jgv.0.000959
- Coulas JA, Smyth R, Openshaw PJ. Respiratory syncytial virus (Rsv): A scourge from infancy to old age. *Thorax* (2019) 74(10):986–93. doi: 10.1136/thoraxjnl-2018-212212
- Shi T, McAllister D, O'Brien K, Simoes AEAF, Madhi SA, Gessner BD, et al. Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in young children in 2015: A systematic review and modelling study. *Lancet* (2017) 390(10098):946–58. doi: 10.1016/S0140-6736(17)30938-8
- Shi T, Denouel A, Tietjen AK, Campbell I, Moran E, Li X, et al. Global disease burden estimates of respiratory syncytial virus-associated acute respiratory infection in older adults in 2015: A systematic review and meta-analysis. *J Infect Dis* (2020) 222(Suppl 7):S577–s83. doi: 10.1093/infdis/jiz059
- Domachowski JB, Anderson EJ, Goldstein M. The future of respiratory syncytial virus disease prevention and treatment. *Infect Dis Ther* (2021) 10(Suppl 1):47–60. doi: 10.1007/s40121-020-00383-6
- Mathieu M, Martin-Jaular L, Lavieu G, Théry C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat Cell Biol* (2019) 21(1):9–17. doi: 10.1038/s41556-018-0250-9
- Parimon T, Garrett NE3rd, Chen P, Antes TJ. Isolation of extracellular vesicles from murine bronchoalveolar lavage fluid using an ultrafiltration centrifugation technique. *J Vis Exp* (2018) (141):e58310. doi: 10.3791/58310
- Wang X. Isolation of extracellular vesicles from breast milk. *Methods Mol Biol* (2017) 1660:351–3. doi: 10.1007/978-1-4939-7253-1_28
- Arraud N, Linares R, Tan S, Gounou C, Pasquet JM, Mornet S, et al. Extracellular vesicles from blood plasma: Determination of their morphology, size, phenotype and concentration. *J Thromb Haemost* (2014) 12(5):614–27. doi: 10.1111/jth.12554
- Iwai K, Yamamoto S, Yoshida M, Shiba K. Isolation of extracellular vesicles in saliva using density gradient ultracentrifugation. *Methods Mol Biol* (2017) 1660:343–50. doi: 10.1007/978-1-4939-7253-1_27
- Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (Misev2018): A position statement of the international society for extracellular vesicles and update of the Misev2014 guidelines. *J Extracell Vesicles* (2018) 7(1):1535750. doi: 10.1080/20013078.2018.1535750
- Pitt JM, Kroemer G, Zitvogel L. Extracellular vesicles: Masters of intercellular communication and potential clinical interventions. *J Clin Invest* (2016) 126(4):1139–43. doi: 10.1172/jci87316
- Gupta R, Radicioni G, Abdelwahab S, Dang H, Carpenter J, Chua M, et al. Intercellular communication between airway epithelial cells is mediated by exosome-like vesicles. *Am J Respir Cell Mol Biol* (2019) 60(2):209–20. doi: 10.1165/rcmb.2018-0156OC
- Meckes DG Jr., Raab-Traub N. Microvesicles and viral infection. *J Virol* (2011) 85(24):12844–54. doi: 10.1128/JVI.05853-11[doi]
- Fleming A, Sampey G, Chung MC, Bailey C, van Hoek ML, Kashanchi F, et al. The carrying pigeons of the cell: Exosomes and their role in infectious diseases caused by human pathogens. *Pathog Dis* (2014) 71(2):109–20. doi: 10.1111/2049-632x.12135
- Izquierdo-Useros N, Naranjo-Gómez M, Erkizia I, Puertas MC, Borrás FE, Blanco J, et al. HIV and mature dendritic cells: Trojan exosomes riding the Trojan horse? *PLoS Pathog* (2010) 6(3):e1000740. doi: 10.1371/journal.ppat.1000740
- Zhu X, He Z, Yuan J, Wen W, Huang X, Hu Y, et al. Ifitm3-containing exosome as a novel mediator for anti-viral response in dengue virus infection. *Cell Microbiol* (2015) 17(1):105–18. doi: 10.1111/cmi.12339[doi]
- Chahar HS, Corsello T, Kudlicki AS, Komaravelli N, Casola A. Respiratory syncytial virus infection changes cargo composition of exosome released from

airway epithelial cells. *Sci Rep* (2018) 8(1):387. doi: 10.1038/s41598-017-18672-5 [doi];10.1038/s41598-017-18672-5[pil]

19. Ueba O. Respiratory syncytial virus. i. concentration and purification of the infectious virus. *Acta Med Okayama* (1978) 32(4):265–72.

20. Kisch AL, Johnson KM. A plaque assay for respiratory syncytial virus. *Proc Soc Exp Biol Med* (1963) 112:583. doi: 10.3181/00379727-112-28111

21. Aiello A, Giannesi F, Percario ZA, Affabris E. An emerging interplay between extracellular vesicles and cytokines. *Cytokine Growth Factor Rev* (2020) 51:49–60. doi: 10.1016/j.cytogfr.2019.12.003

22. Fitzgerald W, Freeman ML, Lederman MM, Vasilieva E, Romero R, Margolis L. A system of cytokines encapsulated in extracellular vesicles. *Sci Rep* (2018) 8(1):8973. doi: 10.1038/s41598-018-27190-x

23. Hijano DR, Vu LD, Kauvar LM, Tripp RA, Polack FP, Cormier SA. Role of type I interferon (Ifn) in the respiratory syncytial virus (Rsv) immune response and disease severity. *Front Immunol* (2019) 10:566. doi: 10.3389/fimmu.2019.00566

24. Essaidi-Laziosi M, Geiser J, Huang S, Constant S, Kaiser L, Tapparel C. Interferon-dependent and respiratory virus-specific interference in dual infections of airway epithelia. *Sci Rep* (2020) 10(1):10246. doi: 10.1038/s41598-020-66748-6

25. Rha B, Curns AT, Lively JY, Campbell AP, Englund JA, Boom JA, et al. Respiratory syncytial virus-associated hospitalizations among young children: 2015–2016. *Pediatrics* (2020) 146(1):e20193611. doi: 10.1542/peds.2019-3611

26. Konadu KA, Chu J, Huang MB, Amancha PK, Armstrong W, Powell MD, et al. Association of cytokines with exosomes in the plasma of hiv-1-Seropositive individuals. *J Infect Dis* (2015) 211(11):1712–6. doi: 10.1093/infdis/jiu676

27. Obregon C, Rothen-Rutishauser B, Gerber P, Gehr P, Nicod LP. Active uptake of dendritic cell-derived exovesicles by epithelial cells induces the release of inflammatory mediators through a tnfr-Alpha-Mediated pathway. *Am J Pathol* (2009) 175(2):696–705. doi: 10.2353/ajpath.2009.080716

28. Stanifer ML, Guo C, Doldan P, Boulant S. Importance of type I and iii interferons at respiratory and intestinal barrier surfaces. *Front Immunol* (2020) 11:608645. doi: 10.3389/fimmu.2020.608645

29. Sommereyns C, Paul S, Staeheli P, Michiels T. Ifn-lambda (Ifn-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells *in vivo*. *PLoS Pathog* (2008) 4(3):e1000017. doi: 10.1371/journal.ppat.1000017

30. Weber F. Antiviral innate immunity: Introduction. *Reference Module Biomed Sci* (2014), B978-0-12-801238-3.02608-8. doi: 10.1016/B978-0-12-801238-3.02608-8

31. Rajan A, Weaver AM, Aloisio GM, Jelinski J, Johnson HL, Venable SF, et al. The human nose organoid respiratory virus model: An *ex vivo* human challenge model to study respiratory syncytial virus (Rsv) and severe acute respiratory syndrome coronavirus 2 (Sars-Cov-2) pathogenesis and evaluate therapeutics. *mBio* (2022) 13(1):e0351121. doi: 10.1128/mbio.03511-21

32. González-Sanz R, Mata M, Bermejo-Martin J, Álvarez A, Cortijo J, Melero JA, et al. Isg15 is upregulated in respiratory syncytial virus infection and reduces virus growth through protein isgylation. *J Virol* (2016) 90(7):3428–38. doi: 10.1128/JVI.02695-15

33. Ramakrishnaiah V, Thumann C, Fofana I, Habersetzer F, Pan Q, de Ruiter PE, et al. Exosome-mediated transmission of hepatitis c virus between human hepatoma Huh7.5 cells. *Proc Natl Acad Sci U.S.A.* (2013) 110(32):13109–13. doi: 10.1073/pnas.1221899110[doi]

34. Rozmyslowicz T, Majka M, Kijowski J, Murphy SL, Conover DO, Poncz M, et al. Platelet- and megakaryocyte-derived microparticles transfer Cxcr4 receptor to Cxcr4-null cells and make them susceptible to infection by X4-hiv. *AIDS* (2003) 17(1):33–42. doi: 10.1097/01.aids.0000042948.95433.3d[doi];00002030-200301030-00006[pil]

35. Chahar HS, Bao X, Casola A. Exosomes and their role in the life cycle and pathogenesis of rna viruses. *Viruses* (2015) 7(6):3204–25. doi: 10.3390/v7062770[doi]

36. Alem F, Olanrewaju AA, Omole S, Hobbs HE, Ahsan N, Matulis G, et al. Exosomes originating from infection with the cytoplasmic single-stranded rna virus rift valley fever virus (Rvfv) protect recipient cells by inducing rig-I mediated ifn- β response that leads to activation of autophagy. *Cell Biosci* (2021) 11(1):220. doi: 10.1186/s13578-021-00732-z

37. Bedford JG, Infusini G, Dagley LF, Villalon-Letelier F, Zheng MZM, Bennett-Wood V, et al. Airway exosomes released during influenza virus infection serve as a key component of the antiviral innate immune response. *Front Immunol* (2020) 11:887. doi: 10.3389/fimmu.2020.00887

38. Barnes BJ, Somerville CC. Modulating cytokine production *Via* select packaging and secretion from extracellular vesicles. *Front Immunol* (2020) 11:1040. doi: 10.3389/fimmu.2020.01040

Advantages of publishing in Frontiers



OPEN ACCESS

Articles are free to read
for greatest visibility
and readership



FAST PUBLICATION

Around 90 days
from submission
to decision



HIGH QUALITY PEER-REVIEW

Rigorous, collaborative,
and constructive
peer-review



TRANSPARENT PEER-REVIEW

Editors and reviewers
acknowledged by name
on published articles

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org

Contact us: frontiersin.org/about/contact



REPRODUCIBILITY OF RESEARCH

Support open data
and methods to enhance
research reproducibility



DIGITAL PUBLISHING

Articles designed
for optimal readership
across devices



FOLLOW US

@frontiersin



IMPACT METRICS

Advanced article metrics
track visibility across
digital media



EXTENSIVE PROMOTION

Marketing
and promotion
of impactful research



LOOP RESEARCH NETWORK

Our network
increases your
article's readership