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CHAPTER 2 - MULTI-ORGAN DAMAGE

COVID 19 - RESPIRATORY COMPLICATIONS PART THREE - *Exosomes*

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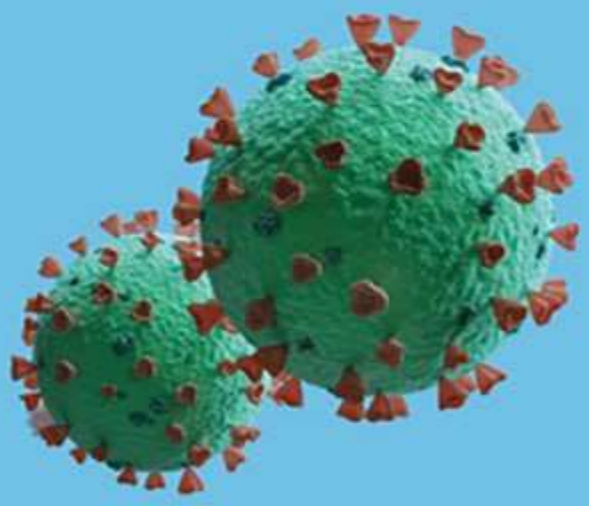

COVID-19

CHAPTER 2 - MULTI-ORGAN DAMAGE

RESPIRATORY COMPLICATIONS - PART THREE - Exosomes

SUMMARY

IN-DEPTH STUDY

Function of exosomes and extracellular vesicles (EVs) [1]

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Large Extracellular Vesicles: Have We Found the Holy Grail of Inflammation?
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The initial discovery of exosomes dates back to the mid-1980s with the identification of small vesicles that sprouted from reticulocytes (immature red blood cells) during their maturation to remove certain proteins bound to the plasma membrane[2] .

These exosomes were originally thought to function as "garbage cans" of the cell and thus received little attention, but further studies indicated that these small exocyte vesicles were not specific to reticulocytes and are released by most mammalian cells.[3]

Subsequently, exosomes have been shown to have immune regulatory effects, and B-cell-derived exosomes are able to stimulate T cells[4] .

Further studies have highlighted the role of exosomes in carcinogenesis, immunomodulatory processes, neurodegenerative diseases, and transfer of infectious agents[5] .

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Jin Wang, IntechOpen, DOI: 10.5772/intechopen.69397.
Available from: https://www.intechopen.com/books/novel-implications-of-exosomes-in-diagnosis-and-treatment-of-cancer-and-infectious-diseases/exosomes-and-their-role-in-viral-infections
https://www.intechopen.com/books/novel-implications-of-exosomes-in-diagnosis-and-treatment-of-cancer-and-infectious-diseases/exosomes-and-their-role-in-viral-infections

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Exosomes: New Tuberculosis Biomarkers - Prospects From the Bench to the Clinic, Understanding Tuberculosis - Global Experiences and Innovative Approaches to the Diagnosis, Pere-Joan Cardona, IntechOpen, DOI: 10.5772/30720.
Available from: https://www.intechopen.com/books/understanding-tuberculosis-global-experiences-and-innovative-approaches-to-the-diagnosis/tuberculosis-biomarkers-prospects-from-the-bench-to-the-clinic
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Exosomes: vehicles of intercellular signaling, biomarkers, and vectors of cell therapy.
Annual Review of Physiology. 2015;77:13–27. doi: 10.1146/annurev-physiol-021014-071641.
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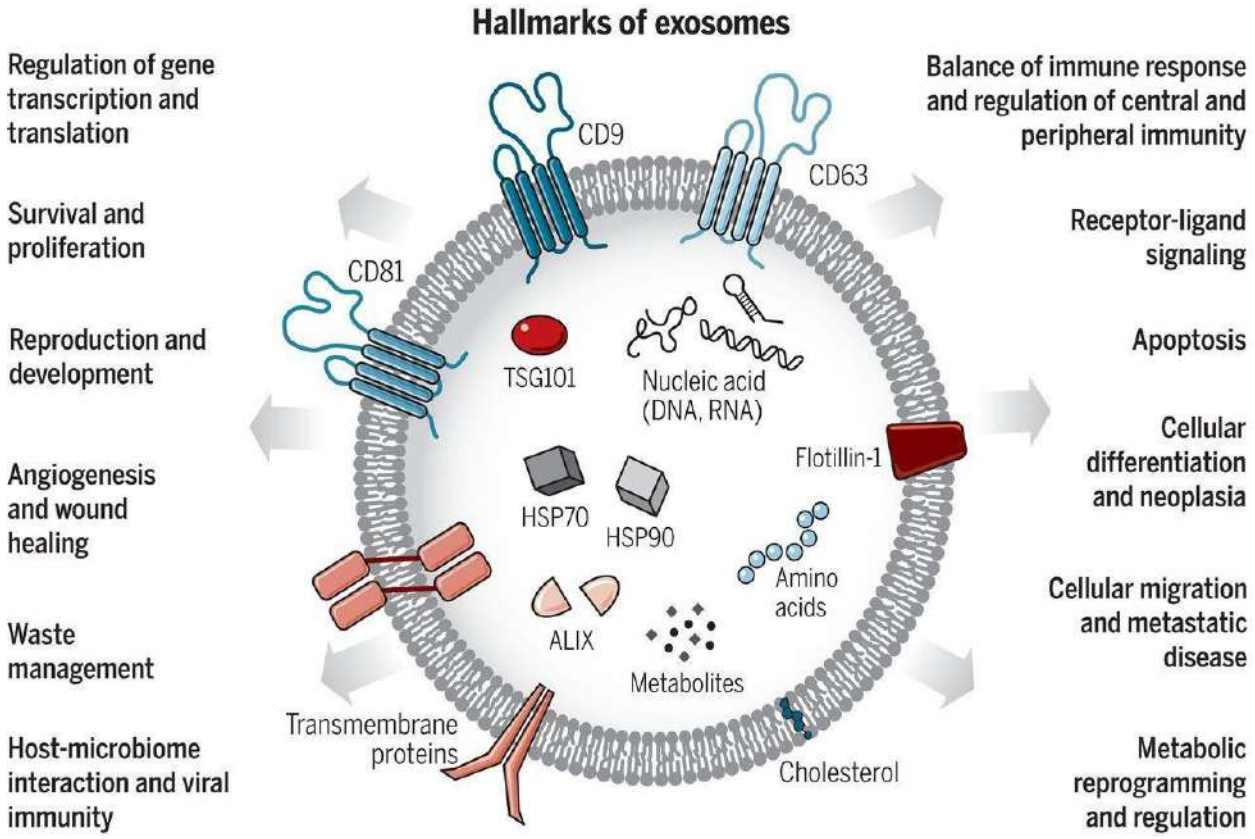
[5] Kim C.-H., Hong M.-J., Park S.-D., et al.
Enhancement of anti-tumor immunity specific to murine glioma by vaccination with tumor cell lysate-pulsed dendritic cells engineered to produce interleukin-12.

The breakthrough in exosome research came in 2007 with the discovery that exosomes released from mast cells contain more than 1200 mRNAs that could be transferred to other cells and be translated into proteins[6] .



https://science.sciencemag.org/content/367/6478/eaau6977.long
Exosomes are extracellular vesicles generated by all cells and transport nucleic acids, proteins, lipids and metabolites. They are mediators of short- and long-distance intercellular communication in health and disease and influence various aspects of cell biology.

Cancer Immunology, Immunotherapy. 2006;55(11):1309–1319. doi: 10.1007/s00262-006-0134-x.
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Singh PP, Smith VL, Karakousis PC, Schorey JS.
Exosomes isolated from mycobacteria-infected mice or cultured macrophages can recruit and activate immune cells in vitro and in vivo.
J Immunol. 2012;189(2):777-785. doi:10.4049/jimmunol.1103638
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Singh PP, LeMaire C, Tan JC, Zeng E, Schorey JS.
Exosomes released from M. tuberculosis infected cells can suppress IFN-γ mediated activation of naïve macrophages.
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The biology, function, and biomedical applications of exosomes.
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[6] Krek A., Grün D., Poy M. N., et al.
Combinatorial microRNA target predictions.
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Valadi H., Ekström K., Bossios A., Sjöstrand M., Lee J. J., Lötvall J. O.
Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells.
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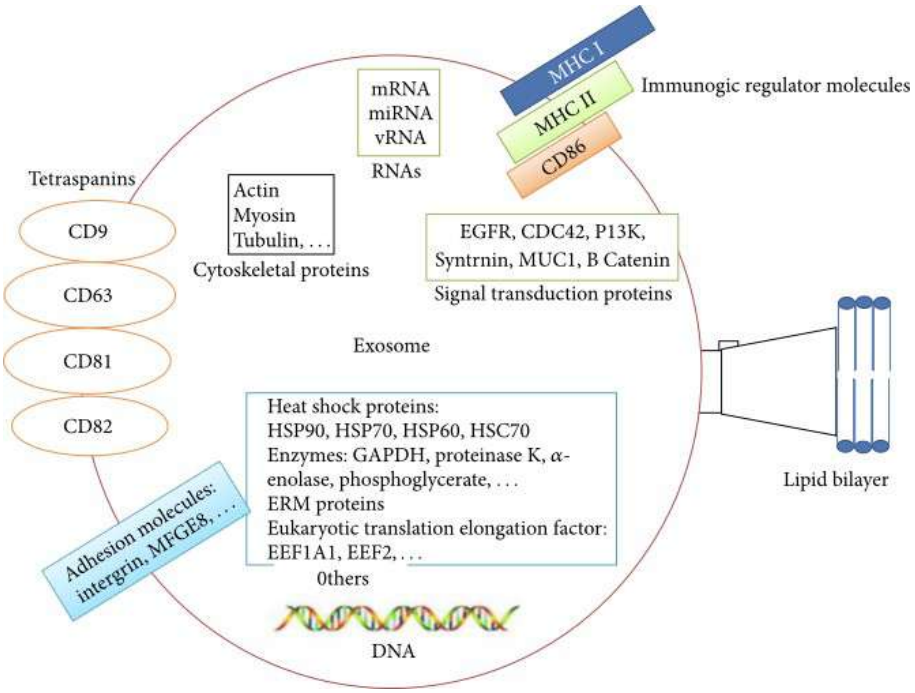
According to ExoCarta, (a database of exosomal proteins, RNAs and lipids[7]) exosomes have a complex composition whose molecular content depends on their cell of origin.
A total of 4,563 proteins, 194 lipids, 1,639 mRNAs and 764 miRNAs were identified in exosomes of different species, with membrane transport proteins and fusion proteins among those most frequently detected.

Some exosomal proteins are always present, such as tetraspanins, CD63, CD81, CD9 and heat shock protein (Hsp70) commonly used as exosomal markers[8] .
Exosomes are rich in lipids such as cholesterol, phospholipids, phosphatidylserine and prostaglandins but lack nuclear, mitochondrial and ribosomal proteins. [9]



https://www.hindawi.com/journals/mi/2016/5628404/
Structure and contents of exosomes: exosomes contain a phospholipid bilayer membrane derived from the plasma membrane. Exosomal contents based on the cell type of origin include mRNA, miRNA and DNA and proteins such as annexins, tetraspanins, MHC molecules, cytoskeletal proteins, enzymes and signal transduction proteins

DEEPENING

INTRACELLULAR COMPARTMENTS THE VESICULAR TRAFFICKING

Membrane proteins destined for degradation are internalized in intraluminal vesicles. The transformation of early to late endosomes is accompanied by the loss of tubular protrusions. The endosome late or multivesicular body (MVB) moves along the microtubules, toward the inside of the cell.

[7] Keerthikumar S, Chisanga D, Ariyaratne D, et al.
ExoCarta: A Web-Based Compendium of Exosomal Cargo.
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[8] Tickner JA, Urquhart AJ, Stephenson SA, Richard DJ, O'Byrne KJ.
Functions and therapeutic roles of exosomes in cancer.
Front Oncol. 2014;4:127. Published 2014 May 27. doi:10.3389/fonc.2014.00127
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4034415/

[9] Alipoor SD, Mortaz E, Garssen J, Movassaghi M, Mirsaeidi M, Adcock IM.
Exosomes and Exosomal miRNAs in Respiratory Diseases.
Mediators Inflamm. 2016;2016:5628404. doi:10.1155/2016/5628404
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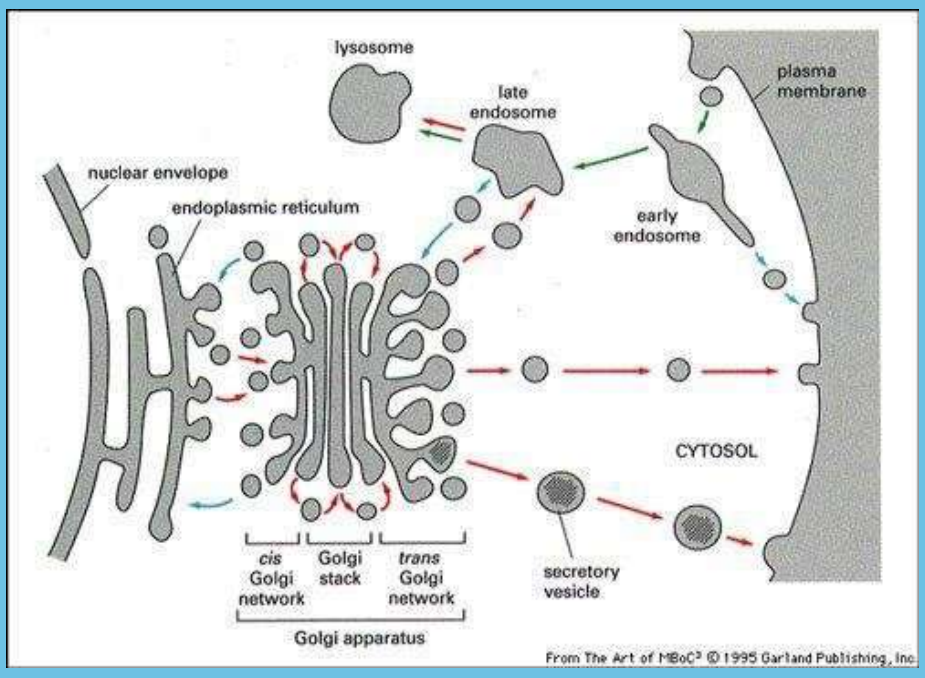
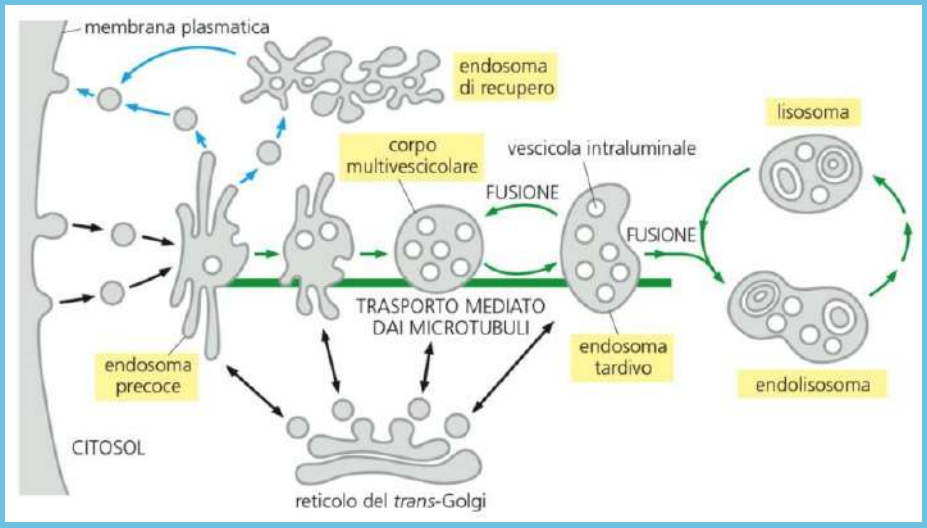
Mature late endosomes no longer transport vesicles to the membrane and fuse with each other and lysosomes to degrade their contents. Maturing endosomes are connected by transport vesicles to the TGN (Trans Golgi Network), continuously supplying newly synthesized lysosomal proteins.





Biogenesis of exosomes

Exosome biogenesis begins with endocytosis and early endosome formation.
The early endosome develops into the late endosome after maturation, characterized by the formation of intraluminal vesicles (ILVs) within the lumen of the endosome.

ILVs, 30-100 nm in diameter, are formed by inward budding of the endosomal membrane, which randomly engulfs portions of the cytosol and incorporates transmembrane and peripheral proteins into the invaginating membrane, leading to the formation of multivesicular bodies (MVBs)[10] .

[10] Keller S, Sanderson MP, Stoeck A, Altevogt P.
Exosomes: from biogenesis and secretion to biological function.
Immunol Lett. 2006;107(2):102-108. doi:10.1016/j.imlet.2006.09.005

Although endocytosis and trafficking of plasma membrane receptors in MVBs are responsible for their degradation upon fusion with lysosomes[11] , the fate of MVBs may vary and not all MVBs are degraded in lysosomes, with a subset fusing with the plasma membrane leading to the generation of exosomes.

The process of exosome biogenesis and cargo sorting is still not well understood, and many studies suggest that the mechanisms of exosome biogenesis may be cell-specific[12] .

Exosomes are secreted mainly by two different mechanisms: **constitutive release** through the Trans-Golgi network and **inducible release** [13]

https://pubmed.ncbi.nlm.nih.gov/17067686/

van Niel G, Porto-Carreiro I, Simoes S, Raposo G.
Exosomes: a common pathway for a specialized
function. J Biochem. 2006;140(1):13-21.
doi:10.1093/jb/mvj128
https://pubmed.ncbi.nlm.nih.gov/16877764/

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Multivesicular bodies: co-ordinated progression to maturity.
Curr Opin Cell Biol. 2008;20(4):408-414. doi:10.1016/j.ceb.2008.04.001
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[12] Perez-Hernandez D, Gutiérrez-Vázquez C, Jorge I, et al.
The intracellular interactome of tetraspanin-enriched microdomains reveals their function as sorting machineries toward exosomes.
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Exosomes as intercellular signalosomes and pharmacological effectors.
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Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologies.
Biochim Biophys Acta. 2014;1841(1):108-120. doi:10.1016/j.bbalip.2013.10.004
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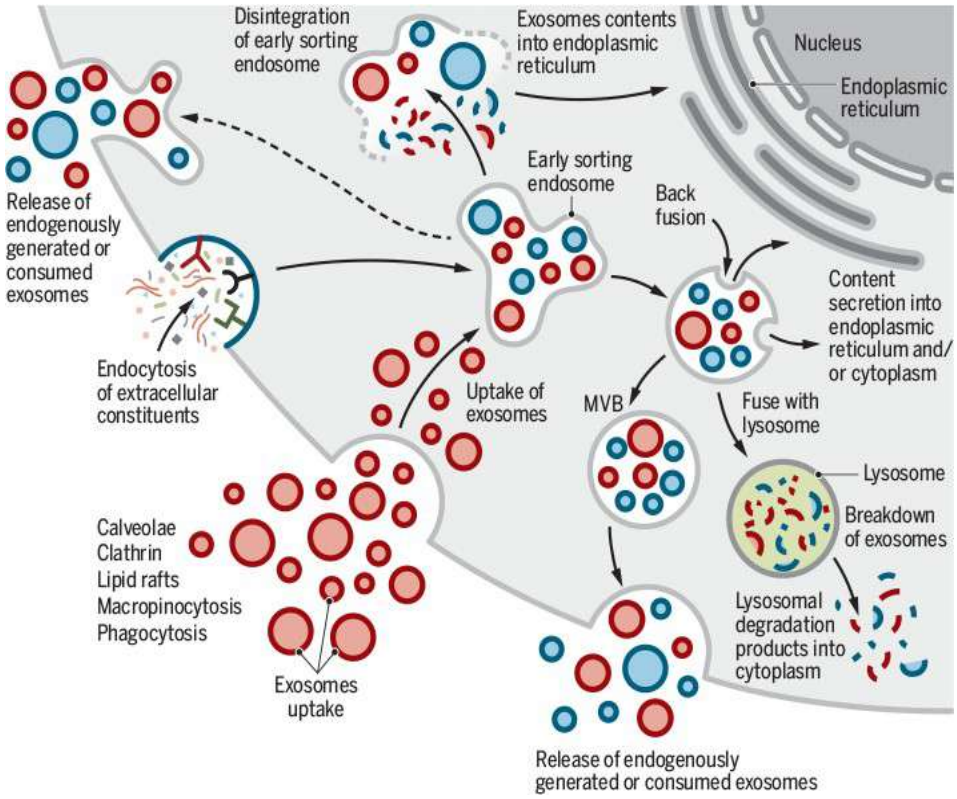
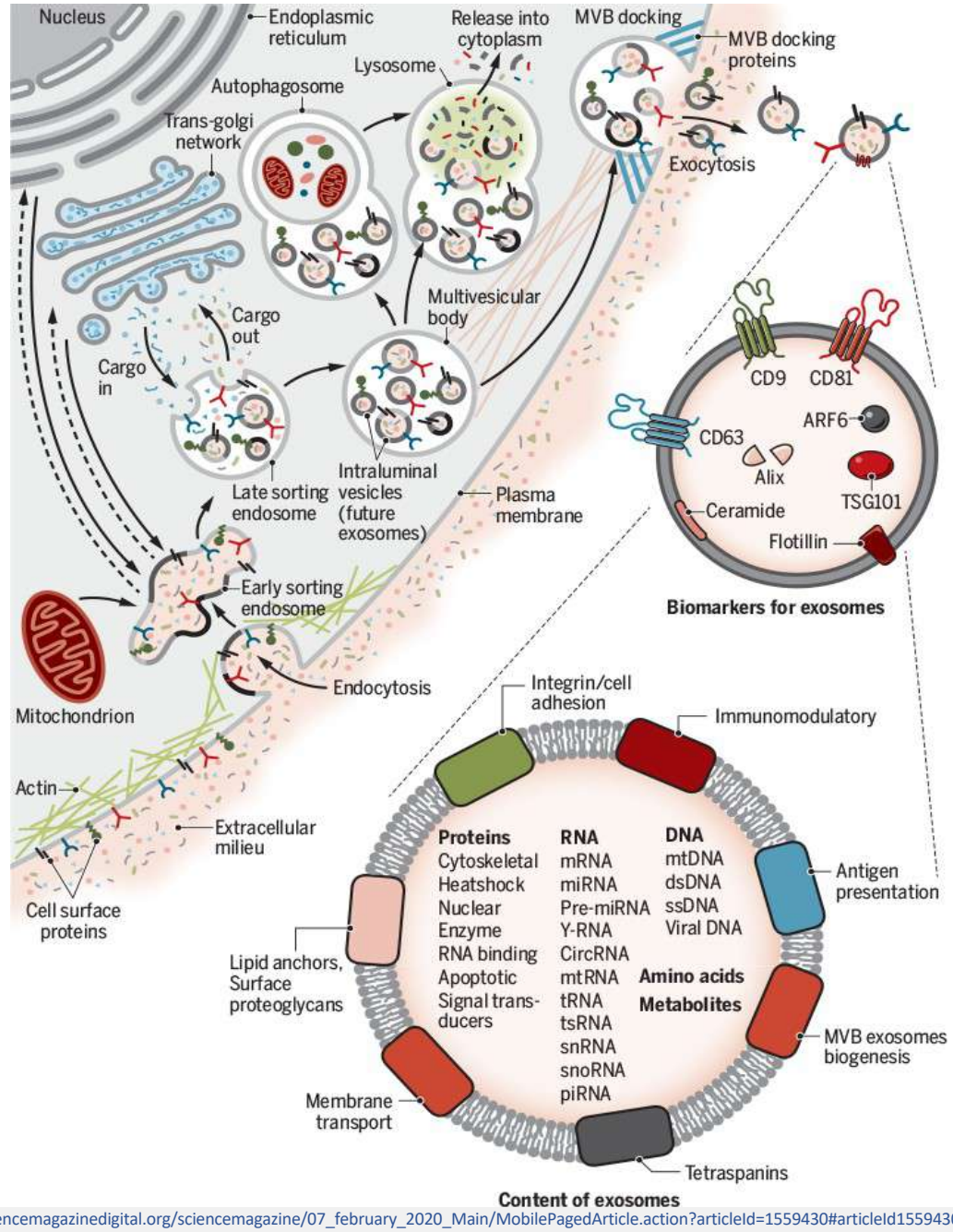


https://www.sciencemagazinedigital.org/sciencemagazine/07_february_2020_Main/MobilePagedArticle.action?articleId=1559430#articleId1559430

Cellular pathway of internalized and endogenously produced exosomes. Exosomes can enter cells directly by different mechanisms (red). Exosomes are generated de novo by cells through the process of endocytosis (blue). Exosomes are continuously generated and taken up by cells. It is likely that they can be secreted as a mixture of the exosomes generated and consumed de novo (red and blue). It is not known whether the release of endogenously generated or consumed exosomes occurs together or separately. Exosomes that are absorbed can be degraded by lysosomes. Exosomes that enter cells may enter or fuse with pre-existing ESEs (early-sorting endosomes) and subsequently disintegrate and release their contents into the cytoplasm. Alternatively, endosomes may fuse back with the plasma membrane and release exosomes outside the cells.



https://www.sciencemagazinedigital.org/sciencemagazine/07_february_2020_Main/MobilePagedArticle.action?articleId=1559430#articleId1559430

Biogenesis and identification of exosomes.

Fluid and extracellular constituents such as proteins, lipids, metabolites, small molecules, and ions can enter cells, along with cell surface proteins, through endocytosis and invagination of the plasma membrane. The resulting plasma membrane budding in the luminal side of the cell occurs with an orientation of the plasma membrane from the outside to the inside. This budding process leads to the formation of ESEs or possible bud fusion with ESEs preformed by constituents of the endoplasmic reticulum (ER), trans-Golgi network (TGN) and mitochondria. ESEs could also fuse with ER and TGN, perhaps explaining how endocytic cargo reaches them.

Some of the ESEs may therefore contain membranes and luminal components that may represent different origins. ESEs give rise to LSEs. The second invagination in the LSE leads to the generation of ILVs, and this step can lead to a further change in the loading of future exosomes, with cytoplasmic constituents entering the newly formed ILV. As part of ILV formation, proteins (which were originally on the cell surface) could be distributed distinctly among ILVs. Depending on the volume of invagination, the process could give rise to ILVs of different sizes with distinct contents. LSEs give rise to MVBs with a defined collection of ILVs (future exosomes). MVBs may fuse with autophagosomes and eventually the contents may undergo degradation in lysosomes.

The degradation products could be recycled by cells. MVBs can also fuse directly with lysosomes for degradation. MVBs that do not follow this trajectory can be transported to the plasma membrane through the cytoskeletal and microtubule network of the cell and anchor on the luminal side of the plasma membrane with the help of MVB-docking proteins. This is followed by exocytosis, which results in the release of exosomes with a lipid bilayer orientation similar to that of the plasma membrane. Several proteins are involved in exosome biogenesis and include Rab GTPases, ESCRT proteins, as well as others that are also used as markers for exosomes (CD9, CD81, CD63, flotillin, TSG101, ceramide, and Alix). Surface proteins of exosomes include tetraspanins, integrins, immunomodulatory proteins and more. Exosomes can contain different types of cell surface proteins, intracellular proteins, RNA, DNA, amino acids and metabolites.

Biogenesis of apoptotic bodies

Apoptosis is characterized by a sequence of steps leading to cell death.
Light and electron microscopic analyses are an effective approach to assess morphological features during the apoptotic process, but only when combined with biochemical study can a complete understanding of the complexity of the mechanism be obtained.
Intrinsic pathways or extrinsic stimuli are capable of leading to activation of apoptotic signaling.

After induction, a caspase-dependent proteolytic cascade is activated.
Caspases are aspartic acid-specific proteases responsible for the degradation of cellular components.
Caspases-8 and -9 act as initiators of the apoptotic signaling pathway, while caspases-3, -6 and -7 act as executor caspases, which actively participate in the degradation of cellular substrates.

Caspase activation can follow two main apoptotic pathways, the **extrinsic** or death receptor **pathway** and the **intrinsic** or mitochondrial **pathway.**

The dying cell is engulfed by professional phagocytes or neighboring cells.
Effective removal of apoptotic cells is driven by interaction with phagocytes through the expression of "*eat me*" signals and the release of "*find me*" signals, which facilitate the attack of the dying cell and its eventual digestion in phagolysosomes [14]

Apoptosis progresses through several stages, the first of which consists of nuclear condensation of chromatin, then followed by nuclear cleavage and the frequent appearance of micronuclei, then *blebbing* (evagination) of the membrane, and, finally, cleavage of the cell contents into distinct vesicles enclosed in the membrane, called apoptotic bodies or, more recently, apoptosomes. [15]
The process of apoptotic cell disassembly and removal of apoptotic material by phagocytes are very rapid, so the presence of apoptotic bodies (ApoBD) is very limited in vivo. [16]

[14] Kerr JF, Wyllie AH, Currie AR.
Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.
Br J Cancer. 1972;26(4):239-257. doi:10.1038/bjc.1972.33
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2008650/

[15] Elmore S.
Apoptosis: a review of programmed cell death.
Toxicol Pathol. 2007;35(4):495-516. doi:10.1080/01926230701320337
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2117903/

Povea-Cabello S, Oropesa-Ávila M, de la Cruz-Ojeda P, et al.
Dynamic Reorganization of the Cytoskeleton during Apoptosis: The Two Coffins Hypothesis.
Int J Mol Sci. 2017;18(11):2393. Published 2017 Nov 11. doi:10.3390/ijms18112393
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5713361/

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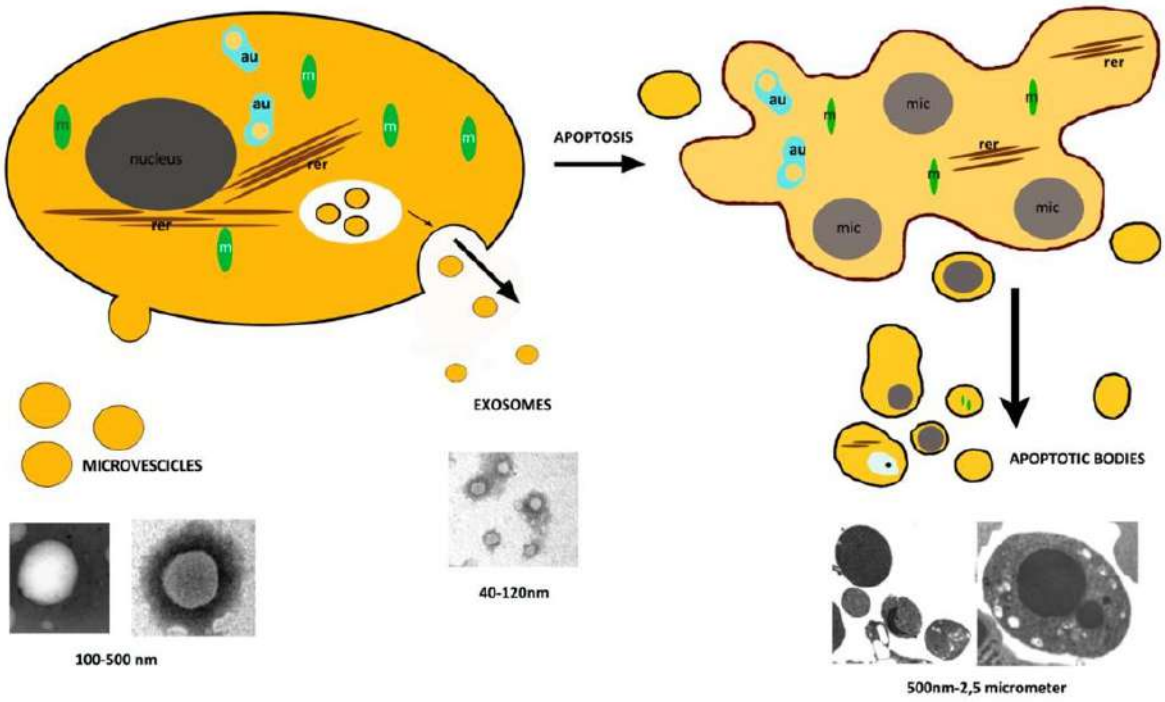
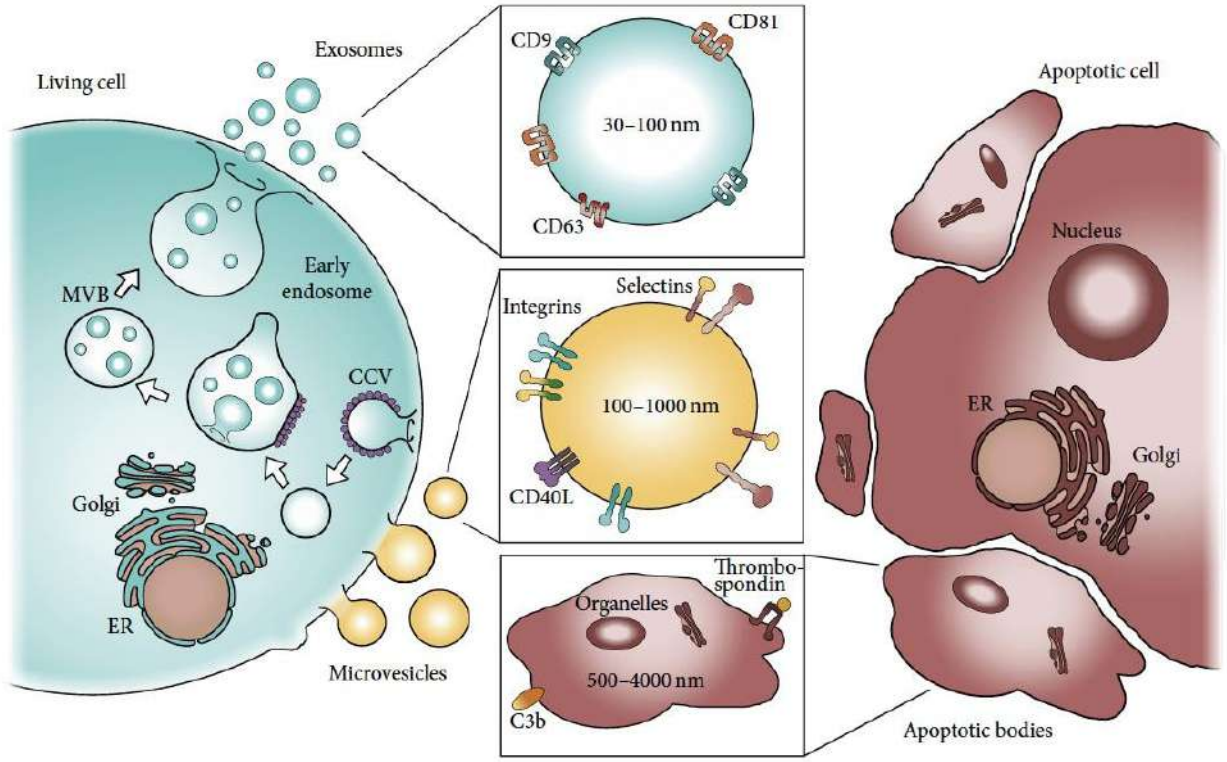
Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies.
J Neurooncol. 2013;113(1):1-11. doi:10.1007/s11060-013-1084-8
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5533094/



https://www.mdpi.com/2079-7737/9/1/21/htm [17]
Schematic diagram of extracellular vesicle formation. This figure shows the biogenesis and release of microvesicles, exosomes and apoptotic bodies. Their morphology was observed by transmission electron microscope (TEM) after negative staining. Apoptotic body extrusion appears in the schematic and sections of conventionally embedded apoptotic cells. m = mitochondria, rer = rough endoplasmic reticulum, mic = micronuclei, Bar = 200 nm.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4681819/ [18]

[17] Battistelli M, Falcieri E.
Apoptotic Bodies: Particular Extracellular Vesicles Involved in Intercellular Communication.
Biology (Basel). 2020;9(1):21. Published 2020 Jan 20. doi:10.3390/biology9010021
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7168913/
[18] Pugholm LH, Revenfeld AL, Søndergaard EK, Jørgensen MM. Antibody-Based Assays for Phenotyping of Extracellular Vesicles. Biomed Res Int. 2015;2015:524817. doi:10.1155/2015/524817

Immune response and exosomes [19]

An effective immune response requires the involvement of host receptors by molecules of pathogenic origin and the stimulation of an appropriate cellular response.
Therefore, a crucial factor in the body's ability to control an infection is the accessibility of immune cells to foreign material.

Exosomes can play a key role in the spread of pathogen- and host-derived molecules during infection, and it has been found that extracellular vesicles produced during an infection can be pathogen- or host-derived.

The former include the membrane vesicles of gram-negative and gram-positive bacteria. The content and function of these bacterially generated vesicles has recently been the subject of extensive investigation[20] .

Although these vesicles probably play an important role in the course of extracellular bacterial infection, their role in intracellular pathogenic infections is less clear, as the mechanisms for transporting the vesicles outside the host cell are not known.
Pathogenic and fungal parasites also release extracellular vesicles that can modulate the immune response.[21]

Host-derived vesicles are present during viral, bacterial, parasitic, and fungal infections. These vesicles have different origins and composition and, based on their biogenesis, are divided into three main categories:

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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4681819/

[19] Schorey JS, Cheng Y, Singh PP, Smith VL.
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https://onlinelibrary.wiley.com/doi/full/10.1111/j.1462-5822.2010.01537.x

Oliveira DL, Rizzo J, Joffe LS, Godinho RM, Rodrigues ML.
Where do they come from and where do they go: candidates for regulating extracellular vesicle formation in fungi
Int J Mol Sci. 2013;14(5):9581-9603. Published 2013 May 2. doi:10.3390/ijms14059581
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3676800/

- **Apoptotic bodies,**
- **exosomes**
- **microvesicles**. [22]

All three of these cell-derived vesicles are enclosed by a lipid bilayer, but they vary in size (30 to 2,000 nm in diameter) as well as in composition.
Unlike microvesicles, which are generated by budding from the plasma membrane[23] , exosomes are derived from the endolysosomal pathway and have a distinctive lipid and protein composition.

Exosomes have been the most studied in the context of infection. An important note, however, is that the purity of the exosomal fraction has not always been analyzed in these studies and, therefore, the vesicle population could be composed of both exosomes and microvesicles, which overlap in size and density.

Recently, the International Society for Extracellular Vesicles updated the guidelines for the analysis of EVs To promote the standardization of EV protocols[24] .

*Larger **microvesicles** (**MVs**, 100 nm-1 µm) and **apoptotic bodies** (**ABs**, 1-5 µm) are distinguished because they sprout directly from the plasma membrane.*

MPs (microparticles) are now replaced with the term MV, and to make it unambiguous, the term "microvesicle (MV)" is used for extracellular vesicles 100 nm^{-1} µm in diameter, and not the term "microparticle (MP)."

***Exosomes**, on the other hand, refer to smaller vesicles (50-100 nm), generated intracellularly by internal budding from multivesicular bodies (MVBs) and released from the cell once MVBs fuse with the plasma membrane.*[25]

https://www.endocrinology.org/endocrinologist/123-spring17/features/how-do-i-measure-extracellular-vesicles-in-my-samples/
https://joe.bioscientifica.com/view/journals/joe/228/2/R57.xml
Three types of extracellular vesicles (EVs). **Apoptotic bodies** (AB, 1-5 µm) are formed by the everting (blebbing) of the cell membrane apoptotic. **Microvesicles** (MV, 100 nm-1 µm) are formed by budding outward from the plasma membrane. **Exosomes** (Exos,

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Microvesicles and exosomes: new players in metabolic and cardiovascular disease.
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https://joe.bioscientifica.com/view/journals/joe/228/2/R57.xml

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Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes.
J Extracell Vesicles. 2013;2:10.3402/jev.v2i0.20677. Published 2013 Sep 12. doi:10.3402/jev.v2i0.20677
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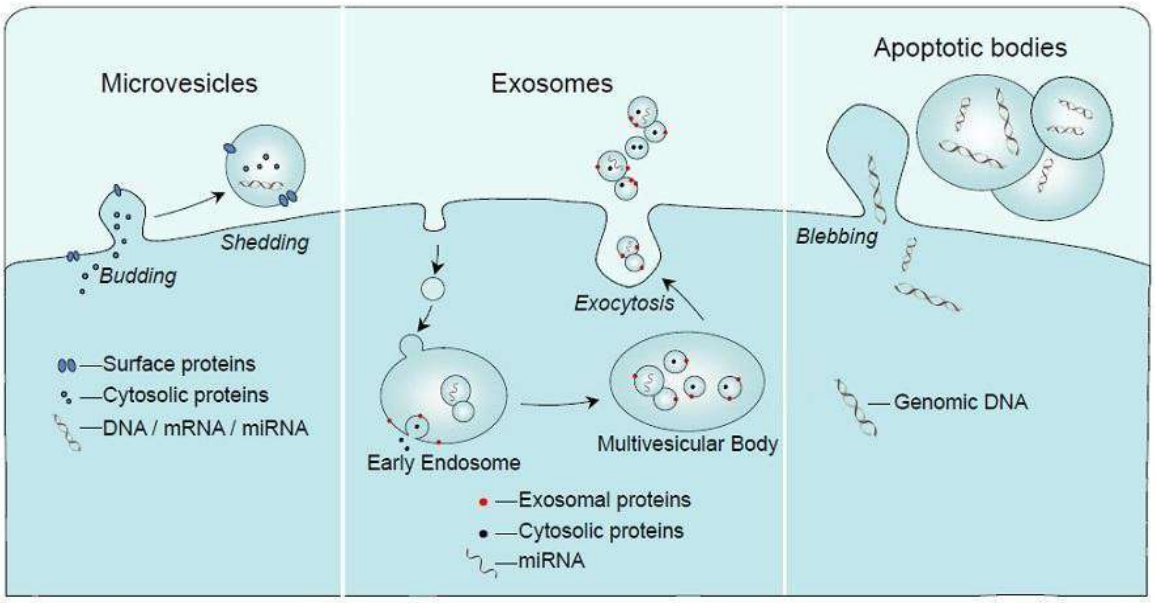
Huang-Doran I, Zhang CY, Vidal-Puig A.
Extracellular Vesicles: Novel Mediators of Cell Communication In Metabolic Disease.
Trends Endocrinol Metab. 2017;28(1):3-18. doi:10.1016/j.tem.2016.10.003
https://www.sciencedirect.com/science/article/abs/pii/S104327601630128X

30-100 nm) are contained in multiple vesicular bodies (MVBs) by internal and reverse budding of an endosomal membrane. The exosomes are released into the extracellular space once the MVBs are fused with the plasma membrane of the cells.



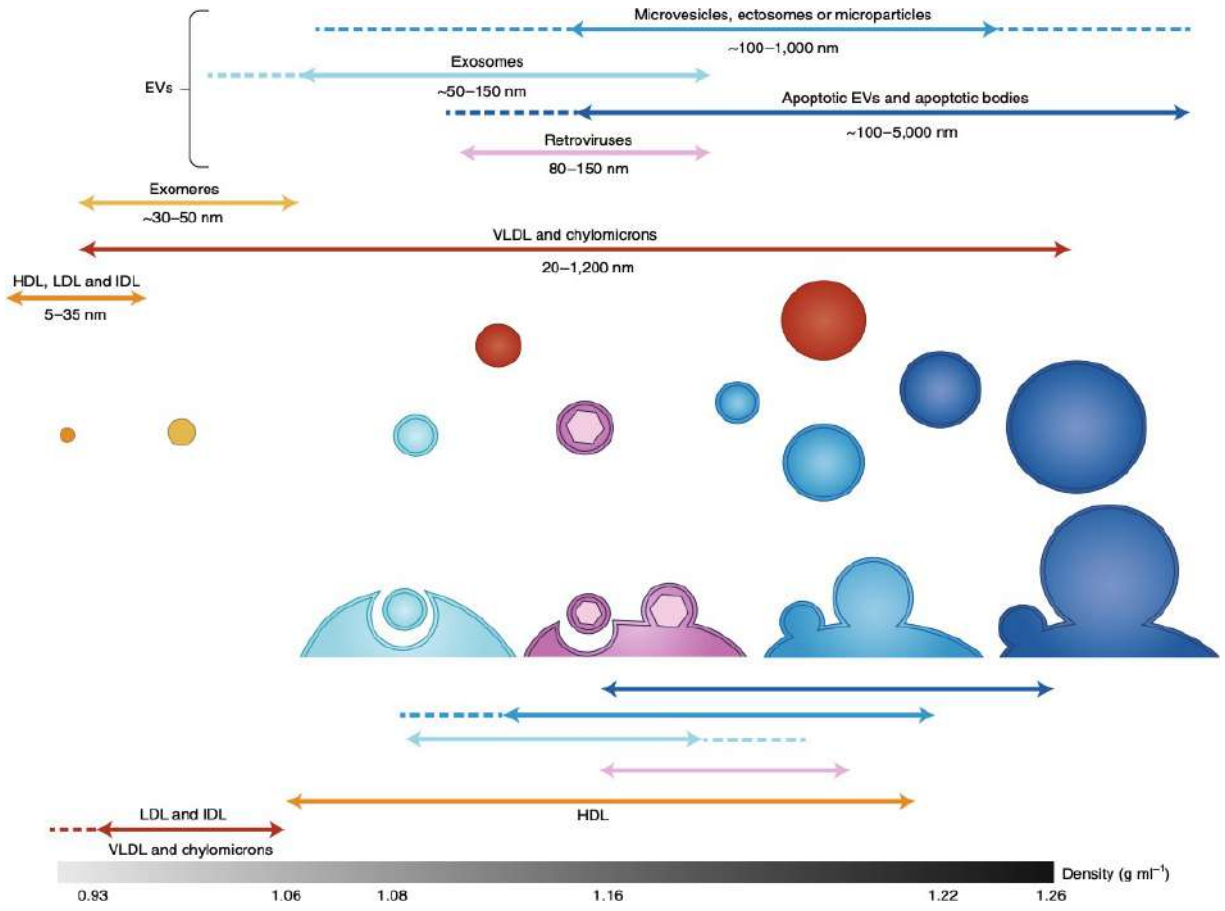
Classification	Exosomes	Microvesicles	Apoptotic bodies
Size	30-100 nm	100-1000 nm	50-5000 nm
Mechanism of formation	Formation of multivesicular bodies by inward budding of endosomal membrane followed by fusion with cell plasma membrane	Outward protrusion (blebbing) of plasma membrane followed by detachment	Fragmentation of cells during the apoptotic process
Characteristics and composition	Rich in lipid rafts, cell endosome-specific proteins (e.g., LAMP1, CD63, TSG 101), cytoplasmic proteins, RNA, miRNA	Externalized phosphatidylserine (annexin V binding), rich in lipid rafts, cell surface-specific molecules, cytoplasmic proteins, RNA, miRNA, other ncRNA, occasionally DNA	Externalized phosphatidylserine (annexin V binding), organelles, DNA, cytoplasmic proteins, RNA, miRNA and other ncRNA
Functional properties	Selective cargo transfer (functional proteins, mRNA, miRNA), receptor interaction	Inflammation, coagulation, thrombosis, angiogenesis, tissue regeneration, tumour cell invasion, metastasis, mRNA, miRNA	Transfer of DNA fragments to the phagocytes, inhibition of inflammatory processes, cell survival

https://fb.cuni.cz/file/5876/fb2018a0015.pdf

Tipo di vescicola	Origine	Dimensione	Marcatore	Contenuto
Esosoma	Via endolisosomiale; formazione intraluminale di corpi multivescicolari e fusione di questi ultimi con la membrana cellulare.	40-120 nm	Tetraspanine (come TSPAN29 and TSPAN30), componenti ESCRT, PDCD6IP, TSG101, flotillin, MFGE8	mRNA, miRNA, e altri RNA non codificanti; proteine citoplasmatiche e proteine di membrana inclusi i recettori e molecole dei complessi di maggiore istocompatibilità
Microvescicola	Superficie cellulare; estroflessione della membrana cellulare	50-1,000 nm	Integrine selectine, CD40 ligand	mRNA, miRNA, RNA non codificanti, proteine citoplasmatiche e proteine di membrana inclusi i recettori
Corpi Apoptotici	Superficie cellulare; estroflessione della membrana di una cellula apoptotica	500-2,000 nm	Vasta quantità di fosfatidilserina	Frazioni nucleari e organelli cellulari

https://ora.uniurb.it/retrieve/handle/11576/2629211/14864/phd_uniurb_257630.pdf

Physical characteristics of different EV subtypes. Different subtypes of EVs are depicted together with other co-isolated particles. The subcellular origin of these EVs (endosomal or from the plasma membrane) is schematized along with size and density ranges. Regardless of the mechanisms of secretion, the different subtypes of EVs cannot be completely separated by size or density because of the overlapping physical characteristics. For example, this applies to small microvesicles, exosomes, and enveloped viruses. Cellular debris, such as apoptotic bodies or small apoptotic vesicles produced when cells undergo apoptosis[26] , may not be distinguishable from other EVs. Other secreted particles that can be co-isolated with EVs include exomers and different types of lipoproteins. After differential centrifugation of fluids containing these vesicles[27] , the larger ones (e.g.,>300 nm) are recovered at low speed (about 2,000 g) and a short period (20-30 min) of centrifugation, followed by recovery of intermediate EV sizes of 150-300 nm at about 10-20,000 g for less than 30 min. Smaller EVs (<15 nm) are mainly recovered after high-speed ultracentrifugation, but such preparations can include smaller non-EV structures (exomers[28] and high-density lipoprotein (HDL)), especially if centrifugation is performed for an extended period of time. However, lipoproteins can be separated from EVs by combining size- and density-based[29] isolation methods. IDL, intermediate density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins.
https://www.nature.com/articles/s41556-018-0250-9?WT.feed_name=subjects_cell-biology



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Detailed analysis of the plasma extracellular vesicle proteome after separation from lipoproteins.
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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6021463/

Importantly, RNA present in exosomes is biologically active and can modulate the protein profile and cellular state of the recipient cell[30] .

However, the exosomal content of RNA is dictated by its cellular origin and the physiological state of the cell[31] , indicating that RNA incorporation into vesicles is a directed event that leads to selective incorporation of RNA into exosomes and other ExMVs.[32]

Once internalized, exosomes can fuse with the membrane of endosomes, resulting in horizontal genetic transfer of their contents into the cytoplasm of target cells.

Bioactive molecules contained in exosomes have been shown to impact target cells through the following mechanisms:

(1) Direct stimulation of target cells through ligands on the surface;

(2) Transfer of activated receptors to recipient cells;

(1) Epigenetic reprogramming of recipient cells through the release of functional proteins, lipids, and RNA[33] .

As a result, progenitor cells can communicate with specific proximal or distal target cells through exosome amplification.

In addition to host RNA, there is clear evidence for the incorporation of viral RNAs into exosomes.

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Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells.
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Montecalvo A, Larregina AT, Shufesky WJ, et al.
Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes.
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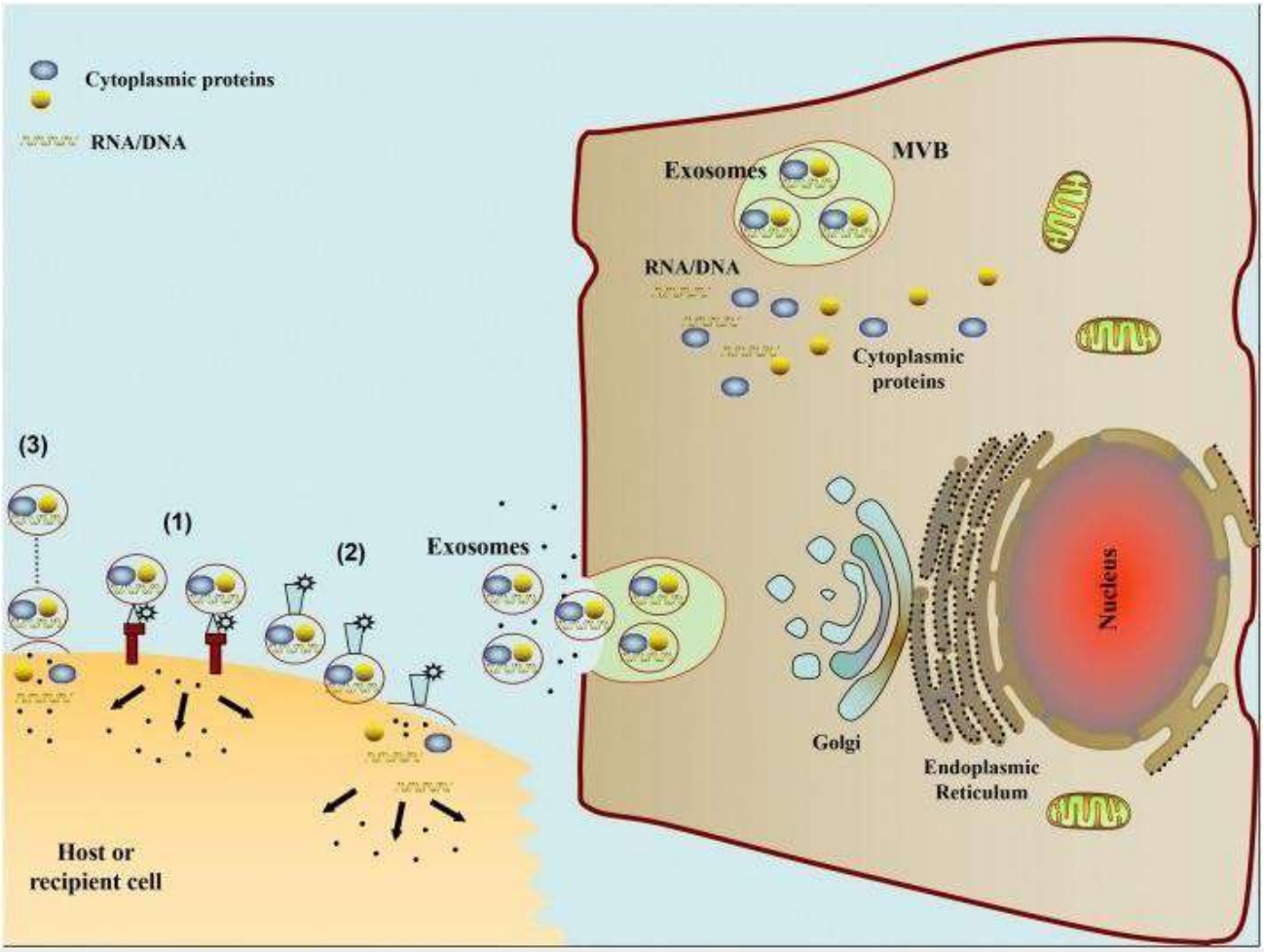


https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6377728/
Schematic diagram of the pathways involved in exosome-mediated cell-cell communication.
(1) Exosomes communicate with recipient cells via ligands on the surface.
(2) Exosomes transfer activated receptors to recipient cells.
(3) Exosomes can epigenetically reprogram recipient cells through the delivery of functional proteins, lipids, and RNAs

The bioactivity of exosomes comes not only from the incorporated proteins and nucleic acids, but also from their lipid components.

Exosomes are normally enriched in phosphatidylserine (PS), phosphatidic acid, cholesterol, sphingomyelin (SM), arachidonic acid and other fatty acids, prostaglandins and leukotrienes, which are responsible for their structural stability and rigidity.

In addition, exosomes also have some functional lipolytic enzymes, which can independently produce units of various bioactive lipids. These exosomal bioactive lipids can be internalized into recipient cells and concentrated within endosomes. [34]

[34] Zhang Y, Liu Y, Liu H, Tang WH.
Exosomes: biogenesis, biological function and clinical potential.
Cell Biosci. 2019;9:19. Published 2019 Feb 15. doi:10.1186/s13578-019-0282-2
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6377728/table/Tab3/

Lipid-related enzymes and bioactive lipids in exosomes

lipid category and description	Lipid related enzymes	Functional effects
LTA4, LTB4, LTC4	LTA4 hydrolase, LTC4 synthase	Triggering polymorphonuclear [131] leukocyte migration
PGE2, 15d-PGJ2	COX-1, COX-2	Immunosuppression, [44] PPARγ ligand
PGE2	PGE synthase	Inflammation [4]
PA	PLD2, DGK	Increasing exosome production [132, 133]
AA, LPC	cPLA2, iPLA2	Accounting for the membrane curvature [44]
/	sPLA2 IIA, sPLA2 V	Prostaglandin biosynthesis [44]
Ceramides	nSMase2	Sorting cargo into MVBs [134]
Cholesterol	/	Regulating exosome secretion [135]
BMP	/	MVB formation [135] and subsequent ILV biogenesis [136]
PS	/	Being involved in exosome fate [13, 122]
SM	/	Triggering calcium influx [135]

LA4, LTB4, LTC4 Leukotriene; *COX-1, COX-2* cyclooxygenases; *PGE2, 15d-PGJ2* prostaglandins; *PLD2* phospholipase D2; *DGK* diglyceride kinase; *PA* phosphatidic acid; *PLA2* phospholipases A2; *cPLA2* calcium-dependent phospholipases A2; *iPLA2* calcium-independent phospholipases A2; *AA* arachidonic acid; *LPC* lysophosphatidylcholine; *sPLA2 IIA; V* secreted phospholipases A2 IIA and V; *nSMase2* neutral sphingomyelinase 2; *BMP* Bis(monoacylglycero)phosphate, also called LBPA; *PS* phosphatidylserine; *SM* sphingomyelin

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6377728/

A key question is whether the functions of ExMVs carrying pathogen-derived molecules drive host defense and pathogen elimination, or mediate the spread of virulence factors to promote pathogen survival and disease. What has been seen is that in some cases, both can occur simultaneously and in equilibrium.

This is a complex question that depends on both host, pathogen, and environmental factors. The answer to this question for various pathogens is not yet available, as many of the necessary tools for investigation are lacking, such as robust methods to specifically inhibit the production of exosomes or other ExMVs in order to assess their role in immune responses and infection control.

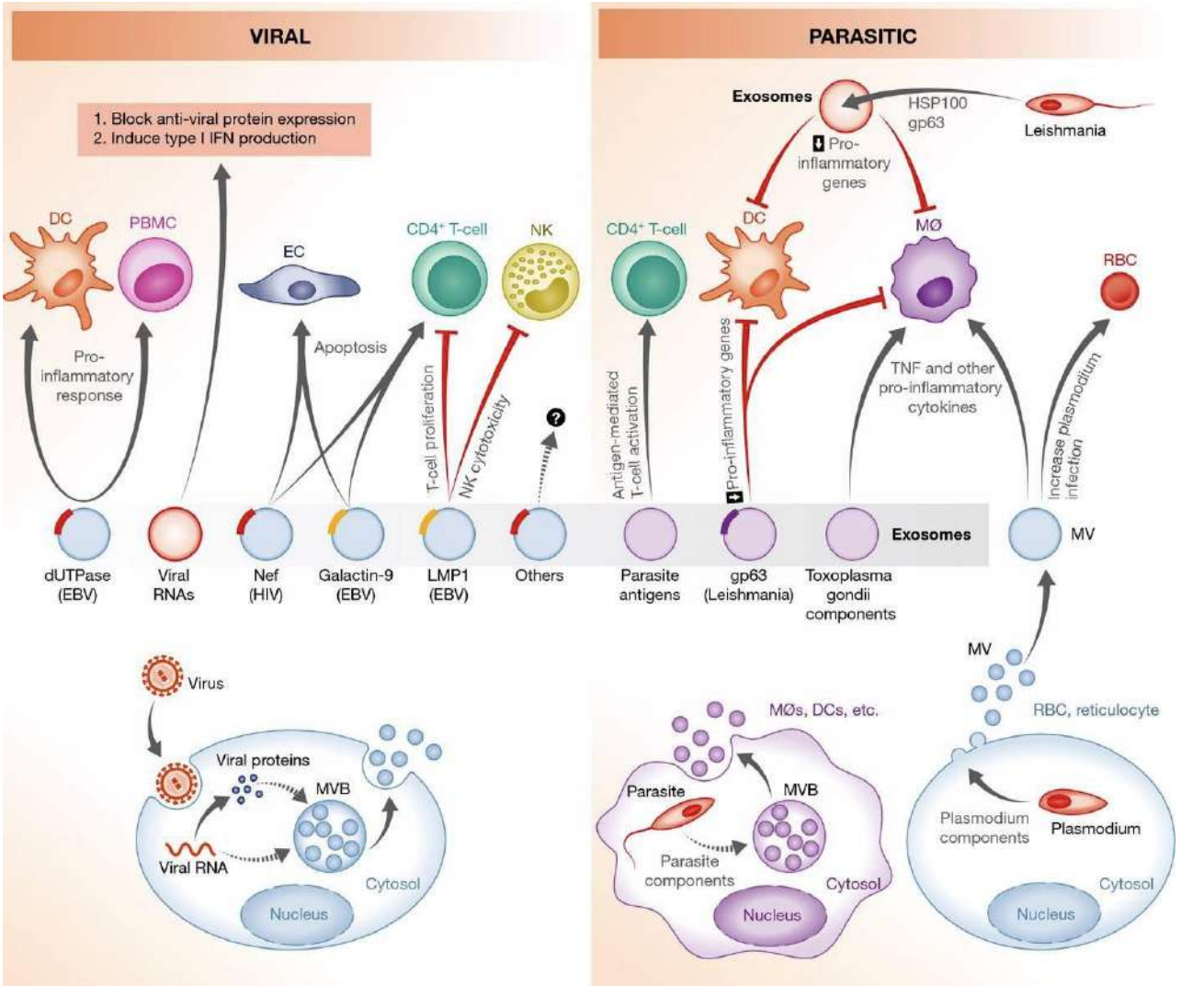
However, studies in various in vitro and in vivo infection models have provided some insight into the function of exosomes and other ExMVs in host defense and immune evasion.

A common observation for many in vivo infection studies is a high concentration of ExMVs in blood, which supports a functional relevance for these vesicles during an infection,[35] indeed, exosomes and other ExMVs have been implicated in the pathogenesis of several viruses.

[35] Nantakomol D, Chimma P, Day NP, et al.
Quantitation of cell-derived microparticles in plasma using flow rate-based calibration.
Southeast Asian J Trop Med Public Health. 2008;39(1):146-153.
https://pubmed.ncbi.nlm.nih.gov/18567455/

Hu G, Gong AY, Roth AL, et al.
Release of luminal exosomes contributes to TLR4-mediated epithelial antimicrobial defense.
PLoS Pathog. 2013;9(4):e1003261. doi:10.1371/journal.ppat.1003261
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3617097/

Upon release, these vesicles are "captured" by cells and the transfer of host and viral proteins and/or RNAs could enhance viral infection and replication in recipient cells or inhibit the immune response through induction of apoptosis or by blocking key antiviral cellular responses.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4304727/

Modulation of host immunity by exosomes during a viral or parasitic infection

Cells infected with viruses or parasites, or the parasites themselves, release exosomes or microvesicles that can stimulate T lymphocyte activation by delivering antigens to APCs. In contrast, exosomes containing microbial molecules, such as HIV Nef or Leishmania GP63, can block T lymphocyte activation or induce apoptosis of effector immune cells. Extracellular vesicles released from virus- or parasite-infected cells can modulate both innate and acquired immune responses. In some cases, this is to the advantage of the pathogen, and in others to the advantage of the host. Dashed lines indicate unknown mechanisms. See glossary for definitions.

Current understanding of the functions of EVs is beginning to fill knowledge gaps on cell-cell communications. For example, EVs can partially answer questions about how cytokines/chemokines achieve the necessary concentration in the microenvironment and reach their target cells.

It has been found that cytokines are not transmitted in free form, but appear to be associated with EVs [36].

[36] Konadu KA, Chu J, Huang MB, et al.
Association of Cytokines With Exosomes in the Plasma of HIV-1-Seropositive Individuals.
J Infect Dis. 2015;211(11):1712-1716. doi:10.1093/infdis/jiu676

Cytokines, chemokines, proteins, and miRNAs are significantly enriched within EVs, suggesting the function of EVs as a vehicle to concentrate and transport these signaling molecules.
In addition, the RNAs in EVs are protected by RNaseA, and thus EVs can be a source of miRNAs for therapeutic purposes and for the detection of disease biomarkers[37] .

Drug delivery by exosomes

EV-based delivery of active ingredients offers numerous advantages over traditional drug delivery systems: EVs exhibit greater stability in the bloodstream, enabling them to travel long distances within the body under physiological and pathological conditions[38] .

- *In plasma they are stable for up to 90 days under various storage conditions[39] . In contrast, peak concentrations of free TNF-alpha occur about 2 hours after administration, followed by a rapid decline in plasma concentration (half-life of about 18.2 minutes)[40] .*
- *They express the same surface markers as their "parent" cells. This functionality potentially offers the opportunity to deliver EV-containing molecules in a cell type-specific manner.*
- *They carry cell type-specific markers and can serve as a diagnostic agent in "liquid biopsy "* to avoid invasive tissue diagnosis. [41]*

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4447830/

[37] Cheng L, Sharples RA, Scicluna BJ, Hill AF.
Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood.
J Extracell Vesicles. 2014;3:10.3402/jev.v3.23743. Published 2014 Mar 26. doi:10.3402/jev.v3.23743
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3968297/

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Exosomes as novel bio-carriers for gene and drug delivery.
Int J Pharm. 2017;521(1-2):167-175. doi:10.1016/j.ijpharm.2017.02.038
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From COPD to chronic systemic inflammatory syndrome?
Lancet. 2007;370(9589):797-799. doi:10.1016/S0140-6736(07)61383-X
http://www.thelancet.com/retrieve/pii/S014067360761383X

[41] Lin J, Li J, Huang B, et al.
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ScientificWorldJournal. 2015;2015:657086. doi:10.1155/2015/657086
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4322857/

Properzi F, Logozzi M, Fais S.
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Biomark Med. 2013;7(5):769-778. doi:10.2217/bmm.13.63
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4322857/

Gámez-Valero A, Lozano-Ramos SI, Bancu I, Lauzurica-Valdemoros R, Borràs FE.

Biofluid	Disease	Associated proteins
Plasma	Chronic hepatitis C	CD81
	Melanoma	CD63, caveolin-1, TYRP2, VLA-4, HSP70, HSP90, (phospho)Met
	Glioblastoma	Epidermal growth factor receptor VIII
	Prostate cancer	Survivin, PSA
	Plasma cell dyscrasias	c-src
	Ovarian cancer	TGFB1 and MAGE3/6
Serum	Glioblastoma	EGFRvIII
	Lung cancer	EGRF
	Pancreatic cancer	KRAS
Urine	Acute kidney injury	Fetuin-A, ATF 3
	Liver injury	CD26, CD81, S1c3A1, CD10
	Bartter syndrome type 1	NKCC2
	Bladder cancer	EGF, α subunit of Gs, Resistin, Retinoic acid-induced protein 3,
	Prostate cancer	PSA, PCA3
	isquemia reperfusion injury	Aquaporin-1, Transcription factor 3
	focal segmentary glomerulosclerosis	Wilms tumor 1, PODXL
	Nephrotic syndrome	Neprilysin, Aquaporin-2, Podocalyxin
Biofluid	**Disease**	**Associated RNAs**
Plasma	Ovarian cancer	miR-21, -141, -200a, -200b, -200c, -203, -205, -214
	Lung cancer	miR-17-3p, miR-21, miR-20b, miR-223, miR-301, let-7f, miR-151a-5p, miR-30a-3p, miR-200b-5p, miR-629, miR-100, miR-154-3p
	Prostate cancer	miR-16, miR-34b, miR-92a, miR-92b, miR-103, miR-107, miR-197, miR-328, miR-485-3p, miR-486-5p, miR-574-3p, miR-636, miR-640, miR-766 and miR-885-5p
	Esophageal squamous cell cancer (ESCC)	miR-21, miR-1246
	Breast cancer	miR-141 and miR195
	Cardiovascular disease	miR-1, miR-133a
	Alcoholic liver disease	miR-122 and miR-155
Serum	Globlastoma	RNU6-1, miR-320, and miR-574-3p
	Colorectal cancer	let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, miR-23a
	Human esophageal cell carcinoma	miR-21
Urine	Renal fibrosis	miR-29c, CD2APmRNA

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5946167/
EVs containing molecules that can potentially serve as biomarkers

Urinary extracellular vesicles as source of biomarkers in kidney diseases.
Front Immunol. 2015;6:6. Published 2015 Jan 30. doi:10.3389/fimmu.2015.00006
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4311634/

Zocco D, Ferruzzi P, Cappello F, Kuo WP, Fais S.
Extracellular vesicles as shuttles of tumor biomarkers and anti-tumor drugs.
Front Oncol. 2014;4:267. Published 2014 Oct 8. doi:10.3389/fonc.2014.00267
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4189328/

* Definitions: THE LIQUID BIOPSY

Studies to date have shown that pathogens are able to hijack the host exosome biosynthetic apparatus as part of their survival strategy.
Viruses such as paramyxovirus, hepatitis C virus (HCV), rhabdovirus, filovirus, herpesvirus, and hepatitis B virus (HBV) use ESCRT endosomal sorting complexes required for transport (ESCRT)[42] to promote their release. [43]

These similarities and the presence of viral proteins in exosomes suggest the shared process for viral protein delivery by exosomes.

Inflammasome and exosomes [44]

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and that "inflammasome-induced" EV can activate inflammatory responses in recipient cells[46] .

Therefore, the activation of EV secretion through various mechanisms is probably the result of the activity Of the inflammasome in evolutionarily determined immune signaling.

In their paper, "*From Inflammasome to Exosome-Does Extracellular Vesicle Secretion Constitute an Inflammasome-Dependent Immune Response?*", Cypryket al suggest a model of inflammasome-dependent EV secretion as a sequential process that serves the release of pro-inflammatory cytokines and several other proteins.

The following figure shows the relationship between inflammasome activity and EV secretion:

A. ***Signal 1****, provided by IL1R, TLR and other PRRs induces transcription and expression of NLRP3, as well as precursors of the inflammatory cytokines IL-1β and IL-18 and to the induction of expression of hundreds of other proteins.*

B. ***Signal 2*** *activates the inflammasome that catalyzes the processing of pro-IL-1β and gasdermin D.*
The N-terminal fragment of gasdermin D inserts into the cell membrane and oligomerizes, forming a pore, which allows direct secretion of small proteins, cytokines, and promotes ion fluxes across the membrane.
In addition, calcium and potassium concentrations are also affected by membrane antiport receptors (e.g.P2X7) or lysosomal leakage, amplifying inflammasome activation and causing the activation of floppases, flippases, and scramblas, which catalyze the transmembrane translocation of phosphatidylserine and phosphatidylethanolamine.*

C. This affects membrane curvature, causing outward sprouting, microvesicle formation, and incorporation of near-membrane proteins (including pro-IL-1β and mature IL-1β) as well as integral membrane proteins into their lumen. The rapid processing of IL-1β may also result in the recruitment of mature IL-1β to intraluminal vesicles of the multivesicular body (MVB), a component of the endosomal pathway, which transfers its contents directly to the membrane, releasing exosomes.

D. Prolonged inflammasome activity leads to pyroptosis, which can cause the release of inflammasome components, cytokines, and other proteins via membrane lysis or larger vesicular structures resulting from cell fragmentation.

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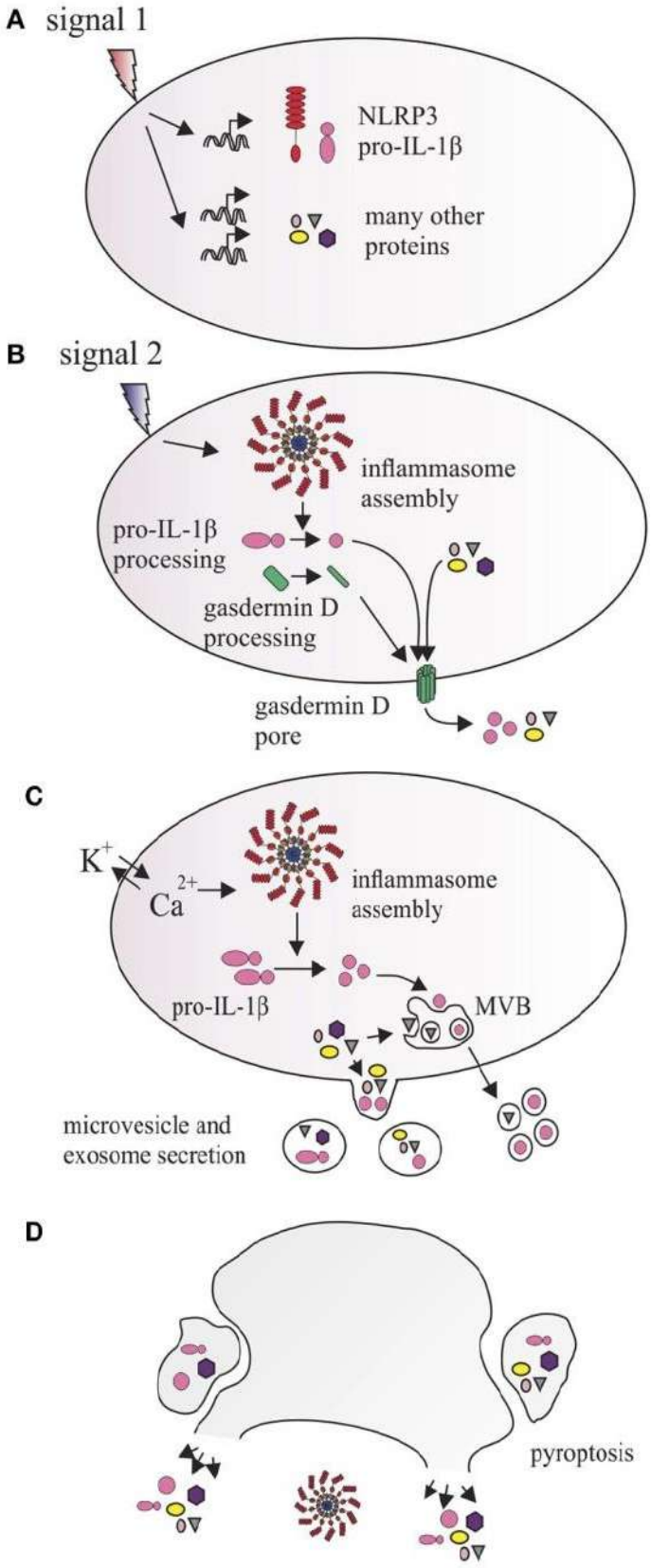

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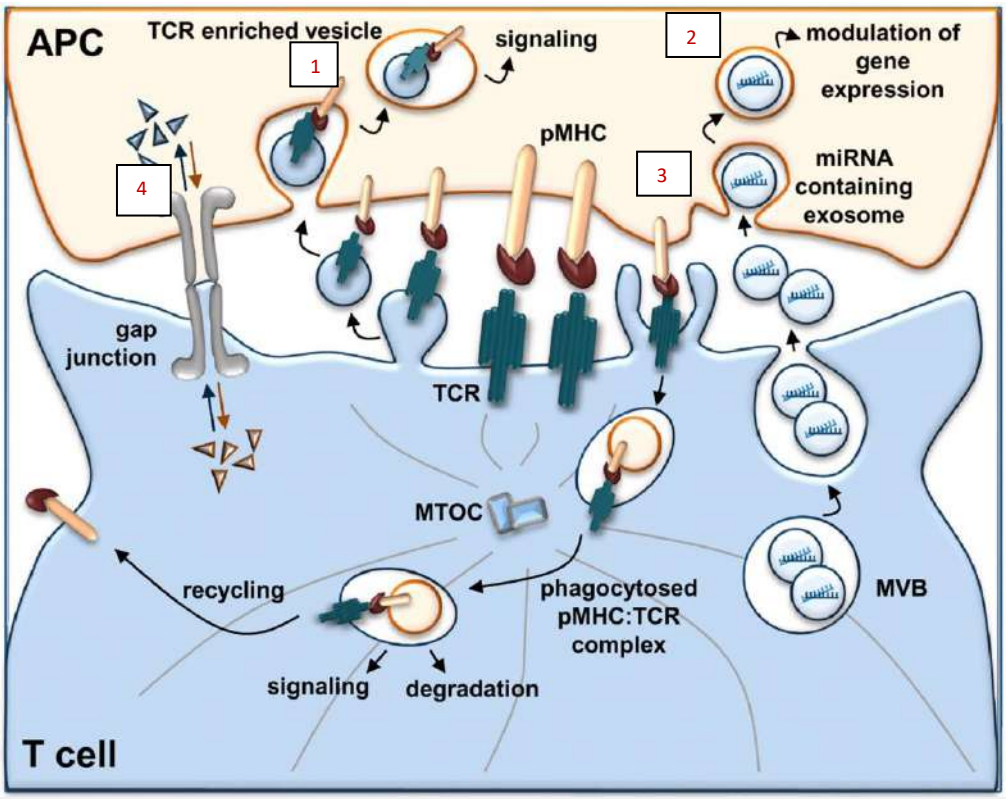
Proposed relationship between NLRP3 inflammasome and vesicular protein secretion

* Definitions: CELL MEMBRANE TRANSPORT AND PERMEABILITY

The immunological synapse and exosomes

The term "**immunological synapse**" was originally coined to highlight the similarities between synaptic contacts between neurons in the central nervous system and related, antigen-dependent interactions between T cells and antigen-presenting cells [47]



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Cell-cell communication at the immunological synapse (IS).
miRNA, microRNA; MTOC, microtubule organizing center; MVB, multivesicular body.

The IS functions as a device for transcellular communication by exploiting several mechanisms:

1. Polarized transfer of T-cell antigen receptor (TCR)-enriched vesicles from T cells to antigen-presenting cells (APCs), which promotes early signaling in the recipient cell;

2. Release of miRNA-containing exosomes from T cells that modulate gene expression in APCs;

3. trogocytosis* of the peptide-MHC:TCR complex (pMHC: TCR) during TCR internalization, associated with both sustained signaling and surface expression of pMHC in T cells, in which the latter confers on T cells the ability to present antigen to other T cells;

4. assembly of the tight junction between T cells and APCs that allows the exchange of soluble molecules at IS.

** **trogocytosis**:[48] transfer of plasma membrane fragments from one cell to another without inducing the cell death. This process is mediated by receptor signaling following cell-cell contact*

EXOSOMES AND ARDS [49]

Cell-cell communication is essential for optimal lung function, and therefore exosomes are important in lung biology and function.

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Exosomes are released from a wide range of cell types within the lung, including endothelial cells, stem cells, epithelial cells, alveolar macrophages, and tumor cells, although **epithelial cells are the main source of lung-derived exosomes**. [50]

Exosomes released from airway epithelial cells contain mucins and alpha-2,6 sialic acid, which have a neutralizing effect on human influenza virus infection[51] .

Mucins influence the structural properties, conformation and surface charge of exosomes and thus contribute to mucociliary defense by the lung's innate immune system. [52]

Exosomes control inflammatory signaling within the airway through intercellular communication[53] and may act as part of the stress response in the airway.

In sarcoidosis, exosomes provoke the initiation and progression of inflammatory responses by enhancing the induction of IL-13, INF-gamma, and CXCL-8 production in the lung microenvironment[54] .

In addition, the secretion of exosomes and their composition may be altered following infection. For example, it has been shown that exosomes derived from alveolar macrophages are enriched for HSP-70 after Mycobacterium infection [66]. [55]

EVs released from airway cells can be found in bronchoalveolar lavage fluid and support inflammation in the lungs, but they can also spill into the circulation and carry a cocktail of pro-inflammatory molecules to recipient cells in distant organs. [56]

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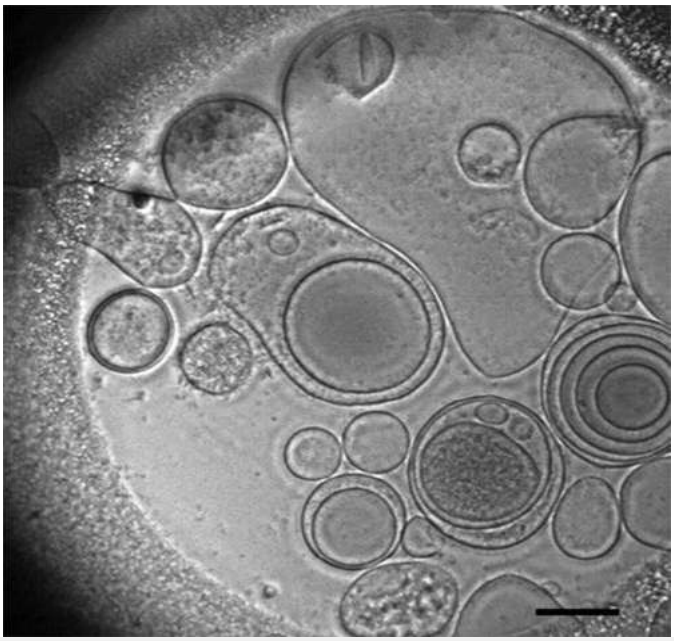

https://www.atsjournals.org/doi/pdf/10.1165/rcmb.2017-0293TR
Morphology of purified extracellular vesicles (EV) from human BAL fluid. A representative phase-contrast transmission electron microscope image is presented. EVs of various sizes and shapes are observed. These EVs were isolated using a conventional ultracentrifugation method. Scale bar: 200 nm.

EV and interaction between the microbiota and the host lung immune response

As already seen, the microbiota is essential for the development of immune responses and airway homeostasis[57] .

Dysbiosis and pathogen colonization have been linked to altered immune responses and disease development in the lungs,[58] and EVs derived from pathogens, including bacteria, have been shown to transfer their contents to host cells and modulate host innate immunity[59] .

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Park et al found that entry of EVs derived from Escherichia coli into the bloodstream induced systemic inflammation that mimicked sepsis,[60] and that EVs derived from Staphylococcus aureus were associated with the pathogenesis of neutrophilic pulmonary inflammation[61] .

In addition, EVs derived from gram-negative bacteria induce Th1 and Th17 cell responses and neutrophilic inflammation in the lungs, which leads to the pathogenesis of airway inflammation diseases such as neutrophilic asthma and COPD[62] .

These data suggest that EVs derived from the gut microbiota may be the key messengers of communication between the gut microbiota and host lung immune responses through the transfer of components contained in EVs.

Kim et al analyzed the pulmonary microbiota from COPD lung tissue and EVs derived from COPD lung tissue,[63] and showed that bacterial-derived EVs possessed distinctive characteristics in the lungs of nonsmokers, healthy smokers and COPD patients.

EVs produced by commensal bacteria can benefit the host by promoting mucosal tolerance and protecting against the onset of lung disease[64] .
Conversely, EVs derived from dysbiosis of the lung microbiota can induce activated pulmonary immune responses and pulmonary inflammatory pathogenesis. [65]

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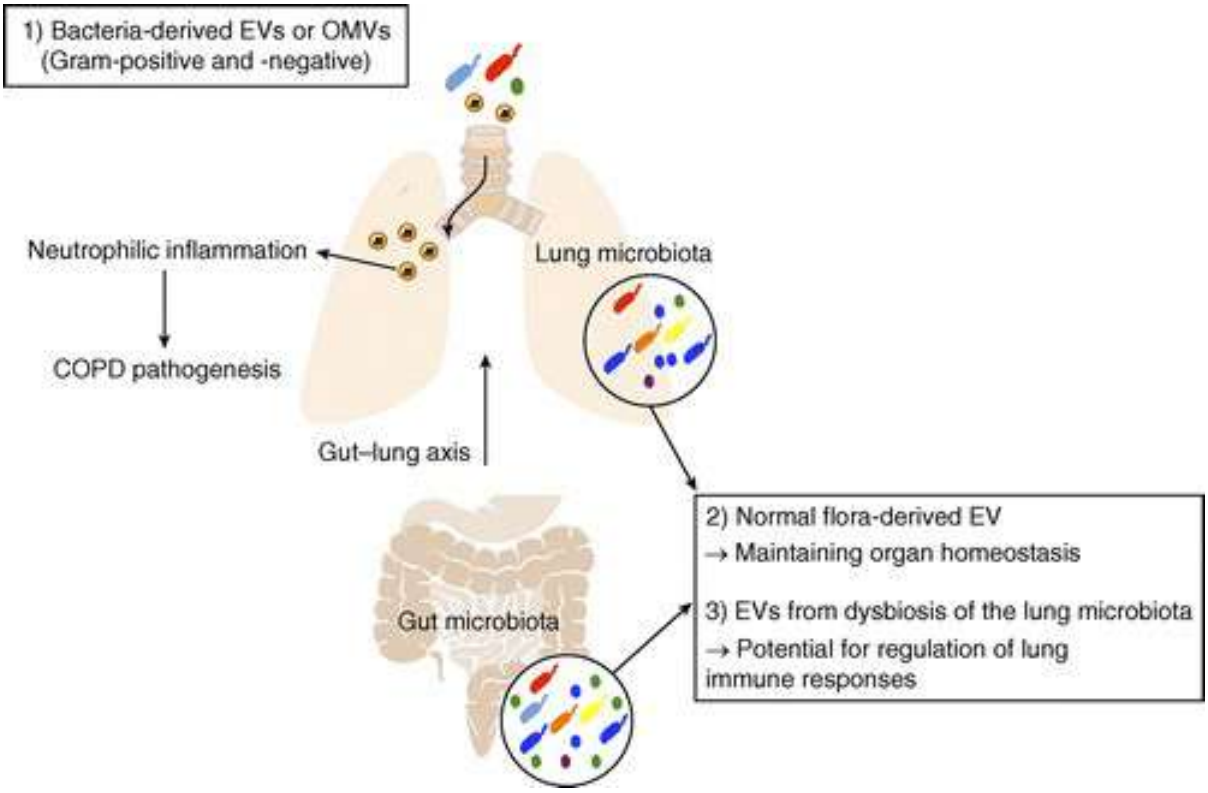

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Microbiota-derived EVs and host pulmonary immune response. It is proposed that the interaction between the microbiota and EV-mediated host lung immune responses has the potential to increase the understanding of fundamental lung immune responses. **1) EVs** derived from bacteria or outer membrane vesicles (OMVs) induce neutrophilic inflammation and the development of chronic obstructive pulmonary disease (COPD). In addition, EVs produced by lung and intestinal microbes can activate pulmonary immune responses**. 2) EVs** produced by normal flora promote mucosal tolerance and protect against the onset of inflammatory diseases**. 3) In** contrast, EVs derived from dysbiosis of the microbiota have the potential to activate pulmonary immune responses

EV contained in bronchoalveolar fluids (BALF).

Large amounts of EV are secreted into the bronchoalveolar lavage fluid by different cell types after infection or lung injury.
BALF EVs contribute significantly to the development of lung inflammation in different models of ALI.
After sterile stimuli, the main source of BALF EVs was from I alveolar type epithelial cells, whereas EVs were mainly derived from alveolar macrophages in BALF induced by infectious stimuli. [66]

Effects of EVs derived from lung tissue

Almost all cell types release EVs that affect neighboring or distant cells and also exert autocrine actions on themselves. These EVs have distinct protective or damaging effects on other cells, which depend strongly on the donor cells, the accepted stimuli of the mother cells, and the variety and composition of EVs.
In lung tissues, communications between alveolar immune and structural cells through EVs contribute to the lung inflammatory response, structural barrier disruption, and modulation of the lung microenvironment.

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Functional Evidence of Pulmonary Extracellular Vesicles in Infectious and Noninfectious Lung Inflammation.
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EV derived from epithelial cells

EVs are critical for the activation and propagation of thrombosis through the exposure of negatively charged phospholipids on their surface and the release of tissue factor (TF, a primary initiator of blood clotting, which also plays a key role in angiogenesis) at the site of thrombosis. [67]

Jin-AhPark et al demonstrated from in vitro and in vivo studies that bronchial epithelial cells under mechanical stress are a source of secreted TF.
The amount of TF in bronchoalveolar lavage fluid from patients with asthma (a disease characterized in part by subepithelial angiogenesis) was found to be at average concentrations that were 5-fold higher than those of the healthy control, and exosomes isolated from normal human bronchial epithelial cells and bronchoalveolar lavage fluid from asthmatic subjects contained TF. [68]

Similarly, following a proinflammatory stimulus, alveolar epithelial cells can release many EVs containing procoagulant tissue factor (TF), which contribute to fibrin deposition in ARDS[69] .

EV derived from alveolar macrophages

macrophage-derived alveolar microvesicles are rapidly released in the early stages of acute lung injury and are potent initiators of inflammation, mediated particularly by TNF (tumor necrosis factor). [70]

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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5099194/

EV derived from endothelial cells

EVs derived from endothelial cells promote the shift to the pro-inflammatory phenotype of endothelial cells[71] .

They induce decreased NO production both in vivo and in vitro, and in vivo pulmonary edema, neutrophil recruitment, and endothelial-alveolar barrier impairment have been found[72] .
Incubation of macrophages with endothelial EVs induced by cigarette smoke (CS) causes significant inhibition of macrophage efferocytosis[73] .

Mechanical ventilation could cause injury and destruction of endothelial cells, even without existing inflammation.
Compared with low tidal volume mechanical ventilation, high tidal volume ventilation increases the score of pulmonary edema and leads to deterioration of gas exchange, associated with an obvious increase in the EV number of circulating endothelial cells [74]

The diagnostic role of EVs in lung damage and inflammation

Molecular components in EVs correlate with certain diseases, indicating that they can also be used to diagnose and predict disease severity[75] .

Leukocyte EVs in BALF and blood (LeuMP) have been identified as biomarkers of favorable prognosis at the onset of ARDS[76] .

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EV derived from other cells

Intratacheal and intravenous administration of blood EVs from LPS-treated rats to normal rats was able to induce ARDS with the significant increase of myeloperoxidase (MPO), TNF-α, IL-1β and IL-10 in BALF and plasma.
The study of lung morphology showed that the alveolar structures were destroyed with a large amount of neutrophils infiltrating the lung tissues. [77]

Studies on stored units of concentrated red blood cells (pRBCs) have demonstrated the presence of EVs in increasing amounts as storage time increases and the association between EVs and several adverse processes, including vascular disease, lung damage, thrombosis, immunomodulation, platelet refractoriness, and neutrophil priming.

In vitro, human neutrophils co-incubated with RBC-MV showed increased CD11b expression and superoxide production, as well as increased phagocytic capacity, which may explain the enhanced inflammatory response observed during transfusion in patients receiving older pRBCs. [78]

VIRUSES AND EXTRACELLULAR VESICLES [79]

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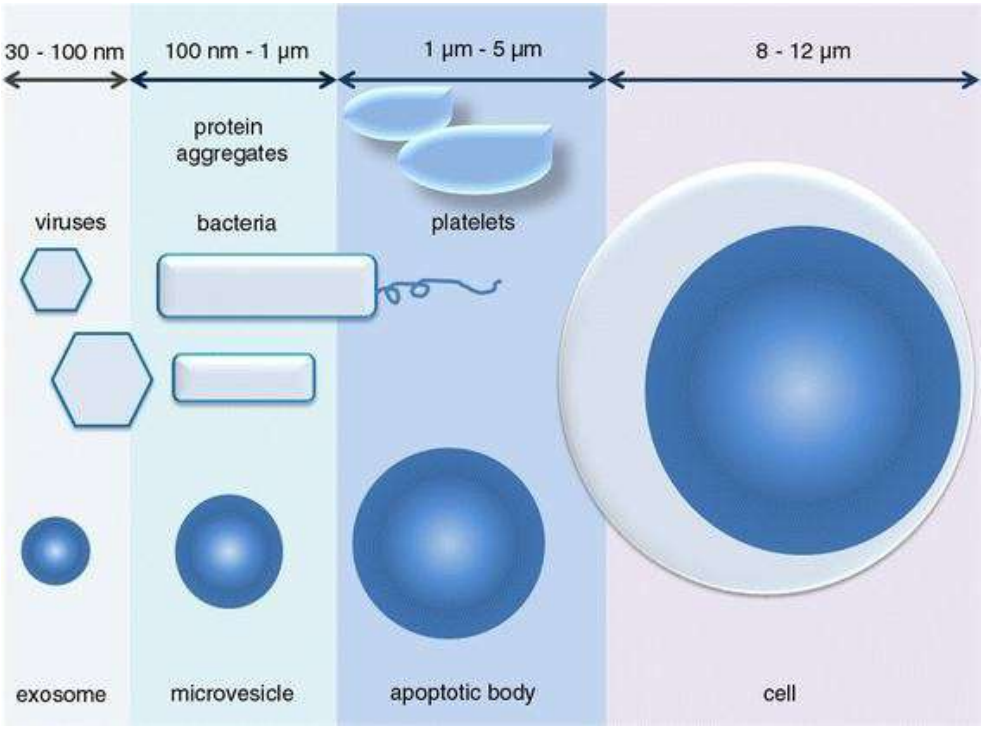
Unlike EVs, the definition of a virus developed by 20th century virologists was quite precise: a virus was "a small infectious agent that can multiply only in living cells." [80]

EVs do not fit this definition because despite their similarity to viruses in many respects, they are fundamentally different in that they **do not replicate**.

However, contemporary virology has distanced itself from this strict definition of viruses with the introduction of the terms noninfectious and defective viruses. Thus, EVs generated from retrovirus-infected cells carrying viral proteins and even fragments of viral genomes essentially fall under the definition of noninfectious viruses.

Based on current knowledge, there are many aspects in which EVs resemble viruses, particularly retroviruses. First, although some EVs may be up to a micrometer in size, most are <300 nm, the size of a typical RNA virus.



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Extracellular vesicles and viruses: Are they close relatives?
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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4995926/

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3142546/ [81]
Size ranges of the main types of membrane vesicles. While exosomes share size distribution with viruses, microvesicles overlap in size with bacteria and protein aggregates (e.g., immune complexes). Both apoptotic bodies and platelets fall within the size range of 1-5 μm

Like envelope viruses, EVs are surrounded by a lipid membrane that also contains cell membrane proteins.

Like many viruses, EVs:

- are formed in the endosomal system or at the plasma membrane through defined biogenesis pathways, for example, involving endosomal sorting complexes required for transport mechanisms (ESCRTs)[82] .

- can bind to plasma membranes of other cells, penetrate them by fusion or endocytosis, and trigger specific reactions in recipient cells[434] .

- carry genetic material that can change the functions of recipient cells[83] .

Especially in the case of retroviruses, EVs generated in infected cells contain selected molecules of viral origin[84] and may be csimilar to noninfectious defective viruses that have lost their ability to replicate, so the difference between them becomes less defined.

In other cases, EVs provide an "envelope" for envelope-less viruses, for example, hepatitis A, and these EV-encapsulated viruses can infect cells[85] .

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A pathogenic picornavirus acquires an envelope by hijacking cellular membranes.
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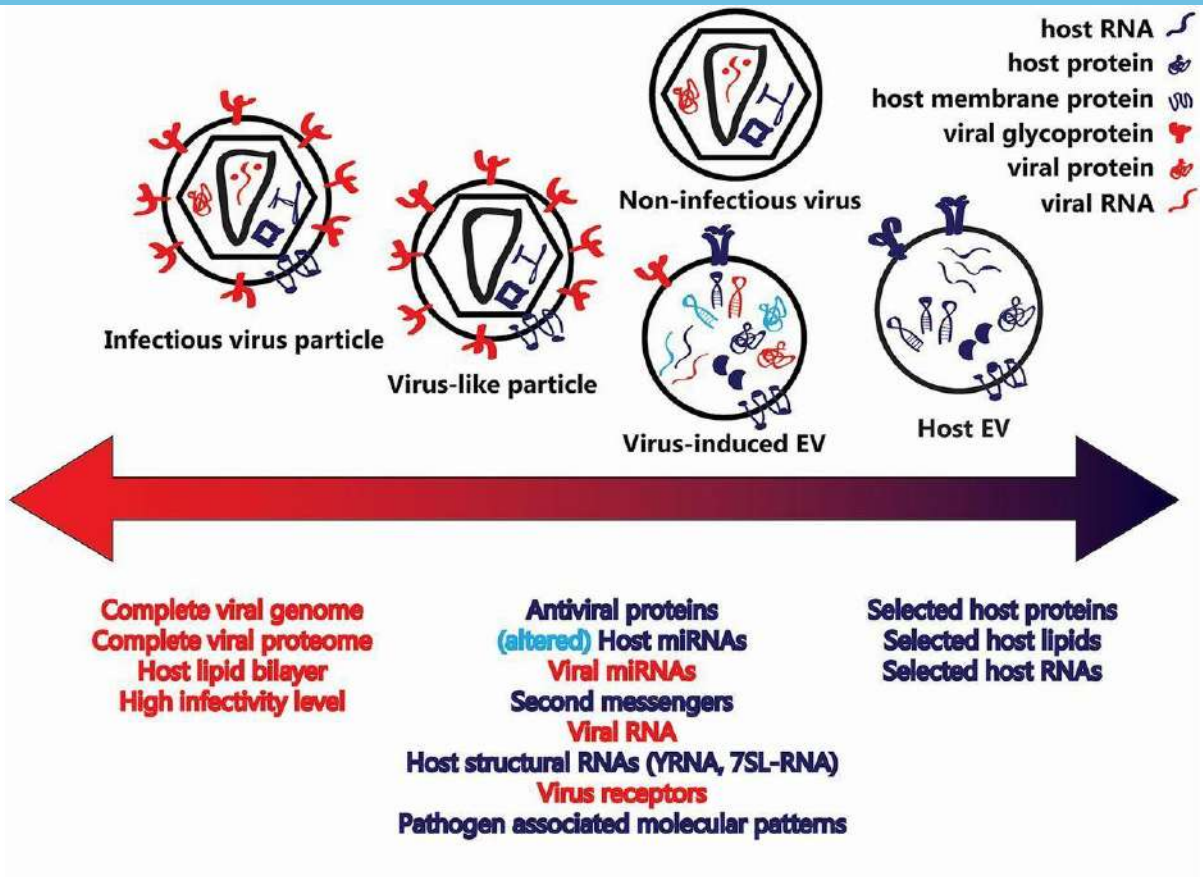
Similarly, EVs released from hepatitis C-infected cells can carry intact and functional viral genomes that in target cells generate new infectious viral particles[86] .

It follows that a variety of different vesicles are released in retrovirus infections, with EVs consisting entirely of host cell components at one extreme and replication-capable viruses at the other.
Between these extremes are nonreplicating particles that can be considered either as defective viruses or EVs containing various amounts of virus-specific molecules .

Of course, unlike true viruses, EVs that contain viral proteins and viral genome fragments do **not cause outbreaks and epidemics.**
However, EVs can interact directly with retroviruses or modulate host cells, thus affecting infection.



https://www.pnas.org/content/113/33/9155
Structural similarities between EVs and virions. Cells infected with enveloped RNA (retro)viruses release vesicles containing a variety of host-derived and viral factors. At one extreme, there are EVs consisting entirely of host cell components (blue), and at the other extreme there are infectious viruses surrounded by a lipid bilayer derived from the host and containing all the virus-specific molecules (red) required for infectivity. In virus-infected cells, EVs incorporate fragments of the viral genome and viral (glyco)proteins. In addition, viral infections modify the incorporation of host proteins and RNAs into EVs (light blue). Such infection-induced EVs, so-called defective viruses and virus-like particles, are intermediate entities, and the boundary between them seems not to exist.

[86] Bukong TN, Momen-Heravi F, Kodys K, Bala S, Szabo G.
Exosomes from hepatitis C infected patients transmit HCV infection and contain replication competent viral RNA in complex with Ago2-miR122-HSP90. PLoS Pathog. 2014;10(10):e1004424. Published 2014 Oct 2. doi:10.1371/journal.ppat.1004424
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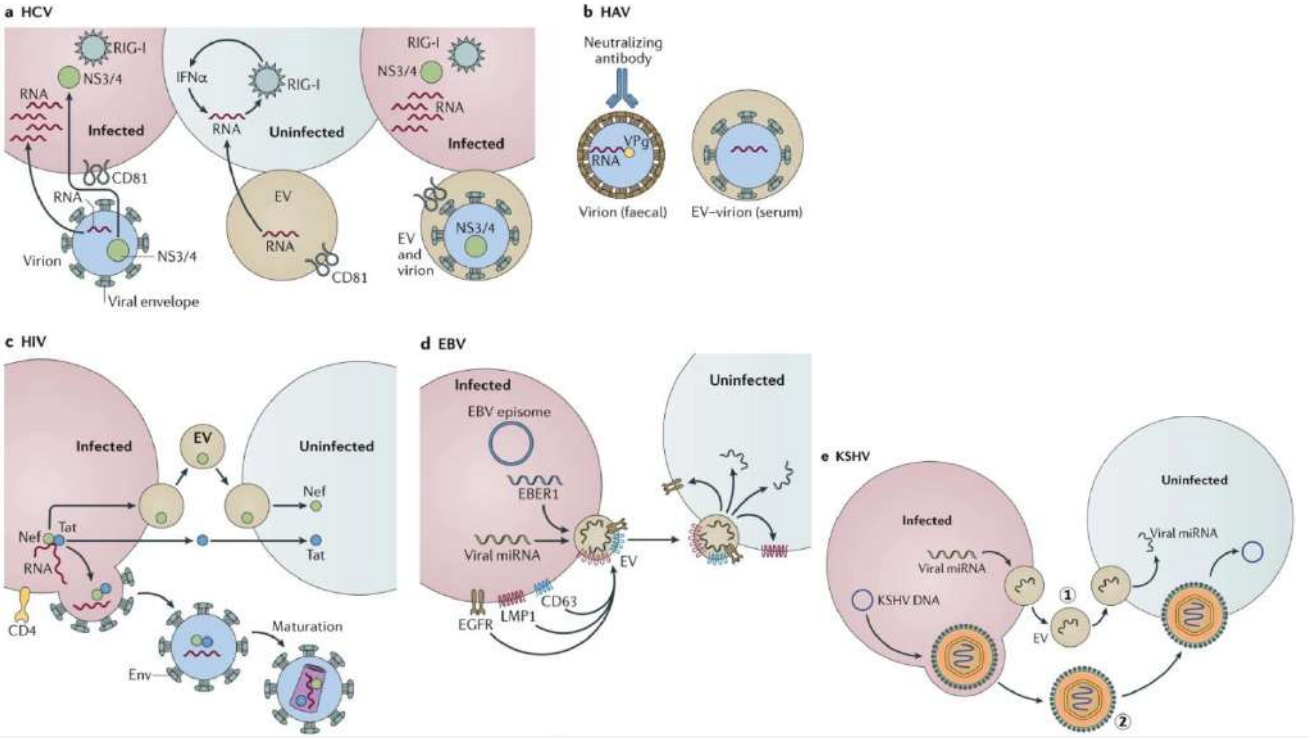

https://www.nature.com/articles/nrmicro.2017.60 [87]
For each virus (hepatitis C virus (HCV), hepatitis A virus (HAV), HIV-1, Epstein-Barr virus (EBV), or Kaposi's sarcoma-associated herpesvirus (KSHV)), a virion-dependent transfer and an extracellular vesicle (EV) dependent transfer steps are shown. Only in the case of HCV (part a) and HAV (part b) were whole virions identified within EVs. For the other viruses, single RNAs or proteins were detected in the EVs. During HIV infection (part c), Nef can be incorporated into EVs and subsequently transported to uninfected cells. The soluble HIV protein Tat can be transported to uninfected cells without being incorporated into EVs. In the case of EBV (part d), cellular and viral proteins and cellular and viral RNAs are transported by EVs from infected cells to uninfected cells. In the case of KSHV (part e), a temporal pattern is shown, whereby viral microRNAs (miRNAs) are transported by EVs before infection (step 1) and can then trigger the recipient cell for infection (step 2). EBER1, Epstein - Barr virus-encoded RNA 1; EGFR, epidermal growth factor receptor; IFNα, interferon-α; LMP1, latent membrane protein 1; NS3 / 4, nonstructural protein 3/4; RIG-I, retinoic acid-inducible gene-I protein; VPg, viral genome-bound protein.

EVs and viruses overlap in biogenesis

Early discussions of the relationships between EVs and viruses [440,441] were largely based on the fact that both EVs and retroviruses use the cellular vesiculation mechanism, with striking similarities in lipid composition (HDL and glycosphingolipids) and protein content (tetraspanins, GPI proteins, and cytoplasmic proteins).

Furthermore, it has been hypothesized that retroviruses exploit pre-existing pathways for intracellular vesicle trafficking (**The Trojan exosome hypothesis**)[88] and could be considered "modified or mutated exosomes."

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Badierah RA, Uversky VN, Redwan EM.
Dancing with Trojan horses: an interplay between the extracellular vesicles and viruses
[published online ahead of print, 2020 Apr 30]. J Biomol Struct Dyn. 2020;1-27. doi:10.1080/07391102.2020.1756409
https://pubmed.ncbi.nlm.nih.gov/32351170/

Other researchers challenged the idea because unlike retroviruses, there was little evidence for an active role of EVs in the functional modification of target cells through the transport of bioactive proteins, lipids and genetic material. [89]
Later, EVs were found to contain genetic material, mainly in the form of small RNAs. [90]
In addition to the involvement of molecular mechanisms for the selection of specific proteins in EVs[91] , numerous studies indicate that the RNA content of EVs does not reflect the RNA content of the EV-producing cell.

Although some RNAs may passively diffuse into EVs in the course of their biogenesis, it has been seen that active selection of specific RNAs depends on defined RNA-binding proteins[92] . In addition, it has been found that miRNAs and mRNAs associated with EVs are enriched in some selection motifs[93] .

Further scientific findings have shown that proteins, lipids, and genetic material associated with EVs can be functionally transferred to target cells [443,94] , implying that EVs and (retro)viruses have

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Extracellular vesicles: exosomes, microvesicles, and friends.
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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3905700/

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Exosomes secreted by human cells largely transport mRNA fragments that are enriched in the 3'-untranslated regions.
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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3732077/

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Regulation of immune responses by extracellular vesicles.
Nat Rev Immunol. 2014;14(3):195-208. doi:10.1038/nri3622
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4350779/

Kowal J, Tkach M, Théry C.
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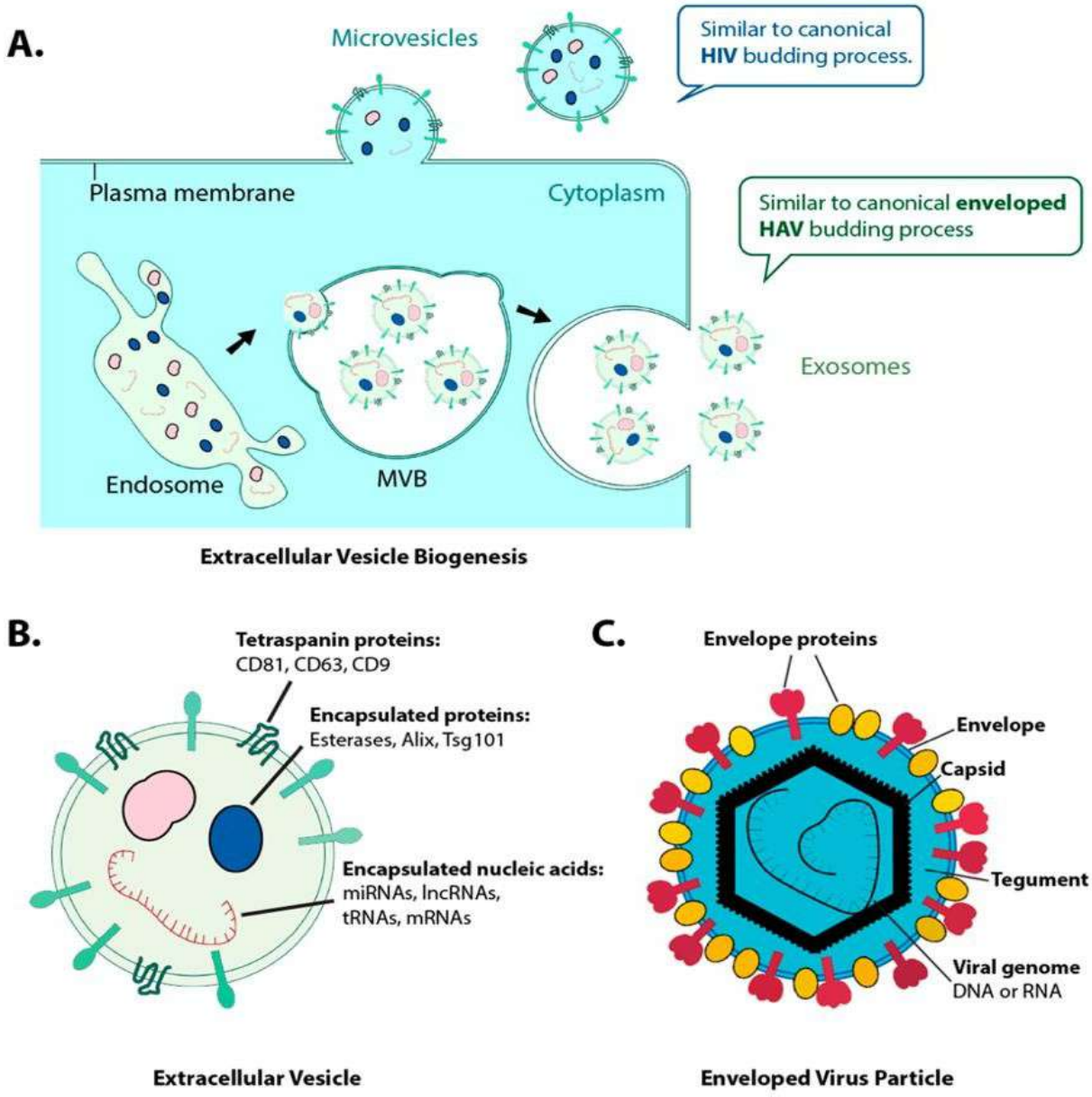
Curr Opin Cell Biol. 2014;29:116-125. doi:10.1016/j.ceb.2014.05.004
https://www.hal.inserm.fr/inserm-02452742/document

Lo Cicero A, Stahl PD, Raposo G.
Extracellular vesicles shuffling intercellular messages: for good or for bad.
Curr Opin Cell Biol. 2015;35:69-77. doi:10.1016/j.ceb.2015.04.013
https://pubmed.ncbi.nlm.nih.gov/26001269/

in common not only structural but also functional aspects because of the similarity in EV and virus biogenesis. [95]



https://www.mdpi.com/1999-4915/12/9/917/htm [96]
Extracellular vesicles (EVs) and viral particles share a similar vesicular budding process and composition, including proteins, nucleic acids and lipids. **(A)** Microvesicles bud on the plasma membrane, similar to the canonical budding process of human immunodeficiency virus (HIV). Exosomes originate from inward budding of the late endosome in the multivesicular body (MVB) and subsequent release onto the plasma membrane, similar to the budding process of hepatitis A virus with canonical envelope (HAV).
(B) Extracellular vesicles can carry factors such as tetraspanins, esterases, Alix and Tsg101. Enclosed nucleic acids are protected by nucleases. **(C).** A viral particle consists of an envelope, capsid, tegument and viral genome.

[95] Gill S, Catchpole R, Forterre P.
Extracellular membrane vesicles in the three domains of life and beyond.
FEMS Microbiol Rev. 2019;43(3):273-303. doi:10.1093/femsre/fuy042
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Purification Methods and the Presence of RNA in Virus Particles and Extracellular Vesicles. Viruses. 2020;12(9):E917. Published 2020 Aug 21. doi:10.3390/v12090917
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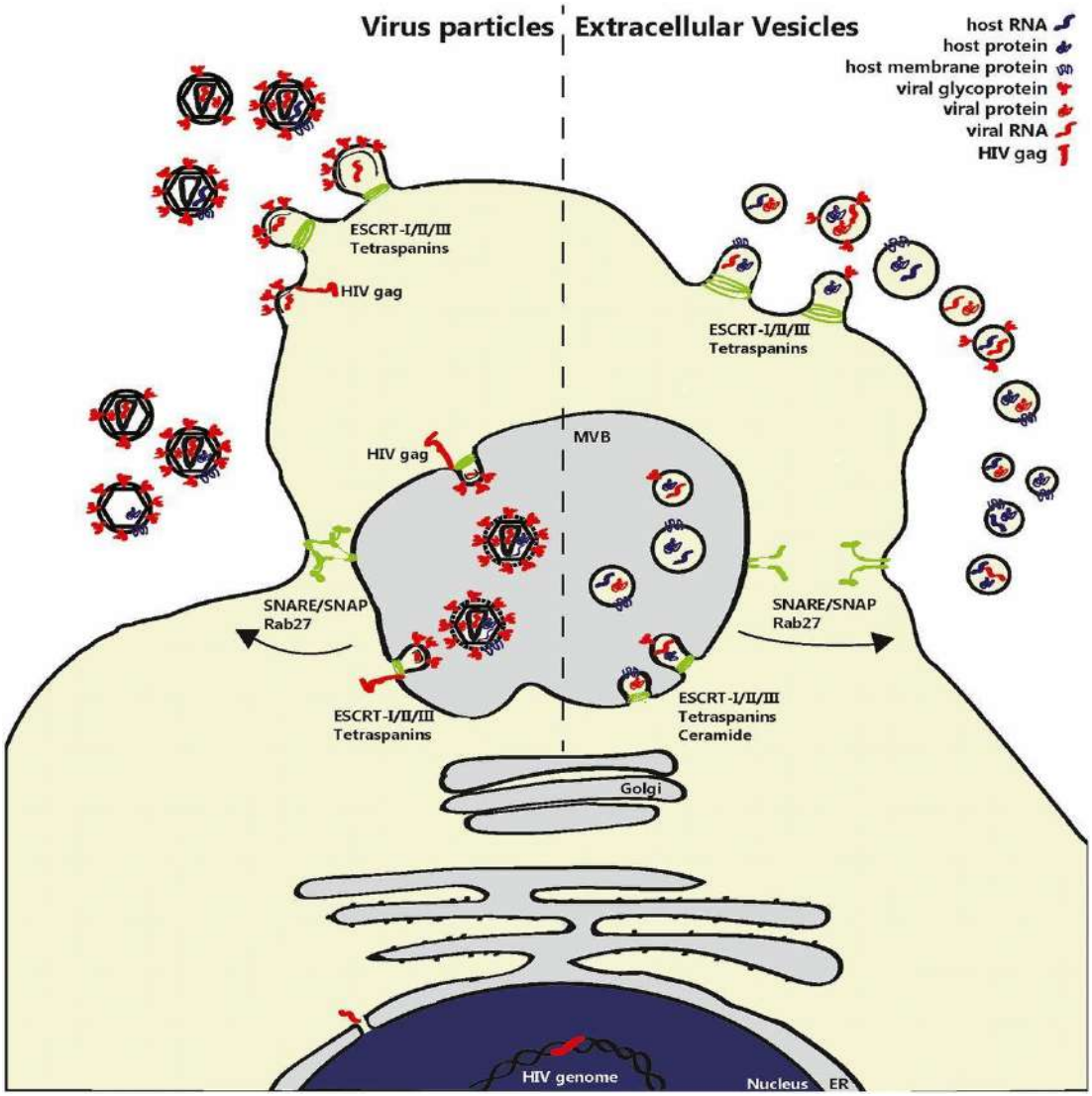


https://www.pnas.org/content/113/33/9155
Similarities between the biogenesis of EVs and virions. EVs and enveloped retrovirus particles (e.g., HIV) are released simultaneously from infected cells and share pathways for biogenesis on the plasma membrane or multivesicular bodies (MVBs). For example, ESCRT complex proteins and tetraspanins are involved in both virion and EV formation. Viral RNA (red) enters the cytoplasm, after w h i c h Gag-mediated virion assembly occurs in the MVB or on the plasma membrane. MVB can contain both virions and EVs and are released from the cell after MVB fusion with the plasma membrane through the action of Rab proteins, SNARE and SNAP.Defective viruses are also formed, but not infectious due to the lack of essential viral components. While specific host proteins and RNA (blue), such as CD63 and APOBEC3G, can be incorporated into virions, viral components (red) are also incorporated into the plethora of EV types released from cells. These include viral genome fragments, viral miRNAs and viral (glyco)proteins, such as Nef and Gag. This intertwining of their pathways for biogenesis blurs the distinction between virions and EVs.

Viral particle entry strategies and exosomes

The **endocytic** pathway is the main cellular entry route for large cargoes and pathogens.

Among the wide variety of specialized lipid structures within endosomes, intraluminal vesicles formed in early endosomes and transferred to late endosomal compartments are emerging as critical effectors of viral infection and immune recognition.

Various viruses carry their genomes in these intraluminal vesicles, which serve as vehicles to transport the genome to the nuclear periphery for replication.
When secreted as exosomes, intraluminal vesicles containing viral genomes can infect permissive cells or activate immune responses in myeloid cells. [97]

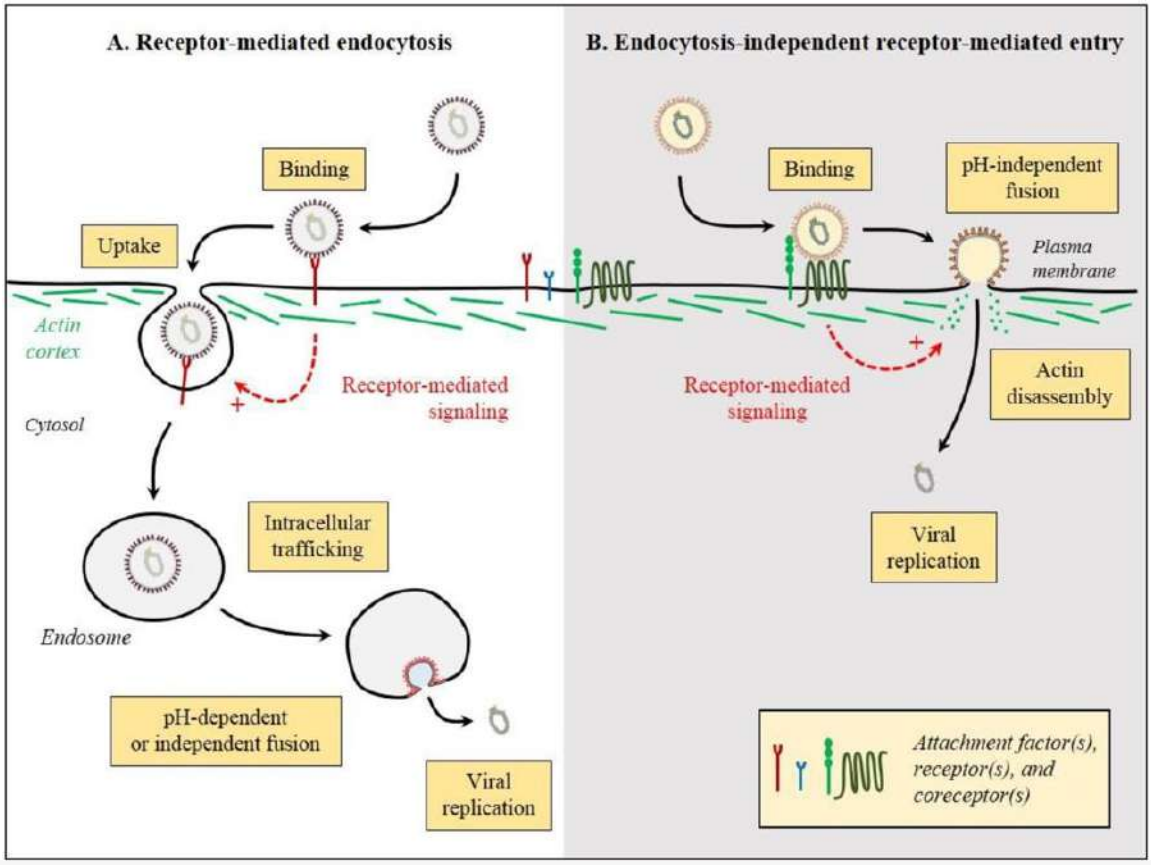
[97] Nour AM, Modis Y.

Virus entry through the endocytic pathway

In order to infect cells and replicate, viruses must have access to the intracellular environment.
This very first step is strictly dependent on the surface-exposed cell receptors to which the viral particles bind.

Viruses can use essentially two different strategies to enter the host. The first, in the classic **viral endocytosis** model, involves that following binding to one or more cellular receptors, viral particles are physically taken up by the endocytic cellular apparatus in a process called receptor-mediated endocytosis.

In a second strategy, virus binding to cellular receptors leads to direct penetration of viral particles from the plasma membrane, bypassing the endocytic mechanism. This process is referred to as receptor-mediated entry independent of endocytosis [98]



https://www.mdpi.com/1999-4915/7/6/2747/htm
Virus entry strategies. To gain access to the cytoplasm of host cells, viruses can employ two main strategies, namely **(A)** t h r o u g h endocytosis and exit from endosomal vesicles in a process referred to as **receptor-mediated endocytosis** or **(B)** by direct penetration from the plasma membrane, referred to as **receptor-mediated entry independent of endocytosis**. Viruses with envelopes are shown; however, viruses without envelopes have developed similar strategies. These are only generalizations, and there are exceptions to these rules. The black arrows represent the sequence of events and the dashed red arrows the potential induced signaling.

Endosomal vesicles as vehicles for viral genomes.
Trends Cell Biol. 2014;24(8):449-454. doi:10.1016/j.tcb.2014.03.006
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4112135/

[98] Boulant S, Stanifer M, Lozach PY.
Dynamics of virus-receptor interactions in virus binding, signaling, and endocytosis.
Viruses. 2015;7(6):2794-2815. Published 2015 Jun 2. doi:10.3390/v7062747
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4488714/

Endocytosis is the mechanism for internalization of molecules of all sizes, plasma membrane components, and nanoparticles by invagination of the plasma membrane and formation of vesicles through membrane fission.

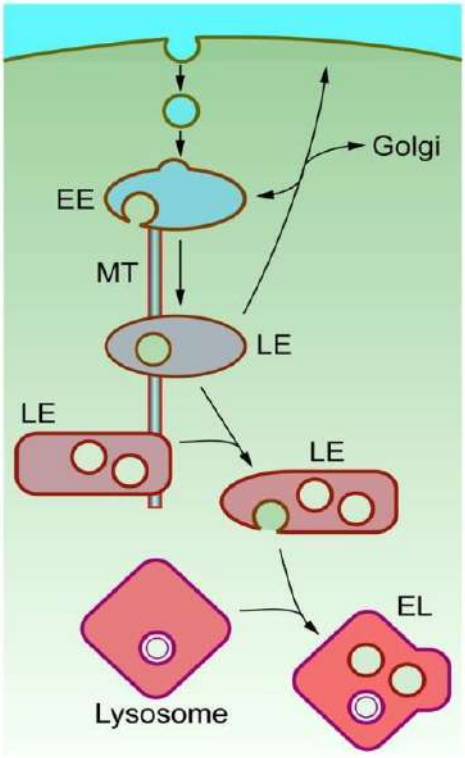
Clathrin-mediated endocytosis is the main pathway for vesicle budding from the plasma membrane, but several clathrin-independent pathways also contribute to endocytosis, including the **caveolin-dependent pathway**.

Endocytic vesicles fuse with early endosomes (EE) in the peripheral cytoplasm, the main sorting station in the endocytic pathway. As extracellular cargoes accumulate in endocytic vesicles, some proteins and membrane components are recycled into the plasma, while others are transferred to the Golgi apparatus; however, most of the cargo is retained within the EEs.

Intraluminal vesicles (ILVs) sprout from the endosomal membrane, starting with EEs, although it remains unclear when ILVs begin to form. EEs and ILVs are transported toward the nucleus via microtubules (MTs). During this period, EEs mature into late endosomes (LEs).
Maturation of LEs results in changes in the lipid and protein composition and pH of the lumen. In addition, vesicles sprout from the outer (limiting) membrane of the maturing endosome to form ILVs. As maturation proceeds, LEs undergo homotypic fusion, grow in size, acquire more ILVs, and receive newly synthesized components from the secretory pathway.

Some ILVs can be secreted onto the cell surface through cleavage of a fragment of the LE and subsequent fusion of the fragment with the plasma membrane. Fusion of an LE with a lysosome generates a transient hybrid organelle, the endolysosome (EL), in which active degradation takes place.

Endolysosomes then mature into lysosomes, storage organelles for hydrolases and membrane components.
Rab family proteins of GTPases define many of the functional attributes of endosomes by regulating the biological activities of effector proteins, which include the ESCRT mechanism responsible for budding and sorting of endosomes and ILVs.



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The **chemical and physical requirements of membrane fusion that direct some viruses into ILVs**
Early and late endosomal membranes and the wide variety of structures they contain are made up of distinct lipid compositions. [99]
Early endosomes, like the plasma membrane from which they originate, are rich in cholesterol, phosphatidylserine (PS) and phosphatidylinositols[100] .

Lipid substructures that originate from early endosomes, including invaginations, tubules, and ILVs, initially have the same lipid composition (lipids may later undergo sorting).

As membranes progress along the endocytic pathway, their cholesterol content decreases. Cholesterol is replaced by ceramide in late endosomes and lysosomes, where it maintains membrane fluidity.[101]

The anionic, acid-resistant lipid BMP (bis-monoacylglycerophosphate) (also known as lysobisphosphatidic acid or LBPA) is enriched in the inner membranes of late endosomes and lysosomes including ILVs derived from them, but not in ILVs carried by early endosomes. [458]

Thus ILVs derived from early or late endosomes form distinct pools. BMP increases the fusogenicity of vesicle membranes at pH <6 and induces internal vesiculation into liposomes* similar to the multivesicular endosomes found in vivo. [102]

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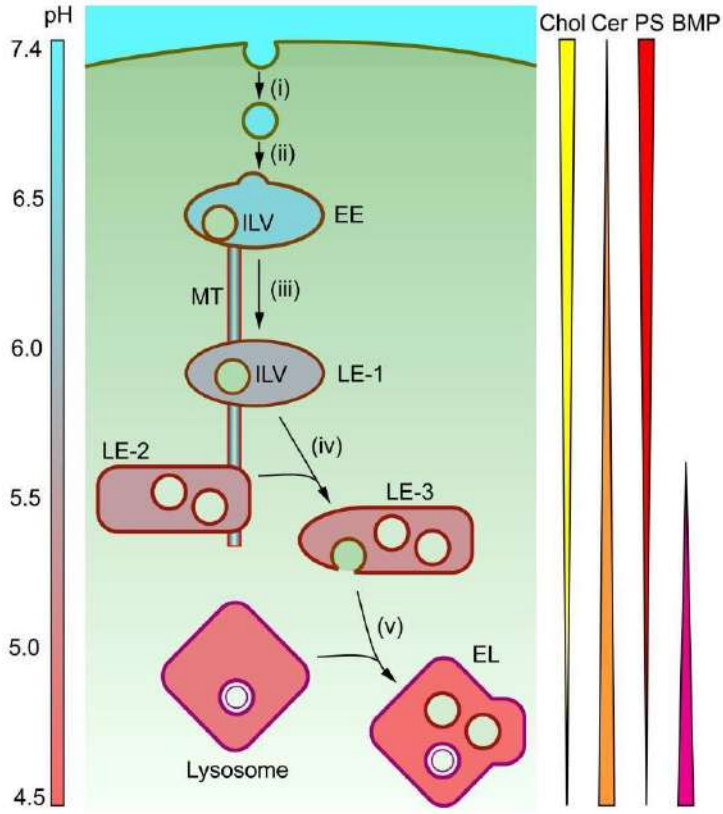
[102] Matsuo H, Chevallier J, Mayran N, et al.
Role of LBPA and Alix in multivesicular liposome formation and endosome organization.

Autoantibodies against BMP cause autoimmune diseases such as antiphospholipid syndrome and Niemann-Pick syndrome type C.
Anti-BMP antibodies cause dysfunction in the sorting and trafficking of late endosomes, [103]
suggesting a critical role for BMP in the function and dynamics of late endosomes.

***Liposomes**: are synthetic vesicles, hollow or containing an aqueous solution, bounded by a single or double layer of phospholipids. Liposomes range in size from 50 nm to 2.5 microns*



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4112135/
The lipid composition of the limiting and inner membranes of the compartments in the endocytic pathway. (i) Intraluminal endocytic vesicles (ILVs) bud inward from the plasma membrane, **(ii)** fuse with early endosomes (EEs), which are transported along microtubules (MTs), **(iii)** as they mature into late endosomes (LEs). **(iv)** Late endosomes fuse with each other and **(v)** with lysosomes to produce endolysosomes (EL). Cholesterol (Chol) decreases in concentration in the endocytic pathway, especially in the endosomal limiting membranes, and is replaced by ceramide (Cer), which is also present in the microdomains of the plasma membrane. Phosphatidylserine (PS) is mainly present in cytoplasmic plasma flaps and EE membranes. Bis (monoacylglycerol) phosphate (BMP) is specific to the inner membranes of late endosomal compartments. The distinct lipid compositions of the different endosomal membranes direct intracellular trafficking and virus entry.

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J Biol Chem. 2002;277(35):32157-32164. doi:10.1074/jbc.M202838200
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Late endosomal membranes rich in lysobisphosphatidic acid regulate cholesterol transport.
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Am J Med. 1989;86(4):391-399. doi:10.1016/0002-9343(89)90335-5
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Recent evidence suggests that these changing lipid compositions in endosomes, combined with the pH fusion threshold, may direct virus entry at specific points along the endocytic pathway.

ILV: transporters of viral genomes to the nuclear periphery

Early endosomes, containing ILVs, travel along microtubules to the perinuclear region, where they fuse with late endosomal membranes, transporting ILVs into the late endosomal lumen[104] .

Viral nucleocapsids contained in endosomal ILVs follow the same pathway. ESCRT proteins in late endosomes mediate ILV back-fusion to the limiting membrane[105] , but the cytosolic localization of ESCRT proteins implies that their effect on back-fusion may be indirect.

Retrofusion allows the recycling of various cellular components including major histocompatibility complex class II (MHC II), mannose-6-phosphate receptors and tetraspannin proteins, all enriched in ILV.

Reaching the perinuclear region is thought to be advantageous for those enveloped DNA viruses that have to replicate their genome in the nucleus such as herpesviruses[106] and for enveloped RNA viruses that assemble their replication complexes in perinuclear organelles such as the endoplasmic reticulum (ER) or ER-derived compartments, as in the case of flaviviruses[107] and HCV[108] .

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4112135/
Pathways into and out of cells of enveloped viruses involving intraluminal endosomal vesicles (ILVs). ILVs are formed in early endosomes, which are then transported along microtubules to late endosomes with which they fuse. Some endosomes are recycled to the cell surface, releasing whatever ILVs they contain into the extracellular environment as "exosomes." Various enveloped RNA viruses release their nucleocapsids (yellow) into ILVs, which may release the nucleocapsids into the perinuclear cytoplasm or transmit the nucleocapsids to another cell by exocytosis. ILV-dependent entry and exit depends on several membrane fission and fusion events: **(i)** budding and fission of an endocytic vesicle from the plasma membrane followed by fusion of the vesicle with an early endosome; **(ii)** fusion of the viral and endosomal ILV membranes; **(iii)** budding and fission of an ILV from the early endosomal membrane and **(iv)** subsequent back-fusion of the ILV to the late endosomal limiting membrane; and **(v)** fusion of an endosome to the plasma membrane to secrete ILV as exosomes.

[104] Huotari J, Helenius A.
Endosome maturation.
EMBO J. 2011;30(17):3481-3500. Published 2011 Aug 31. doi:10.1038/emboj.2011.286
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3181477/

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Molecular assemblies and membrane domains in multivesicular endosome dynamics.
Exp Cell Res. 2009;315(9):1567-1573. doi:10.1016/j.yexcr.2008.12.006
https://pubmed.ncbi.nlm.nih.gov/19133258/

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Endosome-to-cytosol transport of viral nucleocapsids.
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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3360589/

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Entry of herpesviruses into cells: the enigma variations.
Adv Exp Med Biol. 2013;790:178-195. doi:10.1007/978-1-4614-7651-1_10
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The endoplasmic reticulum provides the membrane platform for biogenesis of the flavivirus replication complex.
J Virol. 2010;84(20):10438-10447. doi:10.1128/JVI.00986-10
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2950591/

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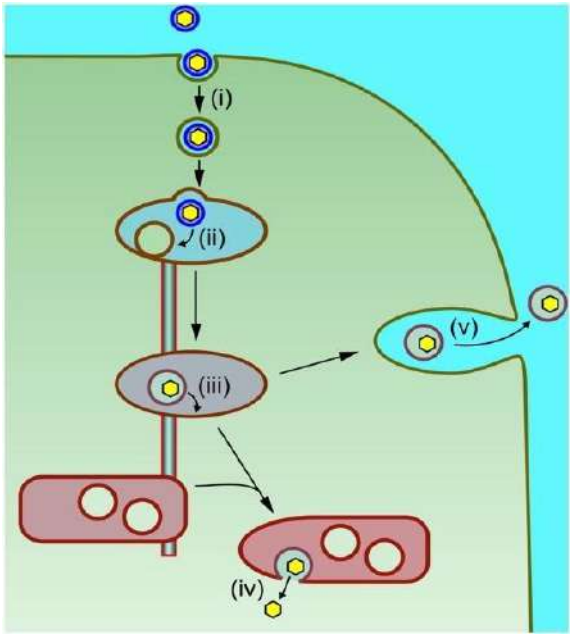
Three-dimensional architecture and biogenesis of membrane structures associated with hepatitis C virus replication.
PLoS Pathog. 2012;8(12):e1003056. doi:10.1371/journal.ppat.1003056
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3516559/



ILV as exosomes: another route to infection

In addition to serving as transport vehicles to the nuclear periphery, recent evidence suggests that ILVs provide a pathway for cell-to-cell transmission.

When late endosomes mature, they mainly fuse with lysosomal compartments, however, a fraction of late endosomes can be induced to fuse with the plasma membrane by releasing endosomal ILVs into the extracellular environment known, as already seen, as exosomes.

Upon release into the bloodstream, lymphatic system, or neural synapse, exosomes can be endocytosed by another cell. If exosomes undergo retrofusion with a late endosomal membrane, their cargo can provide important signals to the cell about the status of surrounding cells. [109]
Although exosomes usually contain cytoplasm from the cell from which they originated, VSVs and flaviviruses can release their genomes directly into endosomal ILVs[110] .
If such ILVs are secreted as exosomes and thus enter the endocytic pathway of a different cell, the viral nucleocapsid within the vesicles could, in principle, be released into the cytoplasm of a recipient cell permissive to infection thus providing a mechanism for intercellular transmission of viral infection.

Indeed, just such a mechanism has recently been reported for HCV. Exosomes isolated from HCV-infected human hepatoma cells were shown to contain full-length viral RNA, along with core and envelope proteins[111] , and were capable of infecting naïve cells.

[109] Simons M, Raposo G.
Exosomes--vesicular carriers for intercellular communication.
Curr Opin Cell Biol. 2009;21(4):575-581. doi:10.1016/j.ceb.2009.03.007
https://pubmed.ncbi.nlm.nih.gov/19442504/

[110] Nour AM, Li Y, Wolenski J, Modis Y.
Viral membrane fusion and nucleocapsid delivery into the cytoplasm are distinct events in some flaviviruses.
PLoS Pathog. 2013;9(9):e1003585. doi:10.1371/journal.ppat.1003585
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Regulation of hepatitis C virus secretion by the Hrs-dependent exosomal pathway.
Virology. 2012;422(2):377-385. doi:10.1016/j.virol.2011.11.009
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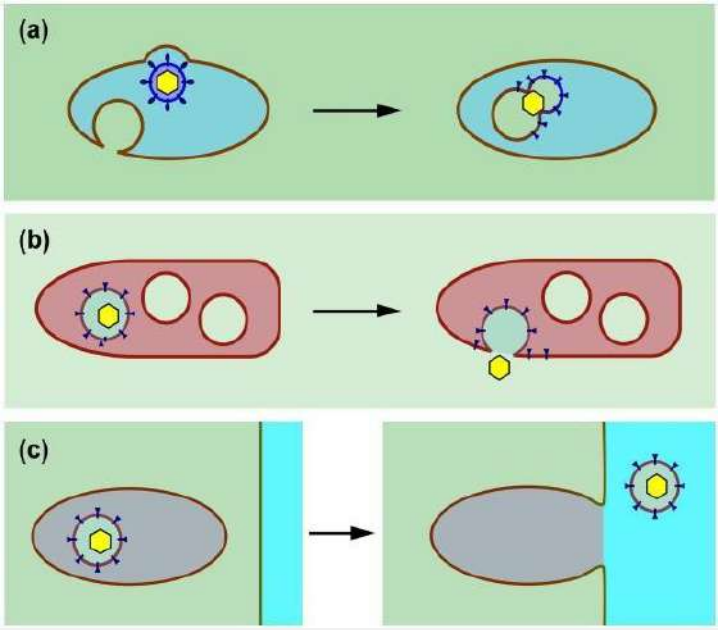
Notably, exosomes containing HCV RNA were partially resistant to antibody neutralization, suggesting that HCV might use transmission via exosomes as a **mechanism of immune evasion**.[112]

In this case, the ESCRT-0 component Hrs is critical for HCV nucleocapsid release through exosomes. Hrs depletion not only reduces exosome production but also inhibits HCV replication and cell-to-cell transmission, suggesting that exosomes are a major pathway for HCV transmission.

Thus, it is possible to state that cell-to-cell transmission of microbial agents encapsulated in exosomes may be a general and important mechanism of pathogenesis that occurs to some extent with all viruses that release their nucleocapsids into endosomal ILVs.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4112135/
Key events in membrane fusion in the transport of viral components by intraluminal endosomal vesicles (ILVs). **(a)** Virus-ILV membrane fusion. The viral nucleocapsid (yellow) is released within the ILV. Viral glycoproteins are released into the ILV membrane, with the glycosylated ectodomains facing the endosome lumen. This fusion event is catalyzed by a conformational change in the viral envelope protein (dark blue). **(b)** Back-fusion of ILVs of the late endosomal limiting membrane. The fusion event transports ILV content and viral nucleocapsid into the cytoplasm. Specific phospholipids and cellular effectors are required for retro-fusion. **(c)** Exocytic fusion of the endosome to the plasma membrane. Any ILV within the endosomal compartment is released into the extracellular environment after fusion. Exosomes released in this way can infect adjacent or distant cells or can be transmitted to a dendritic cell or T lymphocyte via an immunological synapse.

Below are two more figures that further detail the mechanism of formation of extracellular vesicles incorporating RNA (including viral RNA) and their entry into surrounding cells [113]

[112] Ramakrishnaiah V, Thumann C, Fofana I, 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):13109-13113. doi:10.1073/pnas.1221899110
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3740869/

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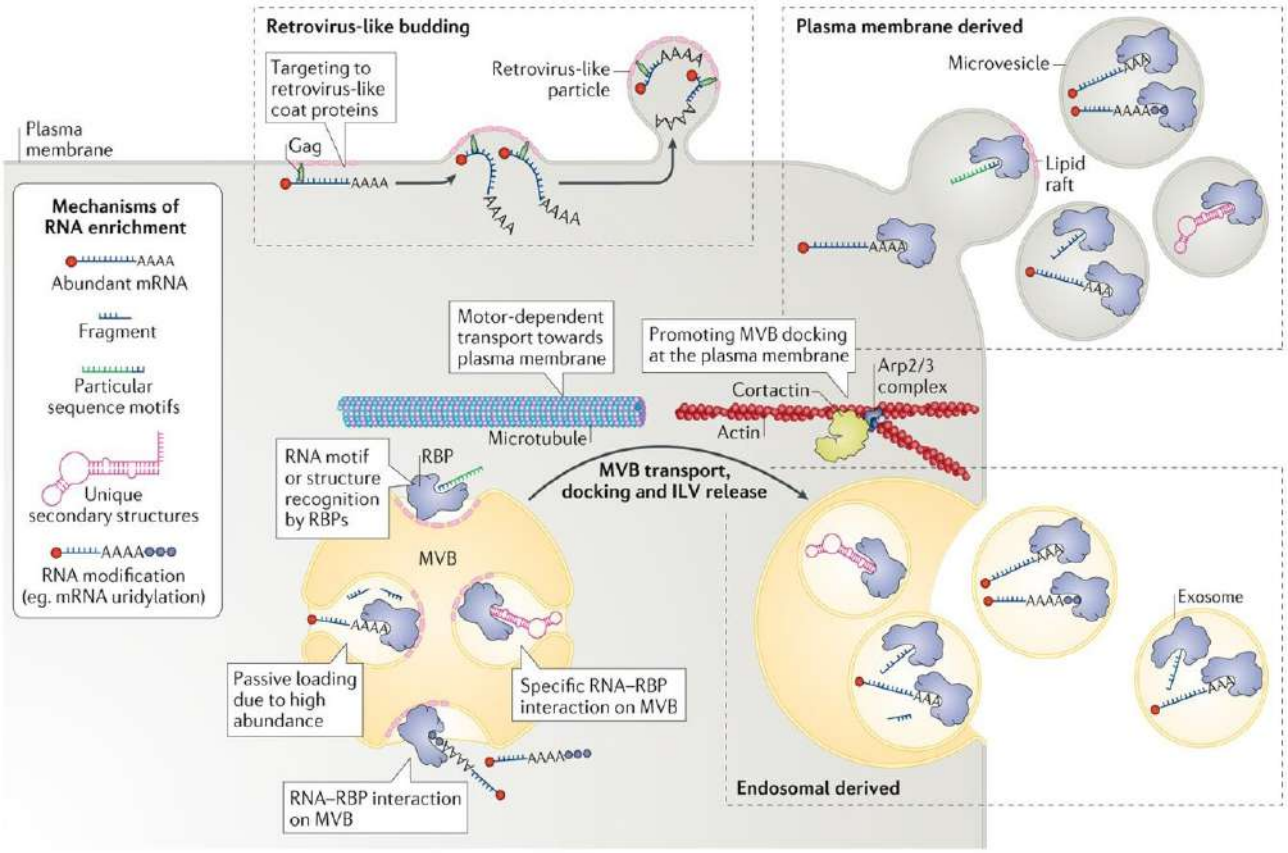
RNA delivery by extracellular vesicles in mammalian cells and its applications
[published online ahead of print, 2020 May 26]. Nat Rev Mol Cell Biol. 2020;1-22. doi:10.1038/s41580-020-0251-y
https://www.nature.com/articles/s41580-020-0251-y.pdf



https://www.nature.com/articles/s41580-020-0251-y.pdf

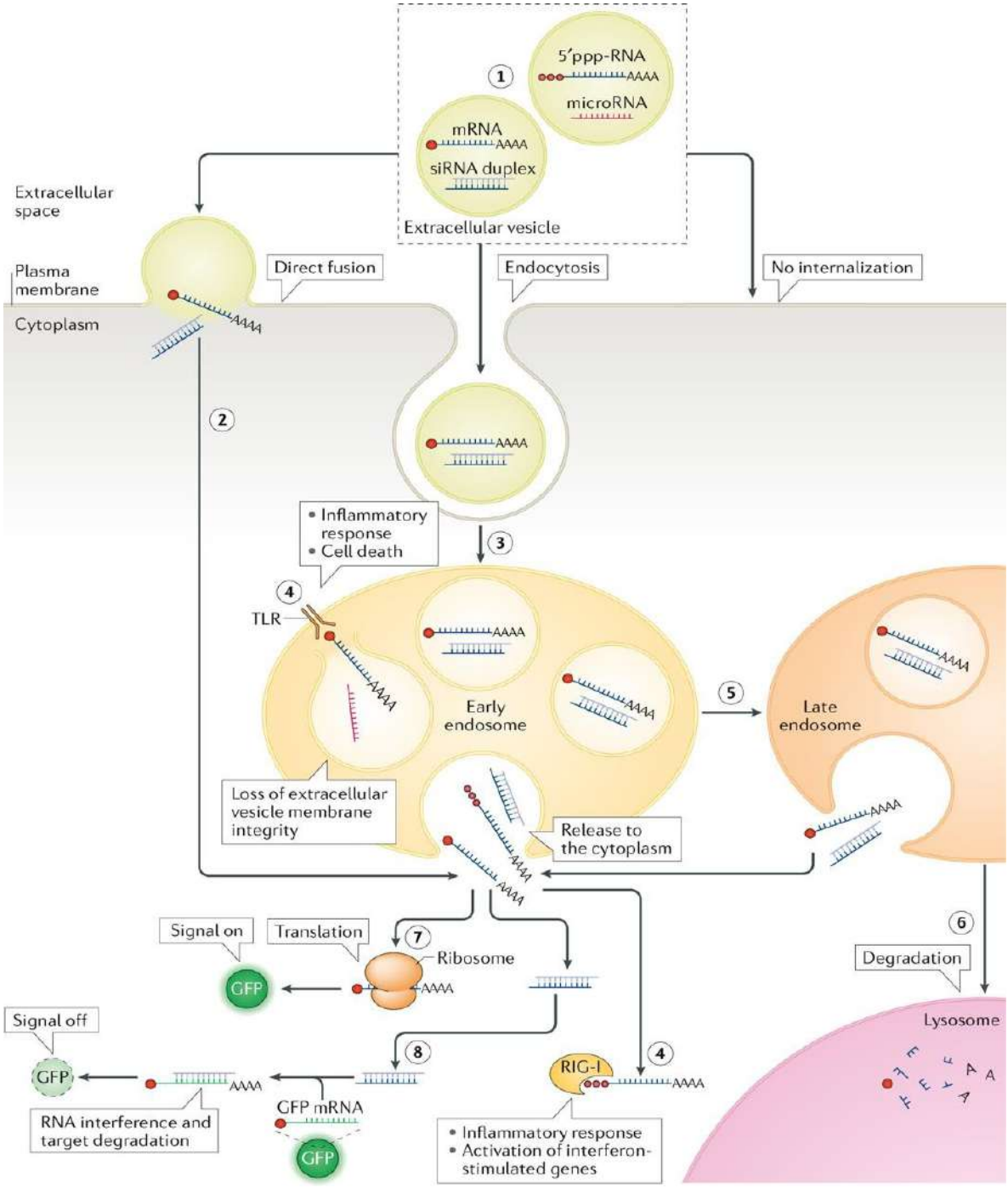
Incorporation of RNA into extracellular vesicles and their release into the extracellular space.

A variety of different RNA species can be incorporated into extracellular vesicles. Several ways have been proposed for the incorporation of (specific) RNAs into extracellular vesicles. First, RNAs can be anchored to the plasma membrane and released as microvesicles. They can also be anchored to the endosomal compartment and incorporated into the intraluminal vesicles (ILVs) of the multivesicular body (MVB), which can then be anchored to the plasma membrane, where they fuse to release ILVs as exosomes. Both of these modes of biogenesis share many factors, and thus vesicle type and vesicle origin are generally difficult to ascertain and control. Membrane microdomains (lipid rafts) have been strongly associated with extracellular vesicle release. In addition, cytoskeletal components are implicated in the biogenesis of extracellular vesicles, particularly for exosomes, which are transported via microtubules, and their docking to the plasma membrane is supported by branched actin filaments generated by Arp2 / 3 stabilized by the actin clustering activity of cortactin . Cortical actin remodeling is also an important event in membrane formation during microvesicle release (not shown). RNA loading into extracellular vesicles can occur through multiple pathways: passively due to the abundance of RNA in the cytosol; by recognition through a series of RNA-binding proteins (RBPs), such as Argonaute, annexin A2, MVP, heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1), YBX1, SYNCRIP and lupus La protein, which bind a particular sequence motif in RNA or recognize unique secondary RNA structures; and through specific modifications, such as uridylation. RNA incorporation into extracellular vesicles may also be promoted by its recognition by retroviral coating proteins such as Gag (and their silent copies found in animal genomes), which effectively anchor RNAs to the plasma membrane (or MVB membrane; not shown), resulting in the release of virus-like particles.

https://www.nature.com/articles/s41580-020-0251-y.pdf

Interaction of RNA incorporated into the extracellular vesicle with recipient cells and its functional delivery.

After encountering the recipient cell, the extracellular vesicle is typically bound to its surface via cell surface receptors (although extracellular vesicles can also be incorporated from the environment in a process known as macropinocytosis; not shown). After establishing an interaction with the cell surface, the vesicle may remain bound to the surface or may be internalized **(1)**. One possible means of internalization is direct fusion with the plasma membrane **(2)**, but the most common mechanism of internalization involves endocytosis, whereby extracellular vesicles are carried to endosomes **(3)**. In the endosome, the RNA content could be released into the luminal space (if the membrane integrity of the extracellular vesicle is disrupted) or it could be released into the cytoplasm (of note, the frequency of these events is low, and endosomal leakage of extracellular vesicle cargo is currently a major bottleneck in the delivery of functional RNA cargo by extracellular vesicles). In both cases, RNAs can be recognized by pattern recognition receptors, such as Toll-like receptors (TLRs) and RIG-I or NOD-like receptors that reside in the endosome and cytoplasm, respectively, augmenting innate immune response signaling **(4)** . Early endosomes will gradually transform into late endosomes with progressive internal acidification and possible RNA release (stimulated by decreasing pH) **(5)** . Further along the endocytic pathway, endosomes will mature into lysosomes, in which cargo that has not been released into the cytoplasm will be degraded **(6)**. The RNA cargo that reaches the cytoplasm can elicit its functional effect. For example, mRNA can be translated into a functional protein, such as green fluorescent protein (GFP), and the resulting fluorescence can act as a reporter of the functional release of extracellular vesicle cargo **(7)**. When a small load of interfering RNA (siRNA) is released into the cytoplasm, it can inhibit the translation of specific transcripts, such as those coding for fluorescent proteins. In this case, the disappearance of fluorescence will report functional release of the extracellular vesicle cargo **(8)**. Extracellular vesicles can be tracked along this pathway with the use of different reagents or labeling strategies (Table 3). 5'ppp-RNA, 5'- triphosphorylated RNA



Published data indicate that EVs share with viruses an important function that has played a critical role in evolution, namely the transport of bioactive material from one cell to another[114] .

[114] Meckes DG Jr, Raab-Traub N.
Microvesicles and viral infection.
J Virol. 2011;85(24):12844-12854. doi:10.1128/JVI.05853-11
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3233125/

Meckes DG Jr.
Exosomal communication goes viral.
J Virol. 2015;89(10):5200-5203. doi:10.1128/JVI.02470-14
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4442506/

Ridder K, Keller S, Dams M, et al.

Specific combinations of lipids and proteins, particularly tetraspanins[115] , in the membrane of EVs may mediate specific targeting of vesicles to recipient cells and may determine the ability of vesicles to fuse with cell membranes.

These molecules, as well as the genetic material and proteins encapsulated in EVs (e.g., transcription factors and cytokines), constitute molecular signals that can influence the function of recipient cells.

It is exactly this characteristic of being multicomponent transport units that EVs share with enveloped viruses.

In some cases, EVs can also deliver genetic material to target cells. After the initial discovery that EVs carry protein-coding mRNAs and small noncoding RNAs involved in the regulation of gene expression [microRNAs (miRNAs)][116] .

Several groups have demonstrated alterations in gene expression of target cells due to the transfer of such RNAs via EV. [117]

In addition to miRNAs, EVs also contain a wide variety of other small noncoding RNAs, such as fragments of protein coding regions and repeated sequences, which could also act as regulatory RNAs by influencing gene expression. [118]

Although most of the genetic material enclosed in virions encodes for viral proteins essential for virus replication, viruses and EVs are united in their ability to transfer RNAs that can activate pathogen recognition receptors (PRRs) in target cells. Indeed, fragments of the viral genome, as well as small viral RNAs, such as those encoded by EBV and some host cell miRNAs, activate PRRs in target cells.

Extracellular vesicle-mediated transfer of genetic information between the hematopoietic system and the brain in response to inflammation [published correction appears in PLoS Biol. 2018 Mar 12;16(3):e1002623]. PLoS Biol. 2014;12(6):e1001874. Published 2014 Jun 3. doi:10.1371/journal.pbio.1001874
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4043485/

Zomer A, Maynard C, Verweij FJ, et al.
In Vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior.
Cell. 2015;161(5):1046-1057. doi:10.1016/j.cell.2015.04.042
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4448148/

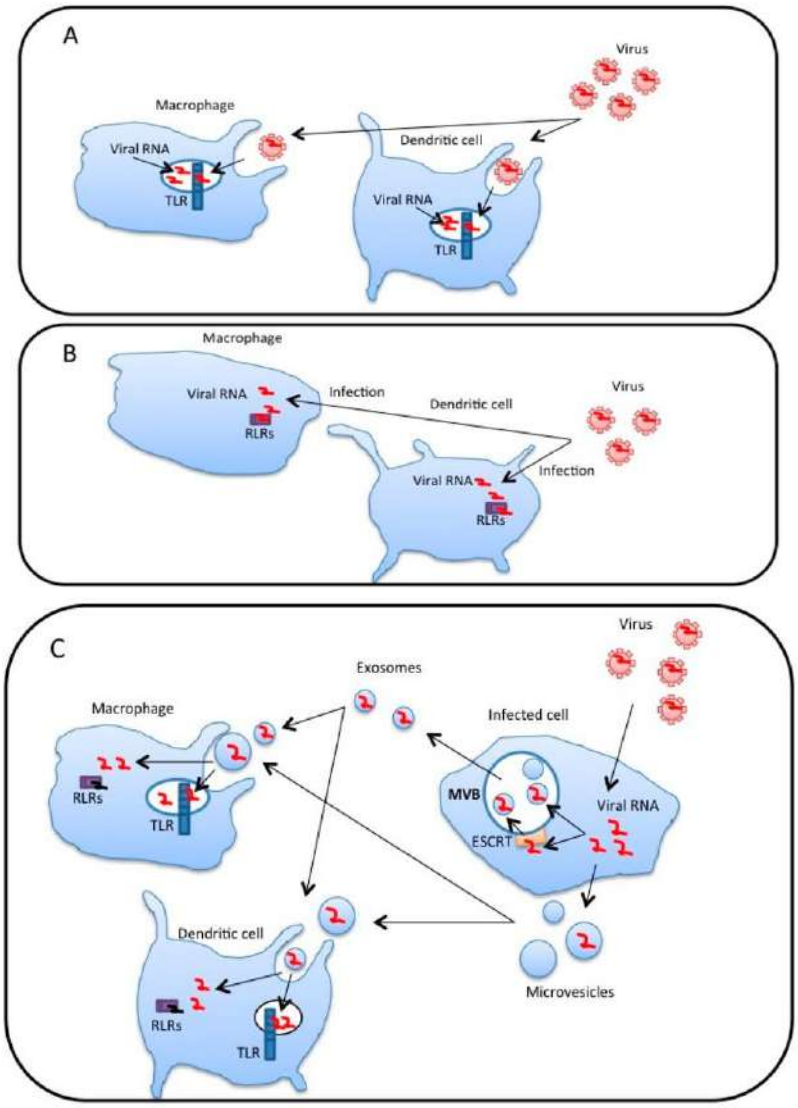
[115] Andreu Z, Yáñez-Mó M.
Tetraspanins in extracellular vesicle formation and function.
Front Immunol. 2014;5:442. Published 2014 Sep 16. doi:10.3389/fimmu.2014.00442
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4165315/

[116] Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO.
Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells.
Nat Cell Biol. 2007;9(6):654-659. doi:10.1038/ncb1596
https://pubmed.ncbi.nlm.nih.gov/17486113/

[117] Yáñez-Mó M, Siljander PR, Andreu Z, et al.
Biological properties of extracellular vesicles and their physiological functions.
J Extracell Vesicles. 2015;4:27066. Published 2015 May 14. doi:10.3402/jev.v4.27066
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4433489/

[118] Nolte-'t Hoen EN, Buermans HP, Waasdorp M, Stoorvogel W, Wauben MH, 't Hoen PA.
Deep sequencing of RNA from immune cell-derived vesicles uncovers the selective incorporation of small non-coding RNA biotypes with potential regulatory functions.
Nucleic Acids Res. 2012;40(18):9272-9285. doi:10.1093/nar/gks658
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3467056/

Although activation of the PRR system results in complex responses, in some cases it can induce an increased state of activation of target cells[119] .



https://www.mdpi.com/1422-0067/18/3/666/htm [120]
Recognition of viral RNA by pattern recognition receptors. **(A**) Dendritic cells and macrophages internalize viral particles through phagocytosis. Viral RNAs are internalized in endosomes, where Toll-like receptors (TLRs) recognize viral RNA and activate the signal to induce innate immune responses; **(B)** Some types of viruses infect dendritic cells and macrophages. Viral RNA is released into the cytoplasm. Cytoplasmic sensors of viral RNA, RIG-I-like receptors (RLRs), detect viral RNA in the cytoplasm and activate innate immune responses; **(C)** In virus-infected cells, viral RNAs are sorted into exosomes and microvesicles via endosomal sorting complexes

[119] Chen X, Liang H, Zhang J, Zen K, Zhang CY.
microRNAs are ligands of Toll-like receptors.
ANN. 2013;19(6):737-739. doi:10.1261/rna.036319.112
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3683908/

Dreux M, Garaigorta U, Boyd B, et al.
Short-range exosomal transfer of viral RNA from infected cells to plasmacytoid dendritic cells triggers innate immunity.
Cell Host Microbe. 2012;12(4):558-570. doi:10.1016/j.chom.2012.08.010
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3479672/

Baglio SR, van Eijndhoven MA, Koppers-Lalic D, et al.
Sensing of latent EBV infection through exosomal transfer of 5'pppRNA.
Proc Natl Acad Sci U S A. 2016;113(5):E587-E596. doi:10.1073/pnas.1518130113
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4747727/

[120] Kouwaki T, Okamoto M, Tsukamoto H, Fukushima Y, Oshiumi H.
Extracellular Vesicles Deliver Host and Virus RNA and Regulate Innate Immune Response.
Int J Mol Sci. 2017;18(3):666. Published 2017 Mar 20. doi:10.3390/ijms18030666
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5372678/

necessary for transport (ESCRT) or unknown factors. Dendritic cells and macrophages internalize extracellular vesicles (EVs) containing viral RNAs, which are released into endosomes and are recognized by TLRs, resulting in innate immune responses. Viral RNAs released into the cytoplasm are recognized by RLRs.

Most of the described EV-mediated effects on the function of other cells are limited to in vitro systems or occur within the same organism.

While viruses transfer between organisms and from cell to cell within an organism, the functional transfer of EVs from one individual to another, unlike EVs derived from breast or seminal milk, has not been demonstrated. [121]

Since EVs are produced by virtually all cells, probably every viral preparation is actually a mixture of virions and EVs.

To study their respective functions, it is necessary to separate EVs and virions. This is very difficult with some viruses, such as retroviruses, because both EVs and retroviruses are of comparable size (EVs range from 50 to 100 nm, virions ~ 100 nm) and density (EVs: 1.13-1.18 g/L; most retroviruses: 1.16-1.18 g/L).
Therefore, density gradients, which are often used to separate EVs from contaminating protein aggregates based on differences in buoyancy densities[122] , are not always reliable for separating EVs from viral particles.

Similar technical obstacles were encountered in the early stages of retrovirus research, when there were longstanding disagreements and controversies about cancer-causing oncoviral particles and their dependence on competent helper viruses for propagation. [123]
In those early days, electron microscopists observed that the ultracentrifuged viruses co-precipitated with other particles enclosed in a 100-nm-sized membrane.

Unless more specifically defined, it is currently **virtually impossible to specifically separate and identify EVs carrying viral proteins**, host proteins and viral genomic elements from viral particles with envelopes carrying the same molecules.

However, high-throughput methods for analyzing single nanometer particles may facilitate the discrimination of different particles in the EV-virus continuum in the future.
For example, recent developments in flow cytometry-based techniques have opened up the possibility of quantifying and characterizing particles as small as 50-200 nm.

[121] Vojtech L, Woo S, Hughes S, et al.
Exosomes in human semen carry a distinctive repertoire of small non-coding RNAs with potential regulatory functions.
Nucleic Acids Res. 2014;42(11):7290-7304. doi:10.1093/nar/gku347
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4066774/

Irmak MK, Oztas Y, Oztas E.
Integration of maternal genome into the neonate genome through breast milk mRNA transcripts and reverse transcriptase.
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[122] Raposo G, Nijman HW, Stoorvogel W, et al.
B lymphocytes secrete antigen-presenting vesicles.
J Exp Med. 1996;183(3):1161-1172. doi:10.1084/jem.183.3.1161
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2192324/

[123] Maeda N, Fan H, Yoshikai Y.
Oncogenesis by retroviruses: old and new paradigms.
Rev Med Virol. 2008;18(6):387-405. doi:10.1002/rmv.592
https://onlinelibrary.wiley.com/doi/abs/10.1002/rmv.592

In vivo, EVs can interact with viruses and each other, either directly or through modulation of host responses, thus participating in a "War and Peace" between virus and host. [124]

Some viruses induce infected cells to release **modified EVs that facilitate infection** by increasing the pool of susceptible target cells (e.g., by increasing the number of activated cells) or their susceptibility to viral infection or by acting as decoys that absorb antiviral antibodies, thereby impairing antiviral immunity.

Conversely, **EVs carrying viral proteins can also be useful to the host, for example,** by providing dendritic cells with viral antigens to facilitate the initiation of adaptive immune responses.

Hypothetically, the ability of EVs to regulate permissive cell lifespan and modify antiviral immune responses may give additional flexibility to the host in responding to viral infection. Therefore, EVs formed during viral infection may play a pro- or anti-viral role.

At present, it is not known whether the different functions attributed to virus-induced EVs can be partly explained by differences in the purity of EV populations used in various studies.
Thus, a general understanding of the parameters that determine the net effect of EVs on viral infections is still lacking. [125]

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The biology of extracellular vesicles: The known unknowns.
PLoS Biol. 2019;17(7):e3000363. Published 2019 Jul 18. doi:10.1371/journal.pbio.3000363
https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.3000363

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Exosomes in Pathogen Infections: A Bridge to Deliver Molecules and Link Functions.
Front Immunol. 2018;9:90. Published 2018 Feb 12. doi:10.3389/fimmu.2018.00090
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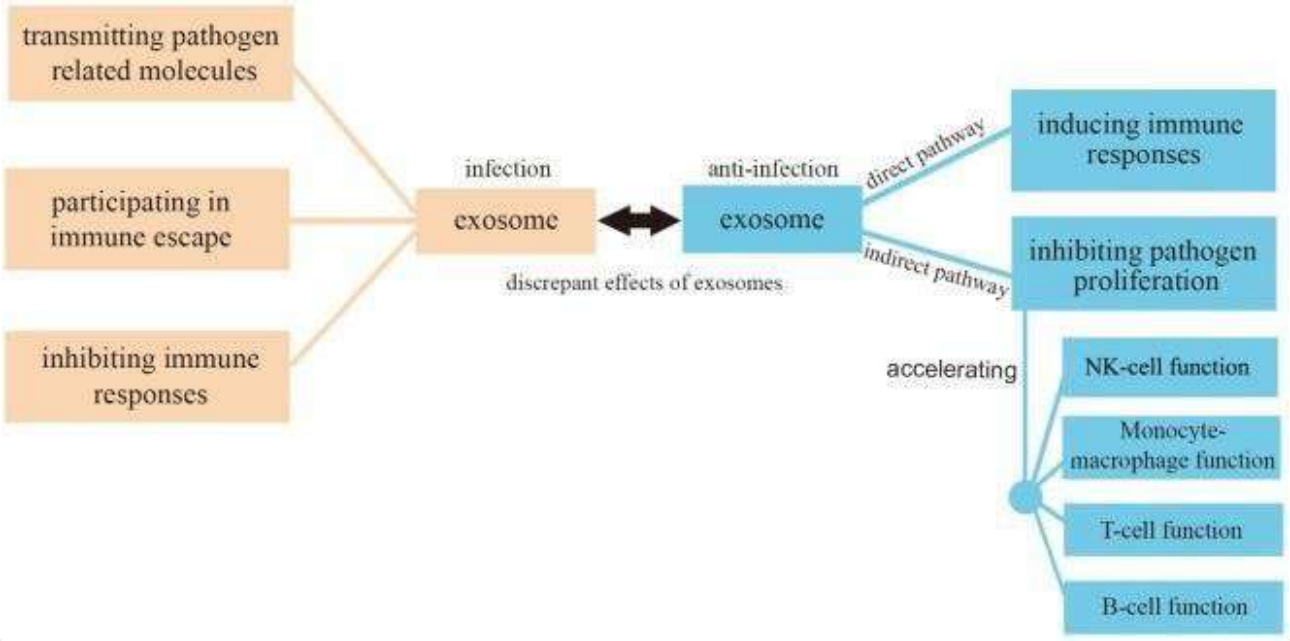
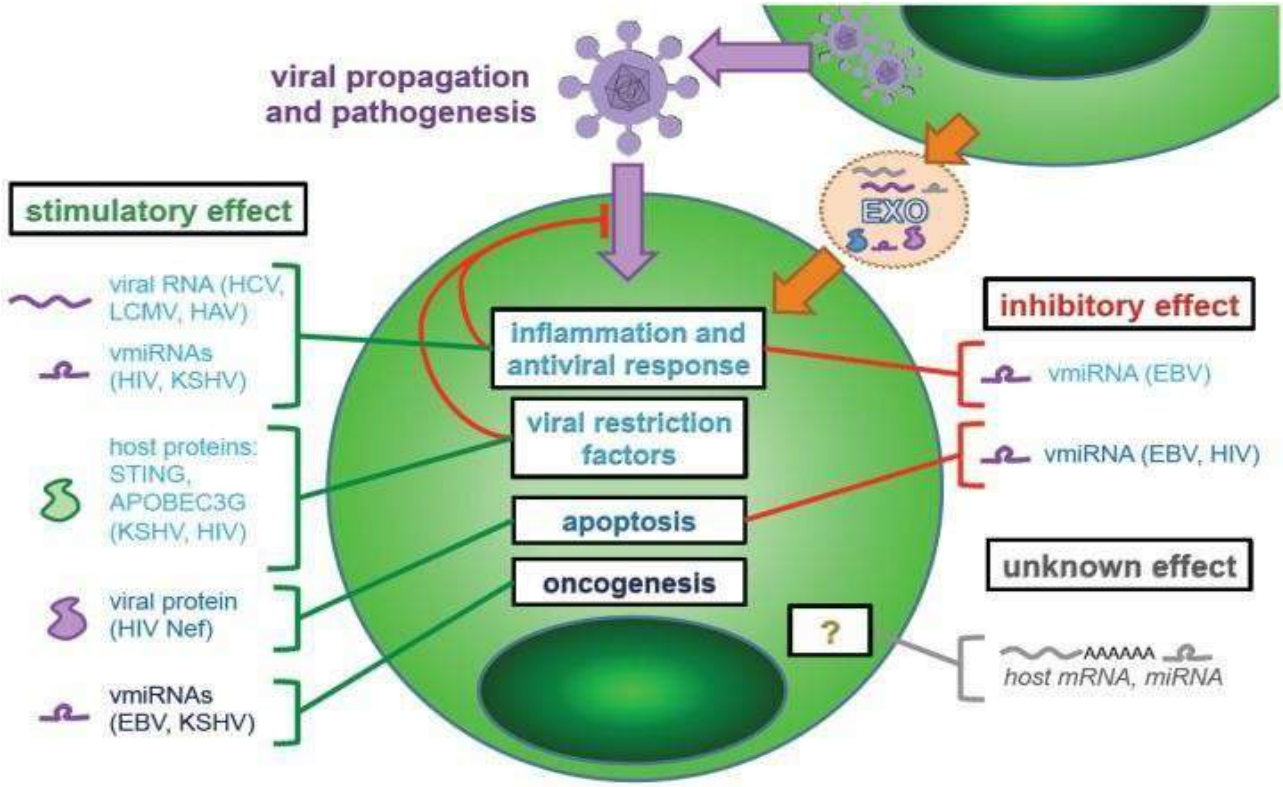
Assil S, Webster B, Dreux M.
Regulation of the Host Antiviral State by Intercellular Communications.
Viruses. 2015;7(8):4707-4733. Published 2015 Aug 19. doi:10.3390/v7082840
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4576201/



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5816030/pdf/fimmu-09-00090.pdf
Exosomes in infection and anti-infection. Exosomes participate in both infection and anti-infection processes ranging from pathogen infection to regulation of immune responses.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4576201/
Exosome-mediated transfer of viral and host components from infected cells to neighboring cells. After infection, the content of exosomes produced by infected cells changes considerably, regarding host proteins, RNAs, and the incorporation of host-derived micro RNAs (miRNAs) or viral genome (viral RNA, vmiRNA). Host- and virus-derived species present in exosomes are transferred to neighboring cells, where they can activate or inhibit a number of different host signaling pathways. These exosome-mediated communications have a broad effect on the functionality of cells in the vicinity of infected cells, including cell types that are refractory to productive viral infection. The schematic shows a selection of different species transferred by exosomes known to influence the course of viral infection and/or pathogenesis targeted to inflammatory, antiviral (via viral restriction factors), apoptotic, and oncogenic pathways. Significant changes occur in the content of host-derived mRNA and miRNA that are encapsulated in exosomes following viral infection, however, to date, the effect of these different RNA species on neighboring cells during viral infection is not fully understood.

Infections in pregnancy and exosomes

A pregnant woman's immune system, far from being in a state of rest, undergoes several changes throughout the gestation period.
Over the years, studies in the field of reproductive biology have approached the multiple interactions at the maternal-fetal interface, which can result in normal or pathological pregnancies. [126]

Initially viewed as a threat to successful pregnancy, inflammation is now recognized as an essential step in the establishment and maintenance of pregnancy, although this immune response must be finely regulated.

Exacerbated inflammation can cause miscarriage and other pregnancy complications, but the absence of inflammation precludes effective implantation due to inadequate tissue remodeling.
During pregnancy there is a transition to a less inflammatory environment, allowing fetal development to occur. Finally, by the end of the third trimester, near delivery, a series of physiological changes occur and a pro-inflammatory environment is again predominant.

In addition, when implantation takes place, paternal antigens are expressed, and the maternal immune system encounters two challenges: to avoid immune activation and rejection of the developing fetus while simultaneously inducing immune activation to avoid pathogen infection.

Fetal tolerance is a complex process that occurs throughout the gestation period and involves the modulation of local immune responses toward an anti-inflammatory profile.

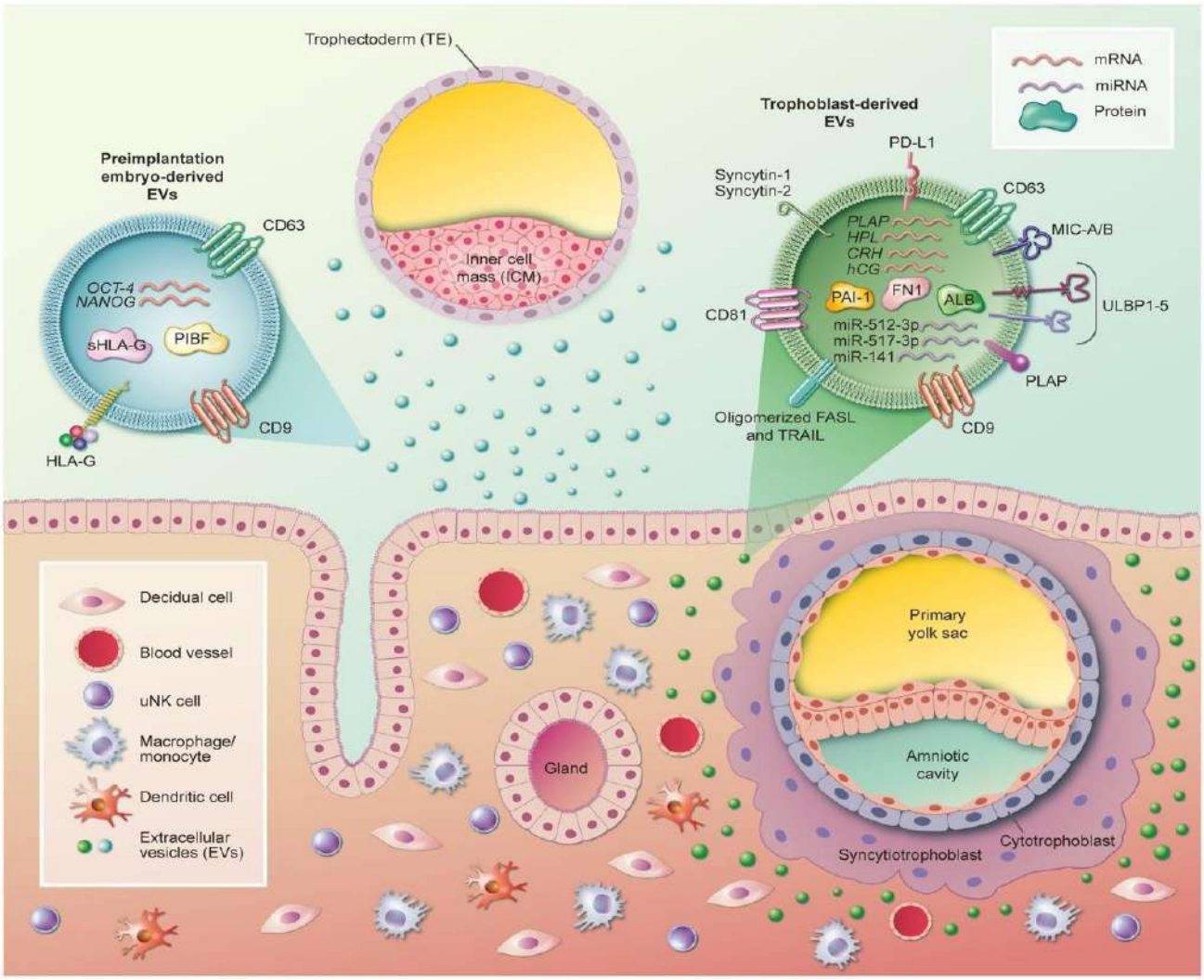
Interestingly, the placenta is a vigorous producer of exosomes and extracellular vesicles that have been described as key players in the regulation of maternal immune responses.

The syncytiotrophoblast has important physical and molecular mechanisms that prevent microbes from bypassing the placenta and reaching the fetus, and these features range from dense, branching microvilli on the apical surface to soluble receptors carried by exosomes. [127]

https://onlinelibrary.wiley.com/doi/full/10.1111/cei.13304
Extracellular vesicle loads released from the preimplanted embryo and trophoblasts with reported effects on local maternal immune cells. PIBF = progesterone-induced blocking factor; ALB = albumin; ULBP1-5 = UL-16 binding protein 1-5; PLAP = placental alkaline phosphatase; PD-L1 = programmed death ligand 1; MIC-A/B = major histocompatibility complex (MHC) class I related protein; PAI-1 = plasminogen activator inhibitor-1; hCG = human chorionic gonadotropin; CRH = corticotropin-releasing hormone; HPL = human placental lactogen; FN1 = fibronectin-1.

[126] Kaminski VL, Ellwanger JH, Chies JAB.
Extracellular vesicles in host-pathogen interactions and immune regulation - exosomes as emerging actors in the immunological theater of pregnancy.
Heliyon. 2019;5(8):e02355. Published 2019 Aug 31. doi:10.1016/j.heliyon.2019.e02355
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6771614/

[127] Giacomini E, Alleva E, Fornelli G, et al.
Embryonic extracellular vesicles as informers to the immune cells at the maternal-fetal interface.
Clin Exp Immunol. 2019;198(1):15-23. doi:10.1111/cei.13304
https://onlinelibrary.wiley.com/doi/full/10.1111/cei.13304



It is worth mentioning that human placenta is unique among normal tissues for having the highest levels of human endogenous retrovirus (HERV) expression.
Endogenous retroviruses are evolutionary fossils inherited in a Mendelian manner and are derived from retroviruses that anciently infected germline cells. A HERV envelope (env) gene belonging to the HERV-W family encodes a protein expressed in the syncytiotrophoblast called as syncytin.

Syncytin-1 has been shown to be involved in human trophoblast cell fusion and is a key factor in the regulation of syncytization during placental formation.

The presence of syncytin-1 in placental exosomes provides a mechanism by which syncytin-1 reaches and interacts with target cells in the maternal immune system and represents a novel mechanism of retroviral-mediated endogenous immunosuppression that may be relevant to maternal immune tolerance.[128]

[128] Toulouse JM, Schjenken JE, Clifton VL, et al.
The endogenous retroviral envelope protein syncytin-1 inhibits LPS/PHA-stimulated cytokine responses in human blood and is sorted into placental exosomes.
Placenta. 2012;33(11):933-941. doi:10.1016/j.placenta.2012.08.004
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Recent studies have revealed the importance of exosomes for successful pregnancy as partners of the immune system at the maternal-fetal interface. [129]

The trafficking of molecules, cells, and pathogens between mother and fetus during pregnancy is currently seen as a natural phenomenon. In this context, exosomes may be important mediators of transplacental infections.

In addition, the immunosuppression induced by seminal exosomes[130] may help explain the persistence of the many viruses in semen.

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[129] Lucia Mincheva-Nilsson
Placental exosome-mediated immune protection of the fetus: feeling groovy in a cloud of exosomes,
Expert Review of Obstetrics & Gynecology, (2010) 5:5, 619-634, DOI: 10.1586/eog.10.43
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Review: Fetal-maternal communication via extracellular vesicles - Implications for complications of pregnancies.
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Placental Exosomes During Gestation: Liquid Biopsies Carrying Signals for the Regulation of Human Parturition.
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Human placenta expresses and secretes NKG2D ligands via exosomes that down-modulate the cognate receptor expression: evidence for immunosuppressive function.
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Stenqvist AC, Nagaeva O, Baranov V, Mincheva-Nilsson L.
Exosomes secreted by human placenta carry functional Fas ligand and TRAIL molecules and convey apoptosis in activated immune cells, suggesting exosome-mediated immune privilege of the fetus.
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Macrophage Exosomes Induce Placental Inflammatory Cytokines: A Novel Mode of Maternal-Placental Messaging.
Traffic. 2016;17(2):168-178. doi:10.1111/tra.12352
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4738478/

Zhang J, Li H, Fan B, Xu W, Zhang X.
Extracellular vesicles in normal pregnancy and pregnancy-related diseases.
J Cell Mol Med. 2020;24(8):4377-4388. doi:10.1111/jcmm.15144
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7176865/

Salomon C, Rice GE.
Role of Exosomes in Placental Homeostasis and Pregnancy Disorders.
Prog Mol Biol Transl Sci. 2017;145:163-179. doi:10.1016/bs.pmbts.2016.12.006
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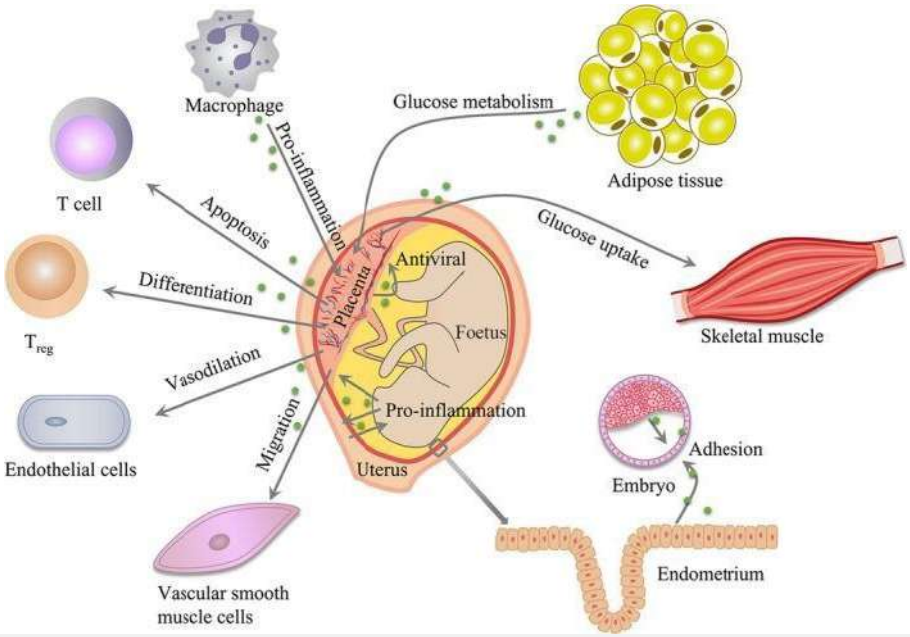
[130] Vojtech L, Woo S, Hughes S, et al.
Exosomes in human semen carry a distinctive repertoire of small non-coding RNAs with potential regulatory functions.
Nucleic Acids Res. 2014;42(11):7290-7304. doi:10.1093/nar/gku347
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4066774/



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7176865/

Effects of EVs in normal pregnancy. EVs mediate maternal-fetal communications in normal pregnancy. EVs contribute to embryo implantation by promoting trophoblast adhesion. The placenta can interact with immune cells via EVs to balance immune activation and suppression during gestation. EVs can activate endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) to promote angiogenesis. EVs can accelerate glucose metabolism in the placenta and skeletal muscles. In addition, maturation inflammation signals in EVs may prepare the uterus for delivery

Madison MN, Roller RJ, Okeoma CM.
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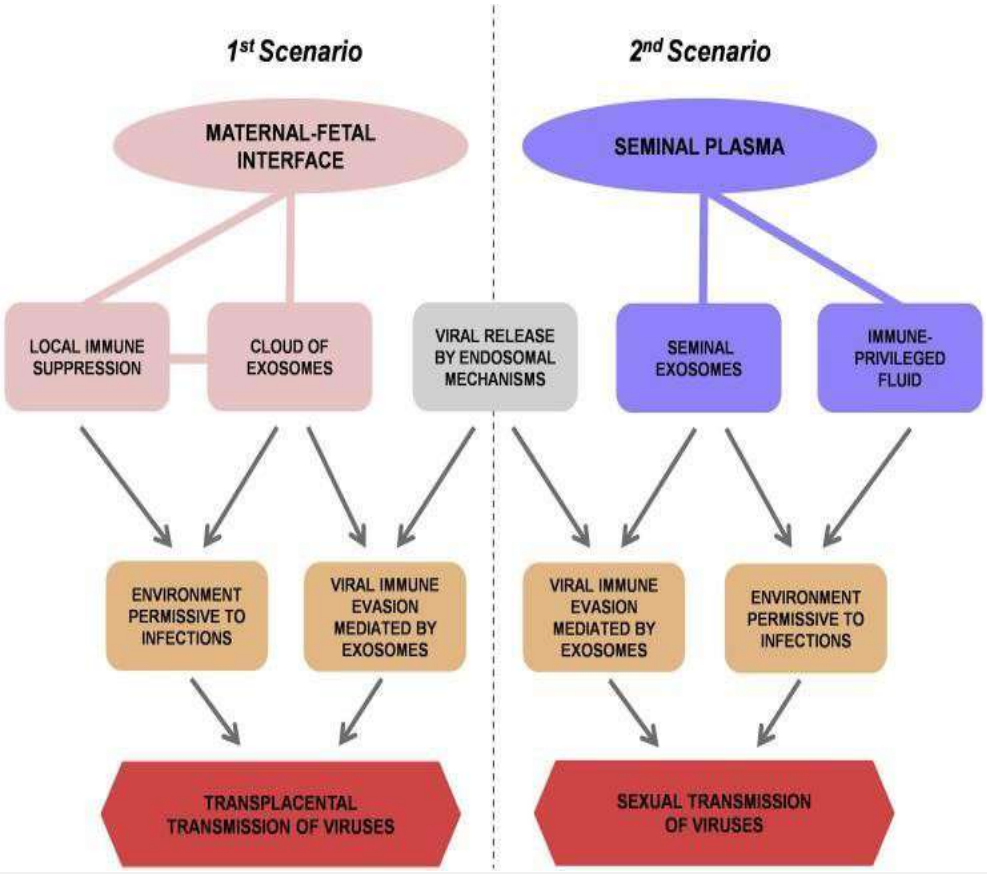
Yang C, Guo WB, Zhang WS, et al.
Comprehensive proteomics analysis of exosomes derived from human seminal plasma.
Andrology. 2017;5(5):1007-1015. doi:10.1111/andr.12412
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5639412/



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6771614/ [131]
Potential roles of exosomes in transplacental (1st scenario) and sexually transmitted viral infections (2nd scenario)

DIFFERENCES AND SIMILARITIES BETWEEN EXOSOMES AND EV AND SARS-COV-2

The presence of exosomes in the tissues of patients affected by SARS-Cov-2 infection has led many researchers to investigate their role in the pathogenesis of COVID-19.

The similarity between exosomes and SARS-Cov-2, has opened a debate on the real existence of SARS-Cov-2 as an infectious agent.
Interesting insights on the topic are given below:

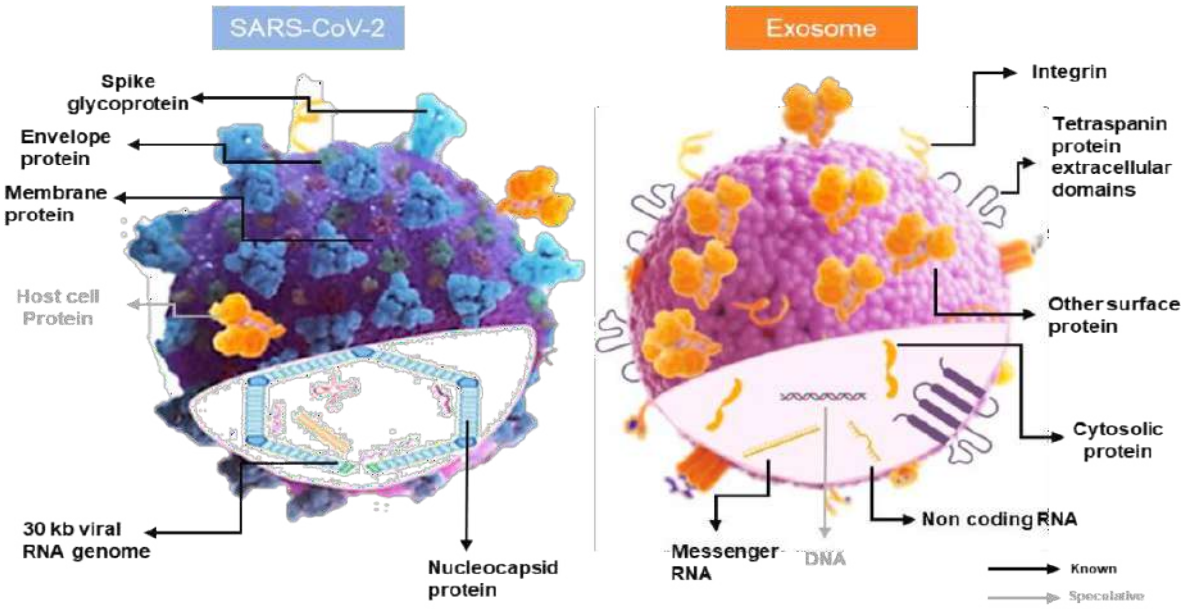
Dr. Fabio Franchi
Pandemic of COVID-19: critical analysis

Dr Andrew Kaufman
COVID 19 Scientific analysis.

Ken Witwer+Jan Lötvall - the extracellular vesicle angle
Is COVID-19 virus an Exosome?

Dr. Pierre Arsène
Lessons for SARS-CoV-2 study (COVID-19 disease) from its exosome relatives

[131] Kaminski VL, Ellwanger JH, Chies JAB.
Extracellular vesicles in host-pathogen interactions and immune regulation - exosomes as emerging actors in the immunological theater of pregnancy. Heliyon. 2019;5(8):e02355. doi:10.1016/j.heliyon.2019.e02355
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6771614/pdf/main.pdf



Key similarities		
80-120 nm	Diameter	50-150 nm
1.16–1.18 g/L	Density	1.13–1.18 g/L
Lipid bilayer envelope	Structure	Lipid bilayer envelope
Protein, RNA, others	Cargo type	Protein, RNA, others
Releasing cell	Cargo origin	Releasing cell
Endocytic pathway, others	Cell entry mechanism	Endocytic pathway, others
Key differences		
Replication & survival	Function	Message delivery
Endoplasmic reticulum–Golgi intermediate compartment (ERGIC)	Intracellular biogenesis	Multivesicular body (MVB)
Host cell budding	Cell exit mechanism	MVB-Plasma Membrane (PM) fusion or surface shedding

Lessons for SARS-CoV-2 study (COVID-19 disease) from its exosome relatives

From the literature review, it is demonstrable **that SARS-Cov-2 and its antigens can be found Within the exosomes**.

The authors of the recent (pre-print) study "*Detection of Viral RNA Fragments in Human iPSC-Cardiomyocytes following Treatment with Extracellular Vesicles from SARS-CoV-2 Coding-Sequence-Overexpressing Lung Epithelial Cells*" found that exosomes containing SARS-CoV-2 RNA represent a pathway of

indirect entry into cardiomyocytes resulting in potential cardiac dysfunction without the need for direct viral infection. [132]

Muthukumar Gunasekaran et al. found that coronavirus infections increased circulating exosomes containing lung-associated self-antigens as well as viral antigens and 20S proteasome[133] .
This fact supports the hypothesis that cells infected with COVID-19 virus produce exosomes containing viral particles.

As detailed in the preceding paragraphs, **the major difference between exosomes and enveloped or exosome-borne viral particles is the ability to replicate**.

In the case of SARS-Cov-2 virus, its **replication ability** in cell culture of clinical specimens was demonstrated by sequencing and its replication kinetics was studied[134] .

Another important feature of SARS-Cov-2 virus that differentiates it from nonreplicating exosomes is the **formation of quasispecies**, that is, populations of mutants that evolve over time by replication in the organism[135] .

---

[132] Kwon Y, Nukala SB, Srivastava S, et al.
Detection of Viral RNA Fragments in Human iPSC-Cardiomyocytes following Treatment with Extracellular Vesicles from SARS-CoV-2 Coding-Sequence-Overexpressing Lung Epithelial Cells.
Preprint. bioRxiv. 2020;2020.05.14.093583. Published 2020 Jul 1. doi:10.1101/2020.05.14.093583
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7337395/

[133] Gunasekaran M, Bansal S, Ravichandran R, et al.
Respiratory viral infection in lung transplantation induces exosomes that trigger chronic rejection. J Heart Lung Transplant. 2020;39(4):379-388. doi:10.1016/j.healun.2019.12.009
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7102671/

[134] Nyayanit DA, Sarkale P, Baradkar S, et al.
Transcriptome & viral growth analysis of SARS-CoV-2-infected Vero CCL-81 cells
[published online ahead of print, 2020 Jul 30]. Indian J Med Res. 2020;10.4103/ijmr.IJMR_2257_20. doi:10.4103/ijmr.IJMR_2257_20
https://pubmed.ncbi.nlm.nih.gov/32773420/

Banerjee A, Nasir JA, Budylowski P, et al.
Isolation, Sequence, Infectivity, and Replication Kinetics of Severe Acute Respiratory Syndrome Coronavirus 2.
Emerg Infect Dis. 2020;26(9):2054-2063. doi:10.3201/eid2609.201495
https://www.researchgate.net/publication/340603961_Isolation_sequence_infectivity_and_replication_kinetics_of_SARS-CoV-2

Liu Z, Zheng H, Lin H, et al.
Identification of Common Deletions in the Spike Protein of Severe Acute Respiratory Syndrome Coronavirus 2. J Virol. 2020;94(17):e00790-20. Published 2020 Aug 17. doi:10.1128/JVI.00790-20.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7431800/pdf/JVI.00790-20.pdf

Ogando NS, Dalebout TJ, Zevenhoven-Dobbe JC, et al.
SARS-coronavirus-2 replication in Vero E6 cells: replication kinetics, rapid adaptation and cytopathology
[published online ahead of print, 2020 Jun 22]. J Gen Virol. 2020;10.1099/jgv.0.001453. doi:10.1099/jgv.0.001453
https://www.microbiologyresearch.org/content/journal/jgv/10.1099/jgv.0.001453/sidebyside

Milewska A, Kula-Pacurar A, Wadas J, et al.
Replication of Severe Acute Respiratory Syndrome Coronavirus 2 in Human Respiratory Epithelium.
J Virol. 2020;94(15):e00957-20. Published 2020 Jul 16. doi:10.1128/JVI.00957-20.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7375387/

[135] Jary A, Leducq V, Malet I, et al.
Evolution of viral quasispecies during SARS-CoV-2 infection
[published online ahead of print, 2020 Jul 24]. Clin Microbiol Infect. 2020;S1198-743X(20)30440-7. doi:10.1016/j.cmi.2020.07.032
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7378485/

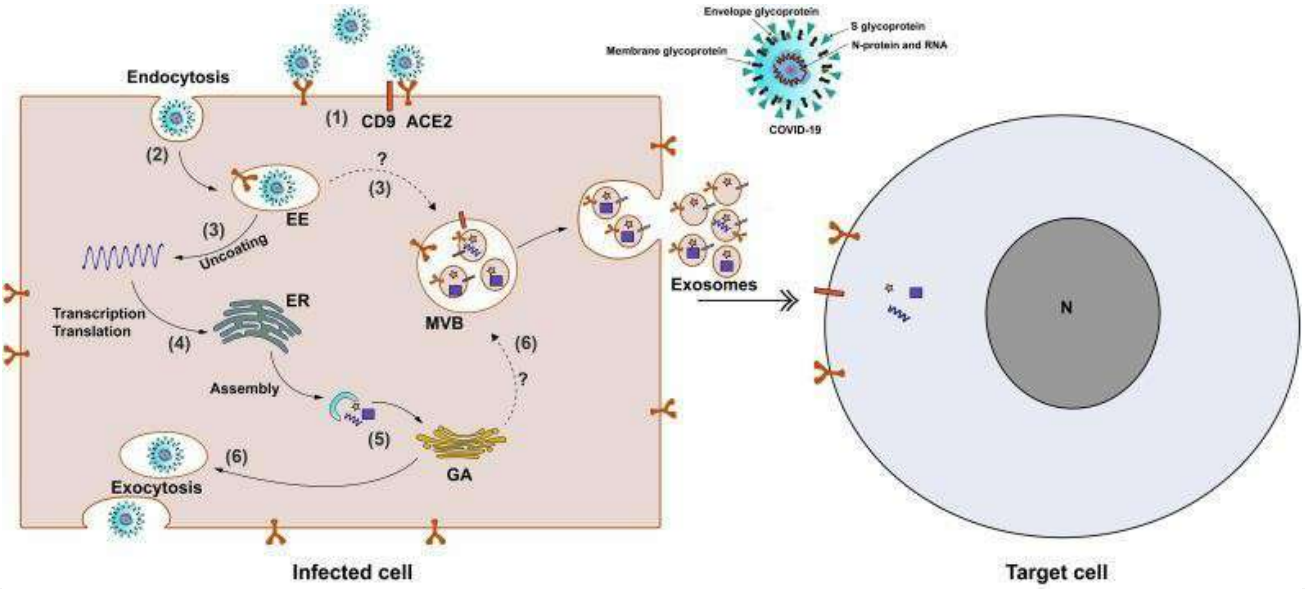
Capobianchi MR, Rueca M, Messina F, et al.
Molecular characterization of SARS-CoV-2 from the first case of COVID-19 in Italy.
Clin Microbiol Infect. 2020;26(7):954-956. doi:10.1016/j.cmi.2020.03.025
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7118617/

There is evidence that **exosomes transfer ACE2** to recipient cells[136] , with a supporting function for internalization and infection of SARS-Cov-2.

Because ACE2s are sorted into exosomes, SARS-Cov-2 can enter cells through the internalization pathway, and consequently its components such as miRNAs and proteins can be packaged into exosomes as with other viruses discussed earlier.

Mechanism of exosome formation in SARS-Cov-2 infection

A mechanism of the life cycle of SARS-Cov-2 in lung cells is proposed below [137]



The life cycle of the virus (COVID-19) in human lung cells. COVID-19 enters cells when the S protein binds to the ACE2 receptor **(1)**. After docking, the conformation of the S protein is changed, which facilitates virus entry into the endosomal pathway **(2)**. Then, COVID-19 virus releases RNA into the cell or/and COVID-19 virus components can be directed into MVBs/exosomes **(3)**. The virus RNA is translated into viral replication polyproteins pp1a and 1ab, which are then cleaved into viral components by viral proteinases. The viral proteins and RNA are subsequently assembled into virions in the endoplasmic reticulum and Golgi **(4 and 5)** and then released out of the cell by exocytosis or oriented into exosomes **(6)**. Upon entry, COVID-19 virus can be directed into the exosomal pathway, and its components are sorted into the exosomes for secretion and diffusion **(passages 3 and 6)**. Extracellular vesicles (exosomes and microvesicles) can help spread this virus as they transfer receptors such as (tetraspanin) CD9 and ACE2, which make recipient cells susceptible to virus attachment. ACE2, angiotensin-converting enzyme 2; EE, early endosome; ER, endoplasmic reticulum; GA, Golgi apparatus; MVB, multivesicular body; N, nucleus https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7293471/

Several research groups have found that coronavirus replication is closely linked to the formation of intracellular vesicles, and the replicative complex binds to the intracellular membrane, leading to the formation of vesicular structures. [138]

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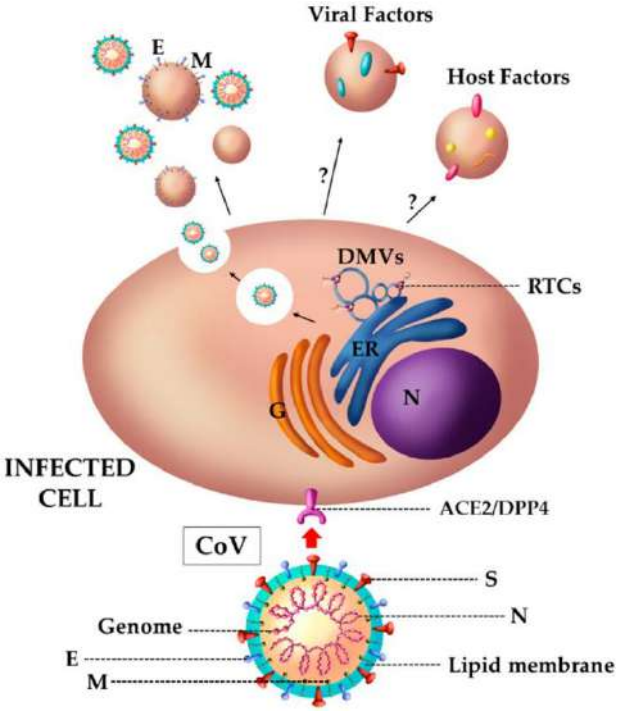
[136] Wang J, Chen S, Bihl J.
Exosome-Mediated Transfer of ACE2 (Angiotensin-Converting Enzyme 2) from Endothelial Progenitor Cells Promotes Survival and Function of Endothelial Cells.
Oxid Med Cell Longev. 2020;2020:4213541. Published 2020 Jan 18. doi:10.1155/2020/4213541
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6995312/

[137] Hassanpour M, Rezaie J, Nouri M, Panahi Y.
The role of extracellular vesicles in COVID-19 virus infection [published online ahead of print, 2020 Jun 13]. Infect Genet Evol. 2020;85:104422. doi:10.1016/j.meegid.2020.104422
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7293471/

[138] Giannessi 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):E571. Published 2020 May 22. doi:10.3390/v12050571
https://www.mdpi.com/1999-4915/12/5/571/htm

Two different vesicular structures were identified:
-The former corresponds to single-membrane spherules formed in membranous organelles, such as ERs, peroxisomes, or endosomes[139] ;

-The second are double membrane vesicles (DMVs) with a diameter of about 200-300 nm, which are often associated with other structures, such as tubules or ER membranes, thus forming a vesicular network in the cytosol.[140]



https://www.mdpi.com/1999-4915/12/5/571/htm
Schematic representation of EVs released from coronavirus (CoV)-infected cells. CoVs hijack the cellular machinery to promote replication. CoVs proteins promote the formation in the cytosol of double membrane vesicles (DMVs) associated with replication and transcription complexes (RTCs) in which viral replication occurs. After the production of structural and nonstructural proteins, budding can occur

[139] den Boon JA, Ahlquist P.
Organelle-like membrane compartmentalization of positive-strand RNA virus replication factories.
Annu Rev Microbiol. 2010;64:241-256. doi:10.1146/annurev.micro.112408.134012
https://pubmed.ncbi.nlm.nih.gov/20825348/
[140] Knoops K, Kikkert M, Worm SH, et al.
SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum.
PLoS Biol. 2008;6(9):e226. doi:10.1371/journal.pbio.0060226
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2535663/

Ulasli M, Verheije MH, de Haan CA, Reggiori F.
Qualitative and quantitative ultrastructural analysis of the membrane rearrangements induced by coronavirus.
Cell Microbiol. 2010;12(6):844-861. doi:10.1111/j.1462-5822.2010.01437.x
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7159092/

Maier HJ, Hawes PC, Cottam EM, et al.
Infectious bronchitis virus generates spherules from zippered endoplasmic reticulum membranes.
mBio. 2013;4(5):e00801-e813. Published 2013 Oct 22. doi:10.1128/mBio.00801-13
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3812713/

Hagemeijer MC, Rottier PJ, de Haan CA.
Biogenesis and dynamics of the coronavirus replicative structures.
Viruses. 2012;4(11):3245-3269. Published 2012 Nov 21. doi:10.3390/v4113245
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3509692/

Qian Z, Travanty EA, Oko L, et al.
Innate immune response of human alveolar type II cells infected with severe acute respiratory syndrome-coronavirus.
Am J Respir Cell Mol Biol. 2013;48(6):742-748. doi:10.1165/rcmb.2012-0339OC
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3727876/

by Golgi and ER membranes. Subsequently, viral particles are released into the extracellular space by exploiting the vesicular network. In addition to viral particles, CoVs induce the release of vesicles carrying viral envelope (E) and membrane (M) proteins. To date, there is no clear evidence of vesicles released from CoV-infected cells carrying other viral or host factors. Nucleus (N); endoplasmic reticulum (ER); Golgi complex (G).

The replicative apparatus of SARS-CoV-2 is similar to that of SARS-Cov and consists of up to sixteen nonstructural proteins (Nsp1 to Nsp16), of which Nsp3, Nsp4, and Nsp6 are involved in DMV induction.[141] Mutations in Nsp4 have been linked to the formation of aberrant DMVs with obvious defects in membrane coupling.

This may explain the appearance of single-membrane vesicles and double-membrane vesicles containing several single-membrane vesicles enclosed within an outer membrane and containing SARS-CoV in Vero E6 cells 9[142] .
Mutations in Nsp6 of several coronaviruses cause single-membrane vesicles to form instead.[143]

These observations indicate that membrane rearrangement (redirecting and rearranging the membranes of host cells for use as part of the viral genome replication and transcription mechanism) represents a strategy used by all known single-stranded RNA viruses with positive polarity.

Several cases reported in the literature have indicated the presence of DMV containing the virus in biopsies, autopsies and endoscopies, and postmortem specimens analyzed by electron microscopy (EM).

By EM histological analysis, coronavirus-compatible viral particles (SARS-CoV) were detected in all examined samples of small intestine tissue obtained by autopsy, as well as in ileal and terminal colon biopsy samples obtained by colonoscopy, which were isolated, cultured in vitro, propagated and confirmed by RT-PCR.

Viral particles were confined to epithelial cells, mainly in apical surface enterocytes and rarely in glandular epithelial cells. At the intracellular level, viral particles were contained within dilated cytoplasmic vesicles consistent with the dilated endoplasmic reticulum.

Vesicles containing the viral particles were often seen toward the apical cytoplasm. Clusters of coronaviruses were also detected on the superficial microvilli, which may suggest virus departure from the luminal surface of enterocytes.
There was no evidence of villus atrophy despite viral adhesion and colonization[144] .

[141] Angelini MM, Akhlaghpour M, Neuman BW, Buchmeier MJ.
Severe acute respiratory syndrome coronavirus nonstructural proteins 3, 4, and 6 induce double-membrane vesicles.
mBio. 2013;4(4):e00524-13. Published 2013 Aug 13. doi:10.1128/mBio.00524-13
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3747587/

[142] Goldsmith CS, Tatti KM, Ksiazek TG, et al.
Ultrastructural characterization of SARS coronavirus.
Emerg Infect Dis. 2004;10(2):320-326. doi:10.3201/eid1002.030913
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3322934/

[143] Al-Mulla HM, Turrell L, Smith NM, et al.
Competitive fitness in coronaviruses is not correlated with size or number of double-membrane vesicles under reduced-temperature growth conditions.
mBio. 2014;5(2):e01107-e1113. Published 2014 Apr 1. doi:10.1128/mBio.01107-13
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3977362/

[144] Leung WK, To KF, Chan PK, et al.
Enteric involvement of severe acute respiratory syndrome-associated coronavirus infection.
Gastroenterology. 2003;125(4):1011-1017. doi:10.1016/s0016-5085(03)01215-0
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7126982/

Free viral particles, viral particles and/or viral particles within single- or double-membrane vesicles, plus viral particles with varying virion diameters within a single DMV have been consistently found in samples derived from SARS-CoV-infected patients,[145]
MERS-CoV[146] and SARS-CoV-2[147] (*see the section on electron microscopy for a more in-depth discussion of imaging*).

The presence of the exosomes in the various tissues, especially the intestinal tissue could explain the prolonged release of viral particles beyond the resolution of clinical symptoms, through the previously mentioned *Trojan horse* immune evasion mechanism, and the eventual flare-up of the disease in cured patients. [148]

In fact, it was reported that in some of these patients, prolonged spread of SARS-CoV-2 RNA occurred with a median duration of 53 days and a maximum of 83 days.[149]

[145] Goldsmith CS, Miller SE.
Modern uses of electron microscopy for detection of viruses.
Clin Microbiol Rev. 2009;22(4):552-563. doi:10.1128/CMR.00027-09
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2772359/

Qinfen Z, Jinming C, Xiaojun H, et al.
The life cycle of SARS coronavirus in Vero E6 cells.
J Med Virol. 2004;73(3):332-337. doi:10.1002/jmv.20095
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7166737/

Shieh WJ, Hsiao CH, Paddock CD, et al.
Immunohistochemical, in situ hybridization, and ultrastructural localization of SARS-associated coronavirus in lung of a fatal case of severe acute respiratory syndrome in Taiwan.
Hum Pathol. 2005;36(3):303-309. doi:10.1016/j.humpath.2004.11.006
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7112064/

[146] Alsaad KO, Hajeer AH, Al Balwi M, et al.
Histopathology of Middle East respiratory syndrome coronovirus (MERS-CoV) infection - clinicopathological and ultrastructural study.
Histopathology. 2018;72(3):516-524. doi:10.1111/his.13379
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7165512/

[147] Menter T, Haslbauer JD, Nienhold R, et al.
Postmortem examination of COVID-19 patients reveals diffuse alveolar damage with severe capillary congestion and variegated findings in lungs and other organs suggesting vascular dysfunction
[published online ahead of print, 2020 May 4]. Histopathology. 2020;10.1111/his.14134. doi:10.1111/his.14134
https://pubmed.ncbi.nlm.nih.gov/32364264/

Martines RB, Ritter JM, Matkovic E, et al.
Pathology and Pathogenesis of SARS-CoV-2 Associated with Fatal Coronavirus Disease, United States.
Emerg Infect Dis. 2020;26(9):2005-2015. doi:10.3201/eid2609.202095
https://wwwnc.cdc.gov/eid/article/26/9/20-2095_article

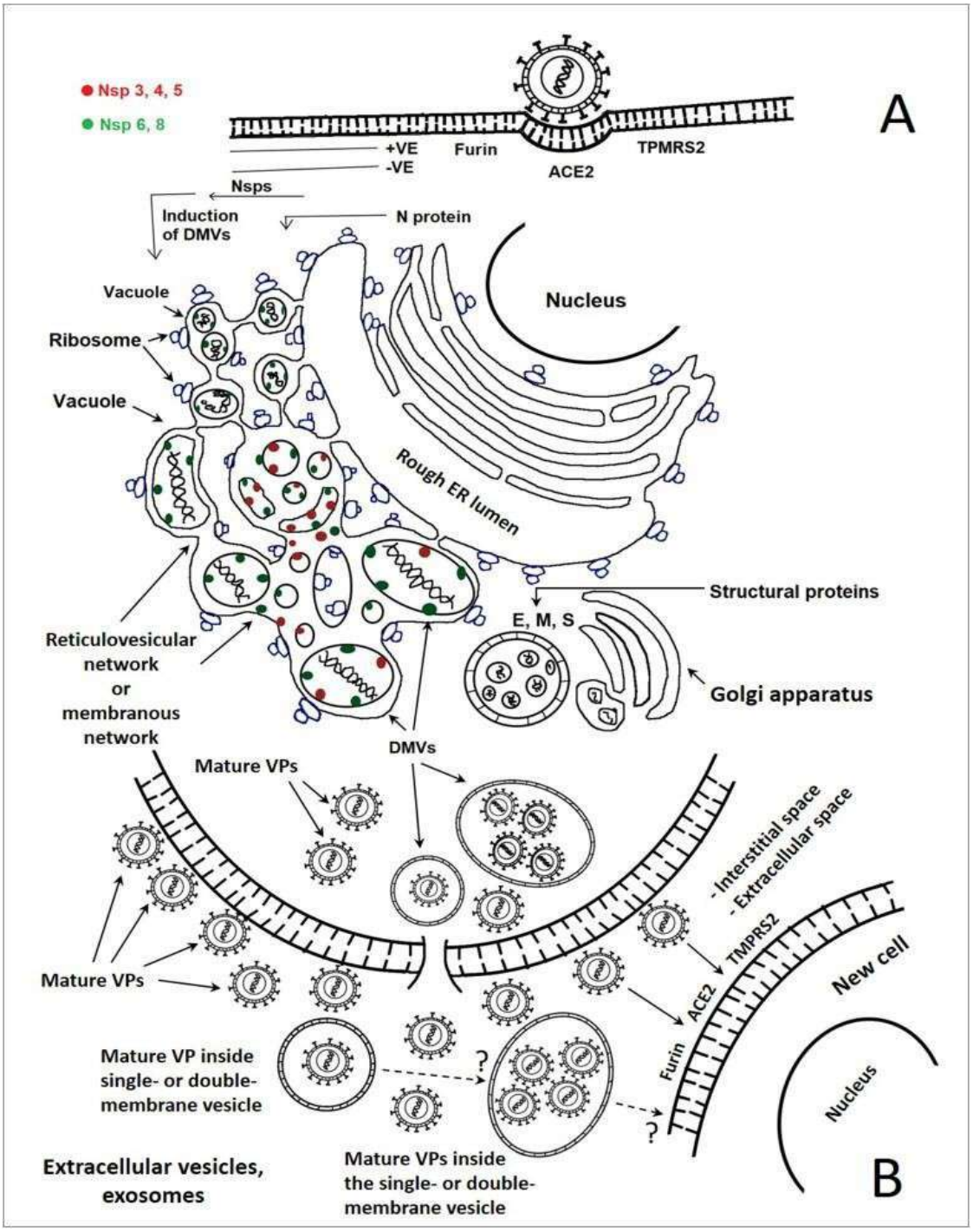
Zhu N, Zhang D, Wang W, et al.
A Novel Coronavirus from Patients with Pneumonia in China, 2019.
N Engl J Med. 2020;382(8):727-733. doi:10.1056/NEJMoa2001017
https://pubmed.ncbi.nlm.nih.gov/31978945/

[148] Irashdy F, Aljaddawi AA, Redwan EM, Uversky VN.
On the potential role of exosomes in the COVID-19 reinfection/reactivation opportunity
[published online ahead of print, 2020 Jul 9]. J Biomol Struct Dyn. 2020;1-12. doi:10.1080/07391102.2020.1790426
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7441802/

Ye G., Pan Z., Pan Y., Deng Q., Chen L., Li J., Li Y., & Wang X.
Clinical characteristics of severe acute respiratory syndrome coronavirus 2 reactivation.
Journal of Infection, (2020). (5), e14-e17. 10.1016/j.jinf.2020.03.001
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7102560/

[149] Li N, Wang X, Lv T.
Prolonged SARS-CoV-2 RNA shedding: Not a rare phenomenon
[published online ahead of print, 2020 Apr 29]. J Med Virol. 2020;10.1002/jmv.25952. doi:10.1002/jmv.25952
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7267144/

In addition to this prolonged carriage of SARS-CoV-2, some patients who had recovered from COVID-19 demonstrated recurrence of SARS-CoV-2. [150]



[150] Xiao AT, Tong YX, Zhang S.
False negatives of RT-PCR and prolonged nucleic acid conversion in COVID-19: Rather than recurrence ù[published online ahead of print, 2020 Apr 9]. J Med Virol. 2020;10.1002/jmv.25855. doi:10.1002/jmv.25855
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7262304/pdf/JMV-9999-na.pdf

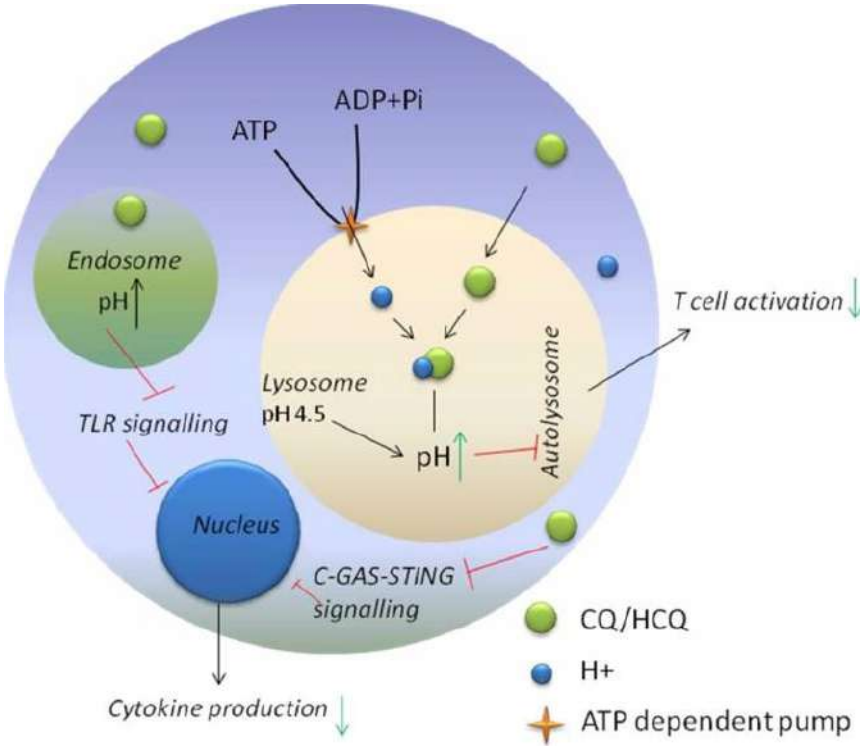
Ye G., Pan Z., Pan Y., Deng Q., Chen L., Li J., Li Y., & Wang X.
Clinical characteristics of severe acute respiratory syndrome coronavirus 2 reactivation.
Journal of Infection, (2020). (5), e14-e17. 10.1016/j.jinf.2020.03.001
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7102560/

Yuan J, Kou S, Liang Y, Zeng J, Pan Y, Liu L.
PCR Assays Turned Positive in 25 Discharged COVID-19 Patients
[published online ahead of print, 2020 Apr 8]. Clin Infect Dis. 2020;ciaa398. doi:10.1093/cid/ciaa398
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7184423/

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7441802/
Putative life cycle of SARS-CoV and SARS-CoV-2 in the human host cell (in vivo) and/or Vero E6 cell (in vitro). Virus-induced double-membrane vesicles in the cytoplasm of infected cells represent platforms for coronavirus replication, assembly, trafficking, extrusion, and dissemination of mature viral particles (free and/or within the vesicles). The virus-infected cell demonstrated the formation of a reticulo-vesicular network of modified membranes, which included single/multiple double-membrane vesicles, representing the site where the virus replicates. All are contiguous with the rough endoplasmic reticulum. Viral RNA+ is released into the cytoplasm and mainly translated into viral polyproteins coding for Nsps, which stimulate/induce DMVs to proceed and complete the virus life cycle in association with the Golgi to produce viral particles in vesicles, which eventually fuse with the plasma membrane. The DMV may contain the mature or immature viral particle or the unassembled viral apparatus. Nsp 3-8 are present on the CM, while some Nsp8 can be detected within DMVs. Histological and ultrastructural analysis of the appearance of specimens from SARS-CoV-2-infected patients demonstrated the presence of mature viral particles as well as immature viral particles or unassembled viral apparatus within DMVs. Mature and immature SARS-CoV-2 viral particles spread/disseminate into new neighboring cells as documented, while the introduction of SARS-CoV-2 virus particles into cells through extracellular vesicles (exosomes) has yet to be documented.

It should be remembered that the RT-PCR test cannot distinguish between infectious viruses and non-infectious nucleic acids of the same virus, which is why study by culture propagation of patient samples is necessary to determine whether the person is capable of transmitting the virus.

*Interestingly, **chloroquine** in its non-protonated form can easily diffuse across cell membranes to acidic vesicles in the cytoplasm [lysosomes, late endosomes, trans-Golgi network (TGN) vesicles] and remains trapped in vesicles after being protonated. Protonated chloroquine is unable to diffuse from the lysosome or endosomes, being retained in cellular compartments with hydrolases. Because chloroquine and its analogs are weak diprotic bases and their nonprotonated form can selectively enter lysosomes and become protonated inversely proportional to pH, they are also known as lysosomotropic agents.*
The drug then alters the acidic environment in the lysosome and, as a result, the cell cannot proceed with endocytosis, exosome release, or phagolysosomal fusion [151]



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7243790/
Schematic diagram of the role of chloroquine (CQ) and hydroxychloroquine (HCQ) in the intracellular space. The drugs increase the pH of endosomes and lysosomes. As a result, activation of T lymphocytes and other cytokines is repressed.

[151] Tripathy S, Dassarma B, Roy S, Chabalala H, Matsabisa MG.
A review on possible modes of action of chloroquine/hydroxychloroquine: repurposing against SAR-CoV-2 (COVID-19) pandemic.
Int J Antimicrob Agents. 2020;56(2):106028. doi:10.1016/j.ijantimicag.2020.106028
https://www.ncbi.nlm.nih.gov/pmc/articles/pmid/32450198/

Identification of SARS-Cov and SARS-Cov-2 by electron microscopy

Electron microscopy study of SARS-Cov-2 requires special caution because of the presence of extracellular vesicles released from all cells in infected and/or inflamed tissues.

In the case of lung tissue as already discussed the criticality is considerable, precisely because of the multiple functions of both pro- and antiviral exosomes and because of the presence of extracellular vesicles that could contain the infectious virus, or viral components of the same size as the virus with envelope giving rise to coronavirus-like structures.

Regarding the difficulty of discerning by microscopy between SARS-Cov-2 viruses and exosomes, please refer to the relevant published technical literature. [152]

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[152] Goldsmith CS, Miller SE.
Caution in Identifying Coronaviruses by Electron Microscopy
[published online ahead of print, 2020 Jul 10]. J Am Soc Nephrol. 2020;ASN.2020050755. doi:10.1681/ASN.2020050755
https://jasn.asnjournals.org/content/early/2020/07/14/ASN.2020050755

Varga Z, Flammer AJ, Steiger P, et al.
Endothelial cell infection and endotheliitis in COVID-19.
Lancet. 2020;395(10234):1417-1418. doi:10.1016/S0140-6736(20)30937-5
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7172722/

Goldsmith CS, Miller SE, Martines RB, Bullock HA, Zaki SR.
Electron microscopy of SARS-CoV-2: a challenging task.
Lancet. 2020;395(10238):e99. doi:10.1016/S0140-6736(20)31188-0
https://www.thelancet.com/pdfs/journals/lancet/PIIS0140-6736(20)31188-0.pdf

Varga Z, Flammer AJ, Steiger P, et al.
Electron microscopy of SARS-CoV-2: a challenging task - Authors' reply.
Lancet. 2020;395(10238):e100. doi:10.1016/S0140-6736(20)31185-5
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7237177/

Miller SE, Brealey JK.
Visualization of putative coronavirus in kidney.
Kidney Int. 2020;98(1):231-232. doi:10.1016/j.kint.2020.05.004
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7206426/

Miller SE, Brealey JK.
Visualization of putative coronavirus in kidney.
Kidney Int. 2020;98(1):231-232. doi:10.1016/j.kint.2020.05.004
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7206426/

Calomeni E, Satoskar A, Ayoub I, Brodsky S, Rovin BH, Nadasdy T.
Multivesicular bodies mimicking SARS-CoV-2 in patients without COVID-19.
Kidney Int. 2020;98(1):233-234. doi:10.1016/j.kint.2020.05.003
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7206432/

Su H, Yang M, Wan C, et al.
Renal histopathological analysis of 26 postmortem findings of patients with COVID-19 in China.
Kidney Int. 2020;98(1):219-227. doi:10.1016/j.kint.2020.04.003
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7194105/

Oshiro LS, Schieble JH, Lennette EH.
Electron microscopic studies of coronavirus.
J Gen Virol. 1971;12(2):161-168. doi:10.1099/0022-1317-12-2-161
https://www.microbiologyresearch.org/docserver/fulltext/jgv/12/2/JV0120020161.pdf?expires=1598814490&id=id&accname=guest&checksum=20E0AA1B64433FE3FA51C922B99F2974

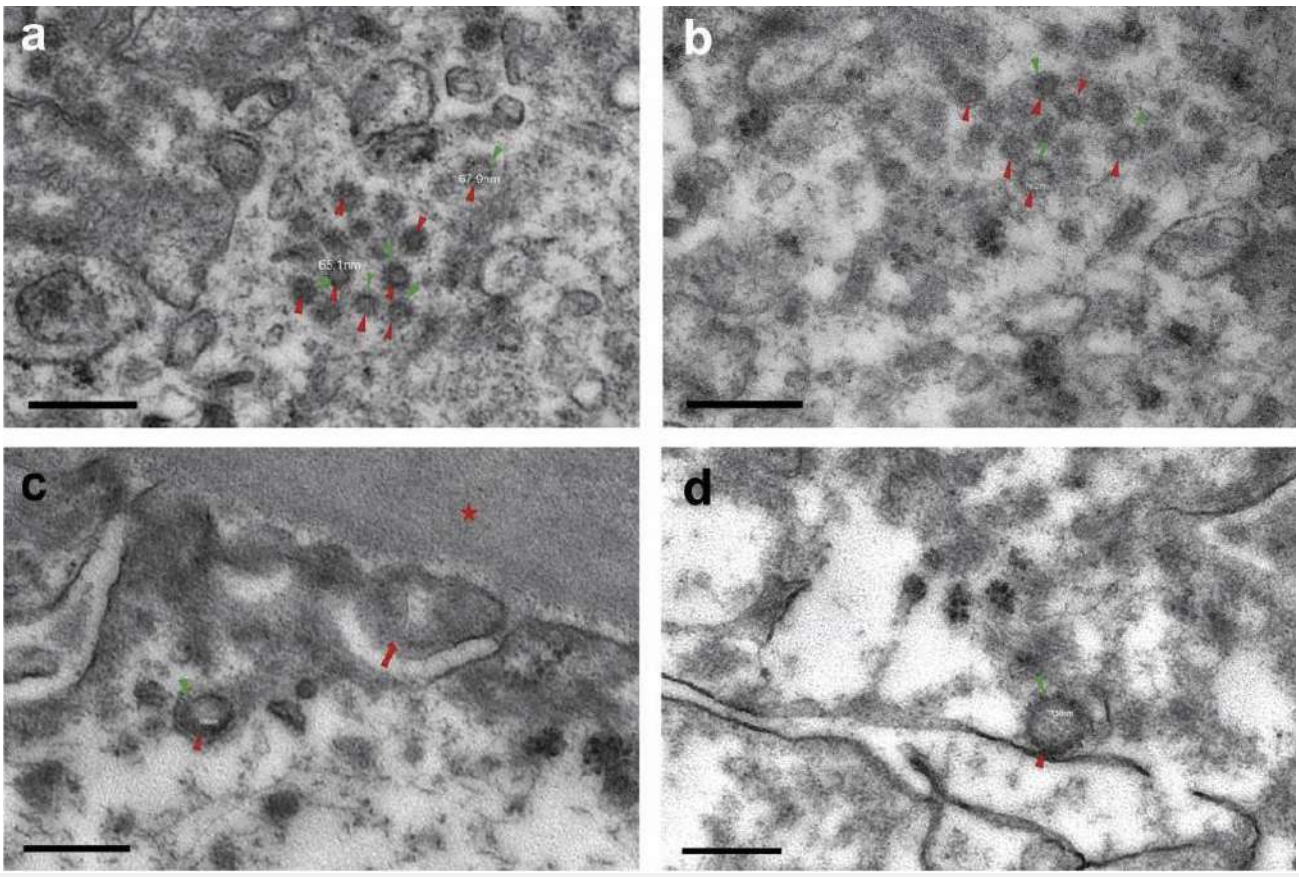
Knoops K, Kikkert M, Worm SH, et al.
SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum.
PLoS Biol. 2008;6(9):e226. doi:10.1371/journal.pbio.0060226
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2535663/

Goldsmith CS, Tatti KM, Ksiazek TG, et al.
Ultrastructural characterization of SARS coronavirus.
Emerg Infect Dis. 2004;10(2):320-326. doi:10.3201/eid1002.030913
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3322934/

Experts agree that transmission electron microscopy (EM) of tissue sections is neither a specific nor a sensitive method for detecting viral particles; there are numerous structures detectable by EM that resemble viruses (so-called virus-like particles), as well as endothelial tubuloreticular inclusions (called myxovirus-like particles), and therefore, they advise caution when identifying a virus by EM in tissue sections.

Immunohistochemistry can also cause nonspecific staining, particularly in renal tubules, leading to false-positive results.

By way of example, the images that have been questioned for the presence of viral particles are shown:

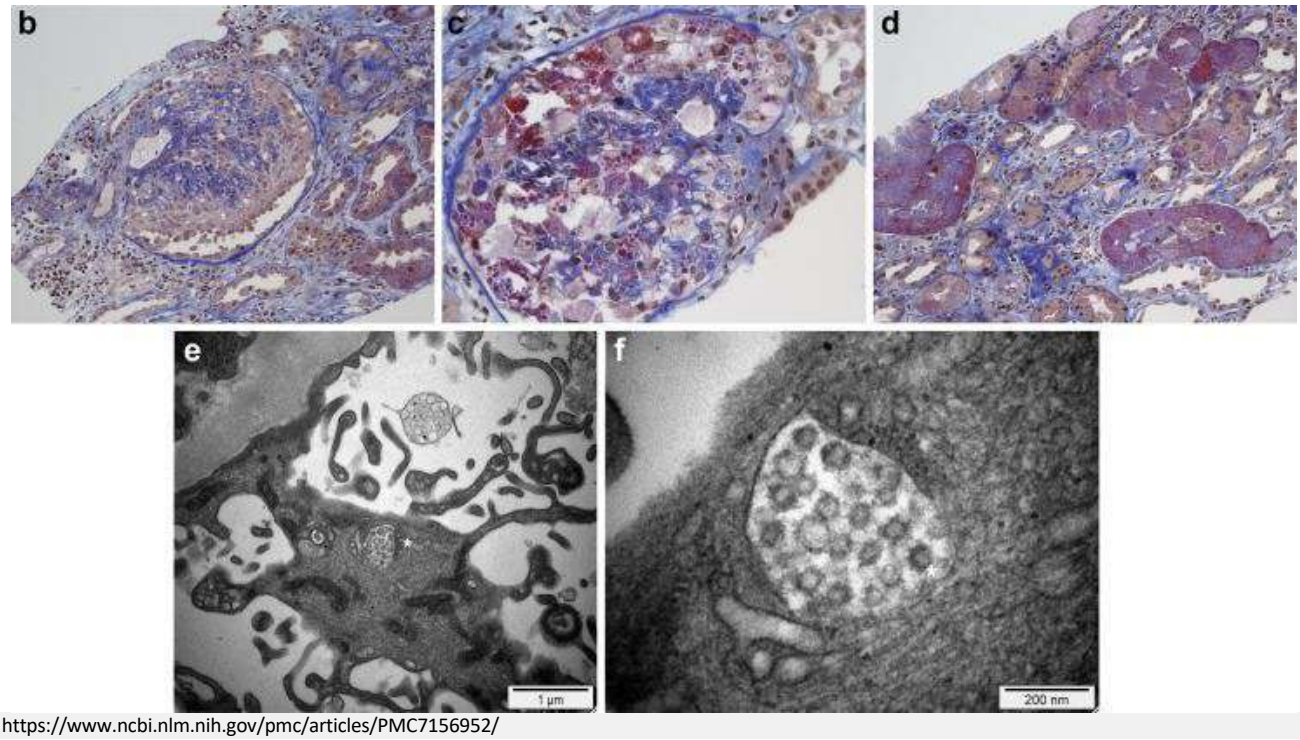


https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7156952/

Su et al. show putative viral particles in the cytoplasm of renal tubular epithelium and podocytes.
These structures are not viral particles, but rather clathrin-coated vesicles, normal cellular organelles involved in intracellular transport.
The objects in their Figures 2a and b (⍰60 nm) are slightly smaller than coronaviruses (⍰80 to 140+ nm), but more importantly, their "peaks" (peplomers) are in contact with the cytosol, like those on the clathrin-coated -vesicles; the largest particle in the
Figure 2d also has tips touching the cytosol and no dense spots within the particles corresponding to the coiled nucleocapsid, cut in cross section.
Coronaviruses, on the other hand, have their projections facing the extracellular space between cells or the space within vacuoles within cells.
This phenomenon is due to the fact that coronaviruses receive their outer coating by blooming in or on cell membranes, thus forming intracellular vacuoles with the viral projections in contact with the vacuolar contents, not the cytosol. During assembly,

Farkash EA, Wilson AM, Jentzen JM.
Ultrastructural Evidence for Direct Renal Infection with SARS-CoV-2.
J Am Soc Nephrol. 2020;31(8):1683-1687. doi:10.1681/ASN.2020040432
https://jasn.asnjournals.org/content/31/8/1683.long

viral structural proteins are incorporated into the endoplasmic reticulum-Golgi complex of the infected cell, and viral RNA, assembled with another protein, sprouts in these membranes, forming a membrane-bound sac containing mature virions; spikes are located outside the virion, but inside the vacuole and not in direct contact with the cytosol.
These virions exit the cell by exocytosis when the vacuole membrane fuses with the plasma membrane and opens its contents to the outside; thus, complete virions with peplomers are seen inside the cell within the vesicle (sequestered from the cytosol) and outside the cells, often still attached to the open vacuolar membrane that has fused with the plasma membrane.
The particles shown in the electron micrographs in the article by Su et al. have their spikes in contact with the cytoplasmic fluid, such as endocytotic vesicles, i.e., clathrin-coated vesicles The presence of coating proteins can cause an electron dense area around these vesicles, giving the appearance of a viral "crown." Su et al. found SARS-CoV nucleoprotein in renal tubules by immunohistochemistry, but the presence of a viral protein does not necessarily mean the presence of complete viral particles. [153]



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7156952/
The particles in Kissling et al. are not coronaviruses.
While they are inside a vacuole, they have spikes and are approximately the correct size, but they do not have the uniform appearance of viral particles with an outer membrane coating and spikes on the inside indicating the nucleocapsid.
These microvesicles are fluctuating and are found within a structure called the multivesicular body. The article by Kissling et al. is troubling because electron microscopy is the only purported evidence presented to support the hypothesis that coronaviruses are indeed present in this kidney tissue; all other tests for coronavirus in the kidney have been negative.
These micrographs do not support the claim that the particles are actually viruses. [154]

[153] Miller SE, Brealey JK.
Visualization of putative coronavirus in kidney.
Kidney Int. 2020;98(1):231-232. doi:10.1016/j.kint.2020.05.004
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7206426/

Calomeni E, Satoskar A, Ayoub I, Brodsky S, Rovin BH, Nadasdy T.
Multivesicular bodies mimicking SARS-CoV-2 in patients without COVID-19.
Kidney Int. 2020;98(1):233-234. doi:10.1016/j.kint.2020.05.003
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7206432/

Su H, Yang M, Wan C, et al.
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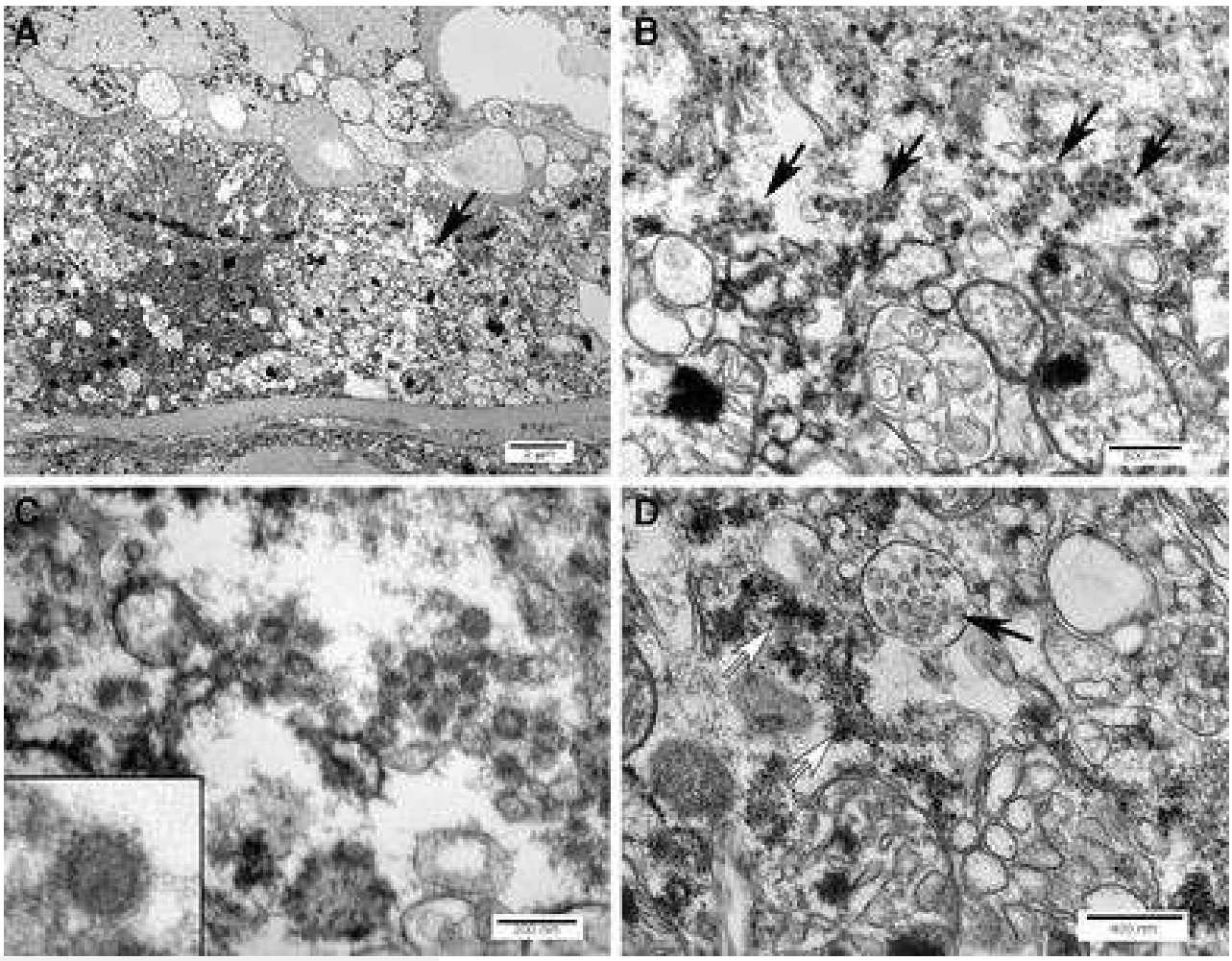
Kidney Int. 2020;98(1):219-227. doi:10.1016/j.kint.2020.04.003
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7194105/
[154] Miller SE, Brealey JK.
Visualization of putative coronavirus in kidney.
Kidney Int. 2020;98(1):231-232. doi:10.1016/j.kint.2020.05.004
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7206426/

Miller SE, Brealey JK.



https://jasn.asnjournals.org/content/31/8/1683.long

In the article by Farkash et al, the electron microscope images in their Figure 3, A-C do not show coronaviruses. Rather, the structures described as viruses are clathrin-coated vesicles (CCVs), normal subcellular organelles involved in intracellular transport.
Figure 3A is a low magnification of a dying cell with nonspecific disorganized cytoplasm with an arrow pointing to an aggregation of CCVs. Panels B and C in their Figure 3 show clusters of CCVs, and the inset for Figure 3C shows a higher magnification.
None of these spherical structures contain cross sections through the nucleocapsid of the viral particles. Moreover, these CCVs are seen free in the cytoplasm, whereas the coronavirus particles are enclosed within a vacuole so that the spikes face inward toward the vacuolar contents, not the cytoplasm. Figure 3D contains a multivesicular body (MVB), which they likened to double-membrane vesicles, the coronavirus replication complex.
The structure shown in the manuscript by Farkash et al. does not have the two closely opposing membranes seen in double membrane vesicles and does not look like what is shown in the reference they cite.
In addition, MVBs can be found in renal tissues observed historically.6 Furthermore, MVBs are formed by invaginations of endosomes and are intermediates in lysosome trafficking. [155]
Furthermore, Farkash et al. document their findings by referring to an article by Su et al. that claims to have identified coronavirus in the kidney. Likewise, that article shows only normal cell structures that, to the virologist not an electron microscopist, may resemble coronavirus.

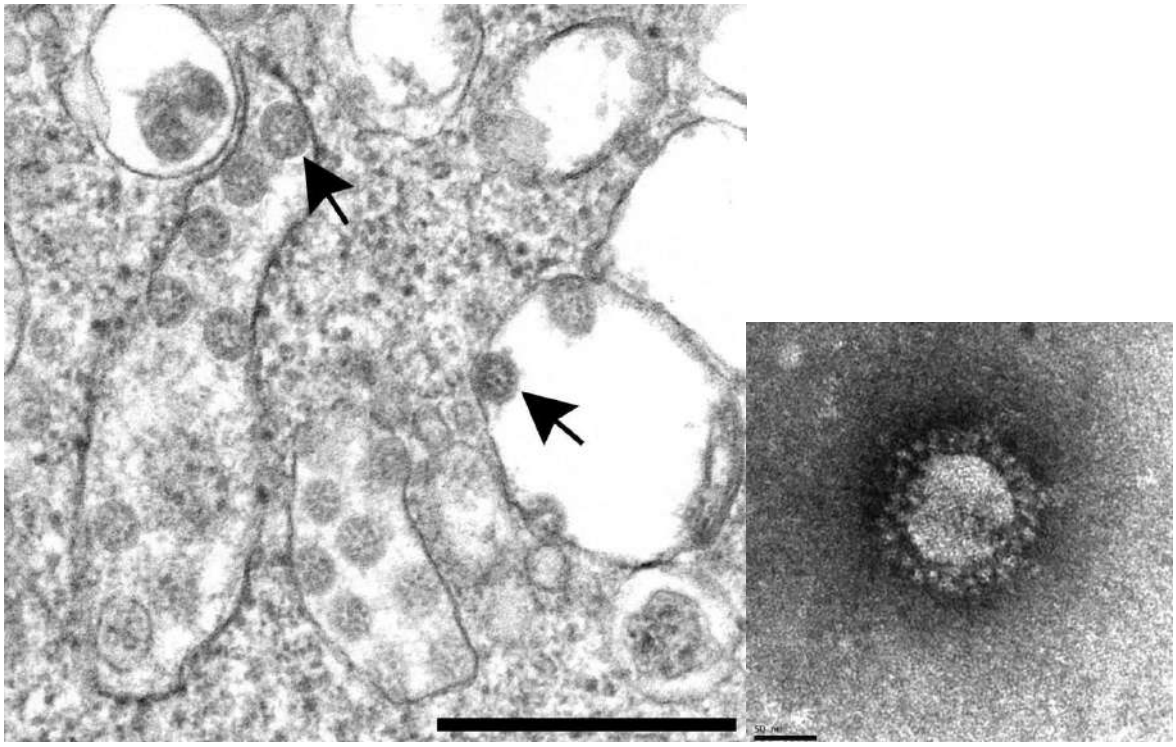
Visualization of putative coronavirus in kidney.
Kidney Int. 2020;98(1):231-232. doi:10.1016/j.kint.2020.05.004
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7206426/

[155] Goldsmith CS, Miller SE.
Caution in Identifying Coronaviruses by Electron Microscopy
[published online ahead of print, 2020 Jul 10]. J Am Soc Nephrol. 2020;ASN.2020050755. doi:10.1681/ASN.2020050755
https://jasn.asnjournals.org/content/early/2020/07/14/ASN.2020050755

Farkash EA, Wilson AM, Jentzen JM.
Ultrastructural Evidence for Direct Renal Infection with SARS-CoV-2.
J Am Soc Nephrol. 2020;31(8):1683-1687. doi:10.1681/ASN.2020040432
https://jasn.asnjournals.org/content/31/8/1683.long

It is then necessary to proceed with confirmation with more in-depth molecular investigations to identify and quantify the virus following propagation in culture (NGS sequencing, reverse genetics) [156]

The following images are taken as reference for EM identification of SARS-Cov-2 and SARS-Cov from infected patient samples



https://jasn.asnjournals.org/content/early/2020/07/14/ASN.2020050755
isolate of SARS-COV-2, propagated in cell culture showing numerous spherical viral particles (arrows) located in the cisternae of the rough endoplasmic reticulum/Golgi complex area of the cell. Note the black dots within the particles, which are cross sections through the viral nucleocapsid. Scale bar: 400 nm.

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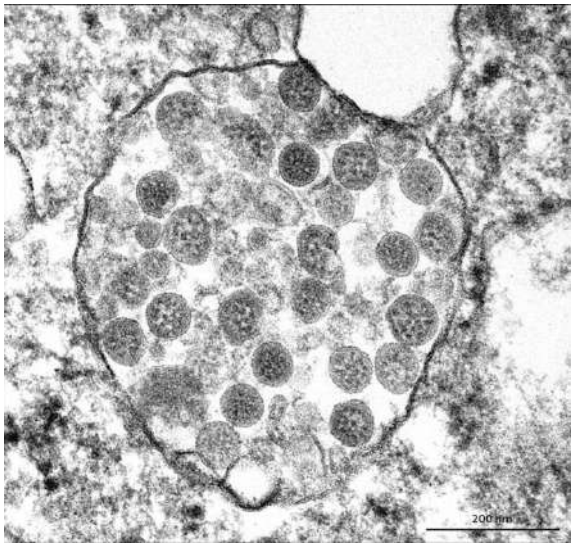


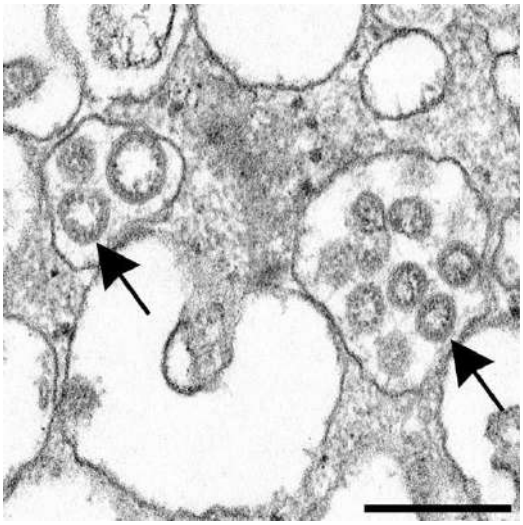
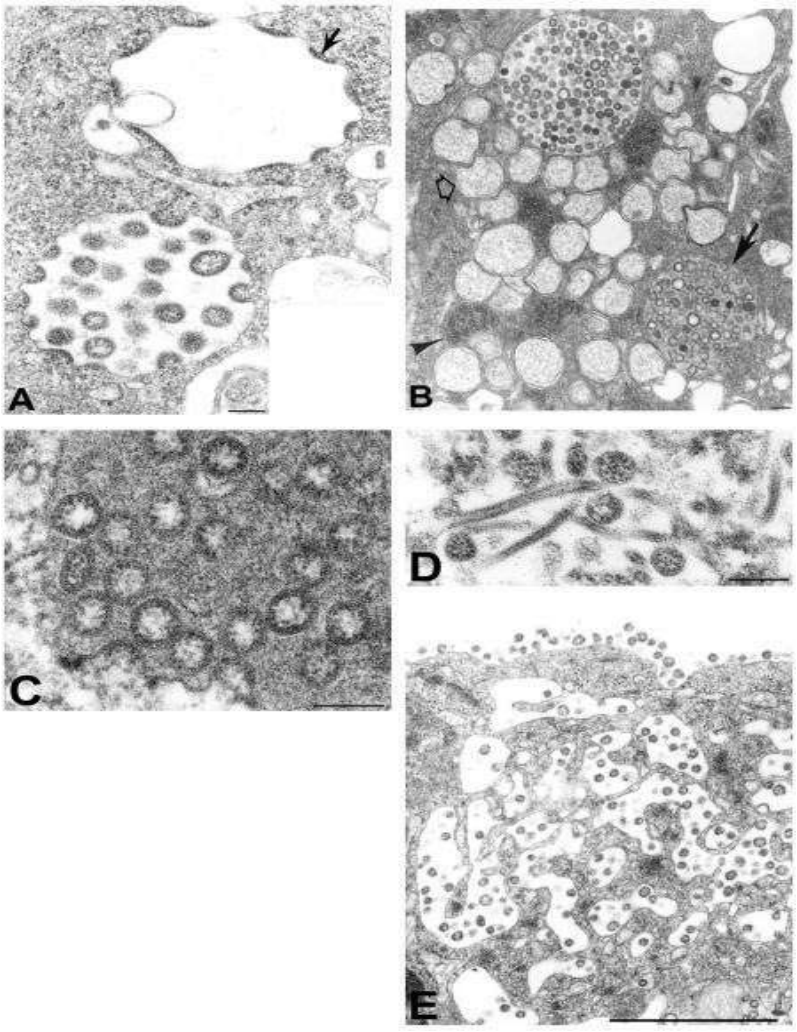


Fig. 1Fig . 2

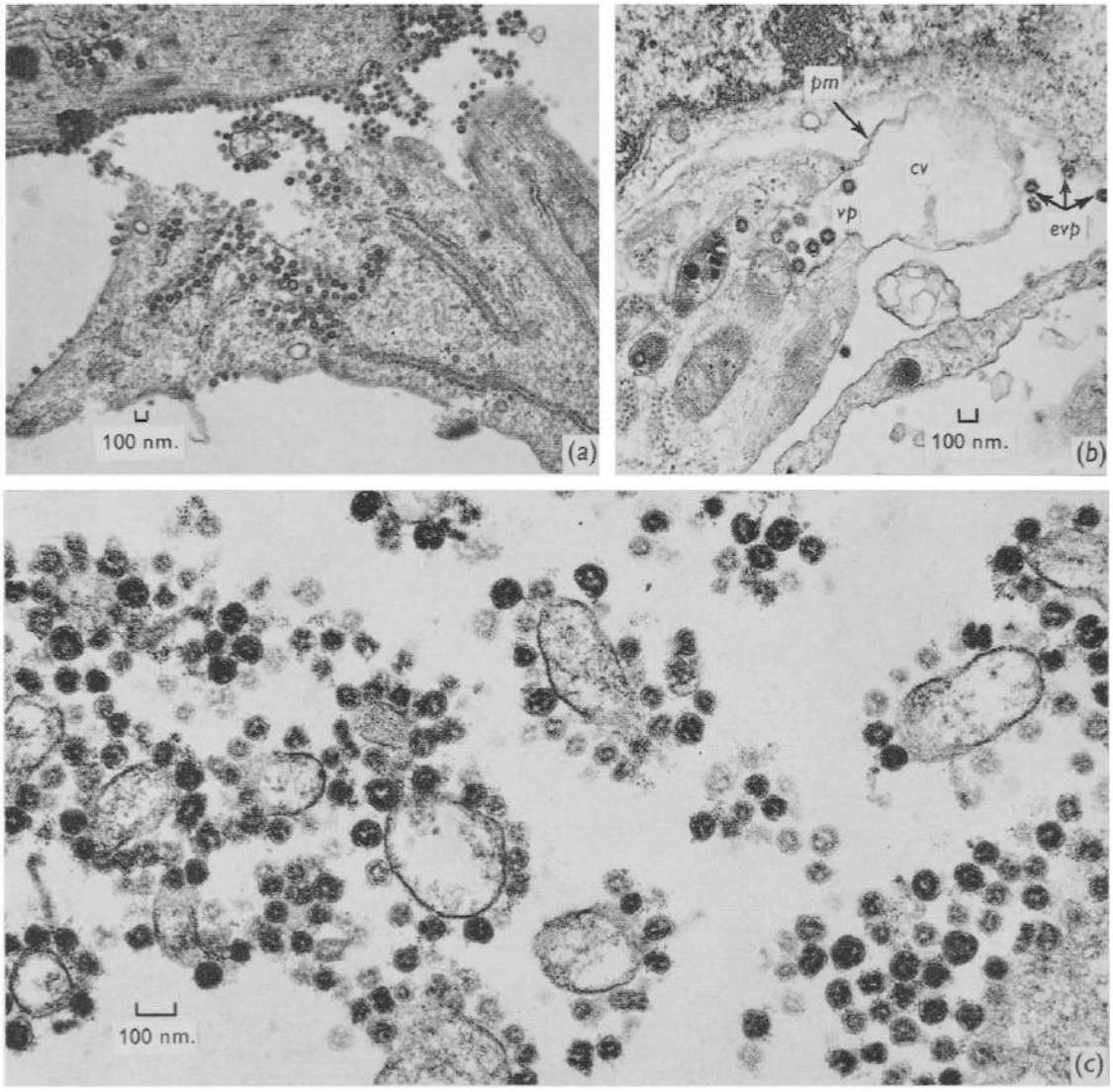
Fig. 1 https://www.thelancet.com/pdfs/journals/lancet/PIIS0140-6736(20)31188-0.pdf
Viral isolate cultured in cell culture Spherical coronavirus particles with cross sections through the nucleocapsid, seen as black dots, are clustered within a membrane separating them from the cytoplasm

Fig. 2 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7206426/
Electron microscope image of an isolate of SARS-Cov-2 seen here inside the vacuoles (arrows). Note the dense membrane coating around the viral particles. This micrograph is of viral particles in a cell culture inoculated with nasopharyngeal and oropharyngeal fluids from infected patients. Bar = 200 nm. Image provided by Cynthia S. Goldsmith, Centers for Disease Control and Prevention.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3322934/

Assembly of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) particles in infected Vero E6 cells. (A) Apposition of nucleocapsids (arrow) along the membranes of the budding compartment when the particles develop and bud. The nucleocapsids measured 6 nm in diameter and were mostly seen in cross section. Some virions had an electron-lucent center, with the nucleocapsid juxtaposed to the envelope, while others were relatively dark when the nucleocapsid was present throughout the particle. Pretreatment with tannic acid improved the visibility of club-shaped viral projections (inset), which had an average length of 14 nm**. (B)** SARS- CoV-infected cell with virus-containing vesicles, double-membrane vesicles (open arrow) and nucleocapsid inclusions (arrowhead). Note the vesicle with granular material interspersed with virions (arrow). **(C)** Higher magnification of a virus-containing vesicle with dark granular material**. (D)** Tubular structures in a virus-containing vesicle**. E)** Virions in the vesicles, which appeared to migrate and fuse with the plasma membrane. You can see the characteristic coating of particles along the cell surface. Bars: A, inset; B - D, 100 nm; E, 1 µm.



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(a) An advanced stage of the lytic process showing viral particles leaving endoplasmic reticulum cisternae through a large break in the cell surface membrane. **(b)** A possible alternative route of virus exit is illustrated by a bubble on the plasma membrane (pm) adjacent to an endoplasmic reticulum cisterna resulting in a cytoplasmic vesicle (cv) containing viral particles (vp). Extracellular virus particles (evp) are also shown. **(c**) Normal serum control for indirect ferritin-labeled antibody labeling experiments. Note some background ferritin granules.

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