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Ph.D Thesis in Cellular and Molecular Biology

CHARACTERIZATION OF SMALL EXTRACELLULAR VESICLES RELEASED BY HUMAN PRIMARY MACROPHAGES AND THEIR ABILITY TO PROPAGATE RESISTANCE TO HIV-1 REPLICATION

Luis Andres Arteaga Blanco

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Pós-Graduação em Biologia Celular e Molecular

M.Sc. LUIS ANDRES ARTEAGA BLANCO

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Advisor: MD, Ph. D. Dumith Chequer Bou-Habib

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Rio de Janeiro, March 19, 2021

Ata da defesa de tese de doutorado acadêmico em Biologia Celular e Molecular de **Luis Andres Arteaga Blanco**, sob orientação do Dr. Dumith Chequer Bou Habib. Ao décimo nono dia do mês de março de dois mil vinte e um, realizou-se às nove horas, de forma síncrona remota, o exame da tese de doutorado acadêmico intitulada: "**Characterization of small extracellular vesicles released by human primary macrophages and their ability to propagate resistance to HIV-1 replication"**, no programa de Pós-graduação em Biologia Celular e Molecular do Instituto Oswaldo Cruz, como parte dos requisitos para obtenção do título de Doutor em Ciências - área de concentração: Farmacologia e Imunologia, na linha de pesquisa: Mecanismos Imunológicos Envolvidos na Patologia de Doenças Autoimunes Infecciosas e Parasitárias. A banca examinadora foi constituída pelos Professores: Drª. Yara Maria Traub Cseko – IOC/FIOCRUZ (Presidente), Dr. Rodrigo Pedro Pinto Soares– IRR/FIOCRUZ, Drª. Luciana Barros de Arruda – UFRJ/RJ, e como suplentes: Drª. Luciana Jesus da Costa- UFRJ/RJ e Drª. Tatiana Maron-Gutierrez - IOC/FIOCRUZ. Após arguir o candidato e considerando que o mesmo demonstrou capacidade no trato do tema escolhido e sistematização da apresentação dos dados, a banca examinadora pronunciou-se pela Aprovado da defesa da tese de doutorado acadêmico. De acordo com o regulamento do Curso de Pós-Graduação em Biologia Celular e Molecular do Instituto Oswaldo Cruz, a outorga do título de Doutor em Ciências está condicionada à emissão de documento comprobatório de conclusão do curso. Uma vez encerrado o exame, o Presidente da Banca atesta a decisão e a participação do aluno e de todos o membros da banca de forma síncrona remota. A Coordenadora do Programa Drª. Leila de Mendonça Lima, assinou a presente ata tomando ciência da decisão dos membros da banca examinadora. Rio de Janeiro, 19 de março de 2021.

Drª. Yara Maria Traub Cseko (Presidente da Banca):

Drª. Leila de Mendonça Lima (Coordenadora do Programa):

DEDICATION

To my family Jose Luis Arteaga Arteaga, Aida Gonzalez Blanco,
Jose Luis Arteaga Blanco, Isabella Arteaga Blanco, and Luz Meza.
FAMILIA SIEMPRE SERA FAMILIA

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"***Somewhere, something incredible is waiting to be known***"

Carl Sagan

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RESUMO

Vesículas extracelulares (VEs) são liberadas por virtualmente todos os tipos de células, e participam da comunicação intercelular por meio do transporte e transferência de moléculas bioativas, como ácidos nucléicos, proteínas, lipídios e moléculas de sinalização, para células receptoras. Nesta tese, nós nos aventuramos por alguns dos desafios atuais no campo das VEs, indo desde o isolamento e caracterização de pequenas sVEs (sVEs) secretadas por macrófagos humanos primários, até o estudo do seu papel na patogênese da infecção pelo vírus da imunodeficiência humana tipo 1 (HIV-1). Até o momento, não há trabalhos metodológicos publicados mostrando em detalhes o isolamento, caracterização e internalização de sVEs liberadas por macrófagos primários humanos derivados de monócitos circulantes (sVEs derivados de MDM). Um dos nossos objetivos foi propiciar um protocolo alternativo, com base na ultracentrifugação diferencial (dUC), para isolar e caracterizar as sVEs dessas células. Assim, macrófagos derivados de monócitos circulantes foram cultivados em meio livre de vesículas durante 24, 48 ou 72 h, e as VEs foram isoladas dos sobrenadantes da cultura por dUC. Os macrófagos secretaram uma grande quantidade de sVEs nas primeiras 24 h, com tamanho variando entre 40 a 150 nm, com pico em 105 nm, segundo avaliações por *nanoparticle tracking analysis* (NTA) e microscopia eletrônica de varredura. Os marcadores proteicos Alix, CD63 e CD81 foram detectados por *immunoblotting* nas amostras de VEs, e a co-localização de CD63 e CD81 após ultracentrifugação por gradiente de densidade de sacarose (S-DGUC) indicou a presença de sVEs de origem endossomal. A microscopia de fluorescência revelou que as sVEs foram internalizadas por macrófagos primários receptores após três horas de co-cultura. Em relação à capacidade das sVEs de propagar resistência anti-HIV-1, vimos que a adição de sVEs às células infectadas diminuiu a replicação do HIV-1 em macrófagos e células mononucleares de sangue periférico (PBMCs), atingindo níveis de inibição de 73% e 74%, respectivamente. As sVEs individuais ou pools dessas vesículas mostraram um efeito inibitório semelhante. Além disso, macrófagos não infectados e expostos ao peptídeo intestinal vasoativo (VIP) liberaram sVEs com atividade anti-HIV-1 em macrófagos ou PBMCs infectados. Encontramos também que o pré-tratamento de PBMCs infectados com HIV-1 com heparina reverteu em quase 80% o efeito antiviral mediado pelas sVEs, sugerindo que as vesículas transferiram a resistência ao HIV-1 para as células receptoras de uma maneira dependente de endocitose mediada por proteoglicanos de heparam sulfato. Nossos dados sugerem que as sVEs derivadas de MDM podem atuar como um componente importante na imunidade inata mediada por macrófagos contra a infecção pelo HIV-1. Acreditamos que as sVEs derivadas de MDM (expostos ou não com VIP) não infectados funcionam como um veículo para transportar mediadores capazes de controlar a replicação viral em células receptoras infectadas pelo HIV-1. Nossos resultados contribuem para a compreensão do papel das VEs de macrófagos na infecção pelo HIV-1, e abrem o caminho para estudos mecanísticos mais profundos e para novas estratégias terapêuticas baseadas nas sVEs.

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ABSTRACT

The nano-sized membrane enclosed extracellular vesicles (EVs) released from various types of cells contribute to intercellular communication via delivering bio-molecules, such as nucleic acids, proteins, lipids, and signaling molecules to recipient cells. In this thesis, we ventured through some of the current challenges in the EV field, moving from isolation and characterization of small EVs (sEVs) secreted by primary human macrophages up to ultimately studying their role in the pathogenesis of human immunodeficiency viruses type 1 (HIV-1). Because, to date, there are no published methodological works showing step-by-step the isolation, characterization and internalization of small EVs released by human primary macrophages derived from circulating monocytes (MDM-derived sEVs), here we aimed to provide an alternative protocol based on differential ultracentrifugation (dUC) to describe sEVs from these cells. Monocyte-derived macrophages were cultured in EV-free medium during 24, 48 or 72 h and, then, EVs were isolated from culture supernatants by (dUC). Macrophages secreted a large amount of sEVs in the first 24 h, with size ranging from 40-150 nm, peaking at 105 nm, as evaluated by nanoparticle tracking analysis and scanning electron microscopy. The markers Alix, CD63 and CD81 were detected by immunoblotting in EV samples, and the co-localization of CD63 and CD81 after sucrose density gradient ultracentrifugation (S-DGUC) indicated the presence of sEVs from late endosomal origin. Confocal fluorescence revealed that the sEVs were internalized by primary macrophages after three hours of co-culture. Regarding the ability of sEVs to propagate anti-HIV-1 resistance, we found that addition of sEVs to infected cells decreased HIV-1 replication in macrophages and peripheral blood mononuclear cells (PBMCs), achieving inhibition percentages 73% and 74%, respectively. Individual sEVs or pools of these vesicles showed a similar inhibitory effect. In addition, uninfected macrophages exposed to the neuropeptide VIP released sEVs with anti-HIV-1 activity in infected macrophages or PBMCs. Importantly, the pre-treatment with heparin on HIV-1 infected PBMCs reduced almost 80% of antiviral effect mediated by sEVs, suggesting, that vesicles probably transfer HIV-1 resistances to recipient cells in a heparan sulfate proteoglycans (HSPGs) dependent manner. Our data suggest that MDM-derived sEVs may act as an important component in macrophage-mediated innate immunity against HIV-1 infection. We believe that MDM-derived sEVs function as a vehicle to transport protective messages from uninfected macrophages (exposed or not with VIP) to the recipient cells and protect them from HIV-1 infection. These data contribute for the understanding of the role of macrophage-EVs in HIV-1 infection and pave the way for new potential EV-based therapeutics strategies.

LIST OF FIGURES

LIST OF TABLES

INDEX

ABBREVIATIONS

ABs	Apoptotic bodies
ADE	Antibody-dependent enhancement
AFM	Atomic force microscopy
AIDS	Acquired immunodeficiency syndrome
Alix	ALG-2 interacting protein X
APC	Antigen-presenting cells
APOBEC	Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3
BSA	Bovine serum albumin
BST2	Bone marrow stromal cell antigen 2
CA	Capsid
cGAS/STING	Cyclic GMP-AMP synthase stimulator of interferon genes
cGAMP	Cyclic guanosine monophosphate–adenosine monophosphate
CM	Conditioned medium
CMV	Cytomegalovirus
CTLs	Cytotoxic T lymphocytes
DAMPS	Damage-associated molecular patterns
DC	Dendritic cells
dNTPs	Deoxyribonucleotides triphosphate
dUC	Differential ultracentrifugation
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ESCRT	Endosomal sorting complexes required for transport
ER	Endoplasmic reticulum
EVs	Extracellular Vesicles
Exo	Exosomes
GAG	Group specific antigen
HBV	Hepatitis viruses B
HCV	Hepatitis viruses C
HHV	Human herpesvirus

HIV-1	Human immunodeficiency virus type I
HSV-1	Herpes simplex virus-1
HSPGs	Heparan sulfate proteoglycans
ILVs	Intraluminal endosomal vesicles
IFN-I	Interferon type 1
ISEV	International Society of Extracellular Vesicles
ISG	Interferon stimulated genes
IEVs	Intermediate extracellular vesicles
LEVs	Large extracellular vesicles
LTNP	Long-term non progression
MDMs	Macrophages derived from circulating monocytes
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
miRNA	Micro RNAs
MMPs	Matrix metalloproteinases
MVBs	Multivesicular bodies
MVE	Multivesicular endosomes
MVs	Microvesicles
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killers
NTA	Nanoparticle tracking analysis
PACAP	Pituitary adenylate cyclase-activating polypeptide
PAMPs	Recognize pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PHA	Phytohemagglutinin
PIC	Pre-integration complex
PM	Plasma membrane
Pol	Polymerase
PS	Phosphatidylserine
PE	Phosphatidyl-ethanolamine

PRR	Pattern Recognition Receptors
RNAi	RNA interference
RT	Reverse transcriptase
SAMHD1 Triphosphohydrolase 1	SAM and HD Domain Containing Deoxynucleoside Triphosphate
sEVs	Small extracellular vesicles
sEVs-Ctr	small EVs secreted from uninfected macrophages
sEVs-VIP	small EVs secreted from uninfected macrophages exposed with VIP
S-DGUC	Sucrose density gradient ultracentrifugation
siRNA	small interfering RNAs
snRNAs	small nuclear RNAs
snoRNAs	small nucleolar RNAs
SEC	Size-exclusion chromatography
SEM	Scanning electron microscopy
TGF-β	Transforming growth factor
TLRs	Toll-like receptor
TNF-α	Tumor necrose factor alpha
TRIM5-α	Tripartite-motif-containing 5α
UC	Ultracentrifugation
UNAIDS	United Nations Program on HIV/AIDS
VCC	Virus-containing compartments
vDNA	Viral DNA
VT-RNAs	Vault RNA
VIP	Vasoactive intestinal peptide
WB	Western blotting
WHO	World health organization

1 INTRODUCTION

1.1 Extracellular Vesicles: History and terminology

The description of small lipid bilayer-enclosed structures released from cell and found in the extracellular space, referred as extracellular vesicles (EVs) (1), began in the late 1960s when researchers imaged vesicle-like material and referred to it as “platelet dust” or matrix vesicles (2,3). In the 1970s and 1980s, some terms, such as microparticles, microvesicles, membrane fragments and membrane vesicles, were proposed to designate vesicles from tumoral and non-tumoral cell lines (4). Two independent groups from North American led by Stahl and Johnstone studying transferrin receptor from reticulocytes, demonstrated that this receptor was secreted from this cell through vesicles originated by the fusion of multivesicular endosomes (MVE) or multivesicular bodies (MVBs) with the plasma membrane (PM) (5,6). Thus MVB-originated EVs, denominated as exosomes, were suggested to be responsible for the elimination of unnecessary proteins (7). These preliminary reports prompted the growth of one of the most studied fields of science nowadays due to the immunomodulatory properties and therapeutic potential of EVs (8). The interest of biomedical researches to study EVs grew randomly up to when it was discovered that some extracellular vesicles released by antigen-presenting cells (APC cells) of the immune system, carried surface molecules of Major Histocompatibility Complex Class II (MHC-II) that could induce signaling in target cells, indicating their potential in inter-cellular communication (9).

To date, the nomenclature of EVs is still a matter of debate. Experts in the field have tried to reach a consensus about how can be catalogued (4,10). The term “exosome” in biological science has been employed to define vesicles from MVB-origin, however, literature have been reported that at least three uses of “exosome” in the history of EVs are available, this including mobile DNA elements and “soma” (11,12) or particle that includes “exo” nucleases and can either processed or degraded RNA (13). This confusing nomenclature are also observed in other EVs types. For example, “Ectosome” refer to outer structures of sponges (14), and/or “microvesicles” defined as structures inside and outside the cell (15), as well as to synthetic particles and vestiges of tissue damage (16). In view of this, the International Society for Extracellular Vesicles (ISEV) proposed a consensus for a specialist and non-specialist in the field to use the designation “extracellular vesicle” as the “generic term for particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate” (1).

The biogenesis (cellular origin) and biophysical properties (size, shape, densities, biochemical markers, protein and lipids composition, etc) are characteristic commonly used on

EVs classification (1). Different EVs subtypes overlap in terms of size, density and molecular signature, suggesting that further studies are need to determine molecules and physical properties to specifics EVs subtypes, thus allowing adequate separation from the other types of vesicles. Such investigations will facilitate a better comprehension of EV role in physiological and pathological states and their therapeutic application in clinical field (17).

1.2 Biogenesis and secretion

Cells can release EVs through membrane-trafficking processes, either by direct outward budding of the plasma membrane (PM) named PM-derived vesicles (also called as microvesicles or ectosomes) or by an intracellular endocytic trafficking pathway involving fusion of multivesicular late endocytic compartments (multivesicular bodies, MVBs) with plasma membrane. This latter called EVs from endosomal origin or exosomes (18) (Figure 1.1). Even though the generation of PM-derived vesicles and endosome-derived EVs occurs at distinct sites within the cell, both entities shared common intracellular mechanisms. For example, all EVs bud away from the cytosol in contrast to other intracellular budding events that are required for trafficking between different compartments (19). This mean that all EVs contain a membrane orientation identical to their parent cell, expose extracellular domains of the transmembrane proteins and carry cytosolic components (18).

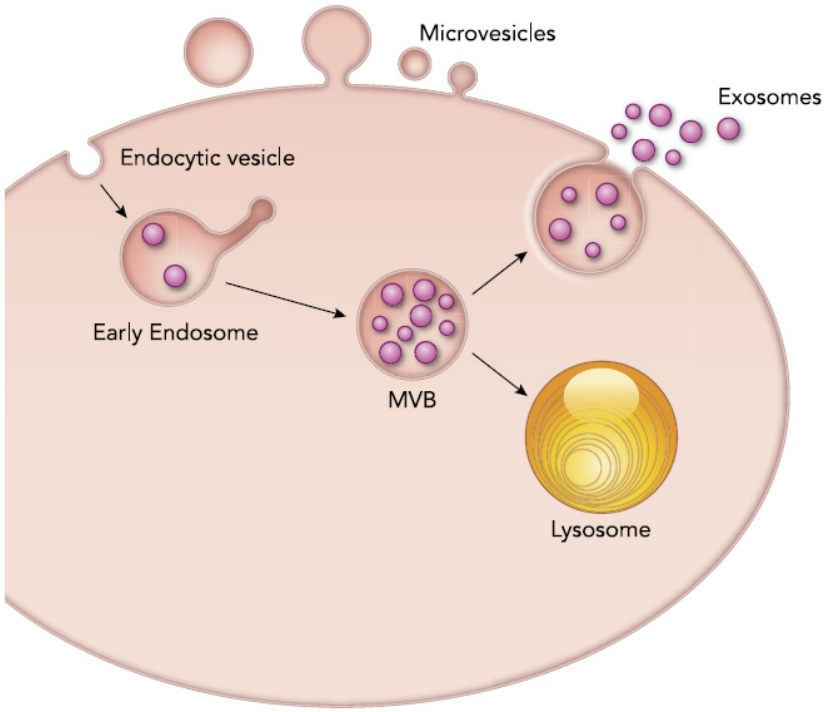

Figure 1.1. Representative graph of EVs biogenesis. PM-derived vesicles or MVs are bud directly from the plasma membrane, sequestering portions of cytosol. Endosome-derived EVs or exosomes are formed during endosomal maturation, a process which intraluminal vesicles of MVBs can fuse with lysosomes (for degradation of their contents) or fuse with the plasma membrane to release small vesicles to the extracellular spaces. Source: (18).

1.2.1 Biogenesis of Microvesicles/Ectosomes

MVs emerge from direct fission of the plasma membrane of healthy cells (19). Nonetheless, vesicles released from the PM during apoptosis are known to produce MVs in the form of apoptotic bodies (20). Several mechanisms including rearrangements of lipids components, proteins compositions and Ca^{2+} levels are required for MVs biogenesis (21). Aminophospholipid translocases (flippase and floppase enzymes) together with scramblases and calpain drive rearrangements in the asymmetry of membrane phospholipids, which contribute to the translocation of phospholipids between the two membrane leaflets, this process is essential in the first steps of the MVs formation (22) (Figure 1.2). Upon lipid redistribution, the fission and release of MVs from cell are led by the cytoskeletal rearrangements of actin and myosin activity regulated via proteins of small GTPase Rho and ARF6 (ADP-ribosylation factor 6) signaling pathway (19). Other set of MVs have been reported to derived from budding events nucleated by the protein arrestindomain-containing protein 1 (ARRDC1)-mediated MVs

(ARMMs), which is recruited to the plasma membrane along with elements of the ESCRT (endosomal sorting complexes required for transport) pathway generating 50 nm vesicles (23).

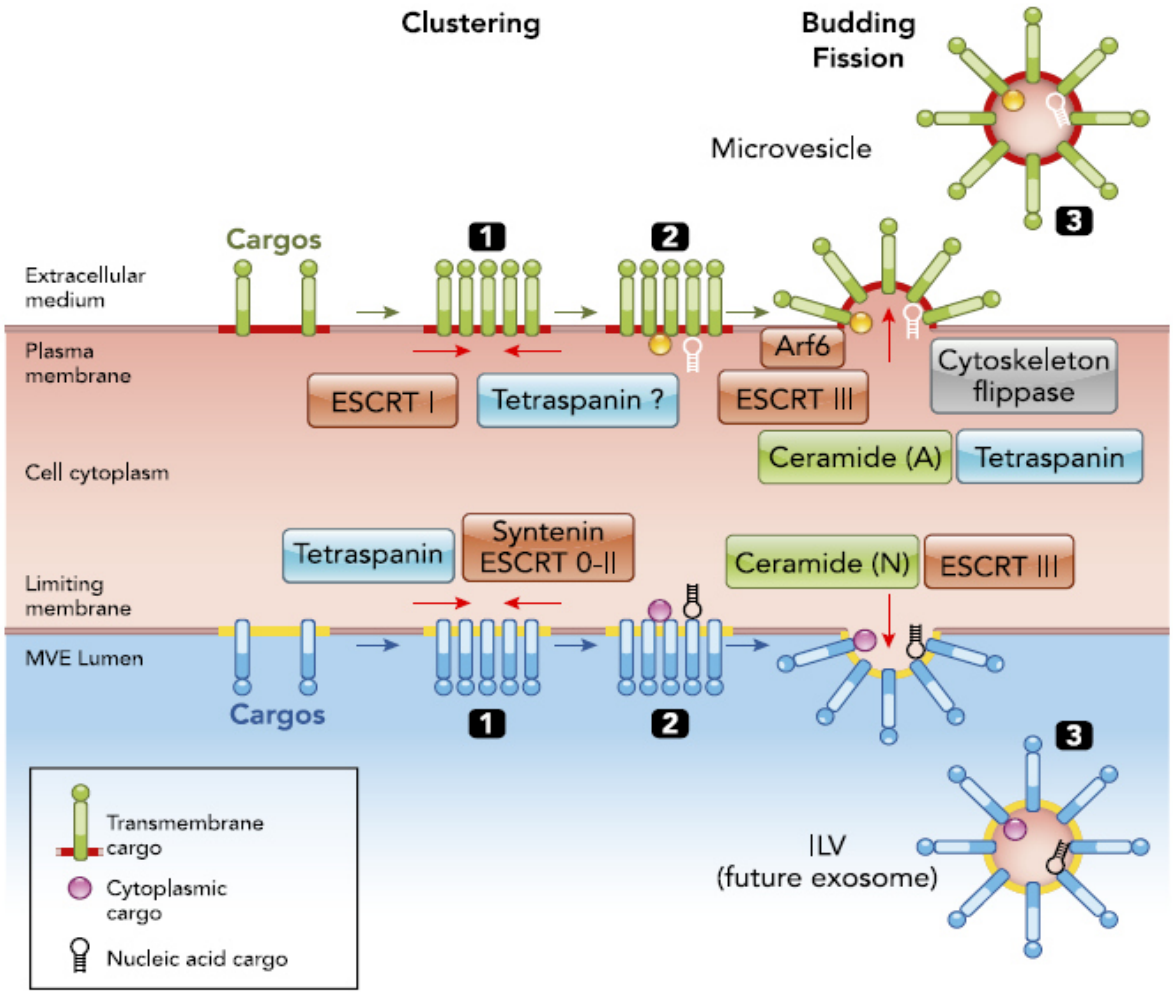

Figure 1.2. Molecular mechanisms involved in the biogenesis of EVs. The formation of MVs and exosomes (sEVs) involves clustering of cargo, budding, and scission, and release into the environment. Transmembrane proteins present on both EVs subtypes maintain the same topology as at the cellular plasma membrane, while cytosolic proteins and genetic material are contained within the bilayer. Abbreviations, ARF6: ADP ribosylation factor 6, ESCRT: endosomal sorting complex required for transport. Source: (18).

1.2.2 Biogenesis of endosomal EVs

The biogenesis of endosome-derived EVs also called small extracellular vesicles (sEVs) or exosomes is closely related to the endosomal pathway (17). sEVs are generated as intraluminal endosomal vesicles (ILVs) by inward budding of the endosomal membrane during their maturation into MVBs (the future sEVs). MVBs can follow either the secretory or lysosomal

pathways (19). In the secretory pathway, MVBs fuse with plasma membrane and released sEVs in the extracellular space. In the lysosomal pathway, MVBs release ILVs in the lumen of lysosomes or autophagosomes to be degraded. The regulation of the balance between secretion and degradation of MVBs remains unexplored (19). The biological pathway involved in ILV generation, including cargo selection, budding, and scission, and this requires the ESCRT family of proteins, lipid rafts enriched in ceramide, tetraspanin-enriched microdomains, syntenin and syndecans proteins (24) (Figure 1.2). The first step in the sEVs biogenesis is the formation of the early endosome containing sEVs-targeted proteins and lipids into the endosomal membrane. During the maturation of endosomes, the ILVs are formed through the invagination of the MVB membrane, a process that regulated by the ESCRT complex, which is composed by four different subcomplexes (ESCRT-0, -I, -II, -III) and other ESCRT-accessory molecules, such as ALG-2 interacting protein X (Alix), vacuolar protein sorting-associated protein 4 (VPS4), and vacuolar protein sorting-associated protein 1 (VTA-1) (25). sEVs-ubiquitinated proteins are capture by ESCRT-0 and ESCRT-I to initiate the clustering and clathrin coating of the cargo proteins, leading to invagination of the endosomal membrane and formation of the MVBs (19). Docking and fusion of the MVBs with the plasma membrane is promoted and regulated by the association of cytoskeleton proteins with specific Rab GTPases and SNARE proteins. Once the fusion is achieved, the budding and scission of the membrane to secrete the sEVs are finalized by the subcomplexes ESCRT-II, -III, and protein VPS4 (19). The use of RNA interference (RNAi) technology allowed to demonstrated that ILVs can be also formed by the presence of lipid molecules ceramide or by the tetraspanin CD63, thus suggesting an alternative mechanisms of ESCRT-independent biogenesis pathways (26,27). Although, progress has been made in recent years in the understanding of basic biology of EVs, further investigations are required to fully clarify the molecular mechanisms of biogenesis, secretion and interaction of EVs with target cells to understand their functional capabilities.

1.3 EV composition

EVs are limited by a phospholipid bilayer and are secreted by different kinds of cells. They are present in every body fluid, for instance, amniotic fluid, cerebrospinal fluid, blood, breast milk, plasma, saliva, semen, etc (28). The lipid bilayer encloses proteins, lipids, nucleic acids, metabolites, cytokines and chemokines to protect them from degradation (10) (Figure 1.3). Lipidome, proteome and transcriptome (including miRNome) data analysis compiled on web-based catalogues such as ExoCarta and Vesiclepedia have revealed that EV compositional repertoire is dependent on the cell origin and its physiological or pathological state (29,30).

Despite of this, EVs subtypes share common features between them, including some proteins and lipids, and, in specific case, nucleic acids (10).

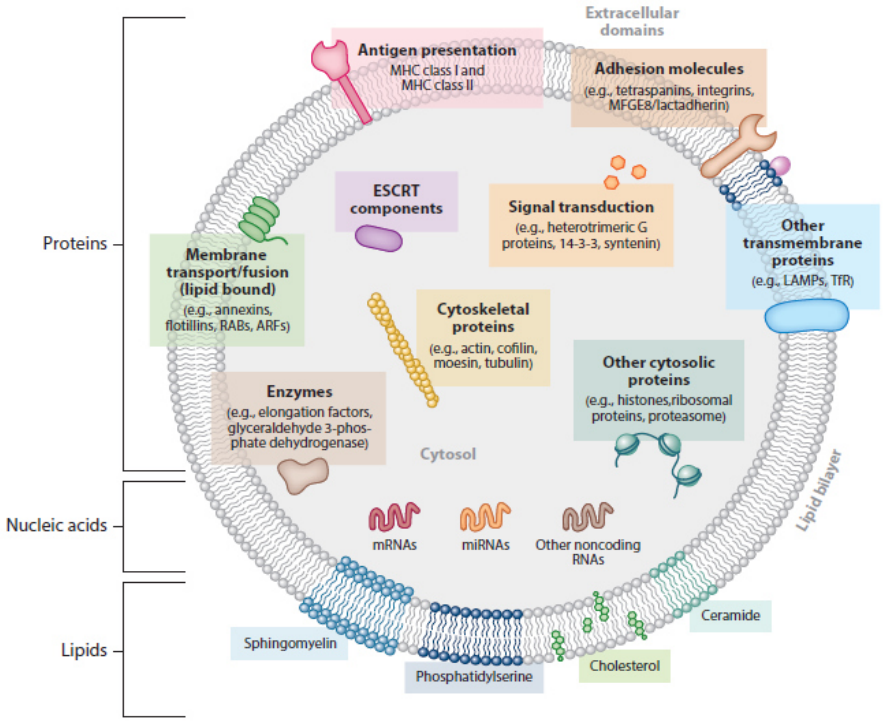

Figure 1.3. Schematic representation of small EVs from endosomal origin. Vesicle are composed by phospholipid bilayer that exposed at its surface extracellular domain of transmembrane proteins and encloses various types of cytosolic proteins. The components listed here may be present in some EV subtypes and not in others. Abbreviations, ARF: ADP ribosylation factor, ESCRT: endosomal sorting complex required for transport, LAMP: lysosome-associated membrane protein, mRNA: messenger RNA, miRNA: microRNA, MHC: major histocompatibility complex, MFGE8: milk fat globule–epidermal growth factor-factor VIII, RAB: Ras-related proteins in brain, TfR: transferrin receptor. Source: (10).

1.3.1 Proteins

Proteins are a major component of the EV cargo and their expression is commonly used for biochemical characterization purposes (1). Proteomics studies have shown that EVs contain specific subsets of cellular proteins, which depends on the cell type that secretes them (10). Generally, EVs proteins comes from endosomes, PM, and the cytosol, whereas proteins from nuclear components, mitochondria, endoplasmic reticulum, and the Golgi complex are mostly absent in small EVs, but may be present in PM-derived vesicles (17). EVs contain proteins of ESCRT involved in MVB biogenesis (e.g., Tsg101 and Alix) which are regularly found in small

EVs from endosomal origin (31). Proteins of the tetraspanin family (CD9, CD63, CD81 and CD82) are highly enriched in sEVs, but some of them are also detected in MVs (31). Other proteins in EVs are involved in the cell adhesion (integrins), antigen presentation (MHC-I, MHC-II) very abundant on antigen presenting cells (APCs)-derived vesicles, stress regulation (heat shock proteins 70 and 90), cell migration (actins, myosin, tubulin), signaling cascades (kinases), transcription and protein synthesis (histones, ribosomal proteins, ubiquitin) (28). Proteins related with transport and membrane fusion (e.g., annexins and Rabs) are also present in EVs (32). EVs can contain several matrix metalloproteinases (MMPs) such as MT1-MMP, MMP13, MMP3, MMP2, MMP9, a disintegrin and metalloprotease 10 (ADAM10), and tumor necrosis factor α-converting enzyme (TACE) (19). Proteomic studies of vesicles have shown that specific protein that were supposedly expressed only by endosome-derived EVs are also found in MVs, thus suggesting that more purification methods and refinement analysis are required to clarify the protein composition of each EV subtype (33).

1.3.2 Lipids

EVs are mainly composed of phosphatidylserine (PS), phosphatidyl-ethanolamine (PE), phosphatidylinositol, phosphatidylcholine, cholesterol (abundant in MVs), sphingomyelin and ceramides (e.g., GM3) (10). These two latter components have been shown to supply EVs with membrane stability, rigidity and also participate in cellular signaling pathways (34). In contrast to PM-derived vesicles, endosome-derived EVs are enriched in cholesterol, sphingomyelin, and hexosylceramides at the expense of phosphatidylcholine and phosphatidylethanolamine (35). Lipid composition of EVs may provide useful information about their purity and this knowledge is important to understand some aspects about vesicles biology and their potential in medical applications.

1.3.3 Nucleic Acids

Numerous groups have analyzed the presence of genetic material in EVs, for example, mRNA that could be translated into proteins by recipient cells (36,37). EVs might contain other long non-coding RNA and/or small non-coding RNA species, including micro RNAs (miRNA), small interfering RNAs (siRNA), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), transfer RNAs (tRNAs), RNA transcripts overlapping with protein coding regions, repeat sequences, structural RNAs, vault RNA (VT-RNAs) and Y RNA (35,38). Other investigations have reported the presence of double stranded DNA, mitochondrial DNA and/or

dsDNA-binding histone proteins (39,40). Nevertheless, the detection of DNA in EVs has been considered as a contaminant from inappropriate genetic material isolation (41).

1.4 EV uptake

For an EV exert its function, it must first be released into the extracellular space, and then reach and bind to its recipient cell. The first step for vesicle internalization is the recognition of tetraspanins, integrins and/or proteoglycans on the EV surface (19). Upon interaction with the cell membrane, EVs are internalized either by direct fusion with the plasma membrane or by multiple endocytosis mechanisms including, phagocytosis, micropinocytosis, clathrin-dependent endocytosis, among other (19) (Figure 1.4). Once internalized, EVs delivery their cargo to the cytosol or transfer it to the endoplasmic reticulum (ER), to be further processed and promote functional responses and phenotypic changes, which may modulate the physiological or pathological status of the recipient cell (19). In contrast, EVs may also access to the endosome system via early endosome and be sorted for degradation in the lysosome (42). The targeting ability of EVs may depend on their natural charge, protein (tetraspanins or integrins), lipid (e.g., PS) and glycan composition (43). Routes of EVs internalization are diverse and depend on the donor and recipient cell type (19). In fact, whether a specific route exists for EVs in general or at least for a particular EV subtype, is yet poorly explored.

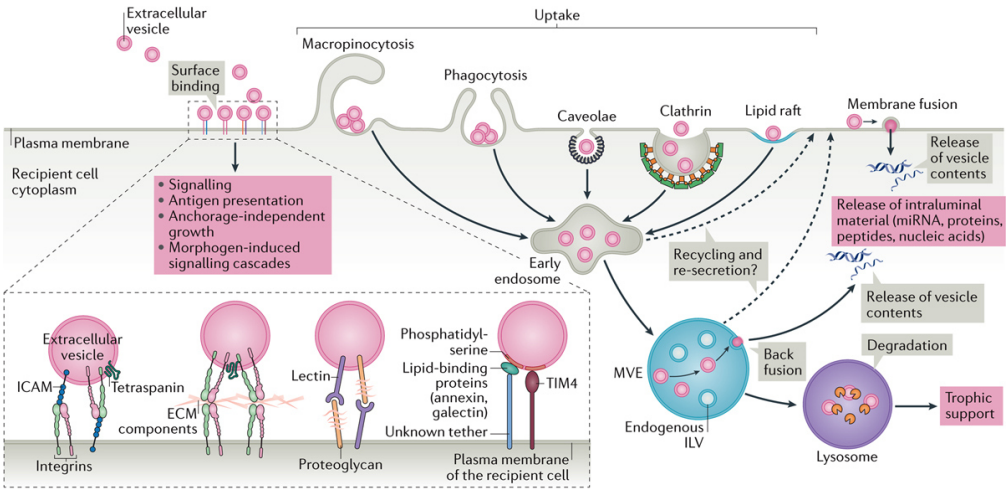

Figure 1.4. EVs internalization routes by recipient cells. In the recipient cell, EVs will bind to the cell surface (see inset) and can undergo various fates. Depending on the cell type, they can remain bound to the surface (e.g., integrins) and can initiate intracellular signaling pathways (e.g., antigen presentation). EVs may also be internalized by multiple endocytosis pathways. Abbreviations, ECM: extracellular matrix, ICAM: intercellular adhesion molecule, TIM4: T cell immunoglobulin mucin receptor 4, MVE: multivesicular endosomes. Source: (19).

1.5 EV isolation and characterization

Despite major advances in EV research and in the understanding of their role in cell-to-cell communication, their clinical use as biomarkers, or extracellular vesicle-based therapies, some aspects related with EV biology and physiology remains unclear (31). The development of a large variety of technologies and strategies for isolation and characterization of EVs has continually been performed (44,45). Currently, methods that allow the detection and separation of EVs influence in the type, purity and yield of the recovered EVs (1). These methods include differential centrifugation (dUC) or ultracentrifugation (UC), polymer-based precipitation, size-exclusion chromatography (SEC), immunoaffinity capture-based techniques, microfluidics, field-flow, and tangential flow (Table 1.1). Each method presents advantages and disadvantages, and the criteria to select an optimal method for a certain study depend on different factors, such as starting material (e.g., cell culture, tissue or biological fluids), volume, purity grade, and isolation purpose (research, therapeutic, or diagnostic use) (1). According to the survey on methods used for isolation and characterization of EVs, generated by the ISEV rigor and standardization subcommittee (45), differential ultracentrifugation was the most widely used technique to separate vesicles, and its use appears to have declined slightly, from 2015 to 2019 (45,46). In contrast, the use of size exclusion chromatography, gradient ultracentrifugation and affinity methods have increased more than double, compared with 2015 reported survey (45,46). Polymer-based precipitation is used by more than 20% of respondents, although it is perhaps the "dirtiest" method available unless combined with other approaches.

Table 1.1. Methods for EVs isolation. Adapted from (47)

Isolation Method	Isolation principle	Advantages/Limitations
Differential centrifugation	EV separation based on particle density, size and shape	- Commonly used; standardized; vesicle enrichment as pellet; EV subtypes isolation by density gradient centrifugation. - Vesicle aggregation; protein and soluble factors. - contamination; low recovery; laborious.
Polymer-based precipitation	EV precipitation using polymers altering solubility	- Easy and inexpensive; high yield; effective with small amount of starting material; preservation of bioactivity -Co-precipitation of protein contaminants and polymeric materials; not suitable for large scale studies; long incubation times

Size-exclusion chromatography (SEC)	EV isolation by gel filtration chromatography based on size	- Inexpensive; reproducible; high yield and purity; preservation of integrity and activity - Specific equipment; not suitable for large scale studies; long run times
Immunoaffinity capture-based techniques	EV immuno-purification using magnetic beads conjugated with antibodies direct toward specific EV surface markers	- Sensitivity; specificity; high purity; EV subtypes isolation. - Expensive; antibody cross-reactivity; low yield.

EVs can be characterized by physical (e.g., size, morphology, concentration) and molecular/biological properties (e.g., protein content, surface marker expression, nucleic acids, lipids, proteins) (1) (Table 1.2) (Figure 1.5). Electron microscopy (EM), atomic force microscopy (AFM), dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), tunable resistive pulse sensing (TRPS), flow cytometry, enzyme linked immune-sorbent assays (ELISA), western blotting (WB), liquid chromatography or mass spectrometry, next generation sequencing (NGS) or microarrays. From 2015 to 2019 survey, WB, single particle tracking, EM, and flow cytometry were the major techniques employed for EV characterization (45,46). In fact, in agreement with the previous survey, Flow cytometer, was the most widespread method, with the potential to provide either population-level or single-particle biochemical and physical information (46). As mentioned in the isolation methods, the characterization techniques also have advantages and limitations (48). Due to the small differences in the physical properties and composition of the different EV subtypes, the combination of diverse techniques is required for qualitative and quantitative EVs characterization. International Society of Extracellular Vesicles (ISEV) recommends to increase the number of characterization methods use of at least three or more independent technologies to characterize individual EVs (1).

Table 1.2. Methods for EVs characterization. Adapted from (47)

Method	Information Acquired	Advantages/Limitations
Electron microscopy (EM)	EV dimension and morphology	- Direct assessment of morphology and size; small sample amount - Time consuming; size and morphology modifications due to sample preparation
Atomic force microscopy (AFM)	EV three-dimensional topography	- No sample fixation and staining; small sample amount - Size and morphology modifications due to sample dehydration on mica surface

Dynamic light scattering (DLS)	EV size distribution	- Fast; no sample preparation; sample preservation for downstream analysis - Inaccurate with polydispersed and size heterogeneous samples
Nanoparticle tracking analysis (NTA)	EV concentration and size distribution	- Fast; no sample preparation; sample preservation for downstream analysis - Inaccurate with size heterogeneous samples and particle aggregates
Tunable resistive pulse sensing (TRPS)	EV concentration, size distribution and surface charge	- Fast; no sample preparation - Difficulties with unknown and heterogeneous size distribution samples (difficult to select the correct nanopore setup); detection of non-vesicular material within size range
Flow cytometry (FC)	EV marker characterization, absolute counting	- Quantitative and qualitative (using specific antibodies) characterization of EVs - Detection limit (>300 nm, flow cytometer dependent); swarming effect (identification of multiple vesicles as a single event); detection of protein/antibody aggregates
ELISA/Western Blot	EV protein quantification	- Standard immunological methods; specific characterization of EV protein markers - Time consuming; possible detection of non-EV proteins; non-specific information on EV concentration/size/distribution

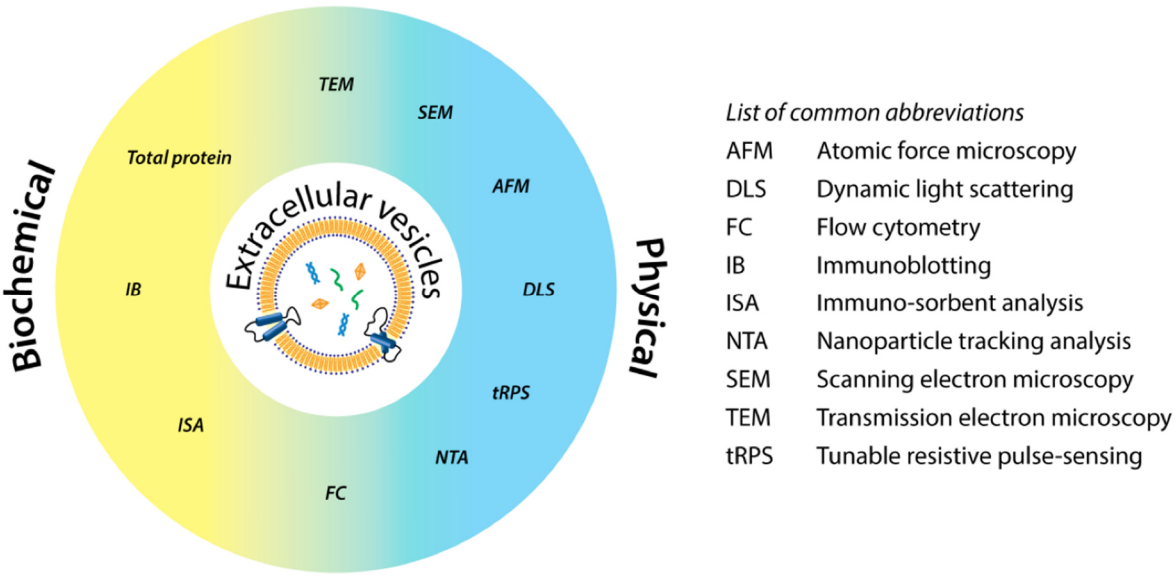

Figure 1.5. Schematic classification of common technologies for EV description. Modified from (48).

1.6 Biological and pathological roles of EVs

As carriers of proteins, lipids and nucleic acids, EVs from donor cell transport at local or distant site (paracrine signaling) bioactive molecules that acts as a multifunctional signaling complexes which modulate biological process in the recipient cell. This EV-mediated intercellular communication is essential to maintain cellular physiology (18). EVs can packaging cellular waste, harmful cytoplasmic DNA, among other undesirable molecules, which can then be removed by phagocytotic cells, thus contributing with the regulation of cellular homeostasis (28). Many studies were published showing that EVs are involved in several cellular processes including angiogenesis regulation, cell differentiation, tissue regeneration, regulation of nervous system, phenotype modulation, inflammation and immune response (28) (Figure 1.6). EVs may also contribute to the pathogenesis of several diseases, such as autoimmune illnesses, neurodegenerative and cardiovascular disorders, blood coagulation, cancer, and bacterial, protozoa, fungal and viral infection (28,49). Although investigations have shown the contribution of vesicles to cellular biology, the physiological and pathological roles of EVs are not completely identified, thus, efforts to developing *in vivo* models to studying endogenous vesicles may contribute to a better compression of their physiology (50).

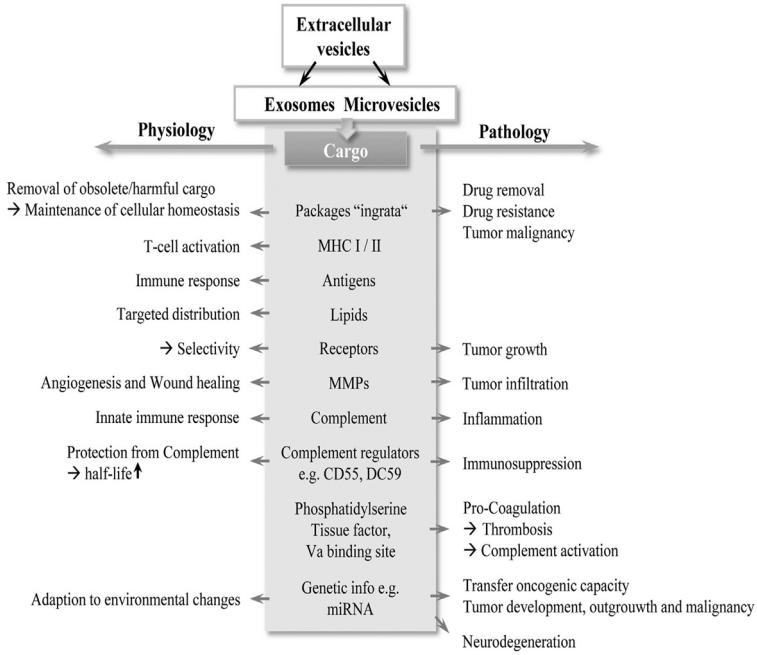

Figure 1.6. schematic overview of EV functions. Depending on their cargo composition, and type (sEVs or MVs) EVs exert bi-directed functions (depicted by arrows) with important contribution to physiology and pathology. Abbreviations, CD: cluster of differentiation, MHC: major histocompatibility complex, MMP: matrix metalloproteinase, miRNA: microRNA, Source: (51).

1.7 EVs role in immune responses

Numerous studies have widely documented the function of EVs in immune responses, mainly in the context of cancer and autoimmunity (28). Cells populations (dendritic cells, monocytes, macrophages, neutrophils, natural Killer, T and B cells, among others) from immune systems can shed EVs with specific cargo. This EV-mediated mechanism of cellular communication is independent cell-cell contact or of soluble factors (e.g., cytokines and chemokines) which is crucial for the regulation of innate and adaptive immunity (52). First studies about EVs and immunity showed that vesicles cargo from immune cell contains MHC class I and class II molecules, and T cell co-stimulatory molecules (53). These findings suggest that EVs can act as antigen-presenting vesicles and effectively stimulating T-cell functions for a proper immunological response (53,54). Here, we summarize and discuss some roles of EVs in determining innate immune responses with special focus on vesicles from human macrophages.

1.7.1 The role of EVs in innate immunity

Many studies have demonstrated that EVs are involved on innate immune response as pro-inflammatory mediators and inducer of inflammatory signals during infections or chronic inflammatory diseases such as atherosclerosis, pre-eclampsia, rheumatoid arthritis, sepsis, type 2 diabetes, among other disease (52,55). Previous evidences suggested that EVs delivery in recipient cells activates the cytosolic DNA-sensor cGAS/STING (cyclic GMP-AMP synthase stimulator of interferon genes), thus triggering the expression of inflammatory genes and IFN type I response, to further enhance antiviral responses (56). Furthermore, EVs can acts as a messenger to activate the complement system (51), and also transport anti-inflammatory molecules. It is also known that, the functional effects of these particles will depend of their cargo and type of target cell (28). For example, EVs released by pathogens or infected cells pack and carry components that may contribute to the propagation of inflammation and, consequently, affect the innate immune response. These signals include damager- or pathogen-associated molecular patterns (DAMPs, PAMPs), double-stranded RNA, lipoarabinomannan, lipopolysaccharide, glycopeptidolipids, miRNAs, DNA, agonists of pattern recognition receptors (e.g., Toll-like receptors, TLRs), and other chemotactic signals (57). In addition, proinflammatory mediators found to be associated with EVs include interleukin (IL)-1α, IL-1β, tumor necrosis factor (TNFα), transforming growth factor (TGF-β), Fas ligand (FASL), and

CD154 (58). These data indicate that the role of EVs in innate immunity is complex and still not well understood.

1.7.1.1 Immunological properties of macrophage-derived EVs

Several studies have reported the immunomodulatory function of EVs from different innate immune cells (59,60). Considering that human macrophages were the cellular model that we chose to carry out our research, we delimited this revision to examine the emerging roles of macrophage-derived EVs, which can modulate directly or indirectly the innate and adaptive immunity.

Early studies showed that EVs derived from monocyte-derived macrophages (MDMs) induced proliferation and activation of CD4+ and CD8+ T cells *in vitro* and *in vivo* after intranasal injection in mice. This EV-mediated stimulus was depended of DCs activity and resulted in the induction of a population of effector memory T cells (61). Furthermore, macrophage-derived vesicles can carry MHC class II and costimulatory molecules, similarly to DC-derived EVs, thus suggesting a role in antigen presentation (58). In addition, macrophage-derived EVs have also been shown to induce the differentiation of monocytes to macrophages, through the transfer of miR-223, which is an important regulator of myeloid cell proliferation and differentiation (62). Emerging evidence has shown that EVs derived from M1 phenotype macrophages induced the release of Th1 cell-promoting cytokines [IL-12, interferon (IFN-γ)] in both macrophages and DC cell lines, and induced a stronger antigen specific cytotoxic T cell response *in vivo* when administered together with a peptide vaccine. In contrast, EVs released by M2 macrophages enhanced the secretion of anti-inflammatory cytokines IL-4 and IL-10 by macrophages and DCs (63).

In infectious scenarios, uninfected macrophages exposed with EVs from macrophages infected with *Mycobacterium tuberculosis* (Mtb), *Mycobacterium bovis* Bacille Calmette–Guerin (*M bovis* BCG), *Salmonella typhimurium* or *Toxoplasma gondii,* stimulated a pro-inflammatory response in a Toll-like receptor and myeloid differentiation factor 88–dependent manner. This intercellular communication during immune response to intracellular pathogens, suggests that EVs containing PAMPs can act as an important mechanism of immune surveillance (64). Other group showed that macrophages infected with *Mycobacterium avium* and M. *smegmatis* stimulated an increase of EV secretion and enhancement of HSP70 expression in these vesicles, to promote *in vitro* macrophage activation and TNFα expression (65). Cronemberger-Andrade and colleagues reported that EVs derived from macrophages infected with *Leishmania amazonensis* induce the production of the pro-inflammatory

cytokines IL-12, IL-1β and TNF-α in naïve macrophages. The immunomodulatory effects of EVs favors the regulation of immune response and contribute with the elimination of the parasite (66). The same group showed that EVs derived from THP-1 macrophages infected with *Trypanosoma cruzi* interact with TLR2 and stimulate the translocation of NF-kB in recipient macrophages which result in the production of proinflammatory cytokines such as TNF-α, IL-6 and IL-1β to maintain the inflammatory response in the course of infection (67). As described above, the role of EVs in innate immune responses is likely more pronounced in the context of an infection, since vesicles may carry both host and pathogen components, meaning that EVs can either enhance or inhibit an infectious process (68). The aforementioned studies and other evidences indicate that macrophage-derived EVs are able to regulate inflammatory reactions and modulate innate immune responses, either controlling or worsening infectious diseases.

1.7.2 Role of EVs in viral infections

It is known that viruses can exploit the functions of EVs in intercellular communication to promote their own replication and persistence (49). EVs have been implicated in the pathogenesis of several viral diseases, including multiple members of the human herpesvirus (HHV) family, hepatitis viruses B, C and E (HBV, HCV, and HEV), human cytomegalovirus (CMV), and human immunodeficiency virus 1 (HIV-1), using EV endocytic pathway and vesicle transport to enter in uninfected cells (49). It has been documented that EVs have both pro- and anti-viral effects in the recipient cell, these outcomes depending on the cellular origin and/or the physiological state (healthy or infected) of the cell that secreted the vesicles (68). The pro-viral effects of EVs consist in 1) spreading the virus from infected to uninfected cells, either by transporting PAMPs or masking viral antigens to avoid immune recognition (69); 2) contributing to the viral replication by reducing innate antiviral responses. 3) dampening the adaptive antiviral immune responses by suppressing Th1 responses or by promoting Treg cell expansion; 4) increasing viral infectivity via elimination of the host protein relevant for antiviral response (70).

On the other hand, EVs are able to increases host antiviral responses to restrict viral infection. For example, EVs can transfer cellular restriction factors like apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3) or soluble host factors (e.g., cGAMP), thereby triggering immune response to control virus replication (71,72). EVs from uninfected primary human trophoblasts, can confer EV-mediated antiviral resistance to recipient cells by inducing the autophagy pathway against the infection of several viruses, including coxsackievirus B3 (CVB), poliovirus (PV), vesicular stomatitis virus (VSV), vaccinia

virus (VV), herpes simplex virus-1 (HSV-1) and CMV (73). Moreover, EVs can transfer cellular factors that enhances an interferon-induced antiviral response against HCV and HSV-1 (74).

In HIV infection, Okeoma's group show that EVs derived from healthy human semen inhibited viral replication *in vitro*, blocked the murine AIDS (mAIDS) virus complex (LP-BM5), and avoided the spread of HIV-1 from vaginal epithelial cells to target cells, thus, suggesting that semen-derived EVs possess anti-retroviral activity (68,75,76). In addition, EVs from CD4+ T cells displayed CD4 molecules on their surface allowing them to bind to HIV-1 envelope protein (gp120) to hinder virus interaction and infection of target cells (77). Another group, documented that monocytic cells exposed to cigarette smoke condensate (CSC) released EVs with anti-HIV-1 activity, likely due to modified incorporation of antioxidant molecules into EVs (78).

In summary, EVs constitute an important messenger that may facilitate or suppress viral infection, as well as an important mediator of antiviral responses, thus suggesting the complex role of extracellular vesicles in the biology of viral infections.

1.8 Human Immunodeficiency Virus (HIV)

1.8.1 Structure and genomic organization of HIV-1

The human immunodeficiency virus (HIV) is the etiological agent of acquired immunodeficiency syndrome (AIDS), a disease characterized by attacking and weakening the immune system (79). HIV-1 is a virus with spherical morphology and size between 90-120 nm in diameter (80). It has a lipoprotein envelope formed by a lipid bilayer from the host cell, a capsid (CA) and protein nucleocapsid (NC) that carries the viral genome and RNA accessory proteins. The viral capsid formed by monomers of a 24 kDa protein, designated (p24), is located inside the envelope and is internalized by the matrix protein (MA, p17) (Figure 1.7). The genetic material of HIV is composed of two single strands of positive polarity ribonucleic acid (RNA) associated with the three viral enzymes: protease (PR), reverse transcriptase (RT) and integrase (IN).

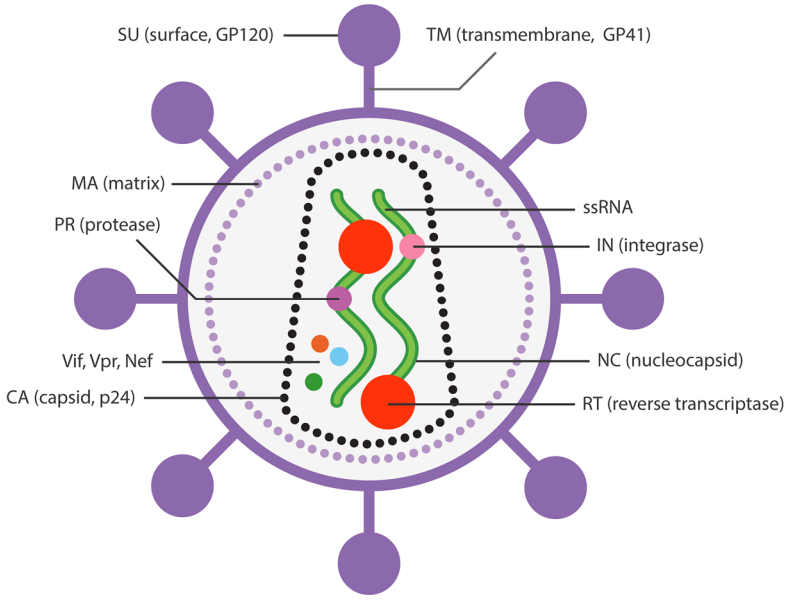

Figure 1.7. Schematic representation of the structure of HIV-1. Structural proteins (MA, CA, NC, SU, TM) accessory proteins (Vif, Vpr and Nef) and viral enzymes (IN, PR and RT) are represented in the scheme. Source: https://old.abmgood.com/The_Lentivirus_System.php.

The genomic structure of HIV-1 consists of two identical single-stranded RNA molecules, at both ends have long terminal repetitions (LTRs), which have identical sequences responsible for the interaction of the viral genome with the host genome and thus give signs of the beginning and end of the transcription of HIV proteins (80). In between the LTR's there are nine genes which codified for proteins with essential roles during the HIV cycle. They can be divided into

3 groups: three structural proteins (env, gag and pol), two regulatory (Tat and Rev) and four accessory proteins (Nef, Vif, Vpu and Vpr) (81) (Figure 1.8). The gag (group specific antigen) gene codes for a polyprotein, Pr55Gag, which is cleaved by the viral protease, giving rise to matrix, capsid and nucleocapsid proteins. The pol (polymerase) gene encodes a precursor polyprotein Pr160GagPol, which is cleaved generating the enzymes PR, RT and IN. The env gene (envelope glycoprotein) codes for a precursor known as gp160, which is cleaved by a cellular protease generating the gp120 and gp41 glycoproteins. The gp120 interacts with the cell receptor (the CD4 molecule) and the cell co-receptors (CCR5 or CXCR4), while the gp41 has the function of anchoring the gp120/gp41 complex to the cell membrane, allowing the virus to enter (79). Several other genes in the HIV genome code products with regulatory or accessory function, acting on the control of viral replication and infectivity (Tat, Rev, Nef, Vif, Vpu and Vpr) (82).

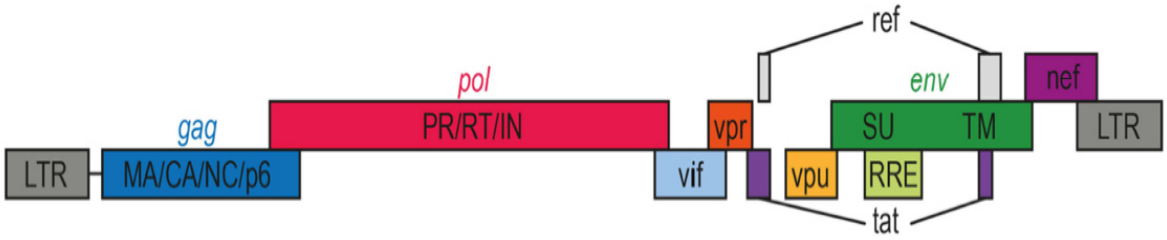

Figure 1.8. Schematic representation of the genomic structure of HIV-1. Blue and green rectangles represent the structural genes. Red rectangles indicate genes encoding viral enzymes. Accessory proteins are also represented in the scheme. Source: (80).

1.8.2 Epidemiological aspects of HIV-1

Reports from the United Nations Program on HIV/AIDS (UNAIDS) and World Health Organization (WHO) indicate that, at the end of 2019, approximately 75.5 million people have become infected with HIV since the start of the epidemic, 32.7 million died from AIDS-related illnesses, while 38 million people globally were living with HIV-1 (83,84). Data also show that 1.7 million new infections occurred over the course of the year and 26 million people were receiving antiretroviral therapy as of the end of June 2020 (83). Therefore, HIV/AIDS requires joint efforts by the scientific community, governments and society for its effective control and prevention. In Brazil, according to the latest statistics carried out by UNAIDS at the end of 2019, the estimate of the number of people living with HIV-1 is 920.000 compared to 640.000 in 2010. In the same year there were 48.000 new infections and 14.000 people died due to

AIDS-related illnesses (83). According to the Brazilian Ministry of Health, National HIV/AIDS/STD Program, from 2007 to June 2020, 342.459 cases of HIV infection in Brazil were reported in Sinan, these including 152.029 (44,4%) in the Southeast, 68.385 (20,0%) in the South region, 65.106 (19,0%) in the Northeast region, 30.943 (9,0%) in the North region and 25.966 (7,6%) in the Midwest region. In 2019, 41.919 new cases of HIV-1 infection were reported, with 4.948 (11,8%) in the North, 10.752 (25,6%) cases in the Northeast, 14.778 (35,3%) in the Southeast, 7.639 (18,2%) in the South and 3.802 (91,2%) in the Midwest region (85).

1.8.3 HIV-1 replication cycle in lymphocytes

HIV-1 infects CD4+ T lymphocytes, macrophages, dendritic cells (DCs) and, in the central nervous system, microglia. The replication of HIV-1, occurs through a series of events that has eight well-planned steps which engage complex relationship between cellular and viral factors (81). The HIV-1 replication cycle begins by the interaction of the viral surface glycoprotein gp120 with the CD4 receptor on the host cell surface, inducing changes in the structural conformation of gp120 and thus increasing its affinity for the CCR5 and/or CXCR4 chemokine co-receptors, forming an intermediate complex. The complex stimulates activation of the gp41 transmembrane glycoprotein, which in turn forms a clamp-like structure, and brings the envelope of the cell membrane closer by promoting fusion and allowing the access of the capsid to the cytoplasm (86) (Figure 1.9). After entry, the virus releases the genome into the cytoplasm, followed by the activation of the enzyme reverse transcriptase, thus the viral RNA is retrotranscribed into complementary DNA (cDNA). Once the viral cDNA is synthesized. It is transported to the nucleus of the host cell by the pre-integration complex (PIC). In the nucleus, the viral DNA (vDNA) will be integrated into the cellular genome by the enzyme integrase. This integrated DNA, named provirus, behaves like a cellular gene that can be transcribed into messenger RNA (mRNA) by the cellular machinery. Proviral transcription is initiated by cellular RNA polymerase II that produces baseline amounts of Tat, which controls the additional transcription of HIV-1 genes. Initially, regulatory and accessory genes are transcribed, then the Rev protein promotes the change to the transcription of the remaining genes, which encode the proteins of the capsid, matrix, viral enzymes and envelope. All these proteins and viral RNA will be packaged in new viral particles. The last stage of the replication cycle is the maturation and budding of the virus, which are released through the cell membrane, forming the viral envelope. This process occurs in the extracellular environment, after proteolytic processing of precursor proteins Gag and Pol (p55, p160) by the action of viral

protease. The new virions spread by infecting new cells, thus continuing the HIV-1 infectious cycle (80).

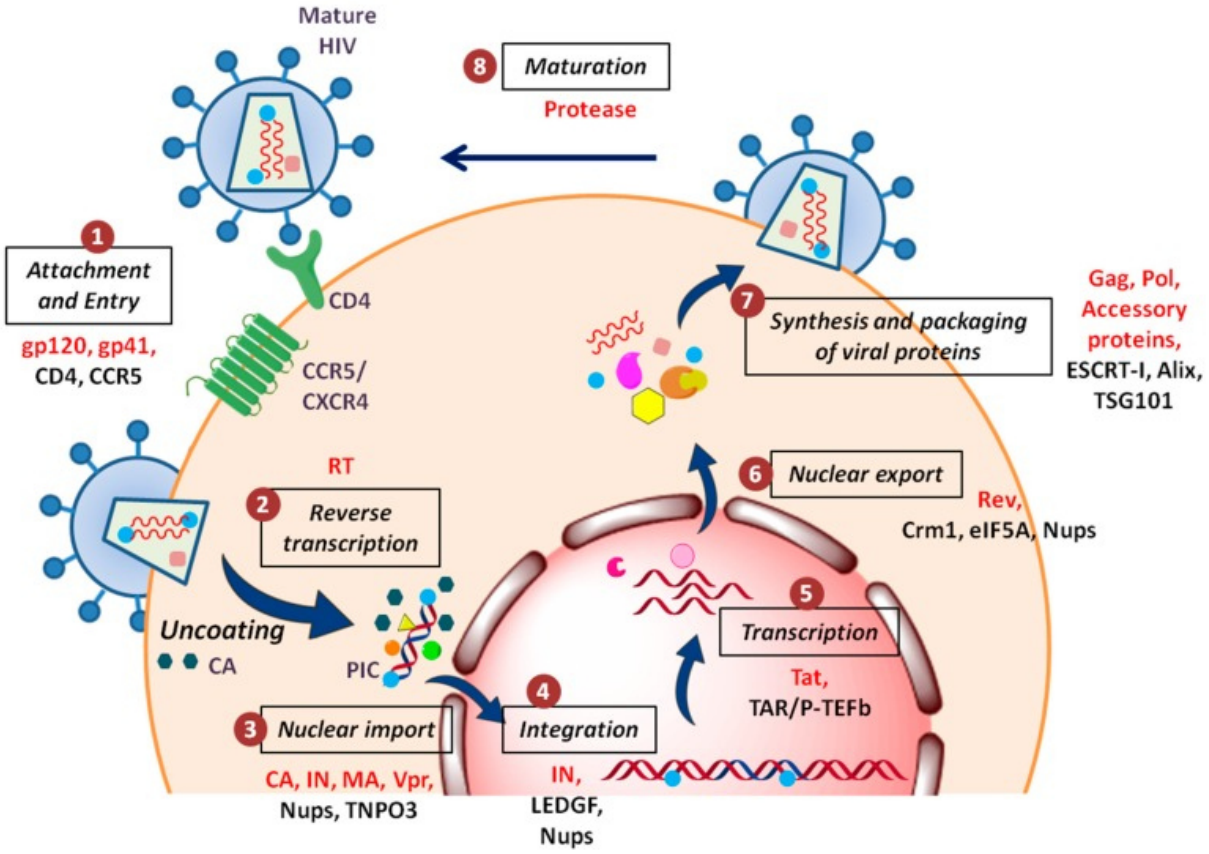

Figure 1.9. HIV-1 replication cycle summary: (1) The HIV-1 particle attaches and fuses itself to the host cell membrane via its surface glycoproteins and enters the cell cytoplasm; (2) viral genomic RNA is reverse transcribed into DNA, accompanied by uncoating of the viral capsid; (3) nuclear entry of pre-integration complex occurs with the help of various host nuclear proteins; (4) integration of viral DNA into the host chromatin; (5) during productive infection, viral transcription takes place; (6) RNA splicing and nuclear export of viral RNA occurs; (7) production and assembly of new virus particles, which bud from the plasma membrane; and (8) virions become infectious after maturation (action of protease). The viral and host cellular proteins involved in the replication cycle of HIV-1 are highlighted at each step in red and black, respectively. Abbreviations, CA: Capsid, LEDGF: lens epithelium-derived growth factor, Crm1: chromosome region maintenance- 1, eIF-5A: eukaryotic translation initiation factor-5A, MA: Matrix, Nups; nucleoporins, IN: Integrase, PIC: pre-integration complex, Rev: regulator of virion expression, RT: Reverse transcriptase, TAR: trans-activation response, TNPO3: transportin 3, Vpr: Virus protein r, Source: (81).

1.8.4 HIV-1 replication cycle in Macrophages

Macrophages are considering the major cellular reservoirs of HIV-1, this cell are more resistant to the cytopathic effects of HIV infection than CD4+ T-cells or DCs, and may support long-term, productive infection as a result of the ability to evade defensive mechanisms of the immune system. Macrophages can spread infection due to their ability to infiltrate in tissues and release virions continuously (87). In addition, macrophages accumulate competent replicating viruses for prolonged periods of time, even in patients receiving antiretroviral treatment. The interruption of antiretroviral therapy produces a rapid resurgence of viral load, with the contribution of this cell type (88). These features indicate, that macrophages play an important role in the establishment of early-stage of HIV-1 transmission, persistence, dissemination and progression to AIDS (89). HIV-1 replicative cycle in macrophages differs from CD4+ T cells in some aspects. For example, the proviral DNA in T cells is synthesized in an approximately 6 h, while in macrophages it takes approximately 36-48 hours (90). The assembly of viral particles in CD4+ T-cells takes place on the plasma membrane, quickly and with cytopathic effects. In contrast, virions in macrophages accumulated in intracellular structures of the late endosome in the cytosol, called multivesicular bodies (MVBs) (91). These structures were later named virus-containing compartments (VCC) and are suggested to be the site of assembly of viral particles in infected macrophages (92).

1.8.5 Immunopathogenesis of HIV-1 infection

The natural course of HIV-1 infection varies between individuals; however, some features are virtually similar in infected people without treatment (93). Infected persons present three clinical phases, including, the eclipse, acute and chronic phase (Figure 1.10). The period between 7-21 days after infection is called the eclipse phase, when the virus can be first detectable in the blood (94). The acute phase of HIV-1 infection corresponds to the period of high viremia accompanied with decrease in CD4+ T cell count, which is usually transient. In addition, high plasma levels of viral RNA copies are detected, reaching a peak of 10^7-10^9 copies of RNA/mL (95). A decrease in the systemic level of central memory CD4+ T cells results in a deficit of effector memory T cells; this process is associated with progression to AIDS (96). The chronic phase can last from several months to many years, which subdivides the patients into different subtypes: *(i)* rapid progression, when AIDS develops within 3 years of infection; *(ii)* intermediate progression, when AIDS develops slowly between a span of 3 and 10 years after seroconversion; and *(iii)* long-term non progression (LTNP), when HIV-1 infected people

remain asymptomatic and maintain high CD4+ and CD8+ T cell counts for more than 10 years (97). Within the LNTPs classification are included the elite controllers, those individuals capable of suppressing HIV replication in such a way that viral load levels remain undetectable in the absence of antiviral therapy. The estimated prevalence of these individuals ranges from 0.15 to 1.5% of all patients living with HIV-1 (98). At the end of chronic phase, the number of CD4+ T cells can be lower than 200/μL, a drastic increase in viremia is observed (with exception of elite controllers) and opportunistic infections and neoplasms may appear, thus initiating the AIDS phase, characterized by the continuous increase in viral load and CD4+ T cell decline until patient's death (99). HIV-1 infection is characterized not only by the development of profound immunodeficiency, but also by chronic immunological activation, including polyclonal B cell expansion, increased T cell turnover, expansion of T cells with activation phenotype, and elevated serum levels of inflammatory cytokines and chemokines (100).

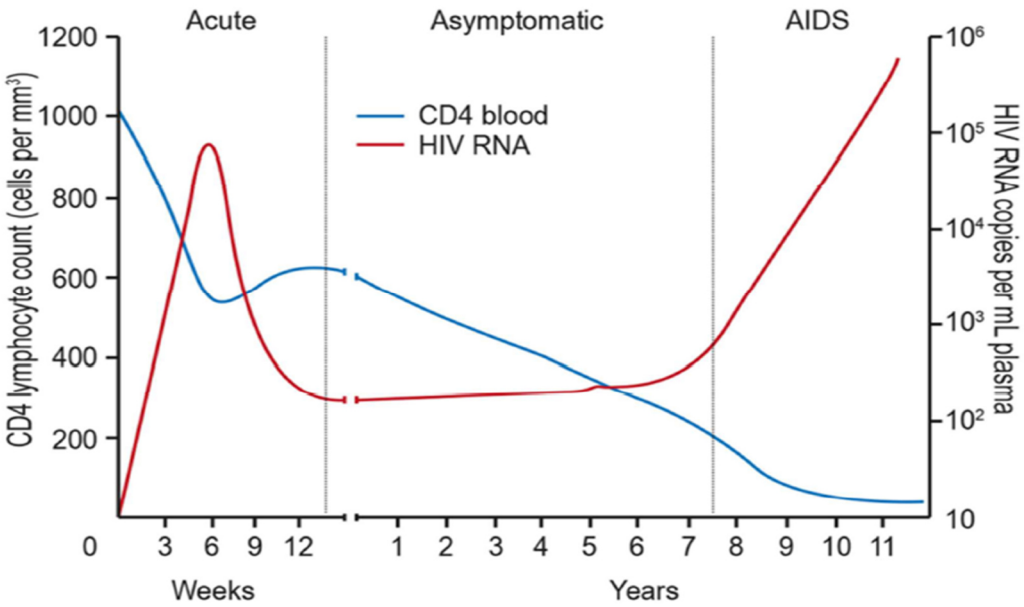

Figure 1.10. The course of untreated HIV infection. In untreated HIV infected patients, the blood CD4+ T cell count progressively declines over the course of infection until patient's death. Adapted from (99).

1.8.6 Immune response to HIV-1 infection

Upon HIV-1 infection, host cells sense the pathogen-associated molecular patterns (PAMPs) in viral products (e.g., structural proteins, p17, p24, and gp41) by pathogen-recognition receptors (PRRs) to initiates innate and adaptative immune response (101). This response stimulates the production of soluble factors such as type I and type III interferon (IFN),

as well as pro-inflammatory cytokines and chemokines that recruit and activate innate immune cells, including macrophages, DCs and natural killer cells (NK) to control infection and trigger adaptive immune response (102).

Cellular response mediated by CD8+ cells (also known as cytotoxic T lymphocytes, CTLs) can inhibit viral multiplication by elimination of infected CD4+ cells, through the interaction of the T-cell receptor (TCR) with viral epitopes presented in the major histocompatibility complex molecules I (MHC-I) of infected cells (103). Besides the innate and adaptive immune system, cell restriction factors are also triggering once PRRs recognize HIV-1 PAMPs (PAMP-PRR interaction) (104). Different cellular restriction factors of HIV-1 infection, induced by Interferon-stimulated genes (ISGs), have been described in different stages of the viral replication process, including 1) APOBEC3G/3F (Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like), a cytidine deaminase, once packaged in the virion, catalyzes the conversion of cytosines into uracils, thus inducing hypermutations of the viral cDNA; 2) SAMHD1 (SAM and HD Domain Containing Deoxynucleoside Triphosphate Triphosphohydrolase 1), enzyme that reduces levels of deoxyribonucleotides triphosphate (dNTPs) in cells that do not undergo mitotic division, consequently the reverse transcriptase is deprived of the substrates necessary for the synthesis of viral cDNA; 3) BST2/CD317 (tetherin/bone marrow stromal cell antigen 2), transmembrane cell protein that prevents the release of budding virions by retaining them on the surface of the infected cell; 4) TRIM5-α (tripartite-motif-containing 5α), which induces early degradation of the viral capsid preventing the synthesis of viral cDNA (104)

In summary, the protective role mediated by the innate, adaptative and intrinsic immune responses may fail to control HIV-1 growth and dissemination in untreated chronic patients, and this event may be related with several adopted strategies of HIV-1 to evade host immune response to achieve viral persistence and dissemination in new uninfected neighboring cells. Viral strategies can be related with: the high error rate of the HIV-1 polymerase to select escape mutations during the viral replication process, modification of its PAMPs, downregulation of complement receptors, increased secretion of inflammatory factors, downregulation of NK cell function to overcome the innate immune response and the use of viral proteins to fight against intrinsic immunity (105). Previously evidences have suggested that HIV-1 may use extracellular vesicles as vehicles to enter in uninfected cells and contribute to viral pathogenesis (68).

1.9 Hypothesis and Justification

Evidences have shown the ability of extracellular vesicles to inhibit or enhance viral infection, and this may depends on several aspects including *i*) the host–pathogen interaction, *ii*) the infection status of the EVs donor cells, *iii*) the source of the EVs (e.g., biofluid, cell culture), and *iv*) the type of producer and recipient cells involved in the EV-mediated cell-to-cell communication (68,70).

Despite the significant advances in the EV field, most of functional and pathogenic studies linking cell communication through vesicles were carried out on HIV-1 infection. However, a great deal of experimental research is still needed to comprehend the implication of EVs in neurocognitive disorders, vascular dysfunction and chronic inflammation during HIV infection, as well as the immunomodulatory mechanisms mediated by these vesicles to control viral replication and spread (49,70).

Given all the information above, the growing number of investigations related to the potential role of EVs as signaling vehicles in immune responses against viral infections, and because, it remains unclear whether EVs secreted by primary human macrophages have the ability to suppress HIV-1 replication in infected primary cells. We developed and challenged the hypothesis that **uninfected macrophages can transfer antiviral resistance to HIV-1-infected cells via small extracellular vesicles.** We choose to investigate EVs from primary human macrophages given the importance of these cells in the immune response against a repertoire of pathogens, their long-live, their ability to recognize and respond to a wide range of stimuli with potent endocytic, phagocytic, secretory functions and involvement in almost every disease, including HIV/AIDS (106). Therefore, understanding the role and mechanisms involved in the inhibition of HIV-1 in primary target cells through the transfer of antiviral molecules from macrophage-derived EVs, may contribute in different aspects; 1) to deepen the comprehension of HIV immunopathogenesis and lead to the development of innovative therapeutic interventions to reduce chronic inflammation in HIV-1-infected patients and 2) to provide new evidences about the role of MDM-derived small EVs on the pathogenesis of HIV-1.

2 AIMS

The overall aim of this thesis is to address some of the challenging topics within the EV field in the context of HIV-1 pathogenesis. Firstly, it outlines the validation of EV isolation method, that allows high recovery yields of small EVs from the endosomal origin derived from primary human macrophages. And secondly, includes an investigation on the role of macrophage-derived small EVs to control HIV-1 infection *in vitro*, to advance the knowledge of EV for potential therapeutic applications.

2.1 General

To assess whether macrophage-derived small EVs from uninfected macrophages can transfer antiviral resistance to HIV-1-infected cells.

2.2 Specific

- To validate a technique to isolate and characterize small EVs from primary human macrophages.
- To evaluate whether macrophage-derived EVs inhibit HIV-1 replication in human primary macrophages or PBMCs.
- To evaluate whether sEVs from macrophages stimulated with the neuropeptide VIP inhibit HIV-1 replication in primary cells.
- To assess the route mechanisms of EV uptake involved in the transfer of anti-HIV-1 response in recipient cells.

3 MATERIALS AND METHODS

3.1 Methodological Considerations

A detailed description of the methods and results performed for macrophage culture, isolation and description of macrophage-derived small EVs can be found in the attached article "Characterization and internalization of small extracellular vesicles released by human primary macrophages", whose content represents the first specific objective mentioned in the previous section of this thesis. Full reference of the attached article: Arteaga-Blanco, L. A., Mojoli, A., Monteiro, R. Q., Sandim, V., Menna-Barreto, R., Pereira-Dutra, F. S., Bozza, P. T., Resende, R. O., & Bou-Habib, D. C. (2020). Characterization and internalization of small extracellular vesicles released by human primary macrophages derived from circulating monocytes. PloS one, 15(8), e0237795.

3.2 Ethics statement

The experimental procedures involving human cells in this study were performed with samples obtained after written informed consent and were carried out under the guidelines and regulations approved by the Research Ethics Committee of the Oswaldo Cruz Institute/Fiocruz (Rio de Janeiro, RJ, Brazil) under the number 397-07

3.3 Cell Culture

The methods for isolation, culture and differentiation of human monocyte-derived macrophages are detailed in the aforementioned article (107). When indicated, activated PBMCs (2×10^6 cell/mL) were stimulated with phytohemagglutinin (PHA, 2 μg/mL) for 48-72h, and then recovered and maintained in RPMI-1640 medium containing 10% fetal bovine serum (Merck Millipore), penicillin-streptomycin (LGC Bio) and recombinant human IL-2 (30 IU/mL), before and during HIV-1 infection experiments.

3.4 HIV-1 expansion

Assays of macrophage and PBMCs infection were performed with the CCR5- dependent isolate HIV-1 Ba-L, which was donated by the AIDS Research and Reference Reagent Program (NIH, MD, USA). This viral isolate was expanded in phytohemagglutinin-activated PBMCs from healthy donors, as described elsewhere (108).

3.5 HIV-1 infection and EVs treatment

Macrophages were exposed for 16–18 h to viral suspensions containing 10-20 ng/mL of HIV-1 isolate, as previously described (108). After incubation, excess virus was washed out, and replenished with fresh medium and maintained under standard culture conditions. PBMCs were incubated with viral suspensions (10 ng/mL) for 2 h at 37 °C and 5% CO2. Cells were washed, resuspended in fresh culture medium and distributed in 96 ($2x10^5$ cell/ well). HIV-1-infected cells were exposed to macrophage-derived EVs from individual donors or pools of vesicles for 16–18 h. Each pool of vesicles used in the study was composed by vesicles obtained from 4 different donors. In some experiments, macrophages or PBMCs infected with HIV-1 were co-cultured overnight with small EVs from unstimulated macrophages or from Vasoactive Intestinal Peptide (VIP)-stimulated macrophages (sEVs-Ctr and sEVs-VIP, respectively), or VIP only (Tocris, 5-10 nM/mL), the latter considered as a positive control of HIV-1 inhibition (109,110) (Vieira, RC, doctoral thesis, 2020), followed by p24 ELISA. When indicated, macrophage cell-conditioned medium centrifuged at 400×g for 10min (referred as conditioned medium, CM) or supernatants free of EVs (referred as EV-free supernatant) colleted from macrophage culture were added to HIV-1 infected PBMCs. EV-free supernatant was obtained by ultracentrifugation at 100,000×g for 20 h and then filtered through 0.22 μm filters (Merck Millipore). HIV-1 replication was evaluated in cell culture supernatants after 6-7 (PBMCs) or 12-14 days (macrophages) using an ELISA kit for HIV-1 p24 antigen (Sino Biological, Beijing, China).

3.6 Inhibition of EVs internalization routes to block anti-HIV-1 response

To study the mechanisms involved in the routes of EVs uptake, HIV-1-infected PBMCs were pre-incubated at 37°C with 20 μg/mL heparin for 30 min and then treated or not with EVs. Virus replication was evaluated as described above.

3.7 Statistical analysis

Statistics of functional assays were performed using GraphPad Prism software version 7. All the numerical variables were tested regarding their distribution using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) was used to compare differences among groups following a normal (parametric) distribution, and Dunnet's post-hoc test was used to locate the differences between the groups. Data are shown as the mean and SD, and the differences between values were considered statistically significant when the *P*-value was ≤ 0.05.

PLOS ONE

RESEARCH ARTICLE

Characterization and internalization of small extracellular vesicles released by human primary macrophages derived from circulating monocytes

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Abstract

Extracellular vesicles (EVs) are small membrane-limited structures derived from outward budding of the plasma membrane or endosomal system that participate in cellular communication processes through the transport of bioactive molecules to recipient cells. To date, there are no published methodological works showing step-by-step the isolation, characterization and internalization of small EVs secreted by human primary macrophages derived from circulating monocytes (MDM-derived sEVs). Thus, here we aimed to provide an alternative protocol based on differential ultracentrifugation (dUC) to describe small EVs (sEVs) from these cells. Monocyte-derived macrophages were cultured in EV-free medium during 24, 48 or 72 h and, then, EVs were isolated from culture supernatants by (dUC). Macrophages secreted a large amount of sEVs in the first 24 h, with size ranging from 40–150 nm, peaking at 105 nm, as evaluated by nanoparticle tracking analysis and scanning electron microscopy. The markers Alix, CD63 and CD81 were detected by immunoblotting in EV samples, and the co-localization of CD63 and CD81 after sucrose density gradient ultracentrifugation (S-DGUC) indicated the presence of sEVs from late endosomal origin. Confocal fluorescence revealed that the sEVs were internalized by primary macrophages after three hours of co-culture. The methodology here applied aims to contribute for enhancing reproducibility between the limited number of available protocols for the isolation and characterization of MDM-derived sEVs, thus providing basic knowledge in the area of EV methods that can be useful for those investigators working with sEVs released by human primary macrophages derived from circulating monocytes.

OPEN ACCESS

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Introduction

Cells can communicate with each other through the secretion of extracellular vesicles (EVs) [1], which are small membrane-limited particles composed by a phospholipidic bilayer naturally released in the extracellular environment by different types of cells [2, 3]. These vesicles are present in many biological fluids, including amniotic and cerebrospinal fluids, blood, breast milk, urine, saliva, and semen [4–10]. Accumulated evidence has demonstrated that EVs participate in cell-to-cell communication through the transport of bioactive molecules, such as antigen-presenting molecules, membrane receptors, proteins, lipids, cytokines, DNA, RNA, mRNAs, and microRNAs [11–13]. The EV-mediated intercellular signaling contributes to the regulation of pathological or physiological cell processes, such as angiogenesis, maintenance of homeostasis, cardiovascular diseases, cell signaling, inflammation, and cancer [13–15]. Moreover, EVs are taken up by macrophages, thus functioning as a vehicle to exchange components among cells of the immune system and to strengthen the immune response against pathogens [16–18].

Although in recent years there has been much progress on the understanding of the fundamental biology of EVs, some aspects related to their biogenesis, secretion mechanisms, interaction with recipient cell and function are not yet clear [13, 19]. One reason is that current protocols for EV isolation do not guarantee the purification of specific EV subtypes, resulting in a mixture of heterogeneous vesicles derived from different subcellular fractions. This limitation hampers a better understanding of the role of a given EV subtype in specific physiological or pathological processes [19, 20]. Therefore, it is essential to know the physical and biochemical characteristics of EVs through several analytical methods that allow assessing their purity, integrity, concentration, and interaction with recipients cells before evaluating the functional properties of these vesicles [13, 21].

Currently, EV types may be classified according to their intracellular origins (endosomal system or plasma membrane), sizes, and density ranges [19]. Based on their size, EVs are subdivided into three groups: large EVs (LEVs, >300 nm in diameter), derived from the outward budding and fission from the plasma membrane of apoptotic or healthy cells [22, 23]; intermediate size EVs (IEVs, 150–300 nm), and small EVs (sEVs, <150 nm), originated from endosomal or non-endosomal systems [19, 20]. sEVs from the endosomal system have been shown to be originated from intraluminal vesicles (ILVs) contained in cytosolic multivesicular bodies (MVBs), which later fuse with the plasma membrane releasing them in the extracellular environment [24, 25]. Moreover, EVs can be co-isolated with other particles, like exomeres and lipoproteins from various densities (high, intermediate and low) when using differential ultracentrifugation (dUC) method [19, 26]. Given that apoptotic bodies are released from cells during the process of apoptosis [27, 28], here the term extracellular vesicles (EVs) will be used to refer only to vesicle preparations containing intermediate and small EVs obtained from healthy cells.

Most of the studies about EVs from human cells have been made with cell lineages, including HeLa [29, 30], THP-1 [18, 31], HEK [32, 33], HMC-1 [34, 35], intestinal cell lines [36], or brain endothelial cells lines [37, 38], and few with primary cells, such as dendritic cells (DCs) [39–41] or neutrophils [42]. However, detailed protocol for isolation, characterization, and analysis of interaction with recipient cells of sEVs released by primary human macrophages derived from circulating monocytes are barely available.

Macrophages play essential roles in the activation and modulation of innate and adaptive immune responses against a repertoire of pathogens, including bacteria, protozoan parasites, fungi and viruses, thus critically contributing for the preservation of the host physiologic homeostasis throughout life [43, 44]. Moreover, macrophages participate in many physiologic

processes, such as brain development [45], bone remodeling [46], erythropoiesis [47], tissue regeneration [48], and the interaction between the immune and neuroendocrine systems [49]. Because few works have described the characterization of EVs from human primary macrophages, and taking into account that the adherence to rigorous criteria for EV isolation is essential for obtaining reliable experimental results, we present here an alternative protocol, based on classical dUC method, for isolation and characterization of small EVs released by human primary macrophages derived from circulating monocytes, and for their interaction with recipient macrophages.

Materials and methods

Ethics statements

The experimental procedures involving human cells in this study were performed with samples obtained after written informed consent and were carried out under the guidelines and regulations approved by the Research Ethics Committee of the Oswaldo Cruz Institute/Fiocruz (Rio de Janeiro, RJ, Brazil) under the number 397–07.

Culture of human primary macrophages

Human monocyte-derived macrophages were obtained from buffy coats of healthy human blood donors, as previously described [50]. In summary, peripheral blood mononuclear cells (PBMCs) that had been isolated by density gradient centrifugation (Ficoll-Paque Premium 1.077; GE Healthcare Biosciences) were plated (4.0×10^7 cells in 4 mL of medium) onto three 25 cm^2 flasks (Greiner Bio-One) in Dulbecco's modified Eagle's medium (DMEM; LGC Bio) containing 10% human serum (Merck Millipore) and penicillin-streptomycin (LGC Bio). Cells were maintained at 37 ˚C in 5% CO_2 for 7–10 days for monocyte differentiation into macrophages. Non-adherent cells were washed out with sterile phosphate-buffered saline (PBS), and the remaining macrophage layer was maintained in DMEM with 5% human serum. Macrophage purity was >90%, as determined by flow cytometry (FACScan; Becton Dickinson) analysis using anti-CD3 (BD Biosciences) and anti-CD68 (Southern Biotech) monoclonal antibodies.

Isolation of sEVs

The macrophage layer was extensively washed with PBS and thus replenished with 10 mL of fresh medium supplemented with 5% EVs-depleted fetal bovine serum (FBS; ThermoFisher; from now on referred to as EV-free medium) and cultured for 24h, 48h or 72h. Then, culture supernatants were collected, transferred to 15 mL conical sterile polypropylene centrifuge tubes, and underwent progressive centrifugation steps to isolate the sEVs, as previously described [51], with minor modifications (S1 Fig). In brief, isolation was set up as follows: 400×g for 10 min to remove floating cells; 2,000×g for 10 min to eliminate dead cells and cell debris; 18,000×g for 40 min to remove some LEVs (including apoptotic bodies and other vesicles >300nm); then, the supernatants were transferred to 13 mL polypropylene tubes and submitted to 130,000×g for 70 min to pellet EVs; finally, the pellet-containing IEVs and sEVs was washed once with PBS at 130,000×g for 70 min. The final pellet was resuspended in PBS (50 μL) and maintained at -80 ˚C for upcoming assays. High centrifugation steps (18,000–130,000×g) were carried out using an SW41 Ti titanium swinging-bucket rotor in an Optima XE-90 centrifuge (Beckman Coulter). Next, isolated sEVs were characterized by nanoparticle tracking analysis (NTA), scanning electron microscopy (SEM), western blot and confocal microscopy, as described below.

Cell viability assay (XTT-based cytotoxicity assay)

Macrophage viability was measured using tetrazolium salts (XTT), Cell Proliferation Kit II (Sigma-Aldrich) according to the manufacturer's instructions. Cells were then cultured with EV-free medium for 24, 48 or 72h, after which the XTT assay was performed. Cell proliferation was spectrophotometrically quantified using a 96 wells plate reader at 450 nm. A decrease in optical density was analyzed by normalization against untreated cells with EV-free medium (control cells). All assays were prepared in triplicates.

Protein extraction and quantification assays

Cellular proteins were extracted by homogenization with 200 μL RIPA Lysis buffer (Sigma-Aldrich) containing protease inhibitor cocktail 1:100 (cOmplete™, Sigma-Aldrich). For sEV proteins, samples (50 μL) were lysed by adding RIPA buffer (30 μL) with a protease inhibitor cocktail and then incubated on ice for 10 min. Samples were sonicated (frequency 60 Hz) in water bath three times for 5 min and vortexed (1 min) between each cycle to ensure protein homogenization and membrane lysis. For protein quantification, DC Protein assay (Bio-Rad) was carried out according to the manufacturer's instructions. Absorbance and readings were obtained at 750 nm on a microplate reader SpectraMax M2 (Molecular Devices), and data were analyzed by SoftMax Pro 6.1 software (Molecular Devices). In parallel, Qubit Protein assay (ThermoFisher) was performed using the Qubit 2.0. Fluorometer. Results obtained using both methods were compared.

ZetaView nanoparticles tracking analysis

EVs sizes were measured using ZetaView nanoparticle tracking analyzer (NTA; Particle Metrix GmbH). For measurements, samples were diluted to 100, 500, 1000, and 2000 in previously filtrated PBS (0.22μm) for optimal concentration range for the NTA software (ZetaView Software version 8.02.31, Particle Metrix GmbH). Software parameters were the temperature at 23˚C, the sensitivity of 30–85 frames per second (fps), a shutter speed of 55, and laser pulse duration equal to that of shutter duration. Acquisition parameters were set to a minimum brightness of 20, a maximum size of 200 pixels, and a minimum size of 5 pixels. Polystyrene particles (Microtrac GmbH) with an average size of 100 nm were used to calibrate the instrument before sample readings. Data were analyzed using ZetaView software and Microsoft Excel 2013 (Microsoft Corp).

Scanning electron microscopy (SEM) analysis

Samples were prepared as previously described [52], with minor modifications. After the last ultracentrifugation, pellets containing sEVs were resuspended (50 μL) in 2.5% glutaraldehyde in cacodylate buffer (0.1 M), pH 7.2, and samples (10 μL) were adhered in glass coverslips, previously covered with Poly-L-lysine (Sigma-Aldrich). After 30 min at 37˚C, coverslips were washed three times in cacodylate buffer and post-fixed with a solution of 1% OsO_4, containing 0.8% potassium ferrocyanide and 5 mM $CaCl_2$ for 20 min at 25˚C. After new washings with the same buffer, samples were dehydrated in ethanol ascending series (50, 70, 90, 100 and 100%), dried using the critical point method, mounted on aluminum stubs, and finally coated with a 20-nm-thick gold layer, and examined with a scanning electron microscope (Zeiss Auriga 45–38, Zeiss).

Western blotting

For assessing the protein profile of macrophages or sEV preparations, western blotting was carried out as described [53], with modifications. After protein extraction, samples were

resuspended in lithium dodecyl sulfate (LSD) buffer (Life Technologies) with or without reducing agent (Life Technologies), when applicable. Samples (40 μg of protein) were boiled at 70 ˚C for 10 min, loaded into 4–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) precast gels, and transferred to polyvinylidene fluoride (PVDF, ThermoFisher) membranes, which were blocked with 5% skimmed milk in Tris-buffered saline-Tween 20 (TBS-T, 0.01%) for 1h at room temperature. Blots were incubated for 18 h at 4 ˚C with anti-Alix, anti-CD63, anti-CD81, anti-Calnexin, anti-Cytochrome C, and anti-β-actin (control) antibodies. After washing with TBS-T, the membrane was exposed to secondary antibodies conjugated to horseradish peroxidase (HRP) anti-mouse or anti-rabbit, as required, for 1h at room temperature, and washed again with TBS-T. Protein bands were revealed using Western Chemiluminescent ECL Luminol substrate (GE Healthcare), and images were captured by C-DiGit Blot Scanner (LI-COR Biosciences). Relative band intensity was calculated using ImageJ software (NIH, USA). The antibodies used for western blotting assay, including their dilutions and supplier, are described in Table 1. Of note, the sEV protein markers were selected taking into account the minimal requirements recommended by the International Society for Extracellular Vesicles [54].

Sucrose density gradient centrifugation

Purification of sEVs was performed using sucrose density gradient ultracentrifugation as previously described [55, 56], with modifications, whose detailed protocol is described in Supporting information.

Interaction of sEVs with human primary macrophages

The protocols used to label sEVs and to evaluate their internalization by macrophages are described in Supporting information. Images of vesicle uptake by macrophages were taken at 63X under a laser scanning confocal microscope (Leica TCS SP8, Mikrosysteme GmbH).

Data availability

We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV200058) [57].

Statistical analysis

All statistical data were performed and analyzed using GraphPad Prism Software version 7.0. NTA, sEV protein kinetics and cell viability data were subjected to two-way analysis of variance (ANOVA) with Tukey *post hoc* correction for determining significant differences between conditions. Mann–Whitney test for comparison of both protein assays and sEV size was applied. Data are shown as the median and quartiles (1st and 3rd), and the differences between values were considered statistically significant when the *P*-value was ≤ 0.05.

Results

Isolation, size, and number of EVs

Macrophages (ranging from 5.0×10^5–1.3×10^6/per flask) were cultured in EV-free medium during 24, 48, or 72h, and the EVs released after each of these time-points were isolated from culture supernatants by ultracentrifugation, as shown in (S1 Fig). The total number and size distribution of the isolated EVs were quantified in samples from six individual donors through NTA. According to NTA measurements, high concentrations of vesicles with size ranging between 40–150 nm, peaking at 105 nm, were obtained from cells cultured in the three

Table 1. Primary and secondary antibodies used for Western blotting to identify sEVs markers.

Antibodies	Molecular weight	Origin	Dilution	Supplier	Catalog Number
Anti-Alix	95 kDa	Mouse	1:1000	Cell Signaling	2171
Anti-CD63	30–60 kDa	Mouse	1:1000	Thermo Fisher	10628D
Anti-CD81	25 kDa	Mouse	1:500	Thermo Fisher	10630D
Anti-Calnexin	90 kDa	Rabbit	1:1000	Santa Cruz	11397
Anti-Cytochrome c	15 kDa	Mouse	1:500	Santa Cruz	13156
Anti-β-Actin	42 kDa	Mouse	1:45.000	Sigma-Aldrich	A3854
Anti-mouse-HRP		Goat	1:2000	Cell Signaling	7076
Anti-rabbit-HRP		Goat	1:2000	Cell Signaling	7074

Supplier information, catalog number are described.

https://doi.org/10.1371/journal.pone.0237795.t001

different time-periods (Fig 1A–1C), despite a slight variation in the total number of vesicles among donors. It can also be observed that, except for D6, the concentration of vesicles harvested was nearly the same at the three time-points (Fig 1D), meaning that 24h is time enough for optimal shedding of EV-macrophages. The proportions of EVs with size <150 nm were equal to the median 79,54% (1st quartile: 77,39 and 3rd quartile: 87,31%), 76,86% (71,5 and 80,43), 85% (80,82 and 89,5) at the same time-periods, respectively (Fig 1E). Similar results were found with EVs obtained from macrophages of extra six donors (S2 Fig). The vesicle median size was equal to 110 nm, with a mode size of 105 nm (Table 2). Moreover, the XTT assay indicated that macrophage survival cultured in EV-free medium during 24, 48, or 72h

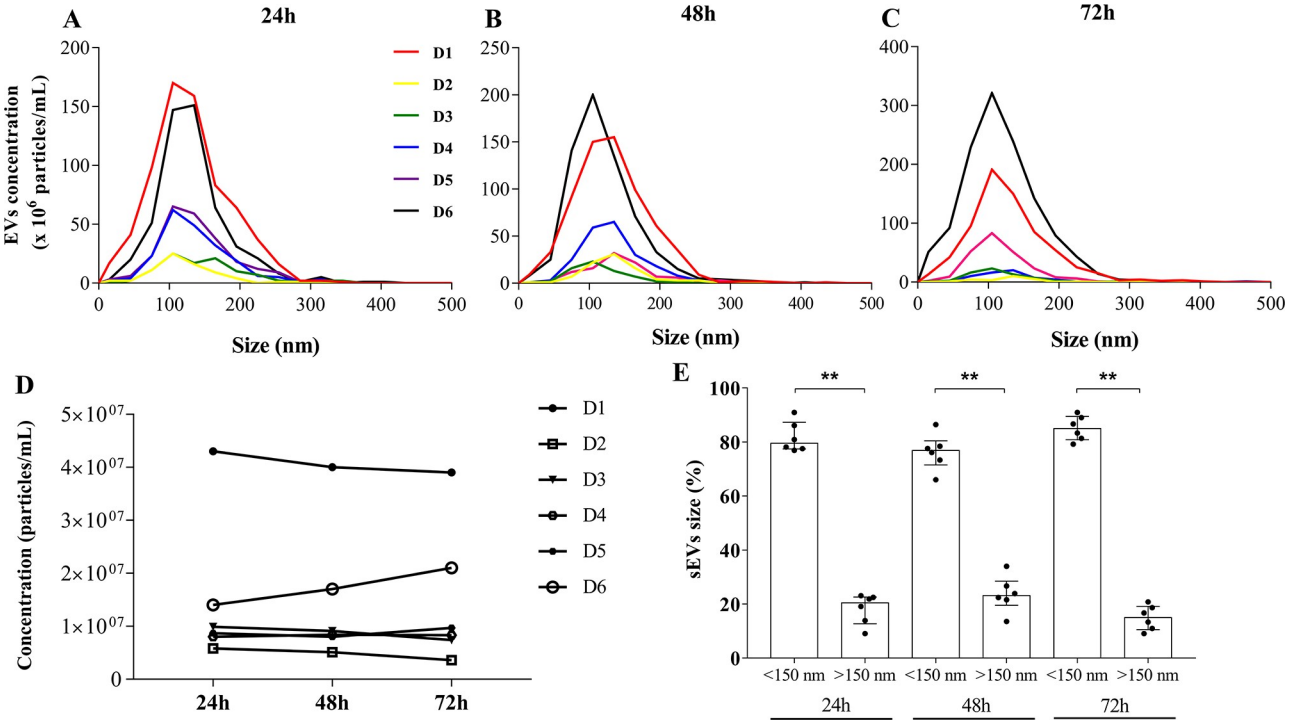

Fig 1. Concentration and size distribution of MDM-derived sEVs. NTA-ZetaView analyzes of total number and size distributions of EVs derived from six donors, isolated after (A) 24h, (B) 48h or (C) 72 h of cell culture. (D) Total concentration of EVs released at the same time-points (one culture flask for each point). (E) Proportion of EVs sizes <150nm or >150nm. Median values are indicated ($n = 6$). Mann–Whitney test was used to evaluate statistical significance: $^{**}p < 0.01$. D: donor.

https://doi.org/10.1371/journal.pone.0237795.g001

Table 2. Sizes and concentrations of sEVs obtained from human primary macrophages.

Donor	Time	Total approximate cell number in three flasks [a)]	Original concentration Particles/cm^3 [b)]	Concentration Particles/mL [b)]	Median size (nm) [b)]	Mode size (nm)
D1	24h	2,2 x 10^6	4.3 x 10^9	4.3 x 10^7	116,2	105
	48h	1,8 x 10^6	4.0 x 10^9	4.0 x 10^7	123,4	135
	72h	2,4 x 10^6	3.9 x 10^9	3.9 x 10^7	121,3	105
D2	24h	2,0 x 10^6	2.9 x 10^9	5.8 x 10^6	111,2	105
	48h	2,1 x 10^6	2.6 x 10^9	5.1 x 10^6	128,6	135
	72h	1,9 x 10^6	1.1 x 10^9	3.6 x 10^6	132,9	135
D3	24h	2,1 x 10^6	9.9 x 10^8	9.9 x 10^6	137,3	105
	48h	2,7 x 10^6	9.1 x 10^8	9.1 x 10^6	107	105
	72h	2,5 x 10^6	7.4 x 10^8	7.4 x 10^6	114,4	105
D4	24h	2,0 x 10^6	4.0 x 10^9	8.0 x 10^6	123,1	105
	48h	1,9 x 10^6	4.2 x 10^9	8.4 x 10^6	122,8	135
	72h	2,3 x 10^6	1.7 x 10^9	8.3 x 10^6	119,2	135
D5	24h	1,7 x 10^6	4.3 x 10^9	8.7 x 10^6	123,3	105
	48h	2,3 x 10^6	4.0 x 10^9	8.0 x 10^6	128,6	135
	72h	2,0 x 10^6	1.9 x 10^9	9.7 x 10^6	104,1	135
D6	24h	2,4 x 10^6	1.0 x 10^{10}	2.1 x 10^7	117,6	135
	48h	2,8 x 10^6	7.8 x 10^9	2.6 x 10^7	113	105
	72h	3,1 x 10^6	1.9 x 10^{10}	6.3 x 10^7	121,9	105

[a)] Three flasks for each time-point

[b)] Calculated by ZetaView Software version 8.02.31

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was not affected. The proportions of viability were equal to the median 95,55% (93,55 and 100,8), 93,1% (89,71 and 100,5), and 91,03% (86,17 and 96,19) at the indicated time-periods, respectively (S2D Fig). Our data show that the proposed dUC protocol allows isolation of a high number of heterogeneous populations of MDM-derived EVs, and that the vast majority of vesicles isolated from individual samples fall within the size expected for sEVs (<150 nm).

Protein quantification of MDM-derived sEVs samples

Samples collected after 24, 48, or 72h of culture were analyzed by colorimetric (DC Protein) or fluorimetric (Qubit) assays to determine EV protein concentration with five μL of EVs as starting volume. All samples had ~500 μg/mL of total protein, with slight variations within the three time-points analyzed (Fig 2). To assure the accuracy of these results, we measured in parallel the protein amount of three known concentrations of bovine serum albumin (BSA), whereas no differences between both methods have been found (S3 Fig). We also measured the protein concentration in three other sEV samples, which provided similar results (S4 Fig). Thus, our analysis shows that both protein quantification assays provide reliable measurements of EV protein concentration. Of note, no significant variation was noticed in the protein content throughout the time points.

Morphology and protein markers of MDM-derived sEVs

We next examined the nanoparticle morphology by SEM, which revealed the presence of sEVs with size ranging from 40–100 nm, as shown in Fig 3. Size measurements of sEVs from two individual donors (number of images analyzed per donor = 3) showed a median size of 64,6 nm (64,59 and 72,84 nm) (D1), 65,24 nm (63,8 and 65,59) (D2) and mode size of 64,34 nm

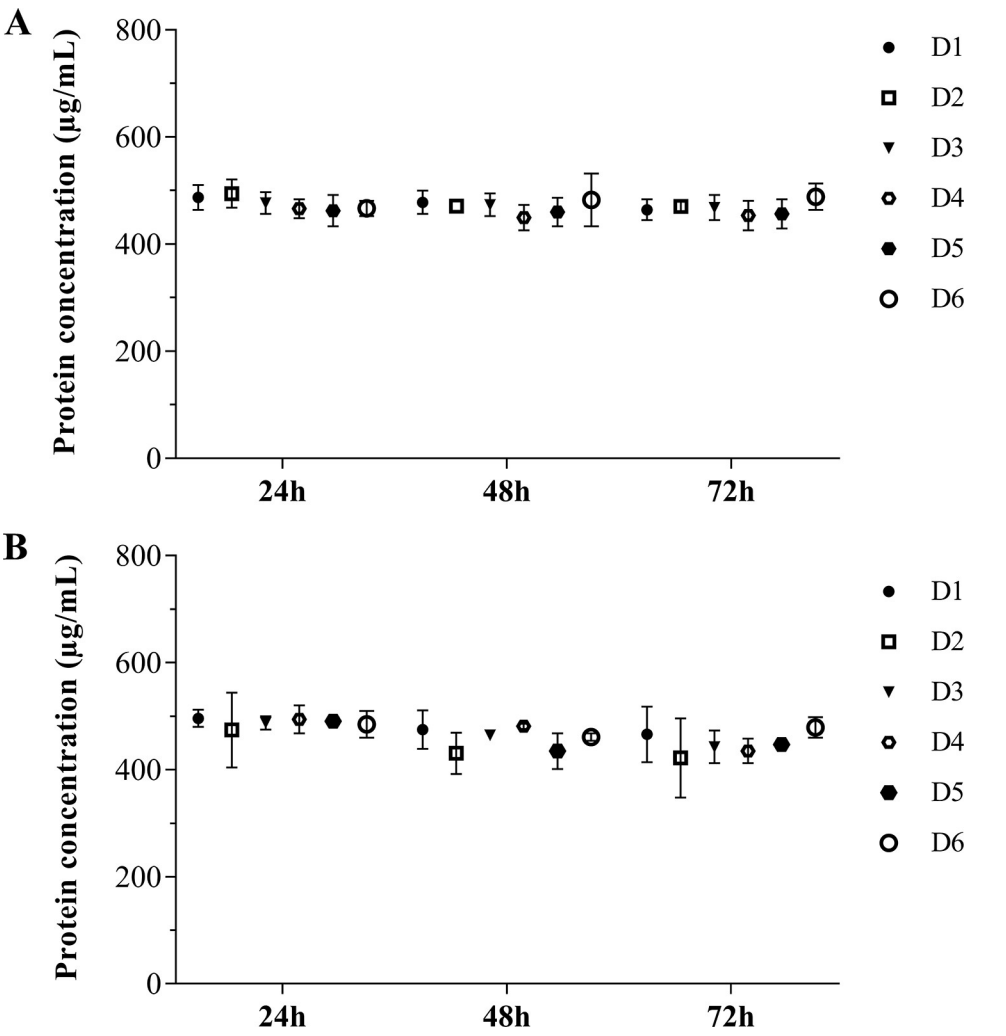

Fig 2. Protein concentration of MDM-derived sEVs. Protein concentration of sEVs obtained after 24, 48 or 72h of macrophage culture, measured by (A) Qubit or (B) DC protein assays ($n = 6$). Median values are indicated. D: donors.

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and 51,98 nm, respectively (Fig 3C and 3D). The proportion of EVs with size <150 nm was 81% (75,33 and 86,67), and with size >150 nm was 19% (13,33 and 24,67) (Fig 3E). Aggregates or clumps of sEVs were observed in SEM analysis, as a result of vesicle isolation with high-speed centrifugation (Fig 3A) [58]. Next, the protein markers of these particles were identified by western blotting assays. We initially observed, as expected, that sEVs exhibited lower protein content compared to their parent cells, as macrophage lysates contained a strong protein expression initiating at 14 kDa, whereas the sEV lysates presented protein content from ~28 kDa (Fig 4A). Next, accessory proteins from the endosomal sorting complex required for transport (ESCRT) and tetraspanins proteins, such as Alix (95 kDa), CD63 (a 30–60 kDa glycosylated protein), and CD81 (25 kDa), were detected in the vesicles as well as in the whole cell lysates (control) (Fig 4B). The absence of markers for cytochrome c (mitochondria) and calnexin (endoplasmic reticulum) in the vesicle lysates indicates that EV preparations were not contaminated with components of the mitochondria and endoplasmic reticulum from dead cells. The complete blotting membranes are shown in S5 Fig. Thus, we identified vesicles with homogeneous round shape morphology in our samples, which are enriched with proteins

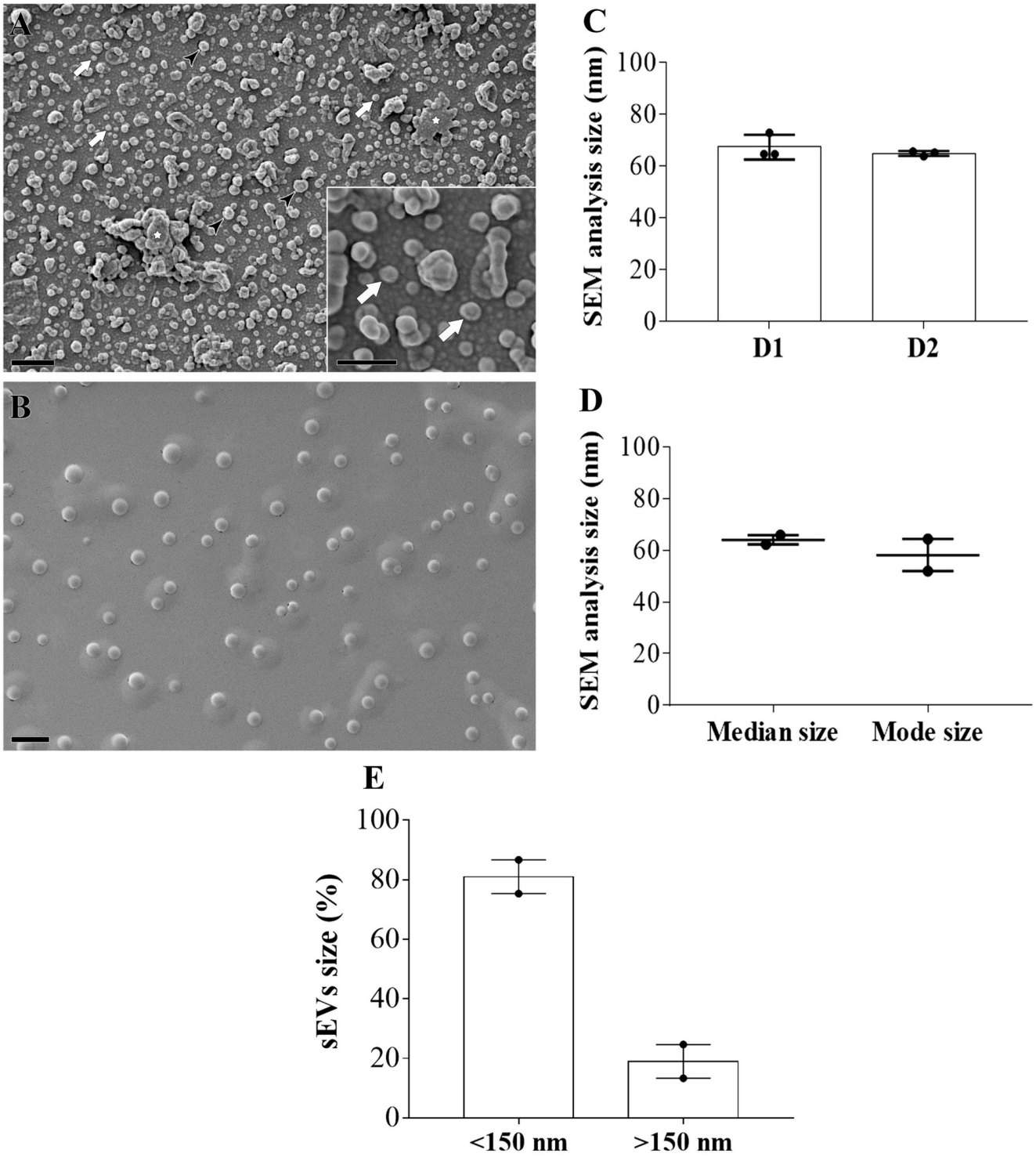

Fig 3. Morphological characterization of MDM-derived sEVs. (A) SEM microscopy of sEVs showing homogeneous vesicle-shaped structures with size ranging from 40–110 nm (Bars = 400 nm) ($n = 2$). White arrows point to sEVs with size around 100 nm, and black arrow heads point to vesicles with size >150 nm; white star shows clumps of sEVs. Inset shows 200 x magnification of sEVs (Bars = 200 nm). (B) Control image containing only fixation solution (Bars = 1000 nm). (C) Size measurement of sEVs from two individual donors (number of images analyzed per donor = 3). (D) Median and mode size of sEVs from the same two donors. (E) Proportion of EVs sizes <150nm or >150nm. Median values are indicated. D: donors.

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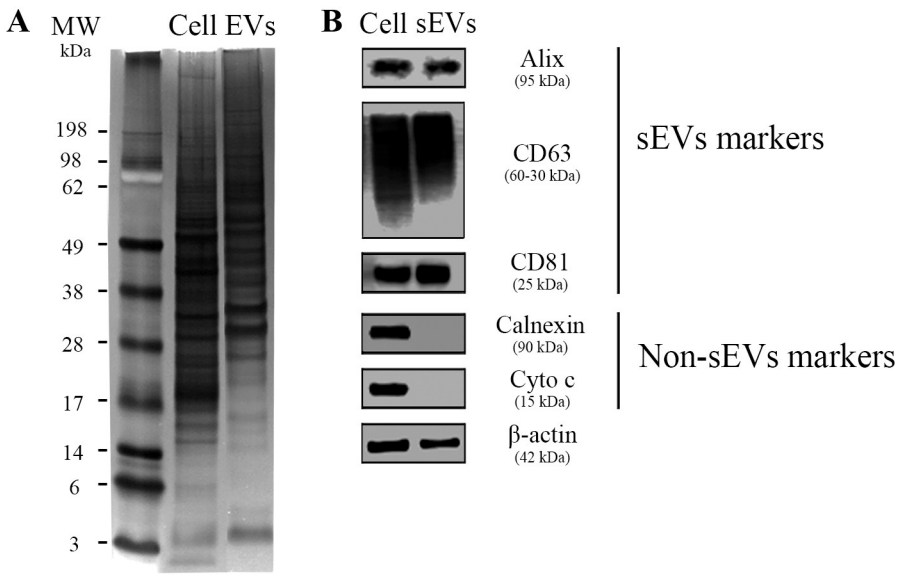

Fig 4. Protein markers of MDM-derived sEVs. (A) Representative image of three independent assays of polyacrylamide gel stained with silver nitrate after separation of 40 μg total protein from cell (Cell) or sEVs pool lysates (EVs; pools comprise samples from four individual donors). (B) Western blot analysis of sEVs markers (Alix, CD63, and CD81) and non-EVs markers (Calnexin and Cytochrome C) ($n = 3$). β-actin = loading control. 40 μg of total protein were loaded onto the gel. MW: molecular weight marker.

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from multi-vesicular endosomes (MVE), features that suggest the presence of sEVs from endosomal origin in our preparations.

Separation of sEVs by sucrose density gradient

To confirm the presence of sEVs from endosomal origin, sucrose density gradient ultracentrifugation (S-DGUC) assays were performed (Fig 5A and S6 Fig). The original pellets from the 130,000 x g spin were then fractionated by S-DGUC at 200,000 x g (Fig 5A), and the resultant fractions, numbered F1 to F6 (which were formed by the contiguous sucrose layers, as detailed in supplementary information) from the top to the bottom of the tube, were subjected to blotting analysis for the EV markers CD63 and CD81. The blotting results were analyzed upon normalization of band intensities, as described elsewhere [61]. We found that CD63 and CD81 were predominantly present in the middle-density gradient F3 (49,28% and 54,92% of the total signal, respectively). Moderate intensity amount of CD63 was detected in F4 (29,82%) and F2 (18,54%), whereas signal reduction was noticed in F5 (2,35%). CD81 labelling F2 (20,997%) and F4 (23,05%) was less intense than F3 (Fig 5B). The raw blotting images from gradient assays are shown in S6 Fig. Our data showed that CD63 colocalized with CD81 in fractions F2 to F4 (densities between 1,117 to 1,181 g/mL), a buoyant property reported for sEVs from endosomal origin [59, 60].

Internalization of sEVs by human primary monocyte-derived macrophages

Next, we analyzed, by confocal microscopy, whether the sEVs would be internalized by macrophages. To this end, sEVs were firstly labeled with the lipophilic dye PKH26 (PKH26-labeled EVs, as described in Supplementary methods) and then fractioned by sucrose gradient. Next, the PKH26-labeled sEVs from fractions F2, F3 and F4 were added separately to recipient macrophages, and the preparations were incubated for 3h. Images show that PKH26-positive

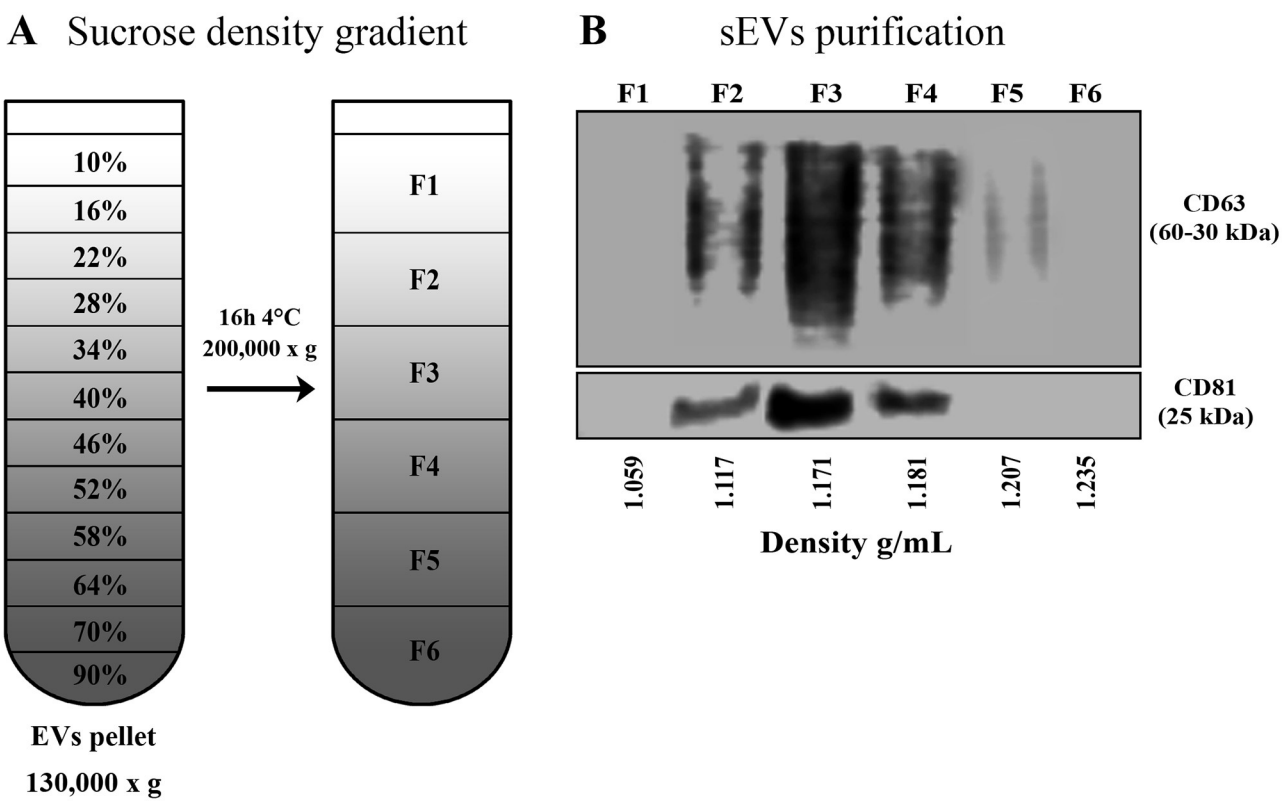

Fig 5. Sucrose density gradient ultracentrifugation in MDM-derived sEVs. (A) Separation of sEVs by sucrose density gradient. The final sEVs pellet 130,000 x g was placed onto 90–10% sucrose gradient layers, then centrifuged for 200,000 x g for 16h, as indicated. Six fractions were collected from the top to the bottom of the gradient for further WB and confocal experiments. (B) Western blot analysis of sEVs recovered at the fractions F1-F6 (n = 3). 15 μg of total proteins were loaded onto the gel. Relative band intensity was calculated by ImageJ.

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vesicles were internalized by macrophages, as evidenced by the red puncta observed in the cells stained with green phalloidin and DAPI (Fig 6). Images of control macrophages treated with EV-free medium labeled with PKH26 (S7 Fig) show a diffuse fluorescence in F4 (PKH26 and merge columns), probably representing contaminants (e.g., lipoproteins, protein complexes, ribonucleoproteins) and/or PKH26 micelles. We also analyzed the interaction of sEVs present in the original pellets without gradient separation, for different periods of times. We observed that macrophages were able to internalize particles as prompt as no more than 15 or 30 min of interaction (S8 Fig), while, as expected, macrophages engulfed a more substantial number of particles after three hours of co-culture. Importantly, the internalization of purified or non-purified PKH26-labeled sEVs by macrophages were confirmed by 3D reconstruction (Fig 7). Thus, our data show that primary macrophages readily interact with and internalize sEVs emitted by other macrophages, and that the presence of some contaminants in the non-purified EV preparations did not impair the uptake of sEVs.

Discussion

We report here a methodical approach for isolation, enrichment and characterization of sEVs released by human primary macrophages from circulating monocytes in culture supernatants. We performed this work taking into account the limited number of studies describing procedures for recovering sEVs from those cells. Due to growing evidence of the critical role of macrophages in a variety of physiological and pathological conditions [43, 44], along with the

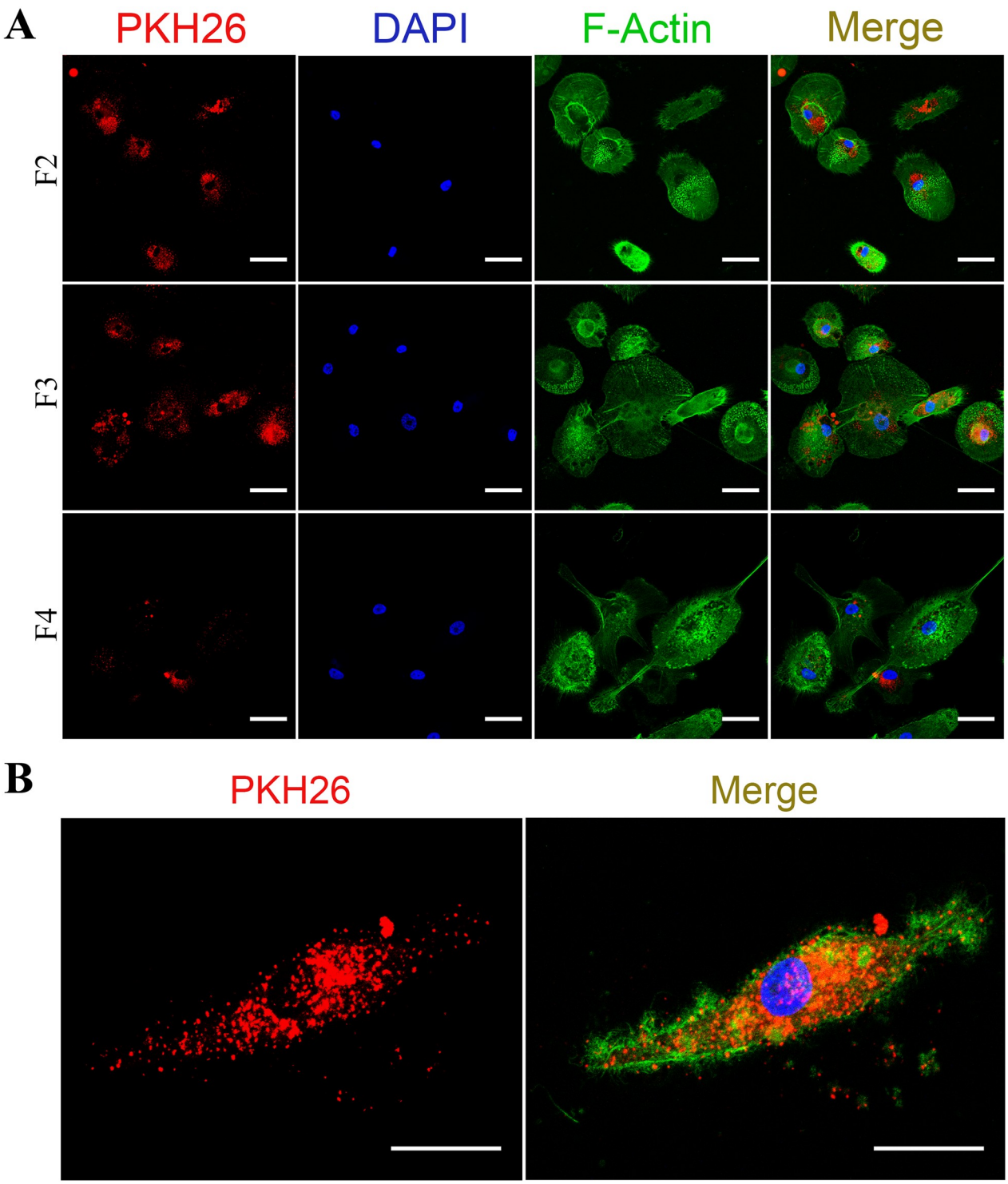

Fig 6. Internalization of sEVs by recipient human primary macrophages. (A) sEVs labeled with PKH26 were separated by sucrose density gradient ultracentrifugation and the fractions F2-F4 were added to recipient macrophages during 3 hours (n = 3) (Bars = 50 μm). (B) representative image of a single macrophage (5x magnification) that internalized labeled sEVs from F3 (Bars = 25 μm). PKH26: sEVs; DAPI: cell nuclei; F-Actin: macrophages.

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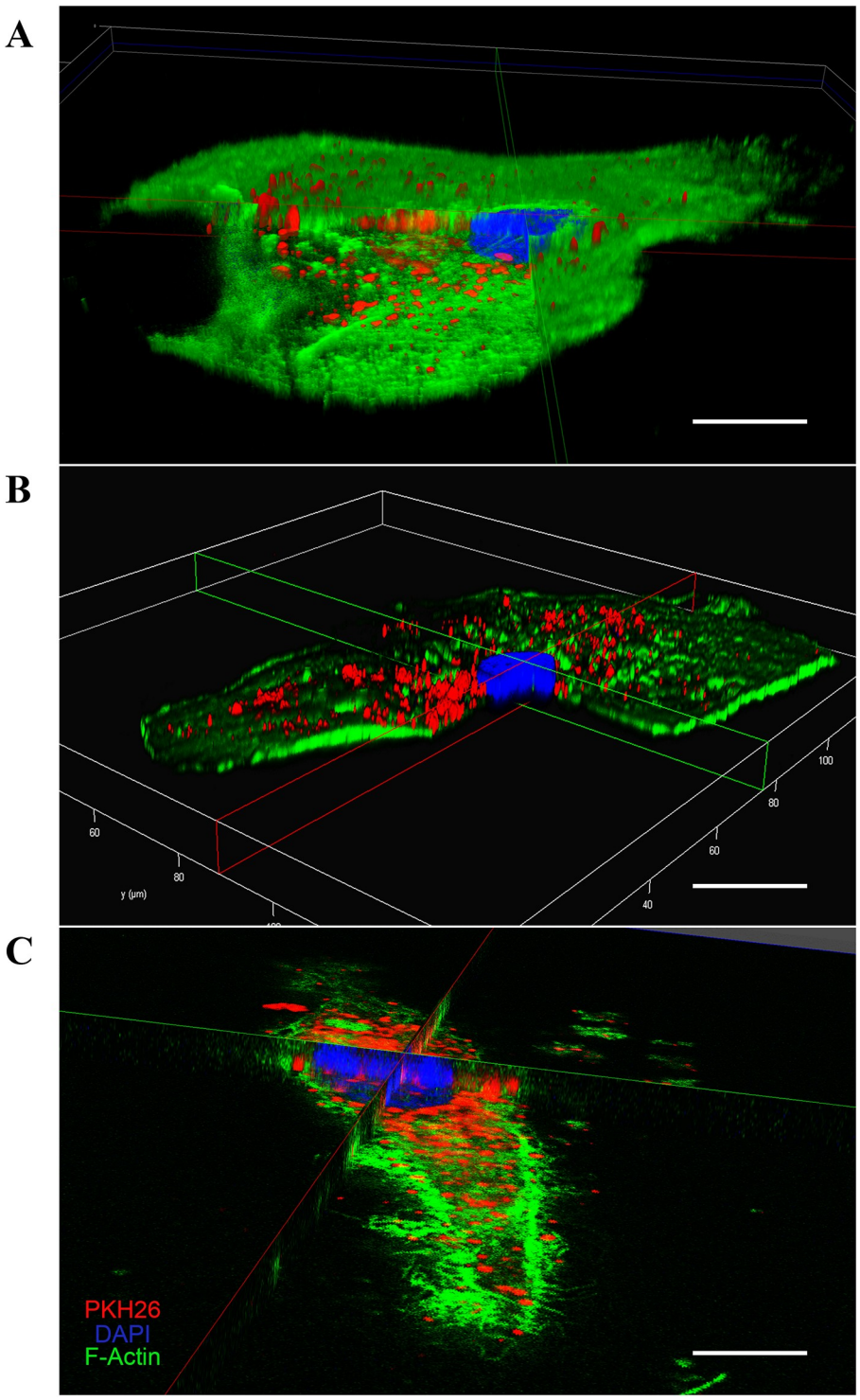

Fig 7. Internalization of sEVs by recipient human primary macrophages. 3D reconstruction image (10X magnification) showing uptake by macrophage of non-purified sEVs after (A) 15 min, (B) 30 min or (C) of sucrose gradient-purified sEVs from fraction F3 after 3h of interaction (n = 3). Scale bar = 25 μm.

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Table 3. Available studies* of EVs secreted by primary human macrophages derived from circulating monocytes.

Research objective	EV isolation methods	EV characterization methods	Filtration as an extra isolation step	Internalization assay	References
Functional study	UC	TEM, FC, WB and Sucrose gradient assay	No	No	[65]
Functional study	UC	TEM, LP, Sucrose gradient assay and PT	Yes	Yes	[66]
Proteomic study	UC	NTA, TEM, WB and PT	Yes	No	[67]
Proteomic study	UC	TEM, WB and PT	Yes	No	[17]
Micro RNA profile	UC	Iodixanol gradient assay, AChE activity and WB	No	No	[68]
Functional study	EVs isolation kit	NTA, TEM and WB	No	No	[70]
Functional study	EVs isolation kit	TEM and WB	Yes	No	[69]
Functional study	UC	NTA, TEM, FC and WB	Yes	No	[18]
Functional study	UC	NTA, TEM and WB	Yes	No	[71]
Functional study	UC	NTA, TEM and WB	Yes	No	[64]
Functional study	UC	NTA, TEM and FC	No	No	[72]

AChE: acetylcholinesterase activity; LP: Lipidome; FC: Flow citometry; PT: Proteomic

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ability of EVs to mediate intercellular communication, the application of reliable methods for the separation of EVs may contribute for a better understanding of the biology of EV-based cellular communications in the macrophage functions. In fact, the use of standardized methods to provide pure populations of EV subtypes is critically important to define the biological functions of EVs in multicellular organisms, as pointed out by other authors [54, 61, 62]. Therefore, several groups in the field are exploring possibilities to improve or design new methods for EV isolation and characterization that allow the understanding of their biogenesis, targeting and physiological role [63].

To date, there are no published protocols detailing step-by-step the isolation and characterization of small EVs secreted by human macrophages derived from circulating monocytes. Available studies about MDM-derived sEVs (Table 3) have applied different procedures for their description, were mainly aimed to functional approaches and not to methodological improvements, or evaluated multiple proteins or microRNA contents from a heterogeneous population of vesicles secreted by macrophages. In fact, some of these works used large volume of culture supernatant or even additional isolation steps before the final centrifugation (e.g., filtration) [17, 18, 64–72]. In contrast, we suggest here a modified protocol based on sucrose density gradient ultracentrifugation with higher speeds that allowed by itself the separation and quantification of large amounts of small EVs enriched with vesicles from the endosomal origin, which is consistent with results reported by other authors [51], who showed that dUC with increasing speed pelleted EVs with decreasing sizes. Furthermore, many studies related to sEVs from human macrophages used the monocytic leukemia cell line THP-1 [18, 31, 73, 74]. The different features between primary and tumor cell lines do not allow a fair comparison of the methods used for isolation and characterization of sEVs from primary cells and their corresponding tumor lines, thus highlighting the importance of applying specific protocols for primary macrophages.

Here, MDM-derived sEVs from healthy human donors were characterized according to their physical and biochemical properties, as well as their interaction and internalization by recipient macrophages. Based on S-DGUC, other authors have considered that sEVs include particles with sizes between ~50–150 nm [19, 20, 59]. According to NTA measurement, our sEVs were in the same size range, peaking at 105 nm. In the method here described, the vesicle

median sizes for all donors analyzed were mostly uniform, fluctuating within the sEVs dimensions already described [59], although slight variations in the total number of vesicles from individual samples were observed. Unlike the donor-to-donor variability in particle concentrations, the number of sEVs from individual donors did not fluctuate throughout the days the sEVs were collected. All samples produced very similar NTA profiles, indicating data reproducibility at these conditions.

Previous findings suggest that EV yield and protein concentration may depend on several factors, including cell type, cell confluence level, cell activation by exogenous compounds (e.g., Ca^{+2} ionophores, cell detachments, hypoxia, etc) [75–77] and culture conditions [40, 78, 79]. In this sense, some specifics of our culture conditions, such as small variations in the number of macrophages (see Table 1) and time of culture (24, 48 or 72 h) probably did not influence the total protein concentration of sEVs, as ascertained by the use of two different protein quantification methods. Moreover, in addition to the conventional assays, such as DC Protein, which is based on the Lowry method, we also confirmed that the Qubit assay is a reproducible and reliable method for measuring proteins of EV samples, which is consistent with previous results of Vergauwen et al, [80], who measured protein concentration of EVs derived from the epithelial breast cancer cell line MCF-7.

The scanning electron microscopy (SEM) analysis revealed a homogeneous population of small EVs with round shape morphology and size smaller than 100 nm, features also found reported in other EV studies [81, 82]. The small amounts of LEV (vesicles larger than 150 nm) detected in our preparations were also expected, since it has been described that separation of EVs by high speed dUC resulted in a heterogeneous population of EVs with different sizes and subcellular origins [20]. Furthermore, NTA and SEM measurements detected a higher proportion (~81%) of vesicles with size <150nm than with size >150nm (~19%), thus suggesting a high enrichment of MDM-derived sEVs with the present protocol.

The identity of vesicles in our samples was further defined by evaluating the expression of small EVs protein markers, such as Alix, CD63, and CD81. These molecules were detected in all samples. Additionally, samples did not express non-EV markers, such as calnexin and cytochrome C, showing that vesicle preparations were not contaminated with components of the mitochondria or endoplasmic reticulum derived from cellular debris. The blotting assays were performed according to recommendations of the International Society for Extracellular Vesicles (ISEV) for an appropriate use and precise documentation for methods related to EV research [54], such that we used three categories of proteins present or enriched in EVs (one cytosolic and two membrane-bound proteins) and two other global proteins «not expected in EVs» (such as mitochondria and Golgi proteins).

To achieve a better specificity of EVs or EV subtype separation, we applied an additional purification technique based on density gradients. We selected the sucrose density gradient taking into account its property to separate membrane-limited vesicles based on their floatation speed and equilibrium density [83, 84]. Moreover, this technique has been proved to be a robust approach for EV purification for consistent functional and structural analyses [20, 59]. Western blot analyses revealed markers for late endosome proteins (CD63 and CD81) in the fractions with low and middle-density gradients (F2-F4; 1.117 to 1.181 g/mL), which is consistent with a previous report that reasoned that the tetraspanins CD63, CD9, and CD81 identify sEVs of endosomal origin from primary dendritic cells [59]. Although we have not used the CD9 marker, we detected the presence of ESCRT-accessory molecules Alix and the colocalization of CD63 and CD81 after sucrose density gradient. Therefore, we propose that the separated preparations after S-DGUC contain sEVs from late endosomal system.

We also found, through confocal microscopy, that recipient macrophages uptake sEVs with three hours of co-culture, whereas other studies reported that internalization occurred after

more extended periods (12-24h) [37, 66, 85]. Other authors may have opted for longer co-culture times to ensure the visualization of the internalized sEVs. In our work, the same origin (human macrophages) and nature (primary cells) of emitting and recipient cells may have contributed to the rapid uptake of the vesicles [13, 86]. Large fluorescent dots were observed in some fractions containing positive PKH26-labeled sEV or control PKH26-labeled sEV-free medium (an unspecific diffuse fluorescence in F4), suggesting the presence of some contaminants (e.g., aggregates, lipoproteins, protein complexes, ribonucleoproteins). This effect could be a consequence of high-speed centrifugation of culture medium in combination with lipophilic dyes such as PKH, which induce the formation of artifacts with different sizes and morphologies that can be detected by fluorescence microscopy [58, 87, 88]. The specialized literature has also reported a multitude of contaminants in vesicles separated by multi-step methods [60, 89, 90]. Moreover, we believe that staining EVs with diameter smaller (<200 nm) than the diffraction limit of light of the confocal microscopy may also have potentially favored the visualization of other large fluorescent dots in our preparations, probably corresponding to clusters of positive PKH26-labeled sEV without the possibility to discriminate one vesicle from another [91, 92]. To isolate highly purified EVs simultaneously with the depletion of non-EV material from a given biofluid or cell conditioned media is difficult using the available tools existing today. In other words, separating sEVs from contaminants that may share biophysical properties with EVs is still a major challenge in the field [60, 89].

During the processes of MDM-derived EV isolation using S-DGUC, there were some methodological advances that should be reported in the modified protocol. First, the protocol permitted us to obtain ~80% of heterogeneous population of sEVs (<150nm) using intermediate samples sizes (10 mL). Second, the proposed method showed reproducibility between macrophage samples from different healthy donors, obtaining sEVs with similar physical and biochemical characteristic according to the analyzes of NTA, SEM and WB. Finally, this is the first study to report the co-localization of CD63 and CD81 markers in the fractions subjected to imunoblotting, indicating the presence of late endosome-derived sEVs of human primary macrophages derived from circulating monocytes.

In conclusion, given the technical difficulties in the EV field, the use of appropriate methodologies for obtaining EVs is critical for understanding their biogenesis and role in cellular communication processes. The protocol that we applied, combining dUC with density gradient purification assays, allowed the isolation of small EVs of endosomal origin released by human primary macrophages. One limitation is necessary to be considered in this study, as we did not use markers to identify the contaminants present in our EVs isolated after the density gradient assay. Since several contaminants have been identified in EVs separated by multi-step methods [90], determining the presence of certain contaminants may also be necessary for specific functional applications of the MDM-derived sEVs. Finally, our work seeks to contribute for enhancing the reproducibility between the limited number of available protocols for the description of MDM-derived sEVs, thus providing an alternative methodology research groups working with EVs released by these cells.

Supporting information

S1 File.
(DOCX)

S1 Fig. Centrifugation steps for isolation of MDM-derived sEVs. The flowchart shows the centrifugation steps applied for sEVs isolation from 10 mL of supernatants from monocytes-derived macrophages cultured in DMEM with 5% EVs-depleted serum. MØ: Macrophage; LEVs: large extracellular vesicles; IEVs: intermediated extracellular vesicles; sEVs: small

extracellular vesicles.
(TIF)

S2 Fig. Concentration, size distribution of sEVs and macrophages viability. EVs were colleted at 72 h of macrophages culture. (A) NTA-ZetaView analyzes of total number and size distributions of EVs derived from other six donors not represented in the main figures ($n = 6$). (B) Total concentrations of EVs released by different donors. (C) Proportion of EVs sizes <150 nm or >150 nm. Mean values ± SD are indicated. $^{**}p < 0.01$ (Mann–Whitney test). (D) Macrophages viability at different time periods ($n = 4$). Decrease in survival was analyzed by normalization against untreated cells with EV-free medium (control cells), represented by red dotted line. Median values are indicated D: donor.
(TIF)

S3 Fig. Bovine serum albumin (BSA) quantification. BSA concentrations at (A) 600 μg/mL, (B) 400 μg/mL, and (C) 200 μg/mL were assessed by Qubit or DC Protein assays ($n = 3$). Identical sample volume (5 μL) were used in all assays. Red dotted line indicates known BSA concentration. Results are expressed as median. D: donor.
(TIF)

S4 Fig. Protein concentration of MDM-derived sEVs. sEVs samples (**A**) 1, (**B**) 2, and (**C**) 3 from other donors colleted at 72 h of cell culture were measured by Qubit or DC protein assay ($n = 3$). Results are expressed as median.
(TIF)

S5 Fig. Protein markers of MDM-derived sEVs. Raw blots of sEVs markers using 40 μg of total protein and specific antibodies for sEVs (Alix, CD63 and CD81) and non-sEVs (Calnexin and Cytochrome C) ($n = 3$). β-actin was used as charge control. All experiments were performed with pools (4 donors) of sEVs.
(TIF)

S6 Fig. Raw blots images of fractions F1-F6 collected of MDM-derived sEVs separated by S-DGUC ($n = 3$). Polyacrylamide gels were loaded with 15 μg of protein and membranes were labeled with sEVs markers (CD63 and CD81). All experiments were performed with pools (4 donors) of sEVs.
(TIF)

S7 Fig. Interaction of EVs-free medium with recipient primary macrophages. EVs-free medium were labeled with PKH26, then separated by sucrose density gradient centrifugation and three fractions (F2-F4) were colleted and added separately to recipient macrophages during 3 hours ($n = 3$). Fluorescent images represent only cells or cells with sEVs, respectively (Bars = 50 μm). PKH26: sEVs; DAPI: cell nuclei; F-Actin: macrophages.
(TIF)

S8 Fig. Internalization of original EVs pellets by macrophages. EVs pellets isolated by dUC (not purified by sucrose gradient) were labeled with PHK26 and added to macrophages during 15 min ($n = 3$), 30 min ($n = 3$), and 3 hours ($n = 4$). Bright field (DIC) and fluorescent images represent only cells or cells with extracellular particles, respectively (Bars = 50 μm). PKH26: EVs and other particles; DAPI: cell nuclei; F-Actin: macrophages.
(TIF)

S9 Fig. Protocol to layer sucrose density gradient. (A) Position of tube angled 90˚ and (B) angled 60˚ during layering sucrose gradients. (C) Correct and (D) incorrect sucrose gradients layers. Black arrows point to layer formation during sucrose gradient; black arrowheads point

to diffuse layers.
(TIF)

S1 Raw image.
(PDF)

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Writing – original draft: Luis A. Arteaga-Blanco, Dumith Chequer Bou-Habib.

Writing – review & editing: Luis A. Arteaga-Blanco, Dumith Chequer Bou-Habib.

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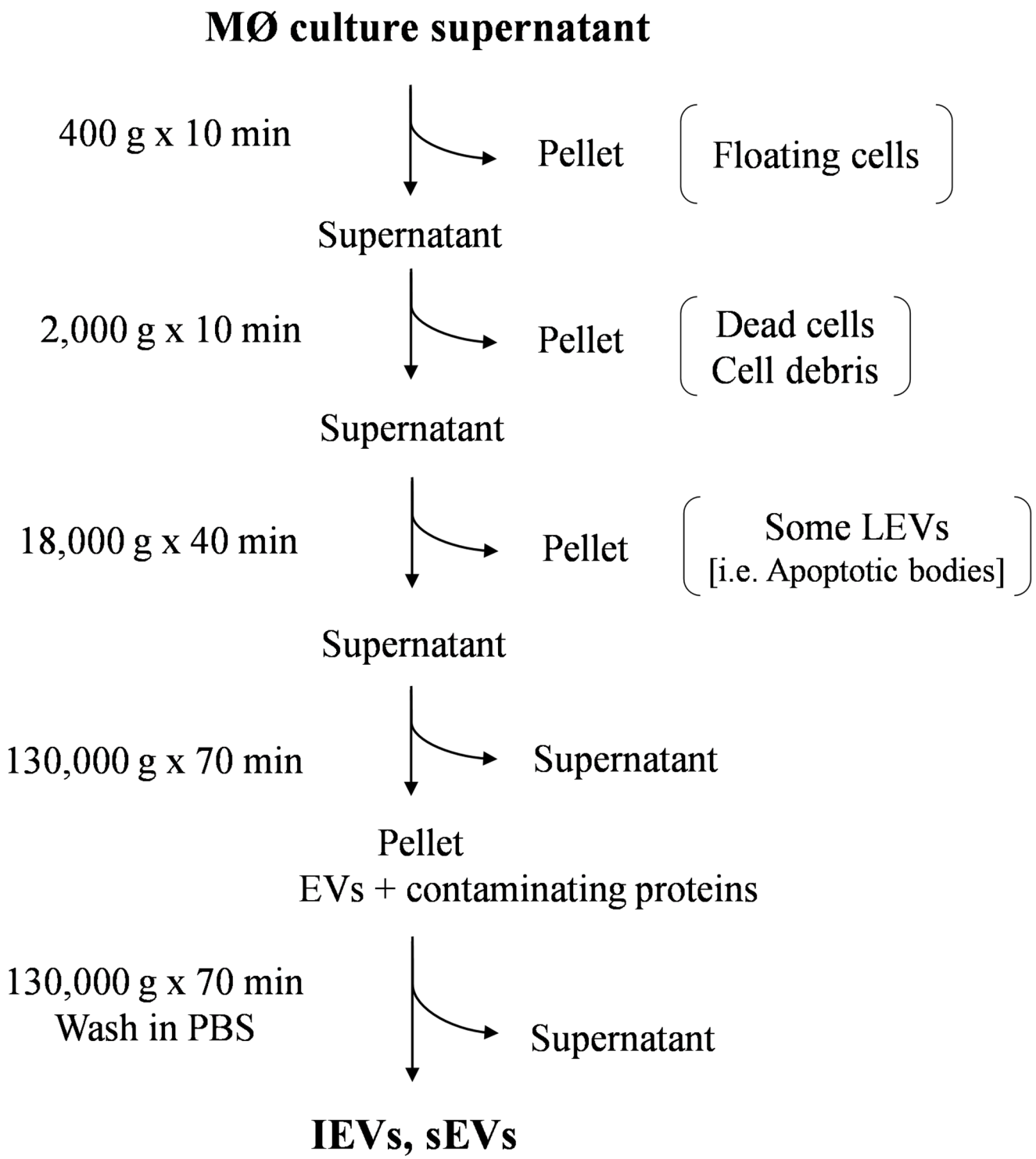
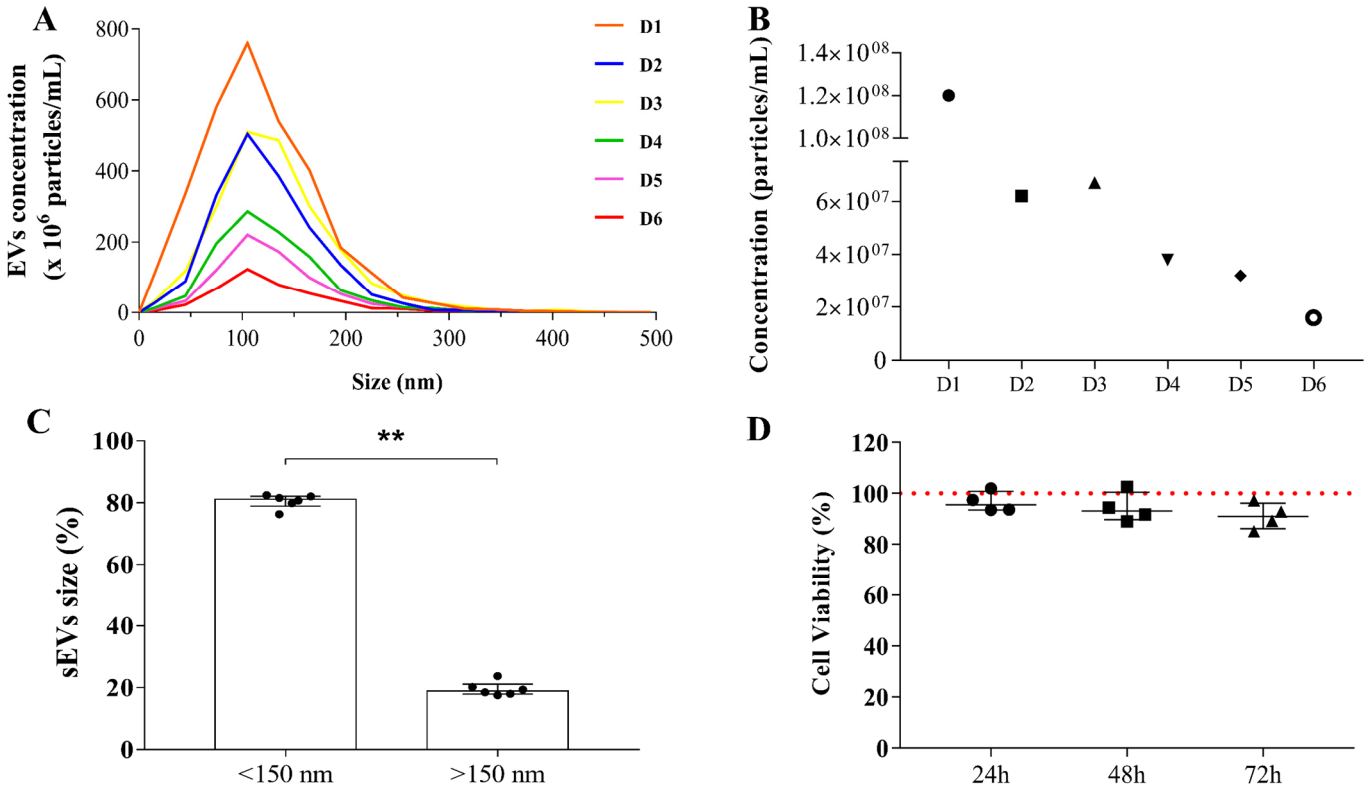
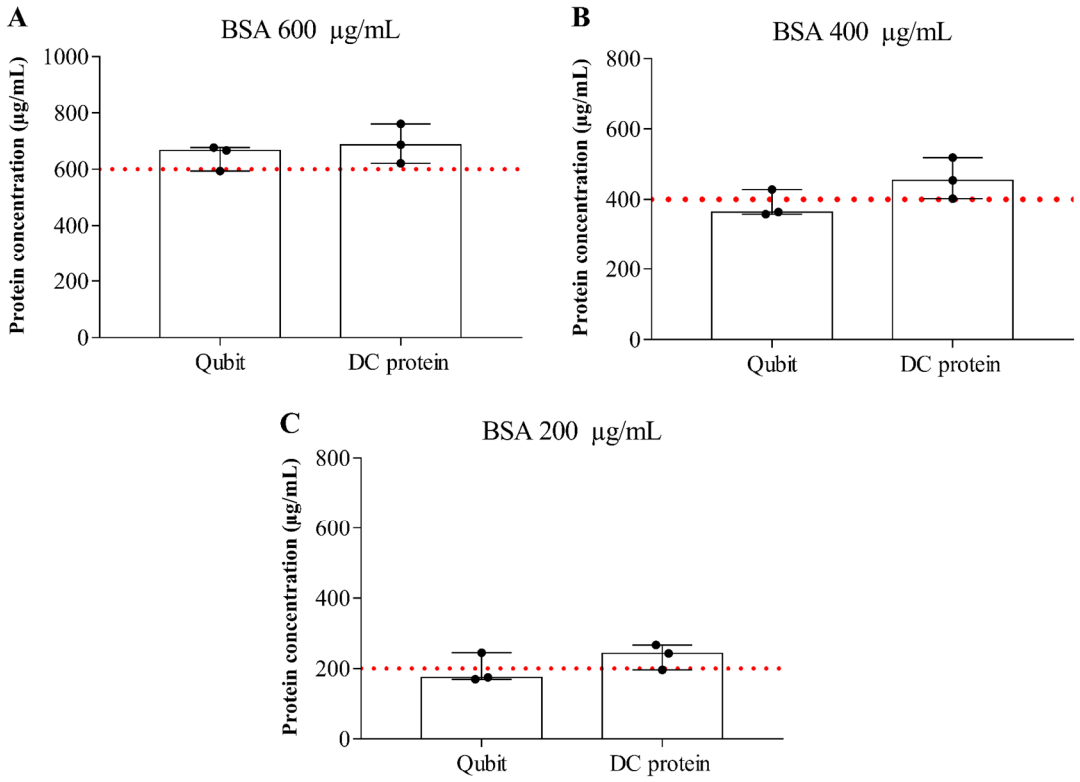
5 MDM-DERIVED sEVs CHARACTERIZATION SUPPLEMENTARY FIGURES AND INFORMATION (ARTICLE)



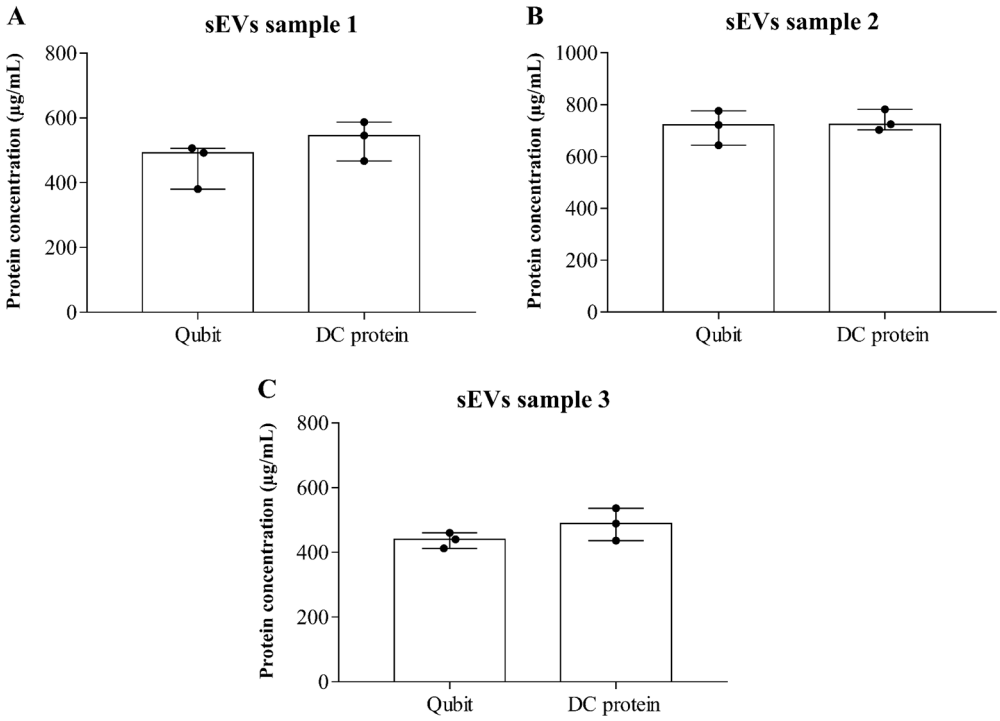
S1 Figure. Centrifugation steps for isolation of MDM-derived sEVs. The flowchart shows the centrifugation steps applied for sEVs isolation from 10 mL of supernatants from monocytes derived macrophages cultured in DMEM with 5% EVs-depleted serum. MØ: Macrophage; LEVs: large extracellular vesicles; IEVs: intermediated extracellular vesicles; sEVs: small extracellular vesicles.



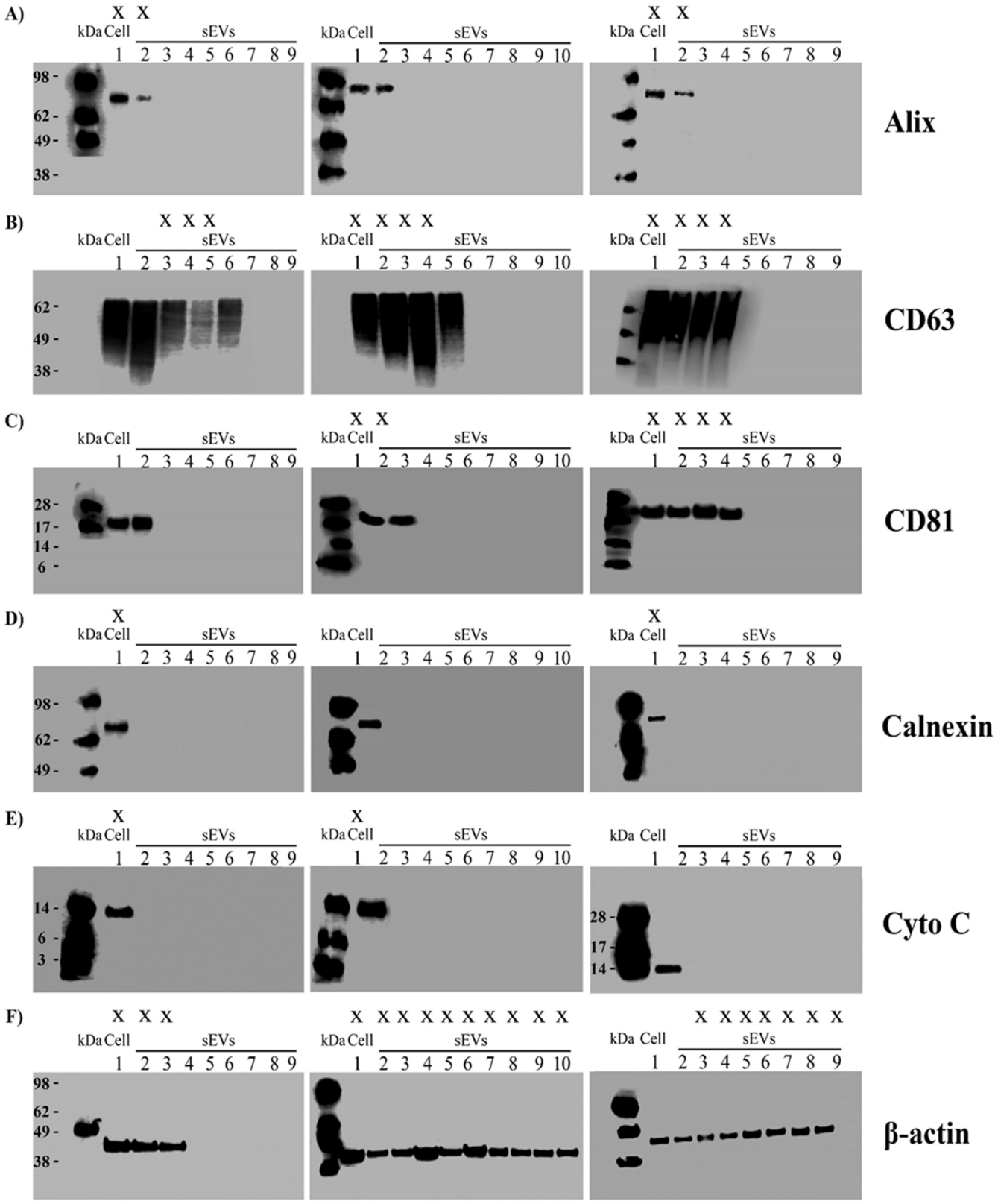
S2 Figure. Concentration, size distribution of sEVs and macrophages viability. EVs were colleted at 72 h of macrophages culture. (A) NTA-ZetaView analyzes of total number and size distributions of EVs derived from other six donors not represented in the main figures (*n*=6). (B) Total concentrations of EVs released by different donors. (C) Proportion of EVs sizes <150 nm or >150 nm. Mean values ± SD are indicated. **p <0.01 (Mann–Whitney test). (D) Macrophages viability at different time periods (*n*=4). Decrease in survival was analyzed by normalization against untreated cells with EV-free medium (control cells), represented by red dotted line. Median values are indicated D: donor.



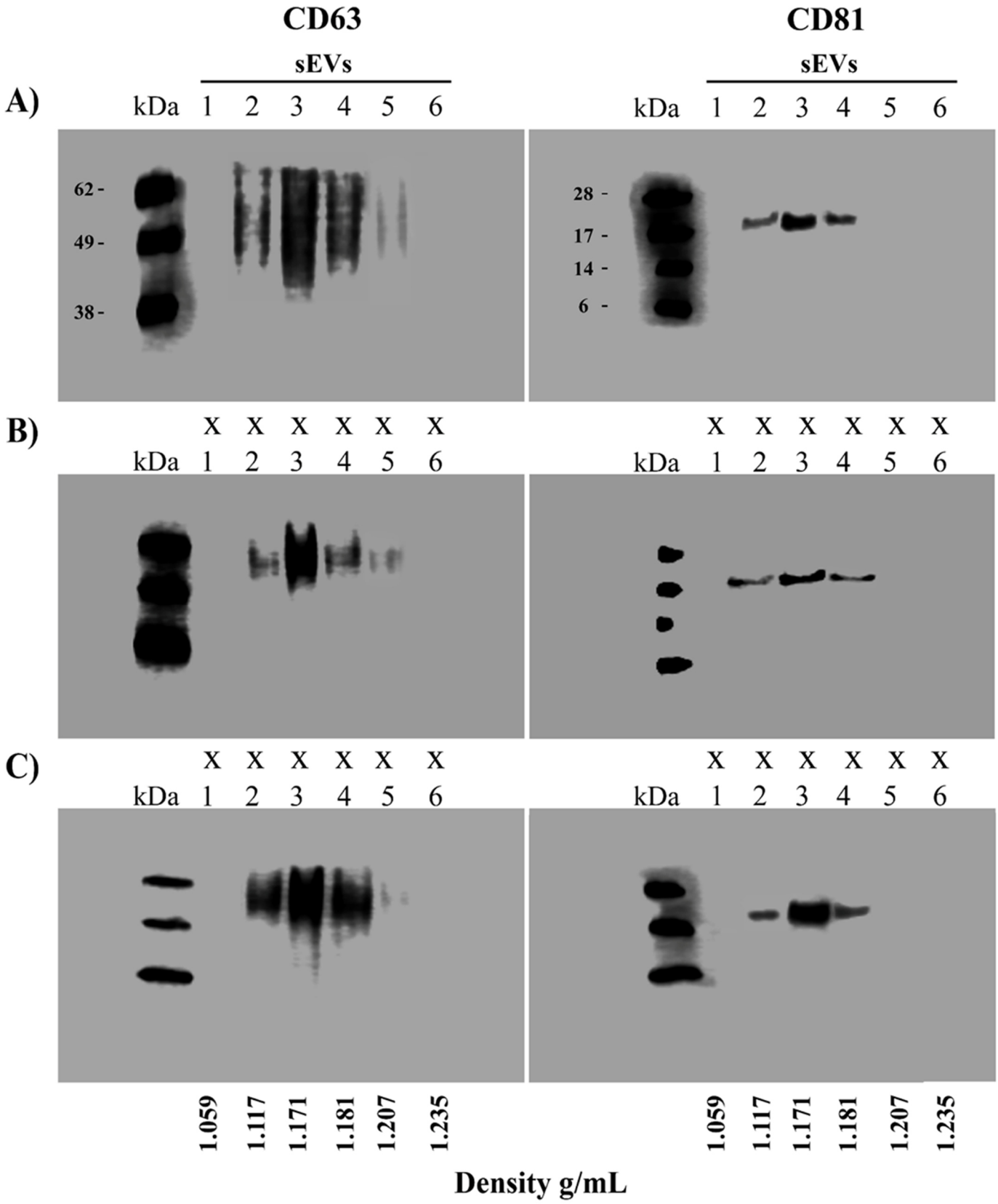
S3 Figure. Bovine serum albumin (BSA) quantification. BSA concentrations at (A) 600 µg/mL, (B) 400 µg/mL, and (C) 200 µg/mL were assessed by Qubit or DC Protein assays (*n*=3). Identical sample volume (5 µL) were used in all assays. Red dotted line indicates known BSA concentration. Results are expressed as median. D: donor.



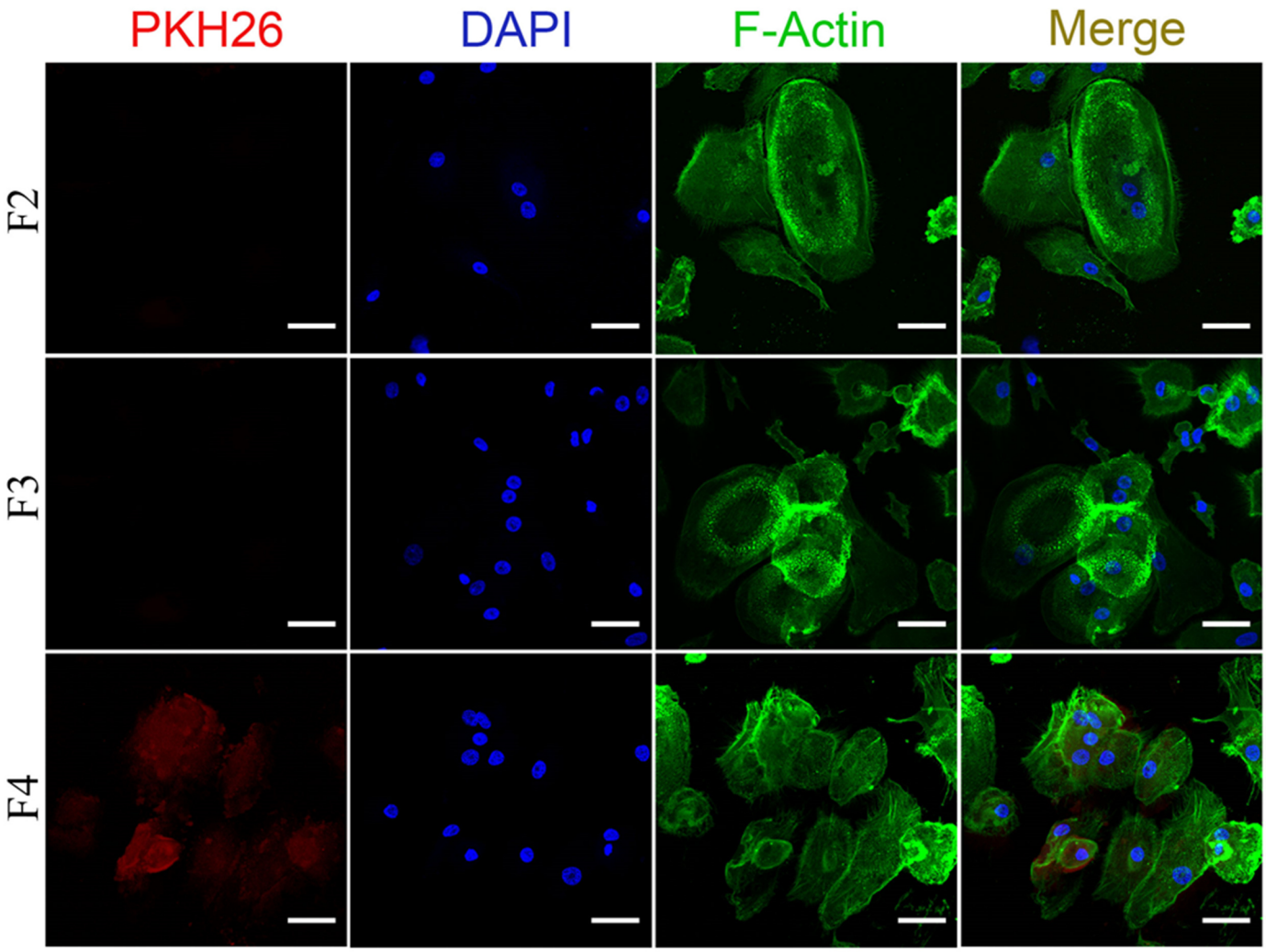
S4 Figure. Protein concentration of MDM-derived sEVs. sEVs samples (A) 1, (B) 2, and (C) 3 from other donors colleted at 72 h of cell culture were measured by Qubit or DC protein assay (*n*=3). Results are expressed as median.



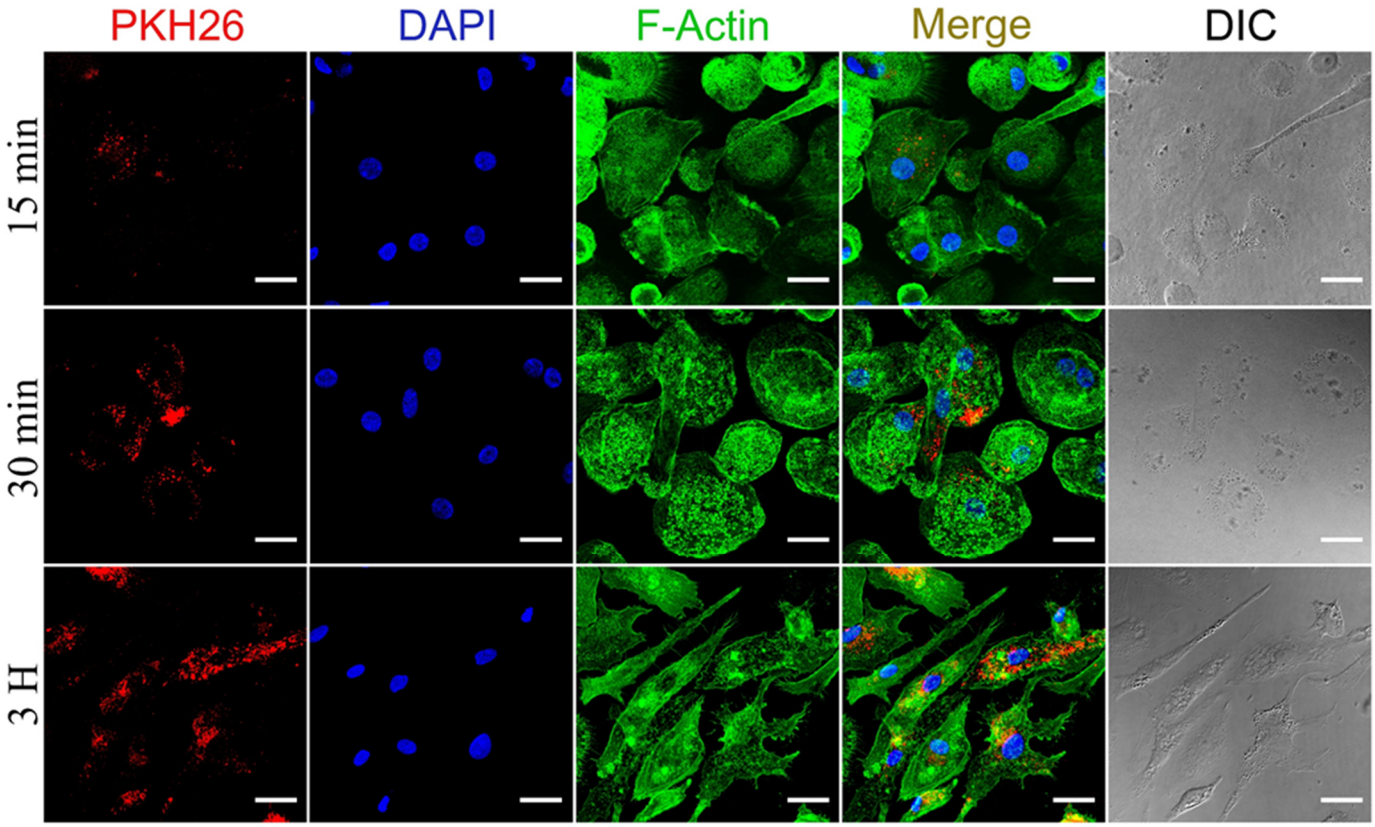
S5 Figure. Protein markers of MDM-derived sEVs. Original blots corresponding to Fig 4 (article). Primary human macrophages (line 1) and pools of MDM-derived sEVs (lines 2-10). Fig 4 was generated from the following panels: Panel A (middle) for Alix; Panel B (left) for CD63; Panel C (left) for CD81; Panel D (middle) for Calnexin; Panel E (right) for Cyto C; Panel F (right) for β-actin. All blot images were captured using Image Studio Software (LI-COR imaging system). Lanes not included in Fig. 4 are marked with "X".



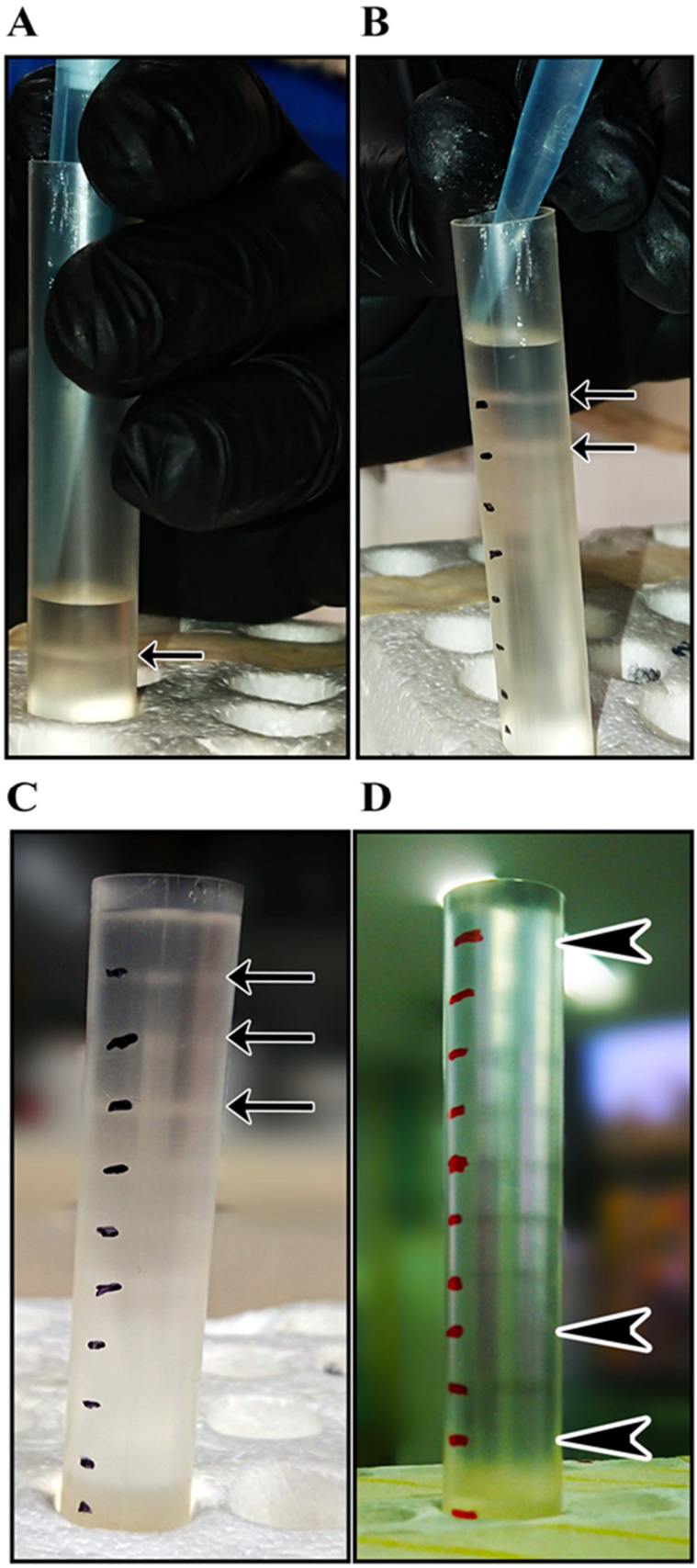
S6 Figure. Raw blot images of fractions F1-F6 collected of MDM-derived sEVs separated by S-DGUC. Original blots corresponding to Fig 5 (article). MDM-derived sEVs (lines 1-6). Fig 5 was generated from panel A of CD63 and CD81 blots. All blot images were captured using Image Studio Software (LI-COR imaging system). Lanes not included in Fig. 5 are marked with "X".



S7 Figure. Interaction of EVs-free medium with recipient primary macrophages. EVs-free medium were labeled with PKH26, then separated by sucrose density gradient centrifugation and three fractions (F2-F4) were colleted and added separately to recipient macrophages during 3 hours (*n*=3). Fluorescent images represent only cells or cells with sEVs, respectively (Bars = 50 μm). PKH26: sEVs; DAPI: cell nuclei; F-Actin: macrophages.



S8 Figure. Internalization of original EVs pellets by macrophages. EVs pellets isolated by dUC (not purified by sucrose gradient) were labeled with PHK26 and added to macrophages during 15 min (n = 3), 30 min (*n*=3), and 3 hours (*n*=4). Bright field (DIC) and fluorescent images represent only cells or cells with extracellular particles, respectively (Bars = 50 μm). PKH26: EVs and other particles; DAPI: cell nuclei; F-Actin: macrophages.



S9 Figure. Protocol to layer sucrose density gradient. (A) Position of tube angled 90˚ and (B) angled 60˚ during layering sucrose gradients. (C) Correct and (D) incorrect sucrose gradients layers. Black arrows point to layer formation during sucrose gradient; black arrowheads point to diffuse layers.

Supplementary materials and methods

1. Equipment

a) **For sucrose density gradient ultracentrifugation (**S-DGUC**):**

- Ultracentrifuge (e.g., Beckman Optima XE-90 centrifuge)
- Ultracentrifuge rotor (e.g, SW41 Ti)
- 13.2-mL polypropylene thinwall tube (Beckman coulter, 331372)
- Refractometer (e.g., Carl Zeiss 120540)

b) **For EVs PKH26-labeling purification in sucrose density gradient ultracentrifugation (**S-DGUC**):**

- Lab-Tek II chamber Slide (ThermoFisher)
- Scanning confocal microscope (e.g., Leica TCS SP8, Mikrosysteme GmbH).

2. Reagents

a) **For sucrose density gradient ultracentrifugation (**S-DGUC**)**

- Phosphate buffer saline (PBS); pH 7.4
- Sucrose (e.g., Sigma-Aldrich)

Stock solution (%)	10	16	22	28	34	40	46	52	58	64	70	90
Sucrose (g)	0.5	0.8	1.1	1.4	1.7	2.0	2.3	2.6	2.9	3.2	3.5	4.5
Add PBS to 5 mL												

b) **For EVs PKH26-labeling purification**

- 100-200 µL of EVs
- PKH26 Red Fluorescent kit (Sigma-Aldrich)
- EVs-depleted fetal bovine serum (FBS; ThermoFisher)
- Bovine serum albumin (BSA)
- Paraformaldehyde (PFA) 4%
- Phalloidin (e.g., Atto 488, Sigma-Aldrich)
- ProLong™ Diamond Antifade Mountant with DAPI (ThermoFisher)

3. Protocol

a) **Purification of EVs in sucrose density gradient ultracentrifugation (S-DGUC)**

- Cool UC rotor to 4°C.
- Prepare sucrose stock concentrations 10-90% as a mentioned above.
- Determine sucrose density on a refractometer.
- Resuspend the EV pellets (100-200 µL) obtained in the final step of UC with 1 mL of 90% sucrose solution and transfer into 13.2 mL polypropylene tube.
- Mount eleven successive gradients with 1 mL of decreasing sucrose concentration starting with 70%. Technical tip: To apply sucrose solutions, touch the center (angled 90°) or side (angled 60°) of the ultracentrifuge tube with the end of 1000 µL pipet tip (cut approx.1cm) containing 1

mL sucrose solution and apply the solution carefully (S9A, B Fig). Avoid turbulence between each sucrose solution added. Note: thin borders or discs between each layer will be observed if layering was careful enough (S9C, D Fig).

- Balance the buckets (SW41 Ti) using 10% of sucrose solution if difference between buckets are noticed.
- Centrifuge samples for 16 h at 4°C for 200,000 x g without breaks. Approximately 3 h is the time the centrifuge takes to reach 0.
- Collect six fractions of 2 mL starting from the top to bottom. Carefully aspirate 1 mL with a 1000 µL pipet tip touching the top of solution and transfer it into another tube. Repeat this step again so that 2 mL fractions are collected.
- Add 9 mL of filtrated PBS (0.22µm) in each fraction, balance the buckets with PBS and centrifuge at 4 °C (130,000 x g for 1 hour) in a SW41 Ti rotor.
- Resuspend the final pellet in 50-100 µL of PBS and store at -80 °C for Western blotting or confocal microscopy assays.

2. EVs PKH26-labeling in sucrose density gradient ultracentrifugation:

- Resuspend the EV pellets (100-200 µL) obtained in the final step of UC with 400 µL of diluent C for 1 min (EVs+dC) into a 13.2 mL polypropylene tube. Separately, in a 1.5 mL microcentrifuge tube dilute 3 µL of dye PKH26 in 400 µL of diluent C (stain solution).
- Transfer the stain solution into 13.2 mL polypropylene tube content the EVs+dC and mix continuously by gentle pipetting. Let stand at room temperature for 3 minutes.
- Quench by adding 800 µL of 5% EVs-depleted FBS or 10% BSA in PBS for 2 min.
- Add 9 mL of fresh medium supplemented with 5% EVs-depleted FBS (referred to as EVs-medium), balance the buckets with medium and centrifuge at 4°C (130,000g for 1 hour) in a SW41 Ti rotor.
- Resuspend 100-200 µL of labeled EVs (referred to as PKH26-EVs) into a 13.2 mL polypropylene tube and repeat the **protocol 1** of sucrose gradient for UC, as a mentioned above. Note: Use labeled EVs as soon as possible to ensure highest possible fluorescent intensity.
- Add 30 µL of the pellet obtained from each fraction into macrophages (1-2 × 10^3/per well, prepared as described in Material and Methods) culture on Lab-Tek II chamber slide and incubate at 37 °C for 3 h.
- After the internalization period, wash macrophages with PBS (twice). Fix the cells with 150 µL of 4% paraformaldehyde (PFA) for 20 min at room temperature. Wash for three time, then stain with 120 µL of Phalloidin-Atto 488 diluted 1:50 in PBS for 50 min and wash again for three time. Mount the slides with 2 or 3 drops of ProLong™ Diamond Antifade Mountant with DAPI and stored at 4 °C overnight. Finally, analyze the slides under a scanning confocal microscope.

6 HIV-1 FUNCTIONAL RESULTS

6.1 Macrophage-derived small EVs inhibit HIV-1 replication in primary cells

After performing the characterization of macrophage-derived sEVs, next we investigated the role of EVs on HIV-1 replication. To test this end, HIV-1-infected macrophages or HIV-1 infected peripheral blood mononuclear cells (PBMCs) were treated with increasing concentrations of macrophage-derived small EVs (from single donors or pools of vesicles) in a post-infection manner, then HIV-1 production was assessed 12-14 or 6-7 days after infection in macrophages or PMBCs, respectively. We found that infected macrophages exposed to the highest concentration (20 μg/mL) of sEVs from individual donors produced significantly less viral titers than control cells incubated only with the virus, a percentage of viral inhibition of 50% (Figure 6.1). Suppression of HIV-1 was not observed with the lower EV concentrations. In order to reduce potential different effects on viral replication of sEVs obtained from different single donors, who might present, different cargos or protein composition, and given that the inhibitory capacity on HIV-1 was similar with the highest concentration of sEVs among the individual samples tested, we evaluated viral replication in macrophages incubated with pools of sEVs, composed of EVs from 4 different donors. Interestingly, all concentration of EV pools promoted a stronger HIV-1 inhibition when compared with the treatment of sEVs derived from individual donors, presenting inhibition percentages of 48%, 66% and 73% for 5, 10 and 20 μg/mL, respectively (Figure 6.1). This result suggests that the antiviral effect of EVs in infected macrophages is dose-dependent.

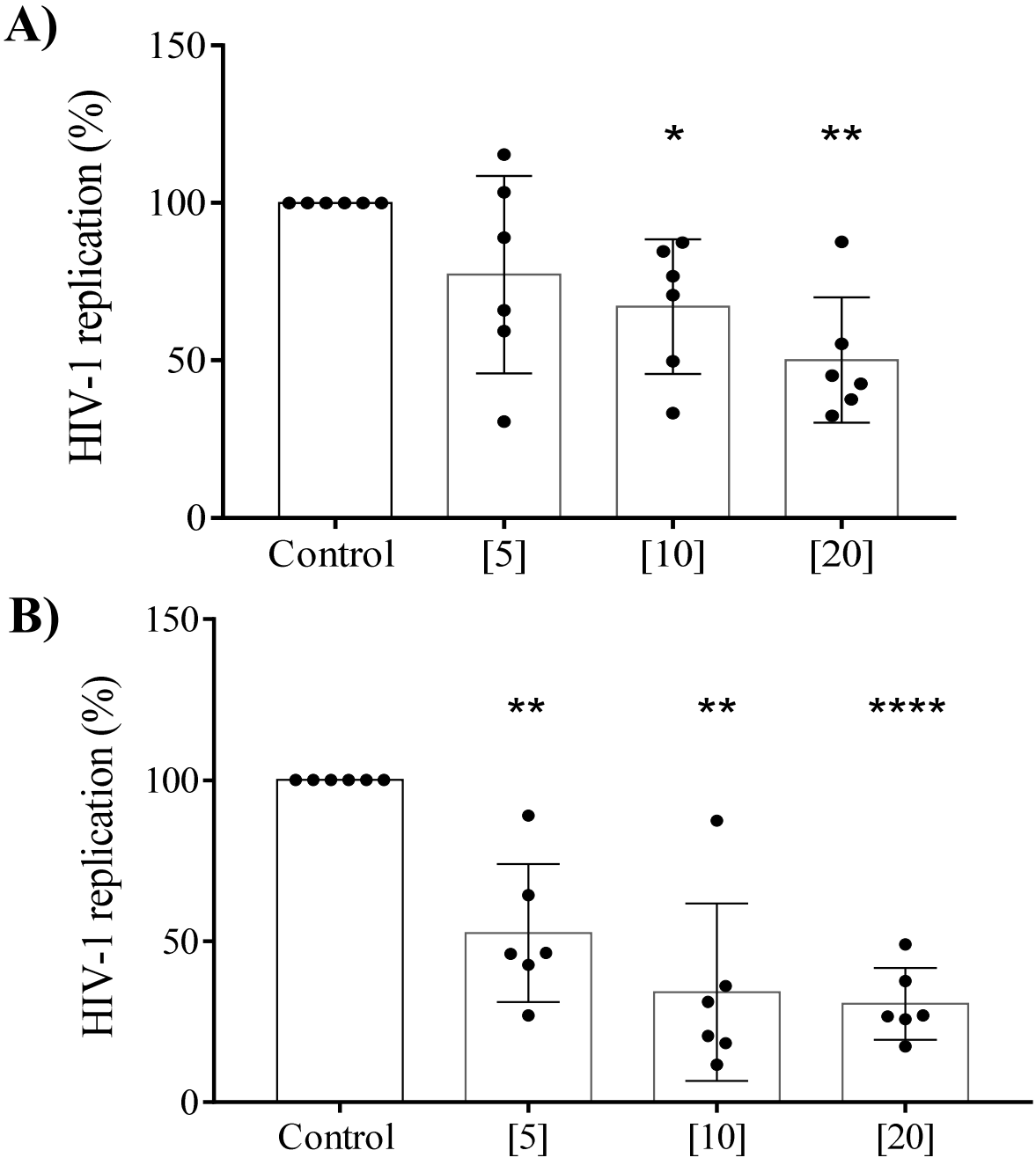


Figure 6.1. Macrophage-derived small EVs inhibit HIV-1 replication in macrophages. (A) Macrophages were infected in vitro and then treated with macrophage-derived small EVs uninfected macrophages obtained from different individuals, at indicated concentrations (5, 10 or 20 µg/mL), and maintained in culture (*n*=6). **(B)** Pools of sEVs were added to infected macrophages at indicated concentrations, and maintained in culture (*n*=6). Virus replication was measured in the culture supernatants by an HIV-1 p24 ELISA 12-14 days after infection. Data are shown as means ± SD. Asterisks indicate adjusted P values: *P ≤ 0.05, **P ≤ 0.01, ****P ≤ 0.0001. One-way ANOVA with Dunnett's multiple comparisons post-test.

Regarding the effects on HIV-1 replication in PBMCs, sEVs from individual donors induced a significant decrease in viral titers: 42%, 40% and 53% for 20, 40 and 60 µg/mL, respectively (Figure 6.2). We also compared the effect of random pools of sEVs, and observed a sharply inhibitory capacity of 74% using 80 µg/mL of protein. Strikingly, lower protein concentrations of vesicles also showed ability to reduce viral replication: 45%, 51% and 67% for 20, 40 and 60 µg/mL, respectively (Figure 6.2).

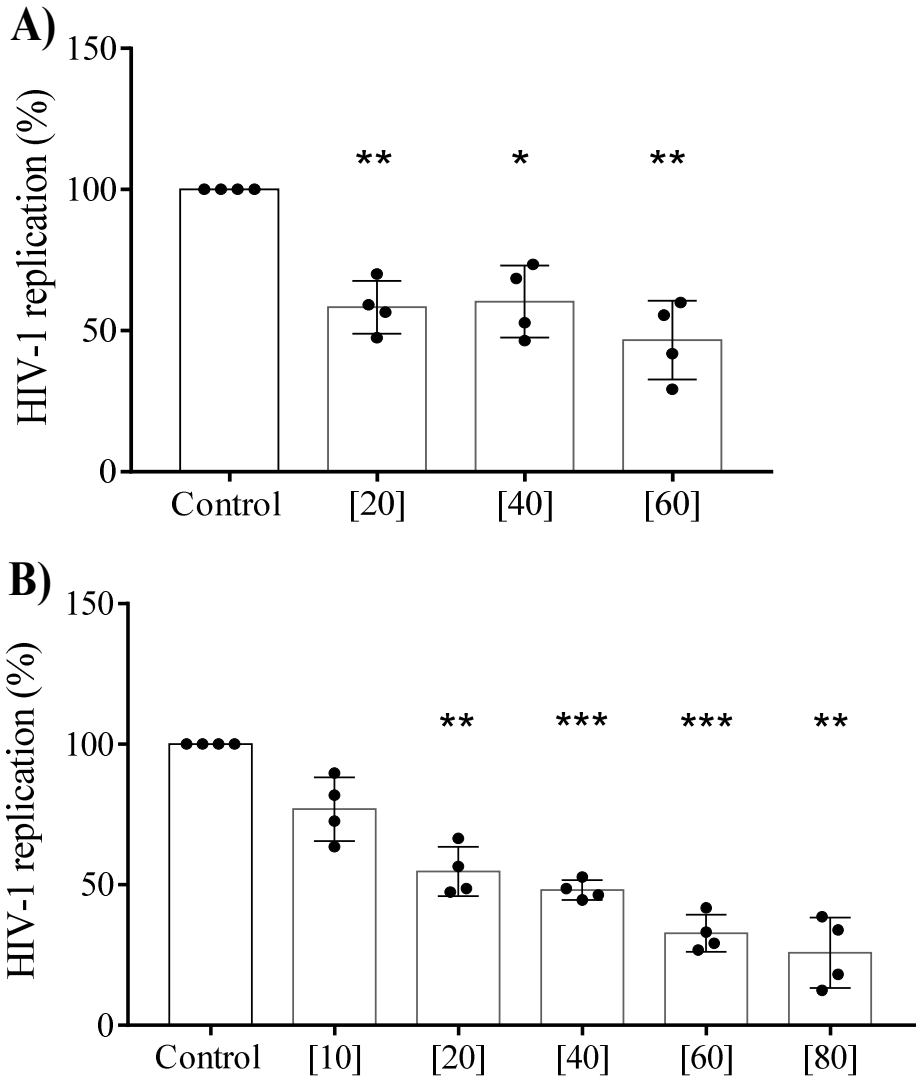


Figure 6.2. MDM-derived sEVs inhibit HIV-1 replication in PBMCs. (A) PBMCs were infected in vitro for 2 hours and then treated with macrophage-derived sEVs from uninfected macrophages obtained from different individuals, at indicated concentrations (10,20, 40, 60 or 80 µg/mL), and maintained in culture (*n*=4). **(B)** Pools of sEVs were added to infected PBMCs at indicated concentrations and maintained in culture (*n*=4). Virus replication was measured in the culture supernatants by an HIV-1 p24 ELISA 7 days after infection. Data are shown as means ± SD. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. One-way ANOVA with Dunnett's multiple comparisons post-test.

Previous studies of our group demonstrated that the neuropeptides Vasoactive Intestinal Polypeptide (VIP) and Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) have the ability to reduce HIV-1 production in human macrophages (109,110) and in PBMCs (Vieira, RC; doctoral Thesis, 2020). The neuropeptides VIP and PACAP belong to the secretin/glucagon family of peptides and were initially discovered due to their vasodilation properties on the gastrointestinal tract and ability to activate rat pituitary adenylate cyclase, respectively (111,112). In addition, these peptides have several regulatory functions in the neuroimmune-endocrine system, including, modulation of cytokine production, activation and cell differentiation (113,114). We initially investigated whether small EVs from uninfected macrophages exposed to the peptide VIP would also affect HIV-1 replication. To test this hypothesis, HIV-1-infected macrophages or PBMCs were treated with small EVs from unstimulated or VIP-stimulated macrophages (sEVs-Ctr and sEVs-VIP, respectively), or VIP only. We found that sEVs-Ctr and sEVs-VIP, at 20 μg/mL of protein concentration, were both able to decrease HIV-1 replication, achieving 50% and 69%, respectively of viral inhibition (Figure 6.3). As expected, the peptide VIP at 10 nM inhibited 59% of viral growth compared with infected cells without treatments. Moreover, small EVs also promoted significant decrease in viral titers in infected PBMCs, equal to 51%, 67%, and 74% for 40, 60 and 80 μg/mL of sEVs, respectively, and 52%, 71%, and 78% for sEVs-VIP, using the same growing protein concentrations (Figure 6.3). Cells treated with VIP alone at 5 nM decreased by 55% the viral replication. These data suggest that both sEVs were similarly effective in their ability to reduce HIV-1 production in macrophages and PMBCs.

Altogether, these findings indicate that macrophage-derived small EVs are endowed with the ability to reduce the HIV-1 production in macrophages and PBMCs in a manner depending on vesicle protein concentration, and that the antiviral effect is more pronounced when pools of vesicles are employed. Considering that the inhibitory effects on viral production using EVs pools were stronger than single sEVs, the subsequent experiments were conducted using pools of vesicles.

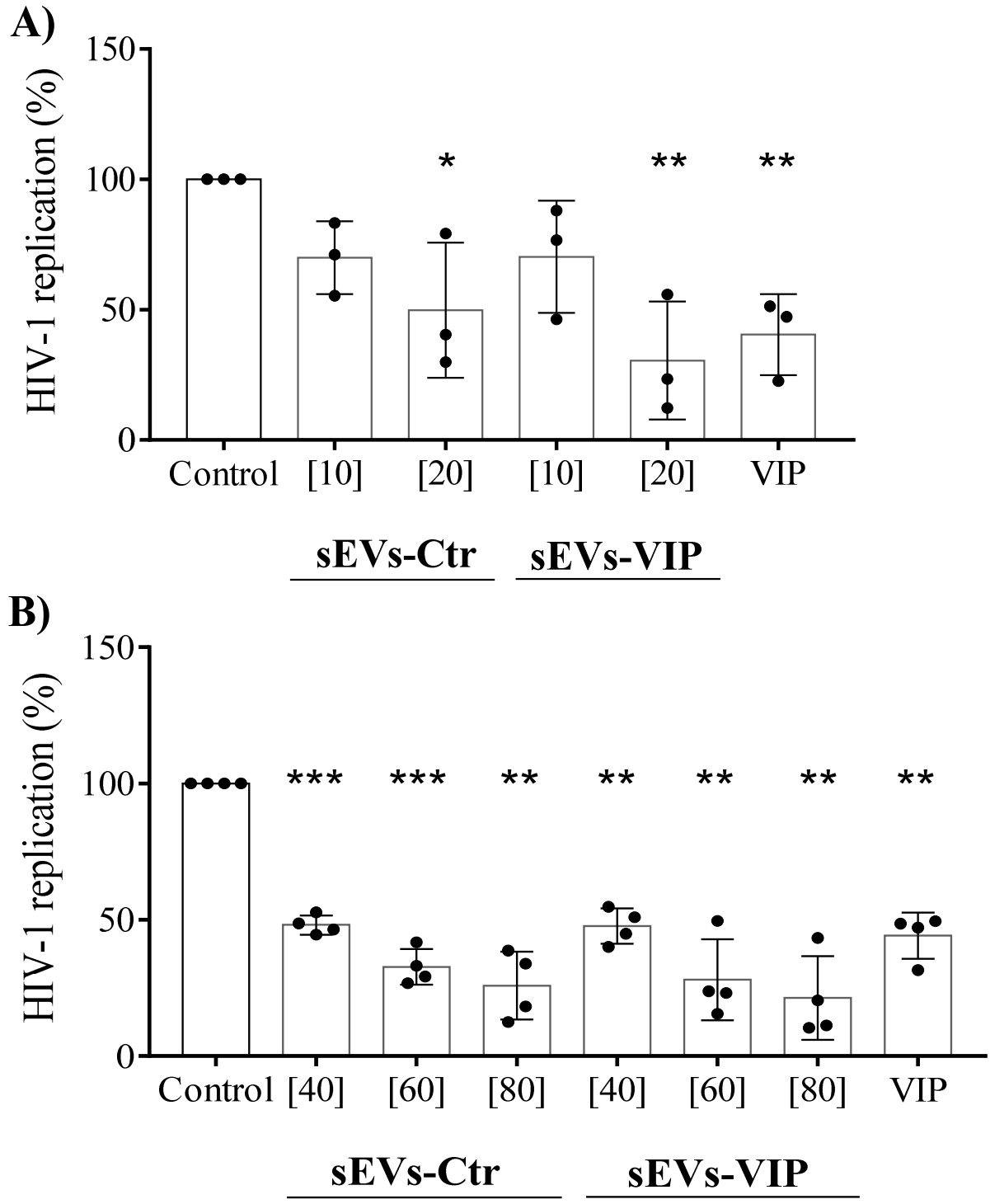


Figure 6.3. Antiviral effect of sEVs-ctr or sEVs-VIP in HIV-1 replication. (A) Macrophages were infected with HIV-1, then cells were treated with pools of small EVs from unstimulated (sEVs-Ctr), or Vip-stimulated macrophages (sEVs-VIP), or VIP only [10 nM] (*n*=3). **(B)** HIV-1-infected PBMCs were exposed with sEVs-Ctr, or sEVs-VIP, or VIP only [5 nM] (*n*=4). EVs were treated at indicated concentrations (40, 60 or 80 µg/mL). Data are shown as means ± SD. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. One-way ANOVA with Dunnett's multiple comparisons post-test.

6.2 EVs do not affect cell viability in recipient primary cells

To investigate whether macrophage-derived sEVs induce cell death in recipient cells, we analyzed mitochondrial activity in uninfected macrophages left untreated (negative control) or treated with increasing concentration of sEVs and maintained in culture for 24 and 48 h. Uninfected PBMCs were also exposed to different doses of sEVs and maintained in culture for 24 h. XTT analysis showed that the viability of macrophages or PBMCs remained unchanged under these conditions (Figure 6.4). Therefore, our results demonstrate that macrophage-derived sEVs did not affect the viability of recipient primary cells targeted with growing protein concentration of vesicles and different time-periods. Thus, we conclude that our observed sEV-mediated inhibition on HIV-1 replication cannot be attributed to cell death during our experimental assays.

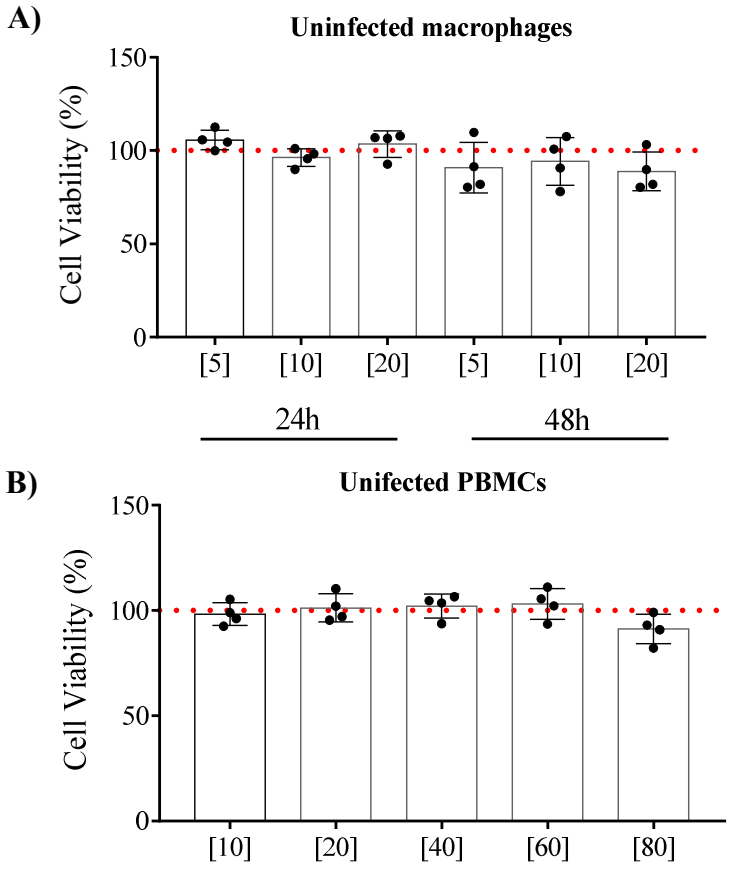


Figure 6.4. Cell viability of primary human cells exposed to MDM-derived sEVs. (A) uninfected macrophages or **(B)** uninfected PBMCs were incubated with growing concentrations of sEVs pools (n=4). EVs were treated at indicated concentrations (5, 10, 20, 40, 60 or 80 µg/mL). Cell viability was assessed 24 or 48 h for macrophages or 24 h for PBMCs after using the XTT method. Data are shown as means ± SD. One-way ANOVA with Dunnett's multiple comparisons post-test.

6.3 Cell conditioned medium and EV-free medium do not confer antiviral effect

Other authors have demonstrated that EV-associated function could be related with the presence of soluble molecules that may (e.g., high or low concentrations) or may not be associated specifically with EVs (1). Moreover, functional activity of an EV preparation may be borne by EVs, or by the additional non-EV components co-isolated with the vesicles, or by a combination of both (1). To determine whether the anti-HIV-1 effect that we observed was associated or not with EV-enriched preparations, HIV-1-infected PBMCs were treated with macrophage culture conditioned medium (referred as CM), or purified macrophage-derived sEVs, or EV-depleted supernatants from the same macrophage culture medium (referred as EV-free medium). We detected that CM and/or EV-free medium failed to suppress HIV-1 production in infected PBMCs. In contrast, purified vesicles inhibited viral replication over 55%, relative to untreated HIV-1-infected cells (Figure 6.5). This result indicates that the anti-HIV-1 effect was associated with functional properties of small EVs from human macrophages, rather than with soluble mediators present in the supernatant of macrophage culture.

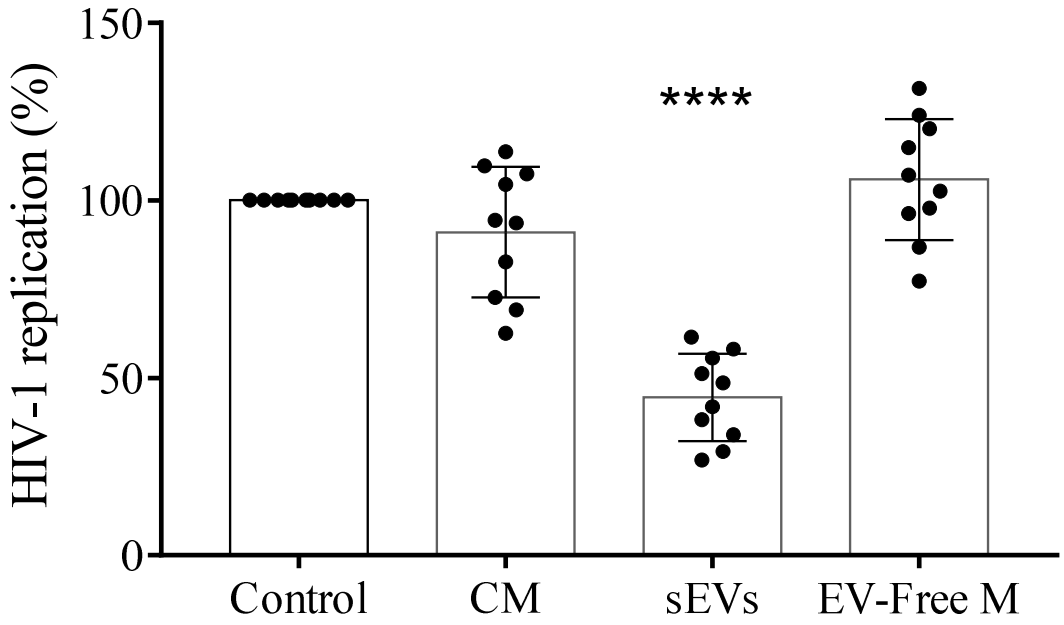


Figure 6.5. Anti-HIV-1 activity is associated with EV-enriched preparations rather than soluble mediators. HIV-1-infected PBMCs were incubated with 60 μg/mL of CM, macrophage-derived sEVs, or EV-Free M (n=10). Untreated cells were used as control. Virus replication was measured in the culture supernatants by an HIV-1 p24 ELISA 7 days after infection. Data are shown as means ± SD. ****P ≤ 0.0001. One-way ANOVA with Dunnett's multiple comparisons post-test. Abbreviation: CM: conditioned medium; EV-free M: EV-depleted supernatant from the macrophages culture medium.

6.4 EVs transfer antiviral response to infected PBMCs in a HSPG-dependent manner.

We next investigated the internalization mechanisms in which macrophage-derived sEVs transfer anti-HIV-1 response to recipient cells. To this end, HIV-1-infected PBMCs were pre-incubated at 37°C with 20 µg/mL heparin for 30 min, and then cells were exposed or not to sEVs, followed by HIV-1 replication evaluation. Under our experimental conditions, sEV-mediated antiviral response was significantly reduced, by to 74% (Figure 6.6). These initial data suggest that macrophage-sEVs transfer anti-HIV-1 resistance to infected cells in heparan sulphate proteoglycans (HSPGs) dependent internalization manner.

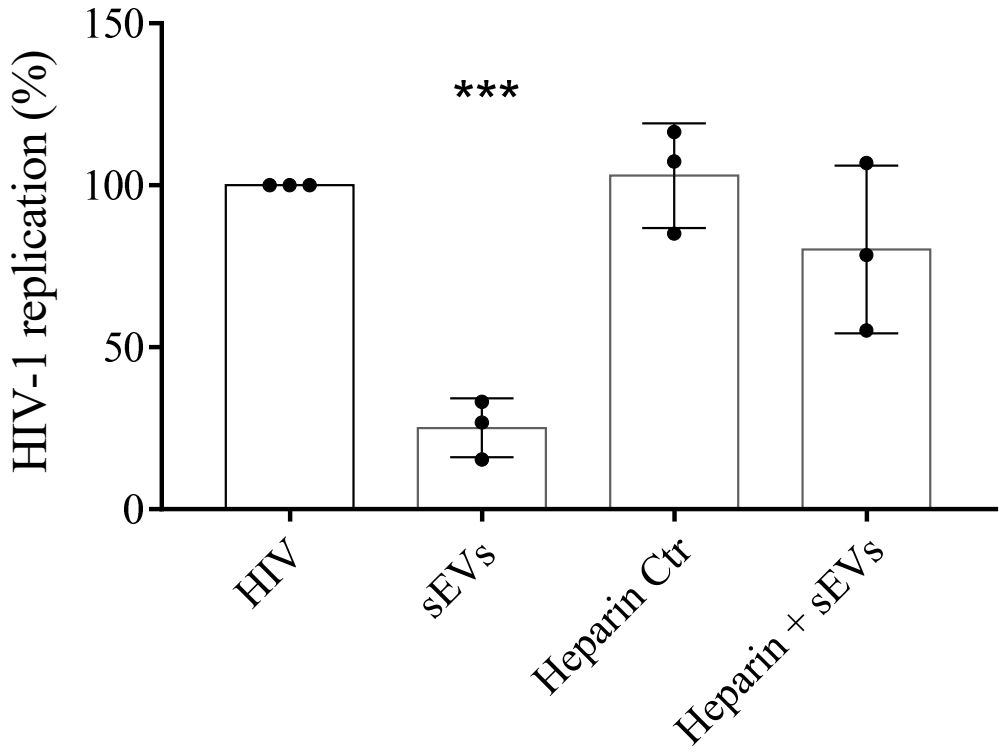


Figure 6.6. The anti-HIV-1 effect mediated by MDM-derived sEVs is reduced by an endocytosis inhibitor. HIV-1-infected PBMCs were treated with 20 µg/mL of heparin for 30 min prior to the addition of small EVs (60 µg/mL) for 16 hours in the presence of the indicated inhibitor. Cells treated with vehicle (Heparin Ctr) or untreated (HIV, medium alone) were used as controls. Virus replication was measured in the culture supernatants by an HIV-1 p24 ELISA 7 days after infection (*n*=3). Data are shown as means ± SD. ***P ≤ 0.001. One-way ANOVA with Dunnett's multiple comparisons post-test.

7 DISCUSSION

The present study aimed to provide evidence as to whether small EVs released by human primary macrophages derived from circulating monocytes (MDM) possess antiviral activity in primary cells infected with HIV-1. This work also showed a comprehensive description of vesicles derived from human macrophages. Initially, MDM-derived sEVs from healthy human donors were characterized according to their physical and biochemical properties through NTA, SEM and WB, as well as their internalization by recipient macrophages. We proposed a modified protocol based on sucrose density gradient ultracentrifugation (S-DGUC) with higher speeds that allowed by itself the separation of large amounts of small EVs enriched with vesicles from endosomal origin (107). Because different separation and characterization methods have their own accuracy and precision in measuring EVs from diverse sources, the evaluation of different methodological parameters and the implementation of several techniques are essential for the success of robust and reliable data in EV field (1,45). Thus, we reported a step-by-step protocol to improve the reproducibility and interpretation of methodological and functional experiments for researches studying EVs from human macrophages. Our protocol demonstrated reproducibility between macrophage samples from different healthy donors, and also were able to recover ~80% of heterogeneous population of MDM-derived small EVs.

Some questions were raised during the preparation of our previous article (107) and are worth to highlight here. For example, (*i*) was the protocol and EV methods adequate enough to make a comprehensive isolation and characterization of small EVs from macrophage culture-conditioned medium? To our knowledge, this is the first study that reported in details the parameters require to recover small EVs from human macrophages from circulating monocytes. The parameters include biophysical description and biochemical description and pre-analytical variables (e.g., culture conditions, medium collection, culture history, percentage of live cells). Because we followed rigorously the minimum requirements recommended by MISEV2018 for the characterization of EVs from cell culture-derived vesicles (1), we understand that our EVs are sufficiently characterized. (*ii*) Why did the study not use western-blot specific markers of contaminants (miRNA/protein complexes marker such as Ago-2 or lipoprotein markers, etc) for both EVs (isolated only by centrifugation and the fractions obtained after S-DGUC), to demonstrate the purity of the preparations? We are aware that our work faced certain limitation. The main idea of using differential ultracentrifugation (dUC) in combination with sucrose density gradient was to identify a specific EV subtype, a goal that, indeed, we reached. We never had in mind achieving "100% purity" when separating these vesicles. In fact, the literature has reported several contaminants in vesicles obtained by multi-step methods, including those

that have gone through “purification techniques” (115–117). While we assume that our isolated EV subtype may present some contaminants, we also understand that the potential presence of contaminants continues to be a limitation common to separation techniques in the EV field (116,117).

MDM-derived small EVs were further added to macrophages or PBMCs infected with HIV-1. Interestingly, sEVs of individual donors or pools of vesicles isolated from uninfected macrophages suppressed HIV-1 replication. The inhibitory effect on viral replication mediated by sEVs was dose-dependent within the range of tested concentrations of 10 to 80 μg/mL. Moreover, the antiviral effect seems to be more effective when pool of vesicles was employed, thus, over 70% of viral inhibition was observed in infected macrophages or PMBCs using 20 or 80 μg/mL, respectively. We proposed that 20 and 60 μg/mL of MDM-derived sEVs were the optimal doses to promote the inhibition of R5-tropic HIV-1 strains in macrophages or PBMCs, respectively. The viral inhibition mediated by sEVs was not related with interferences in cellular viability.

We also evaluated the ability of small EVs secreted from uninfected macrophages exposed with the peptide VIP (sEVs-VIP) to inhibit HIV-1 replication. We selected this neuropeptide taking into consideration that our group previously demonstrated that VIP modulate HIV-1 replication in primary human macrophages, through the secretion of cytokine IL-10, β-chemokines, and the activation of protein kinases A and C (109,110). VIP have also the ability to reduce HIV-1 production in PBMCs, stimulated the production of IL-10, and reduced TAT (40% reduction) and GAG (50% reduction) transcripts of HIV-1 provirus after 72 hours of treatment (Vieira, RC; doctoral thesis,2020). Remarkably, sEVs-Vip inhibited 69% and 78% of viral titers in infected macrophages or PMBCs, respectively. As observed in sEVs from unstimulated macrophages (sEVs-Ctr) the antiviral effect mediated by sEVs-VIP was also dose-dependent of protein concentration. In our experimental model, no differences related to antiviral effect between sEVs-Ctr or sEVs-VIP were observed. Thus, the next functional assays were performed with sEVs-Ctr. Previous findings have demonstrated that the vesicle-producing cell stimuli (e.g., IFN-α-treated cells, Toll-like receptor-3-treated cells) will directly influence in the EV cargoes that those cells produce and such cargo may induce physiological functions in the recipient cells towards controlling viral infections (118–121). In this sense, some specifics of our culture conditions with the neuropeptide, such as, for example, the concentration of VIP (10 nM) used in the macrophage culture probably was not sufficient to induce changes in the cells to secrete vesicles (sEVs-VIP) with a more effective antiviral activity in comparison with cells that were not exposed to the neuropeptide (sEVs-Ctr). Thus, further functional assays comparing different concentration of VIP in macrophage culture, in order to recover sEVs-VIP

with powerful antiviral effect may help to understand the functional differences between the vesicles released by cells exposed or not to VIP observed in this study.

According to literature, the physiological function of a given extracellular vesicles could be displayed by heterogeneous EVs, specific EV subtypes, non-EV components co-isolated with the vesicles, or by a combination of all (1,31). In our study, MDM-derived sEVs suppressed HIV-1 replication in PBMCs, but conditioned medium (CM) or EV-Free medium from supernatants of macrophages cultures did not. This result suggests that the antiviral proprieties observed in our work seems to be predominantly associated by heterogeneous small EVs, rather than soluble mediators presented in the supernatants of macrophage culture. It is worth to note that the particular anti-HIV-1 function found in the macrophage-sEVs could also be present in other EV subtypes, for instance, large EVs >200 nm (e.g. "microvesicles"). Hence, comparing large EVs with small EVs in HIV-1 context, is something that we intend to carry out.

In this investigation, we analyzed only the role of a mixed population of EVs. In other words, our preparations were composed by vesicles from different size and origins (endosomal system and plasma membrane). Furthermore, we did not evaluate the effects of "purified" EV fractions separated from the sucrose gradient-centrifugation referred to as non-EV components or "contaminants" (e.g., ribonucleoprotein aggregates, lipoproteins, exomeres) (1). Based on the above, we propose that the functional activity against HIV-1 infection was mediated by EV-enriched preparations rather than EV-specific activity. Therefore, our findings indicate that heterogeneous population of small EVs in combination with non-EV components have the ability to transfer antiviral factors that can inhibit HIV-1 infection in primary cells.

In our conditions, CM and EV-Free M from supernatants of macrophage cultures had no effect on viral expression in infected PBMCs. Literature have demonstrated that conditioned medium from different cell models can enhanced HIV-1 replication *in vitro*. Vitković *et al*., observed that astrocyte-derived CM stimulated or not with lipopolysaccharide (LPS) enhanced HIV-1 replication in U1 cells, through TNF-α (122). Borghi and colleagues reported that HIV replication in U1 cells was enhanced with conditioned media from human umbilical vein endothelial cells (HUVEC), thus, suggesting that soluble mediators secreted by HUVEC-derived CM (e.g., MCP-1, TNF-a and IL-6) may modulate HIV-1 expression (123). Recent studies have shown that MDM cultures produce pro-inflammatory cytokines such as IL-6, TNF-α or GM-CSF and CC chemokines (MCP-1, CXCL8) with potential to up-regulate HIV replication (124,125). These evidences could be the reason to understand why the EV-Free M showed a tendency to increase viral replication. In contrast, we observed that macrophage-CM did not produce a specific biological effect in HIV-1-infected PBMCs. One could explain that the presence of sEVs together with other non-EV components in the conditioned medium may

be counteracting the proviral activity mediated by the inflammatory cytokines. However, further functional assay comparing macrophage-sEVs and contaminants co-isolated with these vesicles are necessary to understand this phenomenon in the HIV-1 context.

Some authors have demonstrated that the routes and mechanisms of EVs internalization, strongly determine the ability of vesicles to transport their content to elicit a biological function in recipient cells (19,126). The routes and mechanisms of EVs internalization largely depend on surface molecules and glycoproteins on the membrane of the vesicle and the recipient cell, and more than one route is normally involved in this EVs uptake (42). Internalization routes include: micropinocytosis, clathrin-or caveolin-dependent endocytosis, lipid raft and membrane fusion, among other (42). Our preliminary data showed that antiviral effect mediated by MDM-derived sEVs was reduced over 70% in infected PBMCs pre-treated with heparin. The heparin blocks the binding of heparan sulphate proteoglycans (HSPGs), which are expressed on membrane of EVs particles and surfaces of human cells, thus inhibits vesicles endocytosis (127). HSPGs act as internalizing receptors and/or as co-receptors for temporary cell surface attachment to promote internalization of a variety of macromolecules such as DNA, cationic polymers, liposomes, cell-penetrating peptides (CPPs), viruses, protein aggregates, RNases, and EVs (128). One can hypothesize that the inhibitory effect exerted by macrophage-sEVs could be transferred to infected cell in a HSPG-dependent manner. However, our data do not allow us to claim this situation. In fact, other uptake mechanism may be involved and have direct implications for the reduction of HIV-1 replication in target cells. Therefore, more routes of EV-internalization will need further investigations.

The anti-HIV-1 proprieties for EVs from different sources have been reported. EVs can contribute to antiviral responses by delivering host-derived restriction factors to nearby cells. For example, EVs from CD4+ T cell line H9 can transfer cellular restriction factors such as APOBEC3 (A3G), thereby triggering control virus replication in infected cells (71). Furthermore, Toll-like receptor-3 (TLR3) activated human brain microvascular endothelial cells (HBMECs) release EVs that block HIV-1 infection to the central nervous system (CNS) via transport of antiviral factors and IFN-stimulated genes (ISGs), thereby transferring anti-HIV protection to human macrophages. HBMECs-derived EVs contained several antiviral factors, including key IFN-stimulated genes (ISGs; ISG15, ISG56, and Mx2) at mRNA and protein levels (118). Macrophage and CD4+ T cells, enriched in the gastrointestinal system (GI), are protected against HIV-1 by EVs released from TLR3-activated intestinal epithelial cells (IECs) containing HIV-restriction ISGs (ISG15, ISG56, MxB, OAS-1, GBP5, and Viperin) and miRNAs (miRNA-20 and miRNA125b) (119). EVs found in body fluids such as breast milk, semen and vaginal fluids have been described to control HIV-1 infection. Vertical transmission

of HIV-1 can be inhibited by breast milk-derived EVs which bind to monocyte-derived dendritic cells (MDDCs) via the CLR intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN), potentially inhibiting HIV-1 replication in MDDCs and viral transfer to CD4+ T cells (129). Madison and colleagues showed that exosomes from human semen (SE) contain a heterologous population of EVs, enriched in mRNA encoding tetraspanin exosomal markers and various anti-HIV-1 components, which can restrict replication of a wide range of HIV-1 strains and the murine AIDS virus complex LP-BM5 (75). The same group demonstrated that SE avoided the spread of HIV-1 from vaginal epithelial cells to target cells, thus, suggesting that semen-derived EVs possess anti-retroviral activity (130).

Considering the increasing number of researches focusing on the role of EVs in HIV-1 pathogenesis, to our knowledge this is the first investigation showing that primary human macrophages can transfer resistance to primary cells infected with HIV-1 via heterogenous small EVs. Although we have not explored the EV-mediated mechanisms involved in the modulation of HIV-1-infected primary cells, previous investigations suggest that multiple mechanisms may be involved in this phenomenon. For instances, the EVs source, internalization, cargo delivery and activation of several intracellular events in the target cells (68,70,130) may have contributed for the phenomenon that we observed. Therefore, in our experimental conditions, one can hypothesize that sEV-enriched preparations could transfer or induces in targets cells the activation of viral restriction factors (e.g., APOBEC3G, SAMHD1) and/or host´s sensor proteins (cGAS/cGAMP), which up-regulate antiviral response through interferon and innate immune responses to suppress HIV-1 infection *in vitro* (71,72,131). In addition, these vesicles can contain miRNAs that activate NF-kB signaling pathway, and consequently induces inflammatory signals that result in the production of cytokines/chemokines with anti-HIV-1 proprieties (118,119). All these mechanisms could act separately or together, and could explain the HIV-1 inhibitory effect observed here. In the case of vesicles released from uninfected macrophages treated with VIP (sEVs-VIP), one could explain that sEVs may carry components of VIP, which stimulate in target cells the production of the β-chemokines (CCL3 and CCL5) and the cytokine IL-10 to reduce HIV-1 growth (109). Nevertheless, our preliminary data still do not allow us to conclude whether the reduction of HIV-1 replication by sEVs-VIP has a direct implication with the presence of neuropeptide molecules in the vesicle, or was due to a change in the physiological state of macrophages that secreted EVs with antiviral proprieties once these cells were exposed to the peptide. Considering the above, we intend to investigate some mechanisms that will allow us to confirm the antiretroviral activity mediated by MDM-derived sEVs, including the EV cargo (e.g., proteomic and/or miRNome analysis), viral fitness, and the activation and secretion of multiple

anti-HIV-1 factors, such as ISGs, CC-chemokines, and IL-10, in target cell co-culture with sEVs.

8 CONCLUSION

Here we reported a modified protocol based on sucrose density gradient ultracentrifugation with higher speeds that allowed by itself the separation and quantification of large amounts of small EVs enriched with vesicles from endosomal origin. MDM-derived sEVs from healthy human donors were characterized according to their physical and biochemical properties, as well as their interaction and internalization by recipient macrophages. This study seeks to contribute for enhancing the reproducibility between the limited number of available protocols for the description of MDM-derived sEVs, thus providing an alternative methodology to research groups working with EVs released by these cells.

Our findings also suggest that MDM-derived sEVs are likely to function as a vehicle to transport protective messages from uninfected macrophages (exposed or not with VIP) to the naïve recipient infected cells and protect them from HIV-1 replication. Considering the obtained data, we highlight the potential of macrophage-sEVs in modulate viral replication in macrophages and PBMCs. These data advance our understanding of the role of macrophages-EVs in HIV-1/AIDS diseases and pave the way for new potential EV-based therapeutics strategies.

The functional relevance of EVs in HIV-1 infection remains incompletely characterized. EVs are known to play a role in immune response against several viral pathogens. These particles may incorporate and spread both viral and host factors, to inhibit or promote immune responses towards them via a multiplicity of mechanisms. Future studies are necessary to clarify various questions that remain opened and comprehends the involvement of EVs in immune responses and their potential use as agents to modulate viral infection.

Future goals:

- To investigate whether macrophage-derived sEVs induce in recipients cells the release of mediators with anti-HIV-1 activity, such as IL-10 and β-chemokines.

- To examine whether macrophage-derived sEVs alter the transcription of the integrated provirus into the target cell.

- To evaluate the infectivity of HIV-1 particles (viral fitness) released by infected cells exposed with EVs.

- To analyze whether the viral genome integrated in infected cells treated with EVs show G → A mutations, typical of the antiviral activity of the APOBEC family.

- To determine the protein, mRNA and miRNA contents of macrophage-derived sEVs.

At this point, given the SARS-CoV-2 pandemic and the possible role that EVs might play in the physiopathogenesis of COVID-19, we aim also to perform the following studies:

- To evaluate whether macrophage-derived sEVs present anti-SARS-CoV-2 proprieties and protect infected monocytes and human lung cells from virus-mediated cytopathic.

- To analyze whether macrophage-derived sEVs regulated the pro-inflammatory response and reduced and/or prevented lethal cytokine storms in SARS-CoV-2-infected cells.

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9 APPENDICES

Due to the current COVID-19 pandemic situation, the progress of this Ph.D. study was severely affected by the health safety measures determined by Fiocruz and local health authorities. For that reason, some aims proposed in the study are incomplete. In view of this circumstance, we decided to include as appendices other functional assays related with the role of MDM-derived sEVs in SARS-CoV-2 infection.

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a zoonotic virus and the etiological agent of coronavirus disease 2019 (COVID-19), a contagious disease characterized by induce respiratory illnesses. Evidences have demonstrated that SARS-CoV-2 principally targets airway epithelial cells, alveolar epithelial cells, vascular endothelial cells, monocytes and/or macrophages in the lung (132). In later stages of COVID-19 infection, respiratory epithelial cells, DCs and macrophages release high levels of pro-inflammatory cytokines (e.g., IL-1β, IL-6, TNF-α) and chemokines (e.g., CCL-2, CCL-3, CCL-5). The overexpression of these inflammatory factors results in cytokine storm which may induce in some patients the acute respiratory distress syndrome (ARDS) followed by multiple organ dysfunction, septic shock, blood clots and consequently patient death (133).

Previously studies conducted by our group showed that supernatants from monocyte infected with SARS-CoV-2 exposed to neuropeptides VIP or PACAP transferred antiviral resistance to lung cells (134). We hypothesized that the propagation of the protective effect could have been mediated by EVs released into the culture medium by cells stimulated with these neuropeptides. In addition, recent evidences have indicated the immunomodulatory potential of EVs from mesenchymal stem cell to modulate SARS-CoV-2 infection (135,136). Based on the aforementioned evidences, and our preliminary findings showing the anti-HIV-1 activity mediated by macrophage-sEVs to control viral infection in primary cells. We analyzed whether MDM-derived sEVs present antiviral activity and transfer resistance to monocytes and human lung cells infected with SARS-CoV-2.

Human primary monocytes and tumor cell lines.

African green monkey kidney (Vero, subtype E6) and Calu-3 (human lung epithelial cells) cells were cultured in high glucose DMEM with 10% fetal bovine serum (FBS; HyClone, Logan, Utah), 100 U/mL penicillin and 100 µg/mL streptomycin (Pen/Strep; ThermoFisher) at 37 °C in a humidified atmosphere with 5% CO^2. Human monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of healthy human blood donors using density gradient centrifugation (Ficoll-Paque, GE Healthcare), as previously described (137). PBMCs (2 x 10^6 cells) were plated onto 48-well plates (NalgeNunc) in RPMI-1640 with 5% inactivated male human AB serum (Merck) for 2-4 hours. Non-adherent cells were removed by washing and the remaining monocytes were maintained in DMEM (low-glucose, LGC Bio) with 5% human serum (HS; Millipore) and penicillin/streptomycin. The purity of human monocytes was above 90%, as determined by flow cytometric analysis (FACScan; Becton Dickinson) using anti-CD3 (BD Biosciences) and anti-CD14 (BD Biosciences) monoclonal antibodies. The methods for isolation, culture and differentiation of human monocyte-derived macrophages are detailed in the aforementioned article (107).

SARS-CoV-2 isolate and virus expansion

SARS-CoV-2 was expanded in Vero E6 cells from an isolate contained on a nasopharyngeal swab obtained from a confirmed case in Rio de Janeiro, Brazil (GenBank accession no. MT710714). Viral isolation was performed after a single passage in a cell culture in a 150 cm2 flasks with high glucose DMEM plus 2% FBS. Observations for cytopathic effects were performed daily and peaked 4 to 5 days after infection. All procedures related to virus culture were handled in a biosafety level 3 (BSL3) multiuser facility according to WHO guidelines. Virus titers were determined as plaque forming units (PFU/mL). Virus stocks were kept in -80°C ultralow freezers.

Infections, EV treatments and virus titration.

Infections were performed with SARS-CoV-2 at MOI of 0.01 (monocytes) or 0.1 (Calu-3) in low (monocytes) or high (Calu-3) glucose DMEM without serum. Cells were co-cultured overnight with pools of macrophage-derived sEVs, then washed and incubated with complete medium with viral suspensions for 1 h at 37 °C and 5% CO^2. When indicated, macrophage cell-conditioned medium centrifuged at 400×g for 10min (referred as conditioned medium, CM) or

supernatants free of EVs (referred as EV-free medium) collected from macrophage cultures were added to SARS-CoV-2-infected cells. EV-free medium was obtained by ultracentrifugation at 100,000×g for 20 h and then filtered through 0.22 μm filters (Merck Millipore). After 1 h of infection, cells were washed and incubated with complete medium during 24 h (monocytes) or 60 h (Calu-3 cells). For virus titration, monolayers of Vero E6 cells (2 x 10^4 cell/well) in 96-well plates were infected with serial dilutions of supernatants containing SARS-CoV-2 for 1 hour at 37°C. Cells were washed and resuspended in 2.4% carboxymethyl cellulose (CMC)/DMEM (high glucose) semisolid medium with 2% FBS. At 3 days post-infection, cells were fixed with 10% formaldehyde in PBS for 3h. The cell monolayers were stained with 0.04% solution of crystal violet in 20% ethanol for 1h. Plaque numbers were scored in at least 3 replicates per dilution by independent readers blinded to the experimental group and the virus titers were determined by plaque-forming units (PFU) per milliliter.

Molecular detection of SARS-CoV-2 RNA levels.

The total RNA was extracted from supernatants of monocyte cultures 24 h after infection, using QIAamp Viral RNA (Qiagen®), according to manufacturer's instructions. Quantitative RT-PCR was performed using QuantiTect Probe RT-PCR Kit (Quiagen®) in an StepOne™ Real-Time PCR System (Thermo Fisher Scientific). Amplifications were carried out in 25 μL reaction mixtures containing 2× reaction mix buffer, 50 μM of each primer, 10 μM of probe, and 5 μL of RNA template. Primers, probes, and cycling conditions recommended by the Centers for Disease Control and Prevention (CDC) protocol were used to detect the SARS-CoV- 2 (138). The standard curve method was employed for virus quantification. The housekeeping gene RNAse P was amplified as reference to the cell amounts used. The Ct values for this target were compared to those obtained to different cell amounts, 10^7 to 10^2, for calibration.

Measurements of inflammatory mediators and cell death.

The levels of TNF-α, IL-6, and IL-8 were quantified in the supernatants from uninfected and SARS-CoV-2-infected monocytes (24h) and Calu-3 cells (72h) by ELISA (R&D Systems), following manufacturer's instructions, and results are expressed as percentages relative to uninfected cells. Cell death was determined according to the activity of lactate dehydrogenase

(LDH) in the culture supernatants using a CytoTox® Kit (Promega, USA) according to the manufacturer's instructions. In brief, cell culture supernatants were centrifuged at 5,000 rpm for 1 minute, to remove cellular debris, then 25 µL were placed into 96-well plates and incubated with 5 µL of ferric alum and 100 µL of LDH substrate for 3 minutes at 37º C. Nicotinamide adenine dinucleotide (NAD, oxidized form) was added followed by the addition of a stabilizing solution. After 10 minutes, the reaction was read in a spectrophotometer at 492 nm.

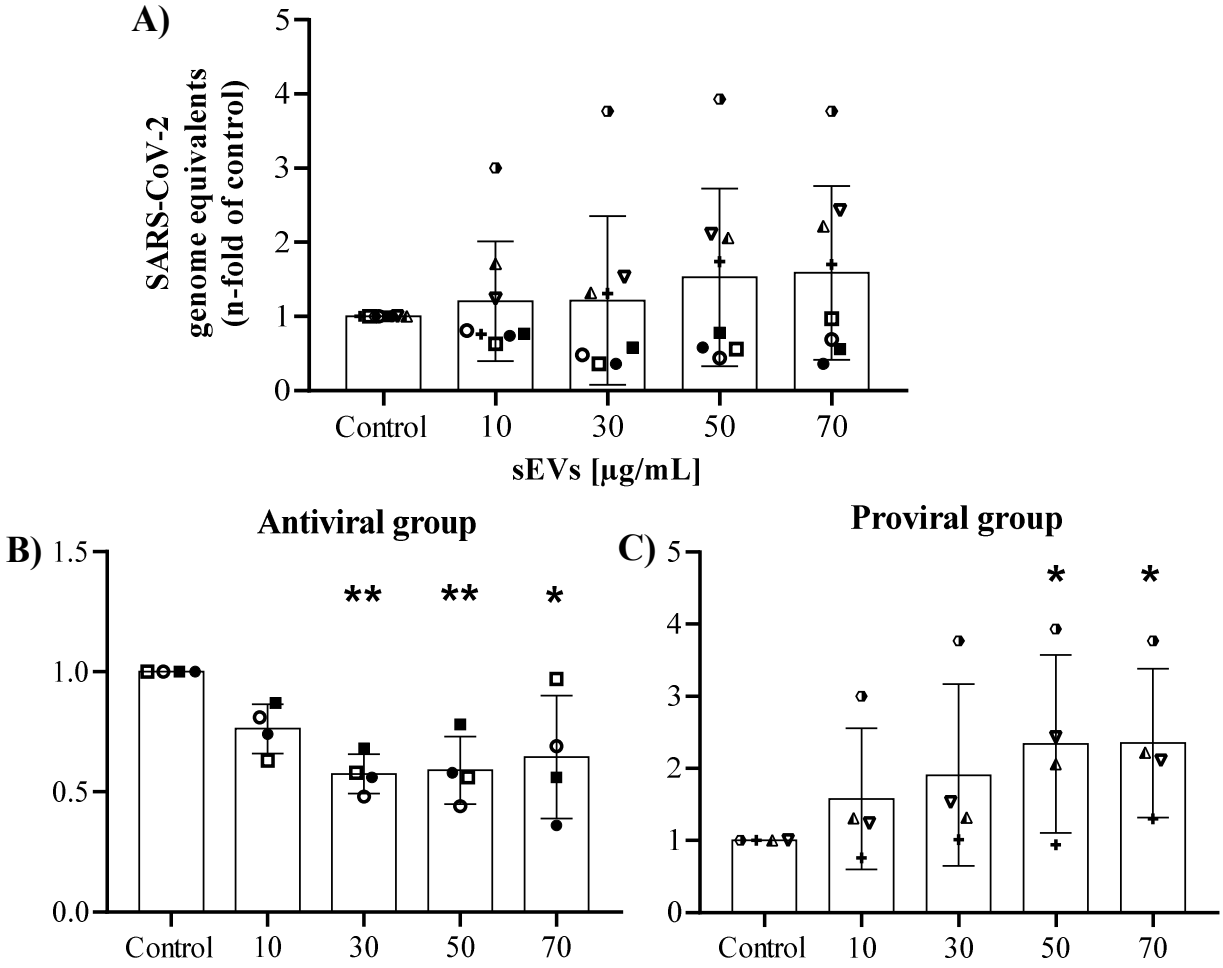
Statistical analysis

Statistics of functional assays were performed using GraphPad Prism software version 7. All the numerical variables were tested regarding their distribution using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) was used to compare differences among groups following a normal (parametric) distribution, and Dunnet's post-hoc test was used to locate the differences between the groups. Data are shown as the mean and SD, and the differences between values were considered statistically significant when the *P*-value was ≤ 0.05.

RESULTS

Dual role of MDM-derived sEVs in SARS-CoV-2-infected monocytes

Based on our preliminary results showing that MDM-derived small EVs presented antiviral effects on primary cells infected with HIV-1, we investigated whether macrophage-sEVs could inhibit viral life cycle in SARS-CoV-2-infected monocytes. To this end, human primary monocytes were exposed with pools of sEVs overnight, then infected with SARS-CoV-2, and the replication of viral genome was measured 24 h later. In our conditions, we did not observe a specific effect of MDM-derived sEVs on SARS-CoV-2 replication in 8 donors (Appendix 9.1). However, when we compared the effect of vesicles between groups of donors, some pools of sEVs decreased viral production in one group of donors (composed by 4 donors), presenting a reduction of 43%, 41% and 36% at concentration equivalent to 30, 50 and 70 µg/mL, respectively (Appendix 9.1). On the other hand, the higher concentrations of other pools of sEVs enhanced viral growth in other 4 donors by more than 81% and 87% at 50 and 70 µg/mL, respectively (Appendix 9.1). These results suggest that MDM-derived sEVs may exert dual role in inhibiting or enhancing SARS-CoV-2 replication in monocytes, and this outcome may vary within the pools of vesicles or donors used in the study. The next functional assays in monocytes were performed with the same donors, thus, based on this preliminary result we divided the donors into 2 groups: Antiviral group, which is represented by donors where the vesicles inhibited viral production, and Proviral group represented by donors where vesicles increased SARS-CoV-2 infection.

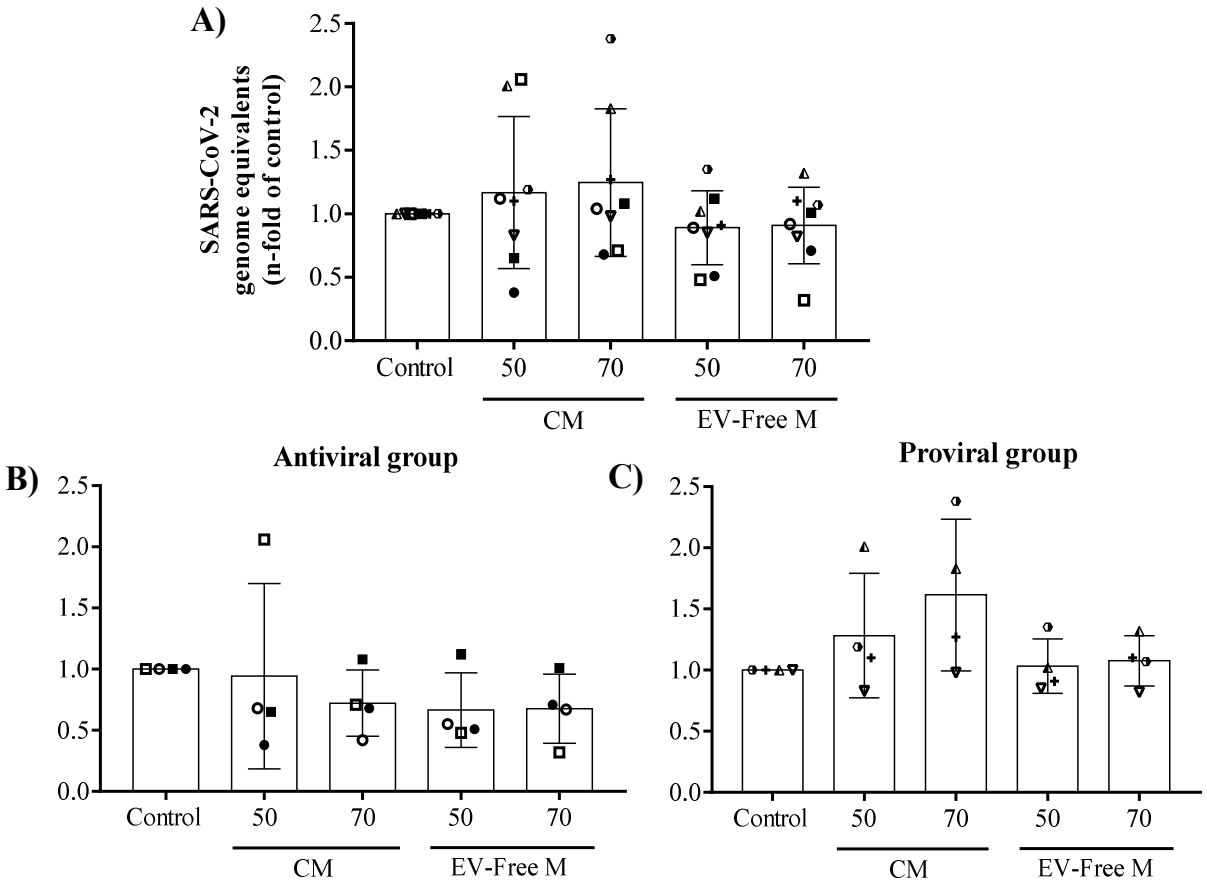


Appendix 9.1. Dual role of MDM-derived sEVs in SARS-CoV-2-infected monocytes. (A) Monocytes were treated with indicated concentrations of pools of macrophage-sEVs, and then infected with SARS-CoV-2 (*n*=8). Virus RNA synthesis was evaluated by qPCR in the culture supernatants 20-24 hours post-infection. Donors represented in A were divided in two group to shown **(B)** the antiviral effects or **(C)** proviral effect mediated by MDM-derived small EVs. Data are shown as means ± SD. Asterisks indicate adjusted P values: *P ≤ 0.05, **P ≤ 0.01. One-way ANOVA with Dunnett's multiple comparisons post-test.

CM and EV-free medium do not affect SARS-CoV-2 replication in monocytes

To determine whether the anti- or proviral effect observed was associated or not with EV-enriched preparations, monocytes were treated with macrophage culture conditioned medium (CM), or EV-depleted supernatants from the macrophage culture medium (EV-free medium), or macrophage-derived sEVs, and then infected with SARS-CoV-2. We found that both CM and EV-free medium failed to suppress or enhance viral replication in infected monocytes

(Appendix 9.2). We used the same analysis strategy and divided the donors in two groups to find any differences in the treatments, but not effects were observed (Appendix 9.2). These findings indicate that the antiviral or proviral activity against SARS-CoV-2-infected monocytes could be associated with the functional properties of sEVs from human macrophages, rather than soluble mediators present in the supernatant of macrophage culture.

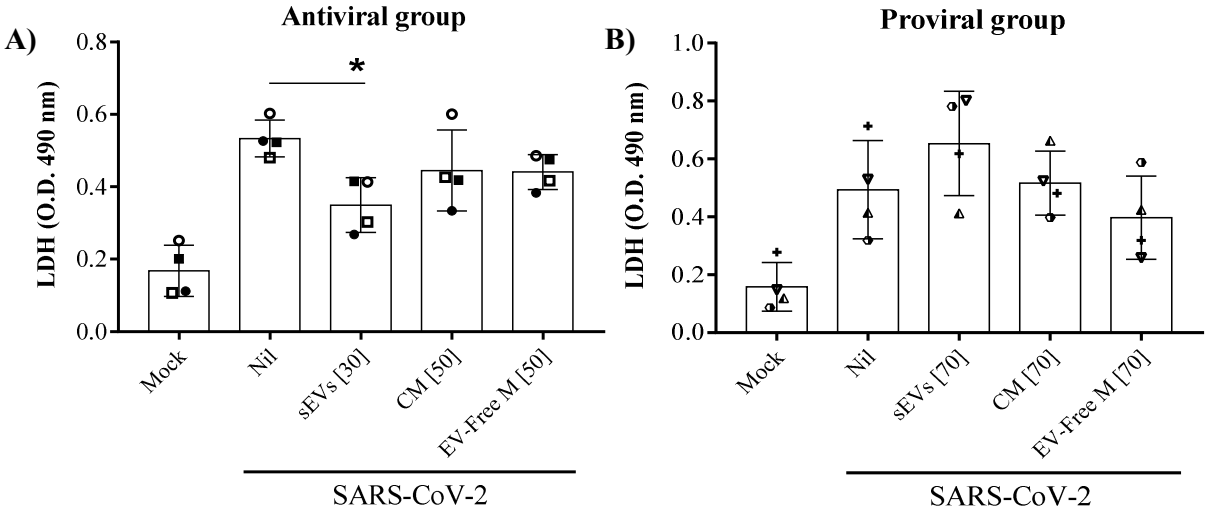


Appendix 9.2. CM and EV-free medium do not affect SARS-CoV-2 replication in monocytes. (A) Monocytes were incubated with indicated concentrations (µg/mL) of CM, or EV-Free M, and then infected with SARS-CoV-2 (*n*=8). Virus RNA synthesis was evaluated by qPCR in the culture supernatants 20-24 hours post-infection. Donors represented in A were divided in two group, **(B)** antiviral or **(C)** proviral group. Data are shown as means ± SD.

MDM-derived sEVs protect monocytes from SARS-CoV-2-mediated cytophaticity.

Severe COVID-19 has been associated with high levels of lactate dehydrogenase (LDH), which result in elevated cellular mortality (139). We asked whether MDM-derived sEVs could protect monocytes from SARS-CoV-2-mediated cytophaticity. Thus, monocytes were exposed with CM, or EV-free medium, or macrophage-derived sEVs, and then infected with SARS-

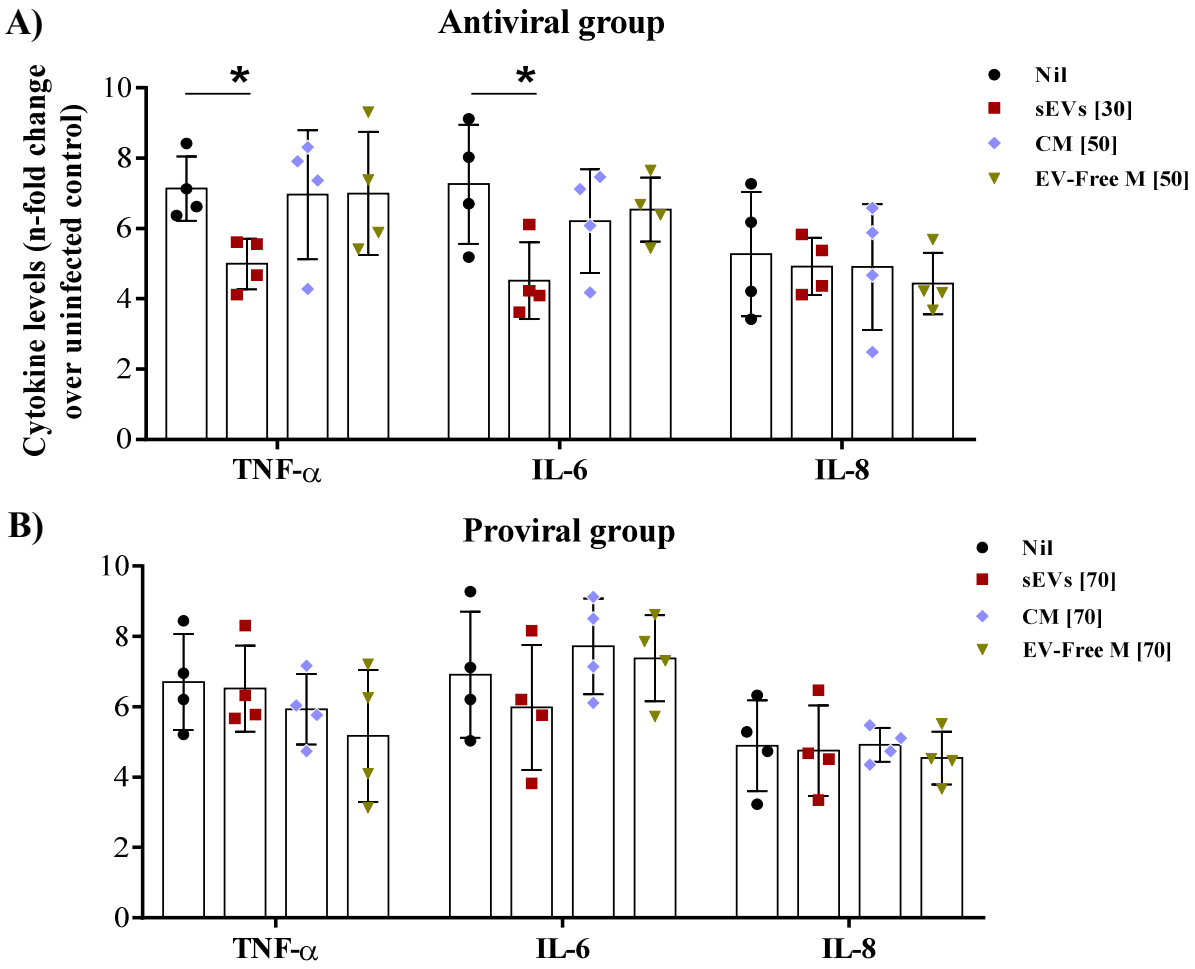
CoV-2. We used 30 and 70 µg/mL for cell viability assay, considering that vesicles were able to induce some biological function (anti- or proviral activity) with these concentrations. Interestingly, in the antiviral group, MDM-derived sEVs significantly reduced 35% of monocyte mortality at 30 µg/mL (Appendix 9.3). However, in the proviral group, sEVs induced an increase over 30% in cell mortality with the highest concentration (70 µg/mL), compared with infected cell without treatment (Appendix 9.3). In addition, lower or higher concentrations of CM or EV-Free M failed to reduce or enhance cellular mortally in both groups (Appendix 9.3). Our results, suggest that MDM-derived sEVs may decrease or increase the SARS-CoV-2-induced monocyte death, and this outcome may vary within the pools of vesicles or donors used in the study.



Appendix 9.3. MDM-derived sEVs protect monocytes from SARS-CoV-2-mediated cytophaticity. Monocytes were incubated with **(A)** low (30 µg/mL), or intermediary (50 µg/mL), or **(B)** high concentrations (70 µg/mL) of macrophage-derived sEVs, CM, or EV-Free M, respectively, and then infected with SARS-CoV-2 (*n*=4). Cellular viability was analyzed by measuring LDH release in the supernatants of uninfected or SARS-CoV-2-infected monocytes, treated or not with sEVs, CM, or EV-Free M. Data are shown as means ± SD. Asterisks indicate adjusted P values: *P ≤ 0.05 compared to untreated infected cells (Nil).

MDM-derived sEVs reduce production of pro-inflammatory mediators by SARS-CoV-2-infected monocytes.

Evidence have demonstrated that reduction in the production of pro-inflammatory mediators may affect SARS-CoV-2 replication (140,141). We evaluated whether macrophage-sEVs could attenuate the production of pro-inflammatory mediators by SARS-CoV-2-infected monocytes. We observed that SARS-CoV-2 infection triggered the increase levels of TNF-α, IL-6, and IL-8, relative to uninfected cells (7,7, and 5 times more, respectively). In the antiviral group, cells treated with sEVs prior SARS-CoV-2 infection, but not CM or EV-free M, significantly reduced the inflammation to 38% and 30% in the cellular production of TNF-α and IL-6, respectively (Appendix 9.4). Changes in IL-8 production was not observed. Moreover, treatments in the proviral group did not affect pro-inflammatory mediators stimulated by the viral infection (Appendix 9.4). These results suggest that MDM-derived sEVs may regulate the inflammatory environment caused by SARS-CoV-2 infection in monocytes.

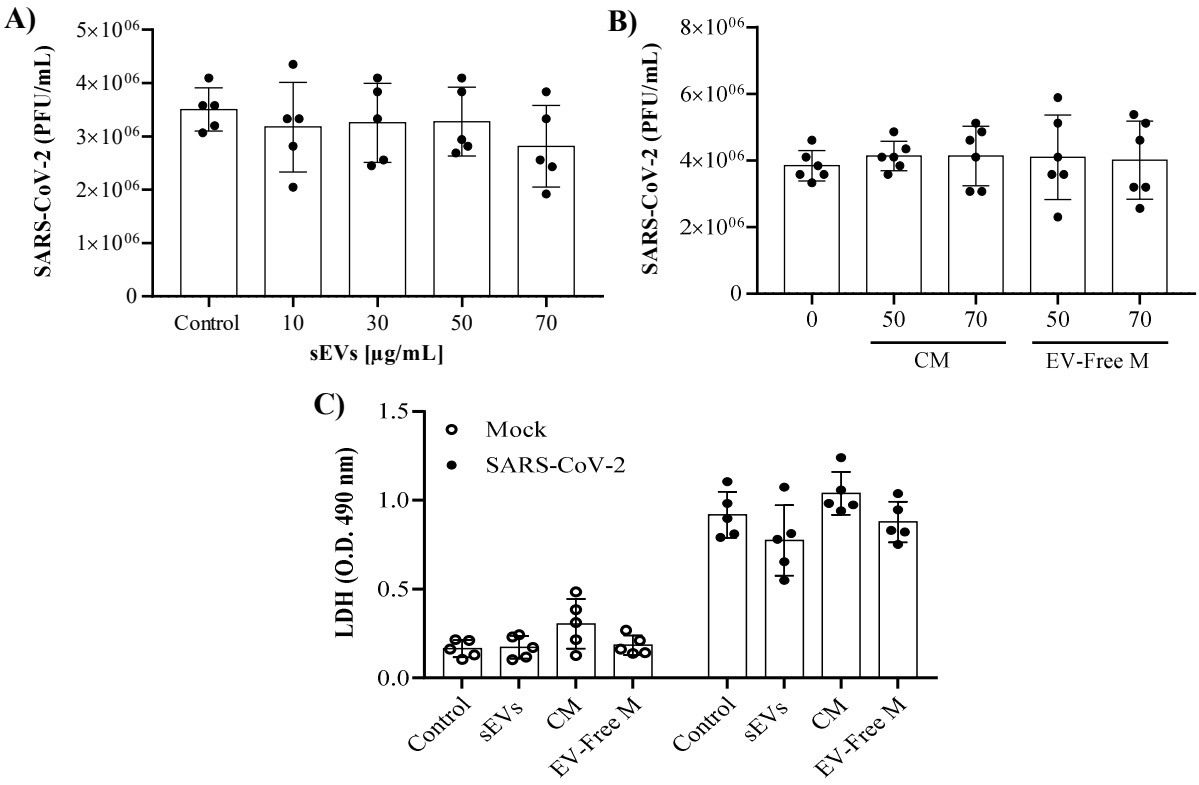


Appendix 9.4. MDM-derived sEVs reduce production of pro-inflammatory mediators by SARS-CoV-2-infected monocytes. Cells were incubated with **(A)** low (30 µg/mL), or intermediary (50 µg/mL), **(B)** or high concentrations (70 µg/mL) of macrophage-derived sEVs, CM, or EV-Free M, respectively, and then infected with SARS-CoV-2 (*n*=4). The levels of TNF-α, IL-6, and IL-8, were measured in culture supernatants of monocytes after 24 hours by ELISA. Data are shown as means ± SD. Asterisks indicate adjusted P values: *P ≤ 0.05 compared to untreated infected cells (Nil).

MDM-derived sEVs role on SARS-CoV-2-infected Calu-3 cells.

We next evaluated whether MDM-derived sEVs could be able to restrict or enhance the SARS-CoV-2 production in human lung epithelial cell line Calu-3. Thus, Calu-3 were exposed with CM, or EV-free medium, or pools of MDM-derived sEVs, and then infected with SARS-CoV-2. Although we did not find significant differences, cell treated with sEVs, but not CM or EV-Free M, were able to reduce 20% of viral replication, at the concentration of 70 µg/mL

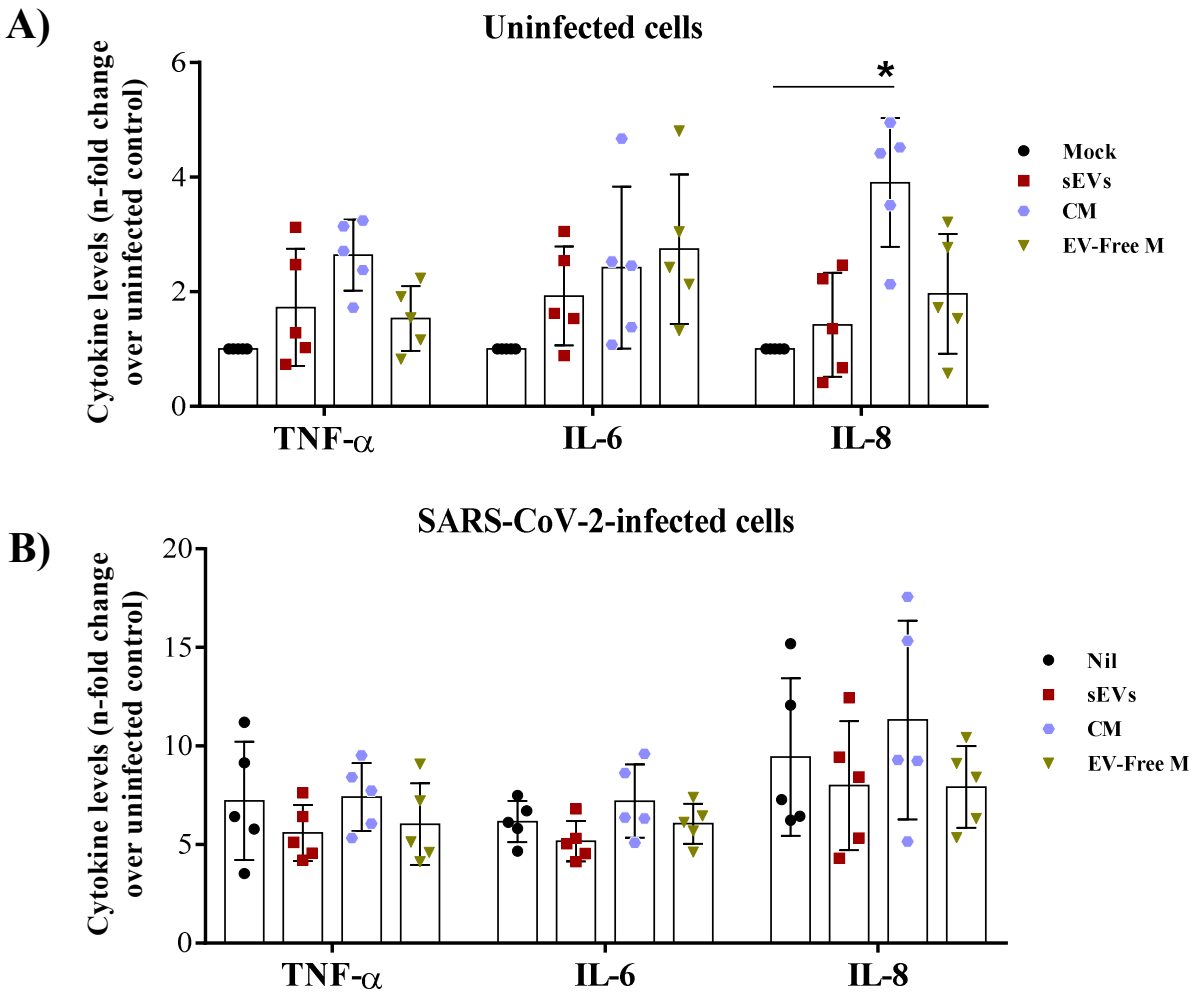
(Appendix 9.5). We also evaluated the cell viability and found that sEVs, but not CM or EV-Free M, reduced cell mortality about 15% (Appendix 9.5).



Appendix 9.5. MDM-derived sEVs do not affect SARS-CoV-2 replication in Calu-3 cells. Calu-3 were treated with indicated concentrations of **(A)** macrophage-sEVs, **(B)** or CM, or EV-Free M, and then infected with SARS-CoV-2 (*n*=5). After 60 hours, supernatants were collected and viral replication was evaluated by quantifying PFUs in plaque assay. **(C)** Cellular viability was analyzed by measuring LDH release in the supernatants of uninfected or SARS-CoV-2-infected Calu-3 cells, treated or not with sEVs (70 µg/mL), or CM (70 µg/mL), or EV-Free M (70 µg/mL). Data are shown as means ± SD.

In addition, we assessed whether MDM-derived sEVs could regulate cytokine storm caused by SARS-CoV-2 infection in Calu-3. We showed that CM, but not sEVs or EV-Free M, was able to sharply increase the levels of TNF-α (164%) and IL-8 (290%) in uninfected cells (Appendix 9.6). Moreover, we found that SARS-CoV-2 infection increased the levels of TNF-α, IL-6, and IL-8, relative to uninfected cells (7, 6, and 9 times more, respectively). However, cells exposed with 70 µg/mL of sEVs prior to infection, reduced inflammation to 23%, 16%, and 15% in the cellular production of TNF-α, IL-6, and IL-8 respectively (Appendix 9.6). In contrast, pre-treatment with CM resulted in slight induction of 17% and 20% of IL-6 and IL-8, respectively. Altogether, our results indicate that MDM-derived sEVs, but not soluble

mediators present in the supernatant of macrophage culture, have a tendency to inhibit viral replication, prevent cellular mortality, and control the inflammatory environment caused by SASR-CoV-2 infection in human lung cells.



Appendix 9.6. MDM-derived sEVs tend to reduce production of pro-inflammatory mediators by SARS-CoV-2-infected Calu-3. (A) Uninfected cells were incubated with the 70 µg/mL of sEVs, or CM, or EV-Free M. **(B)** Cells were incubated with 70 µg/mL of sEVs, or CM, or EV-Free M, respectively, and then infected with SARS-CoV-2 (*n*=5). The levels of TNF-α, IL-6, and IL-8, were measured in culture supernatants of Calu-3 after 60 hours by ELISA. Data are shown as means ± SD. Asterisks indicate adjusted P values: *P ≤ 0.05 compared to untreated not infected cells.

We show for the first time the ability of vesicles from human macrophages to modulate SARS-CoV-2 infection in a primary target cell. We found that MDM-derived sEVs transferred resistance (antiviral activity) to SARS-CoV-2-infected monocytes and protected them for cell death, and regulated the inflammatory environment caused by virus infection. In contrast, sEVs could also induce the opposite effect (proviral activity) in infected cell, thus, suggesting a possibly dual role of macrophage-sEVs in SARS-CoV-2 pathogenesis. In addition, our sEV-enriched preparations showed a slight antiviral activity to protect human lung cells from viral replication. We also demonstrated that the anti- or proviral activity against SARS-CoV-2-infected cells was associated with the functional properties of macrophage-sEVs, rather than soluble mediators present in the supernatant of macrophage culture.

Altogether, our findings suggest potential role of MDM-derived sEVs in SARS-CoV-2 pathogenesis, and also contribute to rise the awareness for studying the mechanism by which cellular communication mediated by macrophage-sEVs regulate the innate immune system during SARS-CoV-2 infection.

Postdoctoral perspectives:

- To evaluate whether sEVs from macrophages stimulated with the neuropeptide VIP inhibit SARS-CoV-2 replication in primary monocytes and Calu-3.

- To determine the miRNA contents of macrophage-derived sEVs.

- To evaluate the routes and mechanisms of MDM-derived sEVs uptake involved in the transference of antiviral and anti-inflammatory response in SARS-CoV-2 infected cells.

- To evaluate whether EVs inhibit the inflammasome pathway and protected SARS-CoV-2 infected monocytes from pyroptosis.

- To assess whether EVs activate endogenous viral restriction factors (e.g., SAMHD1, APOBEC, Viperine) with potential anti-SARS-CoV-2 activity.

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