

## MEMBRANE PROTEIN SCAFFOLDS FOR EXOSOME ENGINEERING

### 1. CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This PCT application claims the priority benefit of U.S. Provisional Application Nos. 62/801,065, filed February 4, 2019; 62/801,636, filed February 5, 2019; and 62/851,581, filed May 22, 2019, each of which is herein incorporated by reference in its entirety.

### 2. REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY VIA EFS-WEB

[0002] The content of the electronically submitted sequence listing in ASCII text file (Name: 4000\_031PC03\_SeqListing\_ST25.txt; Size: 205,964; and Date of Creation: February 4, 2020) filed with the application is herein incorporated by reference in its entirety.

### 3. FIELD OF THE DISCLOSURE

[0003] The present disclosure relates to engineered extracellular vesicles (EVs) (*e.g.*, exosomes) that comprise one or more exogenous proteins that are not naturally expressed in cells from which the EVs are produced (*i.e.*, heterologous exosomal vesicle proteins), and methods of producing and using such EVs.

### 4. BACKGROUND OF THE DISCLOSURE

[0004] Extracellular vesicles (EVs) (*e.g.*, exosomes) are important mediators of intercellular communication. They are also important biomarkers in the diagnosis and prognosis of many diseases, such as cancer. As drug delivery vehicles, EVs (*e.g.*, exosomes) offer many advantages over traditional drug delivery methods as a new treatment modality in many therapeutic areas.

[0005] The use of EVs (*e.g.*, exosomes) for therapeutic purposes requires that EVs (*e.g.*, exosomes) be free or mostly free of impurities including but not limited to contaminant proteins, DNA, carbohydrates, and lipids. Current purification methods do not offer sufficient selectivity to remove significant amounts of these impurities so additional processes are desired to improve purity.

[0006] Furthermore, as EVs (*e.g.*, exosomes) become more frequently used in the treatment of human disease, they may struggle to meet clinical expectations because of heterogeneity in their physicochemical parameters that confer molecular targeting, immune evasion, and controlled drug release. This is mainly due to the heterogeneity and complexity of EV (*e.g.*, exosome) properties (*e.g.*, composition, size, shape, rigidity, surface charge, hydrophilicity, stability, and ligand type and density), payload properties (*e.g.*, drug type,

solubility, loading, potency, dosing, immune response, and release kinetics), and *in vivo* physiological barriers to EV (*e.g.*, exosome) trafficking (*e.g.*, immune surveillance, particle extravasation, tissue targeting, tissue penetration, and cellular uptake). Although a considerable amount of effort has been made, effective methods for obtaining discrete sub-populations of therapeutic EVs (*e.g.*, exosomes) with desired properties, *e.g.*, EVs (*e.g.*, exosomes) containing therapeutic payloads and having appropriate targeting moieties, are not yet readily available.

[0007] Suitable methods for generating, isolating and purifying discrete sub-populations of EVs (*e.g.*, exosomes) are needed to better enable therapeutic use and other applications of EV (*e.g.*, exosome)-based technologies.

## 5. BRIEF SUMMARY OF THE DISCLOSURE

[0008] An aspect of the present disclosure relates to novel EV (*e.g.*, exosome) compositions, methods for preparing these compositions, and therapeutic methods of using the compositions. Specifically, the compositions and methods relate to extracellular vesicles comprising a heterologous extracellular vesicle protein (HEVP) or a fragment thereof, wherein the extracellular vesicle is produced from a producer cell which does not naturally express the HEVP, and wherein the HEVP is produced naturally by a donor cell. In addition, the present disclosure relates to the use of HEVPs that are enriched on the surface of the donor EVs (*e.g.*, exosomes). Examples of HEVPs described herein include CD13, MME, ENPP1, and NRP1, particularly as recombinantly expressed on the surface of EVs (*e.g.*, exosomes) produced by non-mesenchymal cells (*e.g.*, by CHO cells in the case of CD13, MME, ENPP1 and/or NRP1; and/or by HEK cells in the case of CD13, MME, and NRP1). These HEVP proteins can be used to engineer EVs (*e.g.*, exosomes) as described in U.S. Patent No. 10,195,290 (incorporated by reference herein in its entirety).

[0009] In one embodiment, then, the disclosure provides an extracellular vesicle comprising at least one heterologous extracellular vesicle protein (HEVP) or a fragment thereof, wherein the extracellular vesicle is produced from a producer cell which does not naturally express the HEVP, and wherein the HEVP is produced naturally by a donor cell. In a certain embodiment, the extracellular vesicle comprises 2, 3, 4, 5, 6, 7, 8, 9 or 10 heterologous extracellular vesicle proteins (HEVPs) or fragments thereof, wherein the extracellular vesicle is produced from a producer cell which does not naturally express the HEVPs, and wherein the HEVPs are produced naturally by a donor cell. In general, unless otherwise specified, the references to "**at least one HEVP**" and "**2, 3, 4, 5 ... HEVPs**" indicate the presence (if greater

than one) of distinct types or varieties of HEVPs (*e.g.*, HEVPs with different amino acid sequences), and not to the amount of HEVP molecules (of any type) on the EV (*e.g.*, exosome), which will be much larger.

**[0010]** In certain embodiments, the level of the at least one HEVP in the EVs (*e.g.*, exosomes) produced by the donor cell is equal to or greater than about 20, about 80, about 125, or about 200 peptide spectral matches (PSM) measured using liquid chromatography with tandem mass spectrometry (LC-MS/MS). In some aspects, the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of HEVP in the exosomes produced by the donor cell is equal to or greater than about 20 peptide spectral matches (PSM) measured using liquid chromatography with tandem mass spectrometry (LC-MS/MS). In some aspects, the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of HEVP in the exosomes produced by the donor cell is equal to or greater than about 80 peptide spectral matches (PSM) measured using LC-MS/MS. In some aspects, the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of HEVP in the exosomes produced by the donor cell is equal to or greater than about 125 peptide spectral matches (PSM) measuring using LC-MS/MS. In some aspects, the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of HEVP in the exosomes produced by the donor cell is equal to or greater than about 170 peptide spectral matches (PSM) measuring using LC-MS/MS. In some aspects, the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of HEVP in the exosomes produced by the donor cell is equal to or greater than about 200 peptide spectral matches (PSM) measuring using LC-MS/MS. In some aspects, the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of HEVP in the exosomes produced by the donor cell is equal to or greater than about 700 peptide spectral matches (PSM) measuring using LC-MS/MS. In some aspects, the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of HEVP in the exosomes produced by the donor cell is about 177 peptide spectral matches (PSM) measuring using LC-MS/MS. In some aspects, the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of HEVP in the exosomes produced by the donor cell is about 742 peptide spectral matches (PSM) measuring using LC-MS/MS.

**[0011]** In certain embodiments, the level of the at least one HEVP in the EVs (*e.g.*, exosomes) produced by the donor cell is between about 20 and about 80 PSM, or between about

80 and about 200 PSM measured using LC-MS/MS. In some aspects, the level of the at least one HEVP in the exosomes produced by the donor cell is between 20 and 80 peptide spectral matches (PSM) measured using LC-MS/MS. In some aspects, the level of the at least one HEVP in the exosomes produced by the donor cell is between 80 and 200 peptide spectral matches (PSM) measured using LC-MS/MS. In some aspects, the level of the at least one HEVP in the exosomes produced by the donor cell is between about 150 and about 750 peptide spectral matches (PSM) measured using LC-MS/MS. In certain embodiments, the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of the at least one HEVP in the exosomes produced by the donor cell is equal to or greater than about 5% of the total protein content of the exosomes produced by the donor cell.

**[0012]** Additional embodiments include extracellular vesicles such as those described in the Summary above, wherein the at least one HEVP or fragment thereof is a fusion protein. As used herein, the term "**fusion protein**" refers to two or more proteins that are attached to each other. As described herein, in some aspects, a fusion protein comprises a HEVP and an exogenous biologically active molecule (*e.g.*, antigen, targeting moiety, adjuvant, and/or immune modulator). In some aspects, the two or more proteins (*e.g.*, HEVP and an exogenous biologically active molecule) can be fused to one another. In some aspects, the two or more proteins (*e.g.*, HEVP and an exogenous biologically active molecule) can be attached to each other via a linker. In related embodiments, the at least one HEVP or fragment thereof is modified by the addition of a functional moiety. In related embodiments, the functional moiety has affinity to a binding agent. In related embodiments, the functional moiety is an affinity tag. In certain embodiments, the affinity tag is a peptide. In other related embodiments, the functional moiety is a therapeutic compound. In still other related embodiments, the therapeutic compound is selected from the group consisting of a natural peptide, a recombinant peptide, a synthetic peptide, and any combination thereof. In still other related embodiments, the therapeutic compound is selected from the group consisting of nucleotides, amino acids, lipids, carbohydrates, small molecules, and any combination thereof. In still other related embodiments, the therapeutic compound is an antibody or a fragment thereof. In additional related embodiments, the therapeutic compound is an enzyme, a ligand, a receptor, or a fragment thereof. In other related embodiments, the functional moiety is a targeting moiety which, in some embodiments, can be specific to (*e.g.*,) an organ, a tissue, or a cell. In related embodiments, wherein the at least one HEVP is a fusion protein, the fusion protein comprises a

linker. In related embodiments, the EVs (*e.g.*, exosomes) described above further comprise a linker between the at least one HEVP or fragment thereof and a functional moiety attached thereto. In related embodiments, the linker is a flexible linker or a rigid linker. In other related embodiments, the linker is a straight-chain carbon linker, a branched-chain carbon linker, a heterocyclic carbon linker, or a peptide linker. In other related embodiments, the linker is a cleavable linker.

**[0013]** The present disclosure also includes the extracellular vesicles described in the Summary above, wherein the producer cell is selected from the group consisting of HEK, CHO, MB-231, Raji, PER.C6, CAP, MSC cells, and any combination thereof. In related embodiments, the at least one HEVP is selected from the group consisting of a lung fibroblast EVP, an aortic endothelium cell EVP, an acute myeloid leukemia cell EVP, a monocyte EVP, a B-cell lymphoma EVP, a macrophage EVP, a brain endothelium cell EVP, a mesenchymal cell EVP, and any combination thereof. In related embodiments, the at least one HEVP is a human EVP, mouse EVP, rat VEP, dog EVP, or monkey EVP. In still other related embodiments, the at least one HEVP is selected from the group consisting of CD13, MME, ENPP1, and NRP1, or a fragment thereof. In still other related embodiments, the at least one HEVP is selected from the group consisting of CD13, MME, ENPP1, and NRP1, or a fragment thereof, and the producer cell is a CHO cell. In another related embodiment, the at least one HEVP is selected from the group consisting of CD13, MME, and NRP1, or a fragment thereof and the producer cell is a CHO cell or an HEK cell.

**[0014]** In another related embodiment, the at least one HEVP in an extracellular vesicle described above is selected from the group consisting of PTGFRN, BSG, IGSF3, ITGB1, ITGA4, SLC3A2, ATP transporter, or a fragment thereof, and any combination thereof. In some aspects, the producer cell is a cell which does not naturally produce these proteins. In a related embodiment, an extracellular vesicle as described in the Summary above further comprises an EVP selected from the group consisting of PTGFRN, BSG, IGSF3, ITGB1, ITGA4, SLC3A2, ATP transporter, or a fragment thereof, and any combination thereof.

**[0015]** In related embodiments, the disclosure provides an extracellular vesicle (*e.g.*, exosome) disclosed herein, wherein the producer cell is a non-human cell and wherein the at least one HEVP is a human HEVP. Alternatively, the producer cell can be a human cell and the at least one HEVP is a non-human HEVP.

**[0016]** Additional related embodiments include pharmaceutical formulations comprising any of the extracellular vesicle described above. Another aspect of the present disclosure includes a kit comprising an extracellular vesicle or pharmaceutical formulation described in the Summary above, wherein the extracellular vesicle or pharmaceutical formulation is contained in a container or package, and wherein the kit further comprises instructions describing a recommended use of the extracellular vesicle or pharmaceutical formulation.

**[0017]** The present disclosure also includes methods for treating a patient in need thereof, comprising administering to the patient an effective amount of the any of the extracellular vesicle or pharmaceutical formation described above.

**[0018]** The present disclosure further provides a method of expressing a non-naturally occurring protein in an EV derived from a cell, comprising transfecting a nucleic acid encoding at least one heterologous extracellular vesicle protein (HEVP) or a fragment thereof in the cell and isolating an EV comprising the HEVP or a fragment thereof from the cell, wherein the HEVP is not naturally occurring in the EV derived from the cell. In certain aspects, the EV comprises any of the EVs disclosed in the present disclosure. In some aspects, method of expressing a non-naturally occurring protein in an EV can further comprise characterizing the HEVP of the EV.

**[0019]** The HEVPs described herein can be used in various embodiments of the present disclosure. One aspect of the present disclosure relates to generating a fusion protein by conjugating the HEVP with a functional compound (*e.g.*, exogenous biologically active molecules disclosed herein), and producing an engineered EV (*e.g.*, exosome) containing the modified protein on the surface. For example, a native full-length or a biologically active fragment of the therapeutic protein can be transported to the surface of EVs (*e.g.*, exosomes) by being conjugated to the HEVP-enriched proteins or fragments thereof. The methods using the HEVPs described herein are believed to be, in some cases, improved in certain aspects relative to other related systems (*e.g.*, Lamp2B, PDGFR, lactadherin CD9, CD63 and/or CD81, or fragments thereof).

**[0020]** Another aspect of the present disclosure relates to purification of an EV (*e.g.*, exosome) by affinity purification from a heterogeneous solution such as cell culture media or plasma using the HEVPs. Some embodiments relate to isolation of a sub-population of EVs (*e.g.*, exosomes) from the total EVs (*e.g.*, exosomes) by using surface markers specific to a sub-population of EVs (*e.g.*, exosomes).

**[0021]** Another aspect of the present disclosure relates to methods of removing EVs (*e.g.*, exosomes) from a sample when EVs (*e.g.*, exosomes) are a contaminating product. For example, natural or engineered viruses can be purified from contaminating EVs (*e.g.*, exosomes). The HEVPs described herein thus can be used to selectively remove EVs (*e.g.*, exosomes) from biological processes where other particles of similar size, shape, and/or charge are the desirable product.

**[0022]** Another aspect of the present disclosure relates to generation or use of a surface-engineered EV (*e.g.*, exosome) designed for more efficient affinity purification, or for presentation of a targeting moiety or a therapeutically relevant protein (*e.g.*, exogenous biologically active molecules disclosed herein) on the surface. For example, the EV (*e.g.*, exosome) surfaces can be modified to contain the full-length HEVPs and/or a fragment or a modified protein of the HEVPs on the surface at a higher density.

**[0023]** The present disclosure further relates to a producer cell or a method of generating the producer cell for producing such a surface-engineered EV (*e.g.*, exosome). An exogenous polynucleotide (*e.g.*, encoding a HEVP) can be introduced transiently or stably into a producer cell to make the producer cell to generate a surface-engineered EV (*e.g.*, exosome).

**[0024]** Specifically, an aspect of the present disclosure relates to a method of isolating an EV (*e.g.*, exosome), comprising the steps of: (1) providing a sample comprising the EV (*e.g.*, exosome); (2) contacting the sample with a binding agent having affinity to a target protein, wherein the target protein comprises a HEVP (*e.g.*, CD13, MME, ENPP1, or NRP1, or a fragment or a variant thereof); and (3) isolating the EV (*e.g.*, exosome) based on a binding between the target protein and the binding agent.

**[0025]** In some embodiments, the sample is obtained from a cell grown *in vitro*, optionally wherein the cell is an HEK293 cell, a Chinese hamster ovary (CHO) cell, or a mesenchymal stem cell (MSC). In some embodiments, the sample is obtained from a body fluid of a subject.

**[0026]** In some embodiments, the cell is genetically modified to express the target protein. In some embodiments, the cell comprises an expression plasmid encoding the target protein. In some embodiments, the cell is genetically modified to comprise an exogenous sequence expressing a tag having affinity to the binding agent, wherein the exogenous sequence is inserted into a genome of the cell. In some embodiments, the exogenous sequence is inserted in a genomic site located at 3' or 5' end of an endogenous sequence encoding a HEVP (*e.g.*,

CD13, MME, ENPP1, or NRP1). In some embodiments, the endogenous sequence does not encode IGSF8. In some embodiments, the exogenous sequence is inserted in a genomic site located within an endogenous sequence encoding a HEVP (*e.g.*, CD13, MME, ENPP1, or NRP1).

**[0027]** In some embodiments, the target protein is a fusion protein comprising the tag, and CD13, MME, ENPP1, or NRP1, or a fragment or a variant thereof. In some embodiments, the EV (*e.g.*, exosome) comprises the target protein. In some embodiments, the target protein is not IGSF8 or a fragment or modification thereof. In some embodiments, the cell is genetically modified to have a reduced expression of ADAM10.

**[0028]** In some embodiments, the EV (*e.g.*, exosome) comprises the target protein. In some embodiments, the target protein is selected from CD13, MME, ENPP1, and NRP1. In some embodiments, the target protein comprises a fragment or a variant of CD13, MME, ENPP1, or NRP1. In some embodiments, the target protein comprises a polypeptide of SEQ ID NO: 33. In some embodiments, the target protein is a fusion protein comprising CD13, MME, ENPP1, or NRP1 or a fragment or a variant thereof, and an affinity tag, wherein the affinity tag has affinity to the binding agent. In some embodiments, the target protein does not comprise IGSF8 or a fragment or modification thereof.

**[0029]** In some embodiments, the binding agent comprises an immunoglobulin, a protein, a peptide, or a small molecule. In some embodiments, the binding agent is attached to a solid support, optionally wherein the solid support comprises a porous agarose bead, a microtiter plate, a magnetic bead, or a membrane.

**[0030]** In some embodiments, the solid support forms a chromatography column. In some embodiments, the step of contacting the sample with the binding agent is performed by applying the sample to the chromatography column.

**[0031]** In some embodiments, the method further comprises the steps of: (1) contacting a subset of the sample with a different binding agent having affinity to a different target protein; and (2) isolating the EV (*e.g.*, exosome) based on a binding between the different target protein and the different binding agent. In some embodiments, the different target protein comprises CD13, MME, ENPP1, or NRP1 or a fragment or a variant thereof. In some aspects, the target protein comprises CD13 (or a fragment or variant thereof). In some aspects, the target protein comprises MME (or a fragment or variant thereof). In some aspects, the target protein comprises ENPP1 (or a fragment or variant thereof). In some aspects, the target protein comprises NRP1 (or

a fragment or variant thereof). In some embodiments, the different target protein comprises a polypeptide of SEQ ID NO: 33.

**[0032]** Another aspect of the present disclosure relates to an EV (*e.g.*, exosome) produced by the methods provided herein.

**[0033]** In yet another aspect, the present disclosure relates to a pharmaceutical composition comprising the EV (*e.g.*, exosome) of the present disclosure and an excipient. In some embodiments, the pharmaceutical composition comprises a lower concentration of macromolecules than the sample comprising the EV (*e.g.*, exosome) source, wherein the macromolecules are nucleic acids, contaminant proteins, lipids, carbohydrates, metabolites, or a combination thereof. In some embodiments, the pharmaceutical composition is substantially free of the macromolecules.

**[0034]** Another aspect of the present disclosure relates to an EV (*e.g.*, exosome) comprising a target protein wherein at least a part of the target protein is expressed from an exogenous sequence, and the target protein comprises a HEVP (*e.g.*, CD13, MME, ENPP1, or NRP1 or a fragment or a variant thereof). In some aspects, the target protein comprises CD13 (or a fragment or variant thereof). In some aspects, the target protein comprises MME (or a fragment or variant thereof). In some aspects, the target protein comprises ENPP1 (or a fragment or variant thereof). In some aspects, the target protein comprises NRP1 (or a fragment or variant thereof). In some embodiments, the target protein does not comprise IGSF8 or a fragment or a variant thereof. In some embodiments, the target protein comprises a polypeptide of SEQ ID NO: 33.

**[0035]** In some embodiments, the EV (*e.g.*, exosome) is isolated based on a binding between the target protein and a binding agent.

**[0036]** In some embodiments, the EV (*e.g.*, exosome) is produced from a cell genetically modified to comprise the exogenous sequence, optionally wherein the cell is an HEK293 cell, a Chinese hamster ovary (CHO) cell, or a mesenchymal stem cell (MSC). In some embodiments, the cell is genetically modified to have a reduced expression of ADAM10.

**[0037]** In some embodiments, the cell comprises a plasmid comprising the exogenous sequence.

**[0038]** In some embodiments, the cell comprises the exogenous sequence inserted into a genome of the cell. In some embodiments, the exogenous sequence is inserted into a genomic site located 3' or 5' end of a genomic sequence encoding CD13, MME, ENPP1, or NRP1. In some embodiments, the exogenous sequence is inserted into a genomic sequence encoding

CD13, MME, ENPP1, or NRP1. In some embodiments, the exogenous sequence does not encode IGSF8.

**[0039]** In some embodiments, the target protein is a fusion protein comprising CD13, MME, ENPP1, or NRP1, or a fragment or a variant thereof, and an affinity tag, wherein the affinity tag has affinity to the binding agent. In some embodiments, the target protein does not comprise IGSF8 or a fragment thereof.

**[0040]** In some embodiments, the target protein is a fusion protein comprising CD13, MME, ENPP1, or NRP1, or a fragment or a variant thereof, and a therapeutic compound. In some embodiments, the target protein does not comprise IGSF8 or a fragment thereof.

**[0041]** The therapeutic compound can be selected from a group consisting of a natural peptide, a recombinant peptide, a synthetic peptide. In some embodiments, the therapeutic compound comprises a linker. The therapeutic compound can be selected from the group consisting of nucleotides, amino acids, lipids, carbohydrates, and small molecules.

**[0042]** Functional moieties and related compounds, *e.g.*, therapeutic compounds, that can be attached to HEVPs or fragments thereof include antibodies or fragments or variants thereof. A functional compound that is a peptide can be an enzyme, a ligand, a receptor, or a fragment or a variant thereof. A therapeutic peptide can be an antimicrobial peptide or a fragment or a variant thereof.

**[0043]** In some embodiments, the target protein is a fusion protein comprising CD13, MME, ENPP1, or NRP1, or a fragment or a variant thereof, and a targeting moiety. The targeting moiety can be specific to an organ, a tissue, or a cell. In some embodiments, the target protein does not comprise IGSF8 or a fragment thereof.

**[0044]** In some embodiments, the EV (*e.g.*, exosome) further comprises a second, different target protein, wherein the different target protein comprises CD13, MME, ENPP1, or NRP1, or a fragment or a variant thereof. In some embodiments, the EV (*e.g.*, exosome) is isolated based on a binding between the different target protein and a different binding agent. In some embodiments, the target protein does not comprise IGSF8 or a fragment thereof.

**[0045]** In one aspect, the present disclosure relates to a pharmaceutical composition comprising the EV (*e.g.*, exosome) of the present disclosure and an excipient.

**[0046]** In some embodiments, the pharmaceutical compositions are substantially free of macromolecules, wherein the macromolecules are selected from nucleic acids, contaminant proteins, lipids, carbohydrates, metabolites, and a combination thereof.

[0047] In one aspect, the present disclosure is directed to a cell for producing the EV (*e.g.*, exosome) presented herein.

[0048] Specifically, some embodiments relate to a cell for producing EVs (*e.g.*, exosomes), comprising an exogenous sequence inserted into a genomic sequence encoding CD13, MME, ENPP1, or NRP1, wherein the exogenous sequence and the genomic sequence encodes a fusion protein. In some embodiments, the genomic sequence does not encode IGSF8.

[0049] The exogenous sequence can encode an affinity tag.

[0050] The exogenous sequence can encode a therapeutic peptide. The therapeutic peptide can be selected from a group consisting of a natural peptide, a recombinant peptide, a synthetic peptide, or a linker to a therapeutic compound. The therapeutic compound can be selected from the group consisting of nucleotides, amino acids, lipids, carbohydrates, and small molecules. The therapeutic peptide can be an antibody or a fragment or a variant thereof. The therapeutic peptide can be an enzyme, a ligand, a receptor, or a fragment or a variant thereof. The therapeutic peptide can be an antimicrobial peptide or a fragment or a variant thereof.

[0051] The exogenous sequence can encode a targeting moiety. The targeting moiety can be specific to an organ, a tissue, or a cell.

[0052] In some embodiments, the cell line is genetically modified to have a reduced expression of ADAM10.

[0053] In one aspect, the present disclosure provides an EV (*e.g.*, exosome) produced from the cell line of the present disclosure. In some embodiments, the EV (*e.g.*, exosome) includes the fusion protein on the surface at a higher density than a different fusion protein on the surface of a different EV (*e.g.*, exosome), wherein the different EV (*e.g.*, exosome) is produced from a different cell line comprising the exogenous sequence inserted into a different genomic sequence encoding a conventional EV (*e.g.*, exosome) protein, wherein the exogenous sequence and the different genomic sequence encodes the different fusion protein. In some embodiments, the conventional EV (*e.g.*, exosome) protein is selected from the group consisting of CD9, CD63, CD81, PDGFR, GPI anchor proteins, LAMP2, LAMP2B, and a fragment thereof.

[0054] In another aspect, the present disclosure relates to a method of isolating a non-exosomal material, comprising the steps of: providing a sample comprising an EV (*e.g.*, exosome) and the non-EV (*e.g.*, exosome) material; contacting the sample with a binding agent having affinity to a target protein, wherein the target protein comprises CD13, MME, ENPP1, or NRP1 or a fragment or a variant thereof, thereby inducing the EV (*e.g.*, exosome) to bind to the

binding agent; and isolating the non-EV (*e.g.*, exosome) material. In some aspects, the target protein comprises CD13 (or a fragment or variant thereof). In some aspects, the target protein comprises MME (or a fragment or variant thereof). In some aspects, the target protein comprises ENPP1 (or a fragment or variant thereof). In some aspects, the target protein comprises NRP1 (or a fragment or variant thereof).

**[0055]** In some embodiments, the non-exosomal material is virus or a protein. In some embodiments, the non-exosomal material is lentivirus, retrovirus, adeno-associated virus, or other enveloped or non-enveloped virus. In some embodiments, the non-exosomal material is a recombinant protein. In some embodiments, the isolated non-exosomal material is substantially free of EVs (*e.g.*, exosomes).

**[0056]** In some embodiments, the target protein further comprises an affinity tag, wherein the affinity tag has affinity to the binding agent. In some embodiments, the target protein comprises a polypeptide of SEQ ID NO: 33. In some embodiments, the binding agent comprises an immunoglobulin, a protein, a peptide, or a small molecule. In some embodiments, the binding agent is attached to a solid support, optionally wherein the solid support comprises a porous agarose bead, a microtiter plate, a magnetic bead, or a membrane. In some embodiments, the solid support forms a chromatography column. In some embodiments, the step of contacting the sample with the binding agent is performed by applying the sample to the chromatography column.

**[0057]** In some embodiments, the methods of purification described herein are used for purification of nanovesicles. In some embodiments, the compositions and methods described herein are directed to nanovesicles. Additional embodiments of the present disclosure are described in more detail below.

## **6. BRIEF DESCRIPTION OF THE FIGURES**

**[0058]** **FIG. 1** provides a dot graph showing peptide spectrum matches of individual proteins identified in either mesenchymal stem cell (MSC)-derived (y-axis) or HEK293-derived (x-axis) EVs (*e.g.*, exosomes). The individual dots correspond to a different protein. CD13 and MME (shown using arrows) are both enriched in MSC-derived EVs but are not detected in HEK293-derived EVs. The table below the dot graph shows the molecule weight (MW) of CD13 and MME proteins and their relative expression in HEK293 (HEK) and MSC cells.

**[0059]** **FIGs. 2A** and **2B** show expression of CD13 and PTGFRN proteins in EVs (*e.g.*, exosomes) derived from HEK293 producer cells that have been modified to overexpress either

PTGFRN or CD13. **FIG. 2A** provides SDS-PAGE analysis of wild-type (WT) (*i.e.*, non-modified), PTGFRN overexpressing, and CD13 overexpressing HEK293-derived EVs. **FIG. 2B** provides a quantitation of CD13 protein expression on engineered HEK293-derived EVs by ELISA using a recombinant CD13 (rCD13) standard. The graph shown on the left is the standard. The graph shown on the right is for the EVs (*e.g.*, exosomes).

**[0060]** **FIGs. 3A and 3B** provide the bioactivity analysis of CD13 protein expressed on the engineered HEK293-derived EVs (*e.g.*, exosomes). **FIG. 3A** shows the enzymatic activity of recombinant CD13 protein (rCD13) using a commercially available CD13 activity assay kit (BioVision K523). **FIG. 3B** shows the bioactivity of CD13 protein expressed on the engineered HEK293-derived EVs (*e.g.*, exosomes) using the same CD13 activity assay kit. The recombinant CD13 proteins were tested at 6 different concentrations: (i) 880 ng/mL, (ii) 440 ng/mL, (iii) 293 ng/mL, (iv) 220 ng/mL, (v) 110 ng/mL, and (vi) 55 ng/mL. In **FIG. 3B**, the EVs were tested at three different concentrations: (i)  $1.6 \times 10^{10}$  p/mL, (ii)  $7.9 \times 10^9$  p/mL, and (iii)  $4.0 \times 10^9$  p/mL. Non-engineered EVs (*i.e.*, wild-type) were used as control.

**[0061]** **FIGs. 4A and 4B** provide expression analysis of GFP conjugated to CD13 and MME proteins expressed on engineered HEK293-derived EVs (*e.g.*, exosomes). **FIG. 4A** shows GFP expression as measured using SDS-PAGE analysis. EVs expressing GFP conjugated to PTGFRN (PTGFRN-GFP) were used as control. **FIG. 4B** shows a comparison of the expression of GFP conjugated to one of the following scaffold proteins: (i) LAMP2B, (ii) pDisplay, (iii) PTGFRN, (iv) MME, and (v) CD13. GFP was measured spectrophotometrically. The raw fluorescence measurements are shown for each of the different scaffold proteins.

## 7. DETAILED DESCRIPTION

### 7.1. Definitions

**[0062]** Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this disclosure belongs. As used herein, the following terms have the meanings ascribed to them below.

**[0063]** As used herein, the term "**extracellular vesicle**" or "**EV**" refers to a cell-derived vesicle comprising a membrane that encloses an internal space. Extracellular vesicles comprise all membrane-bound vesicles (*e.g.*, exosomes, nanovesicles) that have a smaller diameter than the cell from which they are derived. Generally extracellular vesicles range in diameter from 20 nm to 1000 nm, and can comprise various macromolecular cargo either within the internal space (*i.e.*, lumen), displayed on the external surface of the extracellular vesicle, and/or spanning the

membrane. The cargo can comprise nucleic acids, proteins, carbohydrates, lipids, small molecules, and/or combinations thereof. In some aspects, an EV (*e.g.*, exosomes) comprises one or more cargos or other exogenous biologically active molecules. In some aspects, an EV (*e.g.*, exosomes) can comprise one or more scaffold moieties. In certain aspects, the one or more scaffold moieties are not naturally expressed in cells from which the EVs (*e.g.*, exosomes) are produced. By way of example and without limitation, extracellular vesicles include apoptotic bodies, fragments of cells, vesicles derived from cells by direct or indirect manipulation (*e.g.*, by serial extrusion or treatment with alkaline solutions), vesiculated organelles, and vesicles produced by living cells (*e.g.*, by direct plasma membrane budding or fusion of the late endosome with the plasma membrane). Extracellular vesicles can be derived from a living or dead organism, explanted tissues or organs, prokaryotic or eukaryotic cells, and/or cultured cells. As described herein, in some aspects, the EVs (*e.g.*, exosomes) disclosed herein are produced by cells that have been modified to express one or more transgene products. Accordingly, the EVs of the present disclosure do not comprise naturally occurring EVs (*e.g.*, exosomes).

**[0064]** As used herein the term "**exosome**" refers to a cell-derived small (between 20-300 nm in diameter, more preferably 40-200 nm in diameter) vesicle comprising a membrane that encloses an internal space, and which is generated from a cell (*e.g.*, producer cell) by direct plasma membrane budding or by fusion of the late endosome with the plasma membrane. In certain aspects, exosomes of the present disclosure has a diameter between about 20-290 nm, 20-280 nm, 20-270 nm, 20-260 nm, 20-250 nm, 20-240 nm, 20-230 nm, 20-220 nm, 20-210 nm, 20-200 nm, 20-190 nm, 20-180 nm, 20-170 nm, 20-160 nm, 20-150 nm, 20-140 nm, 20-130 nm, 20-120 nm, 20-110 nm, 20-100 nm, 20-90 nm, 20-80 nm, 20-70 nm, 20-60 nm, 20-50 nm, 20-40 nm, 20-30 nm, 30-300 nm, 30-290 nm, 30-280 nm, 30-270 nm, 30-260 nm, 30-250 nm, 30-240 nm, 30-230 nm, 30-220 nm, 30-210 nm, 30-200 nm, 30-190 nm, 30-180 nm, 30-170 nm, 30-160 nm, 30-150 nm, 30-140 nm, 30-130 nm, 30-120 nm, 30-110 nm, 30-100 nm, 30-90 nm, 30-80 nm, 30-70 nm, 30-60 nm, 30-50 nm, 30-40 nm, 40-300 nm, 40-290 nm, 40-280 nm, 40-270 nm, 40-260 nm, 40-250 nm, 40-240 nm, 40-230 nm, 40-220 nm, 40-210 nm, 40-200 nm, 40-190 nm, 40-180 nm, 40-170 nm, 40-160 nm, 40-150 nm, 40-140 nm, 40-130 nm, 40-120 nm, 40-110 nm, 40-100 nm, 40-90 nm, 40-80 nm, 40-70 nm, 40-60 nm, 40-50 nm, 50-300 nm, 50-290 nm, 50-280 nm, 50-270 nm, 50-260 nm, 50-250 nm, 50-240 nm, 50-230 nm, 50-220 nm, 50-210 nm, 50-200 nm, 50-190 nm, 50-180 nm, 50-170 nm, 50-160 nm, 50-150 nm, 50-140 nm, 50-130 nm, 50-120 nm, 50-110 nm, 50-100 nm, 50-90 nm, 50-80 nm, 50-70 nm, 50-60 nm, 60-300 nm, 60-290

nm, 60-280 nm, 60-270 nm, 60-260 nm, 60-250 nm, 60-240 nm, 60-230 nm, 60-220 nm, 60-210 nm, 60-200 nm, 60-190 nm, 60-180 nm, 60-170 nm, 60-160 nm, 60-150 nm, 60-140 nm, 60-130 nm, 60-120 nm, 60-110 nm, 60-100 nm, 60-90 nm, 60-80 nm, 60-70 nm, 70-300 nm, 70-290 nm, 70-280 nm, 70-270 nm, 70-260 nm, 70-250 nm, 70-240 nm, 70-230 nm, 70-220 nm, 70-210 nm, 70-200 nm, 70-190 nm, 70-180 nm, 70-170 nm, 70-160 nm, 70-150 nm, 70-140 nm, 70-130 nm, 70-120 nm, 70-110 nm, 70-100 nm, 70-90 nm, 70-80 nm, 80-300 nm, 80-290 nm, 80-280 nm, 80-270 nm, 80-260 nm, 80-250 nm, 80-240 nm, 80-230 nm, 80-220 nm, 80-210 nm, 80-200 nm, 80-190 nm, 80-180 nm, 80-170 nm, 80-160 nm, 80-150 nm, 80-140 nm, 80-130 nm, 80-120 nm, 80-110 nm, 80-100 nm, 80-90 nm, 90-300 nm, 90-290 nm, 90-280 nm, 90-270 nm, 90-260 nm, 90-250 nm, 90-240 nm, 90-230 nm, 90-220 nm, 90-210 nm, 90-200 nm, 90-190 nm, 90-180 nm, 90-170 nm, 90-160 nm, 90-150 nm, 90-140 nm, 90-130 nm, 90-120 nm, 90-110 nm, 90-100 nm, 100-300 nm, 110-290 nm, 120-280 nm, 130-270 nm, 140-260 nm, 150-250 nm, 160-240 nm, 170-230 nm, 180-220 nm, or 190-210 nm. The size of the EV (*e.g.*, exosome) described herein can be measured according to methods described, *infra*.

**[0065]** In some aspects, an EV (*e.g.*, exosome) of the present disclosure comprises a bi-lipid membrane, comprising an interior surface and an exterior surface. In certain aspects, the interior surface faces the inner core (*i.e.*, lumen) of the EV (*e.g.*, exosome). In certain aspects, the exterior surface can be in contact with the endosome, the multivesicular bodies, or the membrane/cytoplasm of a producer cell or a target cell.

**[0066]** In some aspects, the EV (*e.g.*, exosome) membrane comprises lipids and fatty acids. In some aspects, the EV (*e.g.*, exosome) membrane comprises phospholipids, glycolipids, fatty acids, sphingolipids, phosphoglycerides, sterols, cholesterol, and phosphatidylserines.

**[0067]** In some aspects, the EV (*e.g.*, exosome) membrane comprises an inner leaflet and an outer leaflet. The composition of the inner and outer leaflet can be determined by transbilayer distribution assays known in the art, *see, e.g.*, Kuypers *et al.*, *Biochim Biophys Acta* 1985 819:170. In some aspects, the composition of the outer leaflet is between approximately 70-90% choline phospholipids, between approximately 0-15% acidic phospholipids, and between approximately 5-30% phosphatidylethanolamine. In some aspects, the composition of the inner leaflet is between approximately 15-40% choline phospholipids, between approximately 10-50% acidic phospholipids, and between approximately 30-60% phosphatidylethanolamine.

**[0068]** In some aspects, the exosome comprises lipid or fatty acid and polypeptide and optionally comprises a payload (*e.g.*, a therapeutic agent), a receiver (*e.g.*, a targeting moiety), a

polynucleotide (*e.g.*, a nucleic acid, RNA, or DNA), a sugar (*e.g.*, a simple sugar, polysaccharide, or glycan) or other molecules. As described herein, in some aspects, exosomes of the present disclosure comprise one or more scaffold moieties. In certain aspects, the one or more scaffold moieties are not naturally expressed in cells from which the exosomes are produced. The exosome can be derived from a producer cell, and isolated from the producer cell based on its size, density, biochemical parameters, or a combination thereof. An exosome is a species of extracellular vesicle. Generally, exosome production/biogenesis does not result in the destruction of the producer cell. In some aspects, exosomes of the present disclosure are produced by cells that express one or more transgene products. The exosomes of the present disclosure are modified (*e.g.*, engineered to overexpress a protein that is not naturally expressed in the producer cell) and therefore, do not comprise naturally occurring exosomes.

**[0069]** As used herein, the term "**heterologous exosomal vesicle protein**" or "**HEVP**" refers to a protein that is heterologously expressed in EVs (*e.g.*, exosomes) produced by a particular cell type where that cell type does not naturally express the protein, but wherein the protein is expressed naturally in the EVs (*e.g.*, exosomes) of a different cell type. The latter cell type is referred to herein as a "**donor cell**" and the former cell type is referred to herein as a "**producer cell**."

**[0070]** As used herein, the term "**nanovesicle**" refers to a cell-derived small (between 20-250 nm in diameter, more preferably 30-150 nm in diameter) vesicle comprising a membrane that encloses an internal space, and which is generated from a cell (*e.g.*, producer cell) by direct or indirect manipulation such that the nanovesicle would not be produced by the producer cell without the manipulation. Appropriate manipulations of the producer cell include but are not limited to serial extrusion, treatment with alkaline solutions, sonication, or combinations thereof. The production of nanovesicles can, in some instances, result in the destruction of the producer cell. In some aspects, populations of nanovesicles described herein are substantially free of vesicles that are derived from producer cells by way of direct budding from the plasma membrane or fusion of the late endosome with the plasma membrane. In some aspects, the nanovesicle comprises lipid or fatty acid and polypeptide, and optionally comprises a payload (*e.g.*, a therapeutic agent), a receiver (*e.g.*, a targeting moiety), a polynucleotide (*e.g.*, a nucleic acid, RNA, or DNA), a sugar (*e.g.*, a simple sugar, polysaccharide, or glycan) or other molecules. In some aspects, nanovesicles described herein disclosure comprise one or more scaffold moieties. In certain aspects, the one or more scaffold moieties are not naturally expressed in cells

from which the nanovesicles are produced. The nanovesicle, once it is derived from a producer cell according to the above-described manipulation, can be isolated from the producer cell based on its size, density, biochemical parameters, or a combination thereof. A nanovesicle is a species of extracellular vesicle. As used herein, nanovesicles have been modified (*e.g.*, engineered to overexpress a protein that is not naturally expressed in the producer cell) and therefore, do not comprise naturally occurring nanovesicles.

**[0071]** As used herein the term "**surface-engineered exosome**" refers to an exosome with a membrane or the surface modified in its composition, so that the membrane or the surface of the engineered exosomes is different from either that of the exosome prior to the modification or that of a naturally occurring exosome. The engineering can be on the surface of the exosome or in the membrane of the exosome so that the surface of the exosome is changed. For example, the membrane is modified in its composition of a protein, a lipid, a small molecule, a carbohydrate, *etc.* The composition can be changed by a chemical, a physical, or a biological method or by being produced from a cell previously or concurrently modified by a chemical, a physical, or a biological method. Specifically, the composition can be changed by a genetic engineering or by being produced from a cell previously modified by genetic engineering. As disclosed herein, in some aspects, a surface-engineered exosome disclosed herein comprises one or more exogenous proteins (*e.g.*, scaffold moieties, *e.g.*, heterologous exosomal vesicle proteins disclosed herein), or a fragment or variant thereof, that can be exposed to the surface of the exosome or can be an anchoring point (attachment) for a moiety exposed on the surface of the exosome. In certain aspects, the one or more scaffold moieties are not naturally expressed in cells from which the exosomes are produced. In some aspects, a surface-engineered exosome comprises a higher expression (*e.g.*, higher number) of a natural exosome protein (*e.g.*, PTGFRN) or a fragment or variant thereof that can be exposed to the surface of the exosome or can be an anchoring point (attachment) for a moiety exposed on the surface of the exosome. While the above definition is provided in the context of exosomes, other types of extracellular vesicles can also be surface-engineered in a similar manner. Therefore, unless indicated otherwise, disclosures relating to surface-engineered exosomes can equally apply to other extracellular vesicles.

**[0072]** As used herein, the term "**lumen-engineered exosome**" refers to an exosome with the membrane or the lumen of the exosome modified in its composition, so that the lumen of the engineered exosome is different from that of the exosome prior to the modification or of the naturally occurring exosome. The engineering can be directly in the lumen or in the membrane of

the exosome so that the lumen of the exosome is changed. For example, the membrane is modified in its composition of a protein, a lipid, a small molecule, a carbohydrate, *etc.* so that the lumen of the exosome is modified. The composition can be changed by a chemical, a physical, or a biological method or by being produced from a cell previously modified by a chemical, a physical, or a biological method. Specifically, the composition can be changed by a genetic engineering or by being produced from a cell previously modified by genetic engineering. In some aspects, a lumen-engineered exosome comprises one or more exogenous biologically active molecules (*e.g.*, scaffold moieties, *e.g.*, heterologous exosomal vesicle proteins disclosed herein). In certain aspects, the exogenous biologically active molecules can comprise an exogenous protein (*i.e.*, a protein that the EV, *e.g.*, exosome does not naturally express) or a fragment or variant thereof that can be exposed in the lumen of the exosome or can be an anchoring point (attachment) for a moiety exposed on the inner layer of the exosome. In certain aspects, the one or more scaffold moieties are not naturally expressed in cells from which the exosomes are produced. In some aspects, a lumen-engineered exosome comprises a higher expression of a natural exosome protein or a fragment or variant thereof that can be exposed to the lumen of the exosome or can be an anchoring point (attachment) for a moiety exposed in the lumen of the exosome. While the above definition is provided in the context of exosomes, other types of extracellular vesicles can also be lumen-engineered in a similar manner. Therefore, unless indicated otherwise, disclosures relating to lumen-engineered exosomes can equally apply to other extracellular vesicles.

**[0073]** As used herein, the term "**a modification**," when used in the context of a protein, refers to a protein having at least about 15% sequence identity to the non-mutant amino acid sequence of the protein. A modification of a protein includes a fragment or a variant of the protein. A modification of a protein can further include chemical, or physical modification to a fragment or a variant of the protein.

**[0074]** The term "**modified**," when used in the context of EVs, *e.g.*, exosomes described herein, refers to an alteration or engineering of an EV, *e.g.*, exosome and/or its producer cell, such that the modified EV, *e.g.*, exosome is different from a naturally-occurring EV, *e.g.*, exosome. In some aspects, a modified EV, *e.g.*, exosome described herein comprises a membrane that differs in composition of a protein, a lipid, a small molecular, a carbohydrate, *etc.* compared to the membrane of a naturally-occurring EV, *e.g.*, exosome (*e.g.*, membrane comprises higher density or number of natural exosome proteins and/or membrane comprises multiple (*e.g.*, at

least two) biologically active molecules that are not naturally found in exosomes (*e.g.*, therapeutic molecules (*e.g.*, antigen), targeting moiety, adjuvant, and/or immune modulator). As used herein, biologically active molecules that are not naturally found in exosomes are also described as "**exogenous biologically active molecules**." Non-limiting examples of such exogenous biologically active molecules include the heterologous exosomal vesicle proteins disclosed herein (*e.g.*, CD13, MME, ENPP1, or NRP1), therapeutic molecules (*e.g.*, antigens), targeting moieties, adjuvants, immune modulators, or combinations thereof. In certain aspects, such modifications to the membrane changes the exterior surface of the EV, *e.g.*, exosome (*e.g.*, surface-engineered EVs, *e.g.*, exosomes described herein).

**[0075]** As used herein, the terms "**scaffold moiety**" and "**scaffold**" can be used interchangeably and refer to a molecule that can be used to anchor a cargo or any other exogenous biologically active molecule of interest (*e.g.*, targeting moiety, adjuvant, and/or immune modulator) to the EV, *e.g.*, on the exterior surface of the EV, *e.g.*, exosome. In certain aspects, a scaffold moiety comprises a synthetic molecule. In some aspects, a scaffold moiety comprises a non-polypeptide moiety. In some aspects, a scaffold moiety comprises a lipid, carbohydrate, or protein that naturally exists in the EV, *e.g.*, exosome. In some aspects, a scaffold moiety comprises a lipid, carbohydrate, or protein that does not naturally exist in the EV, *e.g.*, exosome. In some aspects, a scaffold moiety comprises a heterologous exosomal vesicle protein disclosed herein. In some aspects, a scaffold moiety can be a whole protein or a fragment thereof (*e.g.*, functional fragment, *e.g.*, the smallest fragment that is capable of anchoring another moiety on the exterior surface or on the luminal surface of the EV, *e.g.*, exosome). Non-limiting examples of other scaffold moieties that can be used with the present disclosure include: Scaffold X, Scaffold Y, CD9, CD63, CD81, PDGFR, GPI anchor proteins, lactadherin, LAMP2, and LAMP2B.

**[0076]** As used herein, the term "**Scaffold X**" refers to exosome proteins that have recently been identified on the surface of exosomes. *See, e.g.*, U.S. Pat. No. 10,195,290, which is incorporated herein by reference in its entirety. Non-limiting examples of Scaffold X proteins include: prostaglandin F2 receptor negative regulator ("the PTGFRN protein"); basigin ("the BSG protein"); immunoglobulin superfamily member 2 ("the IGSF2 protein"); immunoglobulin superfamily member 3 ("the IGSF3 protein"); immunoglobulin superfamily member 8 ("the IGSF8 protein"); integrin beta-1 ("the ITGB1 protein"); integrin alpha-4 ("the ITGA4 protein"); 4F2 cell-surface antigen heavy chain ("the SLC3A2 protein"); and a class of ATP transporter

proteins ("the ATP1A1 protein," "the ATP1A2 protein," "the ATP1A3 protein," "the ATP1A4 protein," "the ATP1B3 protein," "the ATP2B1 protein," "the ATP2B2 protein," "the ATP2B3 protein," "the ATP2B protein").

**[0077]** As used herein, the term "**Scaffold Y**" refers to exosome proteins that were newly identified within the lumen of exosomes. *See, e.g.*, International Appl. No. PCT/US2018/061679, which is incorporated herein by reference in its entirety. Non-limiting examples of Scaffold Y proteins include: myristoylated alanine rich Protein Kinase C substrate ("the MARCKS protein"); myristoylated alanine rich Protein Kinase C substrate like 1 ("the MARCKSL1 protein"); and brain acid soluble protein 1 ("the BASP1 protein").

**[0078]** As used herein the term "**a fragment**" of a protein refers to an amino acid sequence of a protein that is shorter than the naturally-occurring sequence, *e.g.*, N- and/or C-terminally deleted and/or any other part of the protein deleted in comparison to the naturally occurring protein. Preferably, a fragment of a heterologous exosomal vesicle protein disclosed herein (*e.g.*, CD13, MME, ENPP1, or NRP1) retains the ability to be specifically targeted to EVs (*e.g.*, exosomes). In some aspects, a fragment of a heterologous exosomal vesicle protein disclosed herein retains the ability to anchor another moiety on the exterior surface or on the luminal surface of the EV (*e.g.*, exosome). Such a fragment is also referred to as "**functional fragment**". As used herein, the term "**functional fragment**" can refer to a protein fragment that retains protein function. In some aspects, the term "functional fragment" refers to a protein fragment that is capable of being expressed in a cell line that does not naturally express the full length protein. Whether a fragment is a functional fragment in that sense can be assessed by any art known methods to determine the protein content of EVs (*e.g.*, exosomes) including Western Blots, FACS analysis and fusions of the fragments with autofluorescent proteins like, *e.g.* GFP. In some aspects, a heterologous exosomal vesicle protein disclosed herein retains at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or about 100% of the ability, *e.g.*, an ability to anchor a moiety, of the naturally occurring heterologous exosomal vesicle protein. In a particular embodiment, the fragment of CD13, MME, ENPP1, or NRP1 retains at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or about 100% of the ability of the naturally occurring CD13, MME, ENPP1, or NRP1 to be specifically targeted to EVs (*e.g.*, exosomes) and/or anchor another moiety on the EVs (*e.g.*, on the exterior surface).

**[0079]** As used herein the term "**variant**" of a protein refers to a protein that shares a certain amino acid sequence identity with another protein upon alignment by a method known in the art. A variant of a protein can include a substitution, insertion, deletion, frameshift or rearrangement in another protein. In a particular embodiment, the variant is a variant having at least about 70% identity to CD13 (also known in the art as, *e.g.*, membrane alanyl aminopeptidase, alanyl aminopeptidase (AAP), aminopeptidase N (AP-N), aminopeptidase M, GP150, LAP1, P150, PEPN, ANPEP, or microsomal aminopeptidase), MME (also known in the art as, *e.g.*, membrane metallo-endopeptidase, neprilysin, neural endopeptidase (NEP), cluster of differentiation 10 (CD10), common acute lymphoblastic leukemia antigen (CALLA), skin fibroblast elastase, atriopeptidase, or enkephalinase), ENPP1 (also known in the art as, *e.g.*, ectonucleotide pyrophosphatase/phosphodiesterase family member 1, phosphodiesterase/nucleotide pyrophosphatase 1, plasma-cell membrane glycoprotein PC-1, membrane component chromosome 6 surface marker 1, alkaline phosphodiesterase 1, Ly-41 antigen, ARHR2, or COLED), or NRP1 (also known in the art as, *e.g.*, neuropilin 1, vascular endothelial cell growth factor 165 receptor (VEGF165R), CD304, BDCA4, NP1, or NRP). In some embodiments, variants or variants of fragments of CD13 share at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% sequence identity with CD13 according to **SEQ ID NO: 47** or with a functional fragment thereof. In some embodiments, variants or variants of fragments of MME share at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% sequence identity with MME according to **SEQ ID NO: 48** or with a functional fragment thereof. In some embodiments, variants or variants of fragments of ENPP1 share at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% sequence identity with ENPP1 according to **SEQ ID NO: 49** or with a functional fragment thereof. In some embodiments, variants or variants of fragments of NRP1 share at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% sequence identity with NRP1 according to **SEQ ID NO: 50** or with a functional fragment thereof. In each of above cases, it is preferred that the variant or variant of a fragment retain a function of the protein (*e.g.*, the ability to be specifically targeted to EVs (*e.g.*, exosomes)).

**[0080]** A "**conservative amino acid substitution**" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid

residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, if an amino acid in a polypeptide is replaced with another amino acid from the same side chain family, the substitution is considered to be conservative. In another aspect, a string of amino acids can be conservatively replaced with a structurally similar string that differs in order and/or composition of side chain family members.

**[0081]** The term "**percent sequence identity**" or "**percent identity**" between two polynucleotide or polypeptide sequences refers to the number of identical matched positions shared by the sequences over a comparison window, taking into account additions or deletions (*i.e.*, gaps) that must be introduced for optimal alignment of the two sequences. A matched position is any position where an identical nucleotide or amino acid is presented in both the target and reference sequence. Gaps presented in the target sequence are not counted since gaps are not nucleotides or amino acids. Likewise, gaps presented in the reference sequence are not counted since target sequence nucleotides or amino acids are counted, not nucleotides or amino acids from the reference sequence.

**[0082]** Methods of alignment of sequences for comparison are well-known in the art. Various programs and alignment algorithms are described in: Smith and Waterman, *Adv. Appl. Math.* 2: 482 (1981); Needleman and Wunsch, *J. Mol. Bio.* 48: 443 (1970); Pearson and Lipman, *Methods in Mol. Biol.* 24: 307-31 (1988); Higgins and Sharp, *Gene* 73: 15 237-44 (1988); Higgins and Sharp, *CABIOS* 5: 151-3 (1989) Corpet *et al.*, *Nuc. Acids Res.* 16: 10881-90 (1988); Huang *et al.*, *Comp. Appl. BioSci.* 8: 155-65 (1992); and Pearson *et al.*, *Meth. Mol. Biol.* 24: 307-31 (1994). The NCBI Basic Local Alignment Search Tool (BLAST) [Altschul 20 *et al.*, *J. Mol. Biol.* 215: 403-10 (1990)] is available from several sources, including the National Center for Biological Information (NCBI, Bethesda, Md.) and on the Internet, for use in connection with the sequence analysis programs blastp, blastm, blastx, tblastn and tblastx. BLAST and a description of how to determine sequence identify using the program can be accessed at the official website of NCBI (National Center for Biotechnology Information) under NIH (National Institute of Health).

[0083] Recitation of any protein provided herein encompasses a functional variant of the protein. The term "**functional variant**" of a protein refers to a variant of the protein that retains the ability to be specifically targeted to EVs (*e.g.*, exosomes).

[0084] The polynucleotide variants can contain alterations in the coding regions, non-coding regions, or both. In one aspect, the polynucleotide variants contain alterations which produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded polypeptide. In another aspect, nucleotide variants are produced by silent substitutions due to the degeneracy of the genetic code. In other aspects, variants in which 5-10, 1-5, or 1-2 amino acids are substituted, deleted, or added in any combination. Polynucleotide variants can be produced for a variety of reasons, *e.g.*, to optimize codon expression for a particular host (change codons in the human mRNA to others, *e.g.*, a bacterial host such as *E. coli*).

[0085] Naturally occurring variants are called "**allelic variants**," and refer to one of several alternate forms of a gene occupying a given locus on a chromosome of an organism (Genes II, Lewin, B., ed., John Wiley & Sons, New York (1985)). These allelic variants can vary at either the polynucleotide and/or polypeptide level and are included in the present disclosure. Alternatively, non-naturally occurring variants can be produced by mutagenesis techniques or by direct synthesis.

[0086] Using known methods of protein engineering and recombinant DNA technology, variants can be generated to improve or alter the characteristics of the polypeptides. For instance, one or more amino acids can be deleted from the N-terminus or C-terminus of the secreted protein without substantial loss of biological function. Ron *et al.*, *J. Biol. Chem.* 268: 2984-2988 (1993), incorporated herein by reference in its entirety, reported variant KGF proteins having heparin binding activity even after deleting 3, 8, or 27 amino-terminal amino acid residues. Similarly, interferon gamma exhibited up to ten times higher activity after deleting 8-10 amino acid residues from the carboxy terminus of this protein. (Dobeli *et al.*, *J. Biotechnology* 7:199-216 (1988), incorporated herein by reference in its entirety.)

[0087] Moreover, ample evidence demonstrates that variants often retain a biological activity similar to that of the naturally occurring protein. For example, Gayle and coworkers (*J. Biol. Chem* 268:22105-22111 (1993), incorporated herein by reference in its entirety) conducted extensive mutational analysis of human cytokine IL-1a. They used random mutagenesis to generate over 3,500 individual IL-1a mutants that averaged 2.5 amino acid changes per variant

over the entire length of the molecule. Multiple mutations were examined at every possible amino acid position. The investigators found that "[m]ost of the molecule could be altered with little effect on either [binding or biological activity]." (See Abstract.) In fact, only 23 unique amino acid sequences, out of more than 3,500 nucleotide sequences examined, produced a protein that significantly differed in activity from wild-type.

**[0088]** As stated above, polypeptide variants include, *e.g.*, modified polypeptides. Modifications include, *e.g.*, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pegylation (Mei *et al.*, *Blood* 116:270-79 (2010), which is incorporated herein by reference in its entirety), proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. In some aspects, Scaffold X and/or Scaffold Y is modified at any convenient location.

**[0089]** As used herein the terms "**linked to**," "**conjugated to**," and "**anchored to**" are used interchangeably and refer to a covalent or non-covalent bond formed between a first moiety (*e.g.*, scaffold moiety, *e.g.*, heterologous exosomal vesicle protein) and a second moiety (*e.g.*, payload).

**[0090]** As used herein the term "**producer cell**" refers to a cell used for generating an EV (*e.g.*, exosome). A producer cell can be a cell cultured *in vitro*, or a cell *in vivo*. A producer cell includes, but is not limited to, a cell known to be effective in generating EVs, *e.g.*, EVs (*e.g.*, exosomes), *e.g.*, HEK293 cells, Chinese hamster ovary (CHO) cells, mesenchymal stem cells (MSCs), BJ human foreskin fibroblast cells, fHDF fibroblast cells, AGE.HN<sup>®</sup> neuronal precursor cells, CAP<sup>®</sup> amniocyte cells, adipose mesenchymal stem cells, RPTEC/TERT1 cells. In certain aspects, a producer cell is not an antigen-presenting cell. In some aspects, a producer cell is not a dendritic cell, a B cell, a mast cell, a macrophage, a neutrophil, a Kupffer-Browicz cell, a cell derived from any of these cells, or any combination thereof. As disclosed herein, in some aspects, a producer cell of the present disclosure has been modified to express one or more transgene products. In some aspects, a producer cell has been modified to express a protein (*e.g.*,

heterologous exosomal vesicle protein) that the producer cell does not naturally express. In some aspects, the EVs, *e.g.*, exosomes, useful in the present disclosure do not carry an antigen on MHC class I or class II molecule exposed on the surface of the EV, *e.g.*, exosome, but instead can carry an antigen in the lumen of the EV, *e.g.*, exosome or on the surface of the EV, *e.g.*, exosome, by attachment to a scaffold moiety.

**[0091]** As used herein, an "**MHC class I molecule**" refers to a protein product of a wild-type or variant HLA class I gene encoding an MHC class I molecule. Accordingly, "**HLA class I molecule**" and "**MHC class I molecule**" are used interchangeably herein.

**[0092]** MHC class I molecules are one of two primary classes of major histocompatibility complex (MHC) molecules (the other being MHC class II) and are found on the cell surface of all nucleated cells in the bodies of jawed vertebrates. They also occur on platelets, but not on red blood cells. Their function is to display peptide fragments of proteins from within the cell to cytotoxic T cells; this will trigger an immediate response from the immune system against a particular non-self antigen displayed with the help of an MHC class I protein. Because MHC class I molecules present peptides derived from cytosolic proteins, the pathway of MHC class I presentation is often called cytosolic or endogenous pathway.

**[0093]** In humans, the HLAs corresponding to MHC class I are HLA-A, HLA-B, and HLA-C. The MHC Class I molecule comprises two protein chains: the alpha chain and the  $\beta$ 2-microglobulin ( $\beta$ 2m) chain. Human  $\beta$ 2m is encoded by the *B2M* gene. Class I MHC molecules bind peptides generated mainly from degradation of cytosolic proteins by the proteasome. The MHC I:peptide complex is then inserted via endoplasmic reticulum into the external plasma membrane of the cell. The epitope peptide is bound on extracellular parts of the class I MHC molecule. Thus, the function of the class I MHC is to display intracellular proteins to cytotoxic T cells (CTLs). However, class I MHC can also present peptides generated from exogenous proteins, in a process known as cross-presentation.

**[0094]** A normal cell will display peptides from normal cellular protein turnover on its class I MHC, and CTLs will not be activated in response to them due to central and peripheral tolerance mechanisms. When a cell expresses foreign proteins, such as after viral infection, a fraction of the class I MHC will display these peptides on the cell surface. Consequently, CTLs specific for the MHC:peptide complex will recognize and kill presenting cells. Alternatively, class I MHC itself can serve as an inhibitory ligand for natural killer cells (NKs). Reduction in

the normal levels of surface class I MHC, a mechanism employed by some viruses and certain tumors to evade CTL responses, activates NK cell killing.

[0095] As used herein, an "**MHC class II molecule**" refers to a protein product of a wild-type or variant HLA class II gene encoding an MHC class II molecule. Accordingly, "**HLA class II molecule**" and "**MHC class II molecule**" are used interchangeably herein.

[0096] MHC class II molecules are a class of major histocompatibility complex (MHC) molecules normally found only on professional antigen-presenting cells such as dendritic cells, mononuclear phagocytes, some endothelial cells, thymic epithelial cells, and B cells. These cells are important in initiating immune responses. The antigens presented by class II peptides are derived from extracellular proteins (not cytosolic as in MHC class I).

[0097] Like MHC class I molecules, class II molecules are also heterodimers, but in this case consist of two homogenous peptides, an  $\alpha$  and  $\beta$  chain, both of which are encoded in the MHC. The subdesignation  $\alpha 1$ ,  $\alpha 2$ , etc. refers to separate domains within the HLA gene; each domain is usually encoded by a different exon within the gene, and some genes have further domains that encode leader sequences, transmembrane sequences, etc. These molecules have both extracellular regions as well as a transmembrane sequence and a cytoplasmic tail. The  $\alpha 1$  and  $\beta 1$  regions of the chains come together to make a membrane-distal peptide-binding domain, while the  $\alpha 2$  and  $\beta 2$  regions, the remaining extracellular parts of the chains, form a membrane-proximal immunoglobulin-like domain. The antigen binding groove, where the antigen or peptide binds, is made up of two  $\alpha$ -helixes walls and  $\beta$ -sheet. Because the antigen-binding groove of MHC class II molecules is open at both ends while the corresponding groove on class I molecules is closed at each end, the antigens presented by MHC class II molecules are longer, generally between 15 and 24 amino acid residues long. Loading of a MHC class II molecule occurs by phagocytosis; extracellular proteins are endocytosed, digested in lysosomes, and the resulting epitopic peptide fragments are loaded onto MHC class II molecules prior to their migration to the cell surface. In humans, the MHC class II protein complex is encoded by the human leukocyte antigen gene complex (HLA). HLAs corresponding to MHC class II are HLA-DP, HLA-DM, HLA-DOA, HLA-DOB, HLA-DQ, and HLA-DR. Mutations in the HLA gene complex can lead to bare lymphocyte syndrome (BLS), which is a type of MHC class II deficiency.

[0098] As used herein, the term "**target protein**" refers to a protein that can be targeted to the surface of an EV (*e.g.*, exosome). The target protein can be a non-mutant protein that is

naturally targeted to an EV (*e.g.*, exosome) membrane, or a fragment or a variant of the non-mutant protein. The target protein can be a fusion protein containing a flag tag, a therapeutic peptide, a targeting moiety, or other peptide attached to the non-mutant protein or a variant or a fragment of the non-mutant protein. The target protein can comprise a transmembrane protein, an integral protein, a peripheral protein, or a soluble protein attached to the membrane by a linker.

**[0099]** As used herein, the term "**contaminant protein**" refers to a protein that is not associated with an EV (*e.g.*, exosome). For example, a contaminant protein includes a protein, not enclosed in the EV (*e.g.*, exosome) and not attached to or incorporated into the membrane of the EV (*e.g.*, exosome).

**[0100]** As used herein, the terms "**isolate**," "**isolated**," and "**isolating**" or "**purify**," "**purified**," and "**purifying**" as well as "**extracted**" and "**extracting**" are used interchangeably and refer to the state of a preparation (*e.g.*, a plurality of known or unknown amount and/or concentration) of desired EVs (*e.g.*, exosomes), that have undergone one or more processes of purification, *e.g.*, a selection or an enrichment of the desired EV (*e.g.*, exosome) preparation. In some embodiments, isolating or purifying as used herein is the process of removing, partially removing (*e.g.*, a fraction) of the EVs (*e.g.*, exosomes) from a sample containing producer cells. In some embodiments, an isolated EV (*e.g.*, exosome) composition has no detectable undesired activity or, alternatively, the level or amount of the undesired activity is at or below an acceptable level or amount. In other embodiments, an isolated EV (*e.g.*, exosome) composition has an amount and/or concentration of desired EVs (*e.g.*, exosomes) at or above an acceptable amount and/or concentration. In other embodiments, the isolated EV (*e.g.*, exosome) composition is enriched as compared to the starting material (*e.g.*, producer cell preparations) from which the composition is obtained. This enrichment can be by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.9%, at least about 99.99%, at least about 99.999%, at least about 99.9999%, or greater than about 99.9999% as compared to the starting material. In some embodiments, isolated EV (*e.g.*, exosome) preparations are substantially free of residual biological products. In some embodiments, the isolated EV (*e.g.*, exosome) preparations are about 100% free, about 99% free, about 98% free, about 97% free, about 96% free, about 95% free, about 94% free, about 93% free, about 92% free, about 91% free, or about

90% free of any contaminating biological matter. Residual biological products can include abiotic materials (including chemicals) or unwanted nucleic acids, proteins, lipids, or metabolites. Substantially free of residual biological products can also mean that the EV (*e.g.*, exosome) composition contains no detectable producer cells and that only EVs (*e.g.*, exosomes) are detectable.

**[0101]** The term "**excipient**" or "**carrier**" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of a compound. The term "**pharmaceutically-acceptable carrier**" or "**pharmaceutically-acceptable excipient**" encompasses any of the agents approved by a regulatory agency of the US Federal government or listed in the US Pharmacopeia for use in animals, including humans, as well as any carrier or diluent that does not cause significant irritation to a subject and does not abrogate the biological activity and properties of the administered compound. Included are excipients and carriers that are useful in preparing a pharmaceutical composition and are generally safe, non-toxic, and desirable.

**[0102]** As used herein, the term "**payload**" refers to a therapeutic agent that acts on a target (*e.g.*, a target cell) that is contacted with the EV (*e.g.*, exosome). Non-limiting examples of payload that can be included on the EV, *e.g.*, exosome, are a therapeutic molecule (*e.g.*, antigen or immunosuppressive agent), an adjuvant, and/or an immune modulator. Payloads that can be introduced into an EV (*e.g.*, exosome) and/or a producer cell include therapeutic agents such as, nucleotides (*e.g.*, nucleotides comprising a detectable moiety or a toxin or that disrupt transcription), nucleic acids (*e.g.*, DNA or mRNA molecules that encode a polypeptide such as an enzyme, or RNA molecules that have regulatory function such as miRNA, dsDNA, lncRNA, siRNA, antisense oligonucleotide, a phosphorodiamidate morpholino oligomer (PMO), or a peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO)), amino acids (*e.g.*, amino acids comprising a detectable moiety or a toxin or that disrupt translation), polypeptides (*e.g.*, enzymes), lipids, carbohydrates, and small molecules (*e.g.*, small molecule drugs and toxins).

**[0103]** As used herein, the term "**biologically active molecule**" refers to an agent that has activity in a biological system (*e.g.*, a cell or a human subject), including, but not limited to a protein, polypeptide or peptide including, but not limited to, a structural protein, an enzyme, a cytokine (such as an interferon and/or an interleukin) an antibiotic, a polyclonal or monoclonal antibody, or an effective part thereof, such as an Fv fragment, which antibody or part thereof can

be natural, synthetic or humanized, a peptide hormone, a receptor, a signaling molecule or other protein; a nucleic acid, as defined below, including, but not limited to, an oligonucleotide or modified oligonucleotide, an antisense oligonucleotide or modified antisense oligonucleotide, cDNA, genomic DNA, an artificial or natural chromosome (*e.g.* a yeast artificial chromosome) or a part thereof, RNA, including mRNA, tRNA, rRNA or a ribozyme, or a peptide nucleic acid (PNA); a virus or virus-like particles; a nucleotide or ribonucleotide or synthetic analogue thereof, which can be modified or unmodified; an amino acid or analogue thereof, which can be modified or unmodified; a non-peptide (*e.g.*, steroid) hormone; a proteoglycan; a lipid; or a carbohydrate. In certain aspects, a biologically active molecule comprises a therapeutic molecule (*e.g.*, an antigen), a targeting moiety (*e.g.*, an antibody or an antigen-binding fragment thereof), an adjuvant, an immune modulator, or any combination thereof. In some aspects, the biologically active molecule comprises a macromolecule (*e.g.*, a protein, an antibody, an enzyme, a peptide, DNA, RNA, or any combination thereof). In some aspects, the biologically active molecule comprises a small molecule (*e.g.*, an antisense oligomer (ASO), a phosphorodiamidate morpholino oligomer (PMO), a peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO), an siRNA, STING, a pharmaceutical drug, or any combination thereof). In some aspects, the biologically active molecules are exogenous to the EVs, *i.e.*, not naturally found in the EVs.

**[0104]** As used herein, the term "**therapeutic compound**" refers to any molecule that can treat and/or prevent a disease or disorder in a subject (*e.g.*, human subject).

**[0105]** In some aspects, a therapeutic molecule comprises an antigen. As used herein, the term "**antigen**" refers to any agent that when introduced into a subject elicits an immune response (cellular or humoral) to itself. In some aspects, an antigen is not expressed on major histocompatibility complex I and/or II molecules. In other aspects, while an antigen in the EV, *e.g.*, exosome, is not expressed as MHC class I or II complex, the EV, *e.g.*, exosome, can still contain MHC class I/II molecules on the surface of the EV, *e.g.*, exosome. Accordingly, in certain aspects, EVs, *e.g.*, exosomes, disclosed herein do not directly interact with T-cell receptors (TCRs) of T cells to induce an immune response against the antigen. Similarly, in certain aspects, EVs, *e.g.*, exosomes, of the present disclosure do not transfer the antigen directly to the surface of the target cell (*e.g.*, dendritic cell) through cross-dressing. Cross-dressing is a mechanism commonly used by EVs, *e.g.*, exosomes, derived from dendritic cells (DEX) to induce T cell activation. *See* Pitt, J.M., *et al.*, *J Clin Invest* 126(4): 1224-32 (2016). In other

aspects, the EVs, *e.g.*, exosomes, of the present disclosure are engulfed by antigen presenting cells and can be expressed on the surface of the antigen presenting cells as MHC class I and/or MHC class II complex.

**[0106]** In some aspects, a therapeutic molecule comprises an immunosuppressive agent. As used herein, the term "**immunosuppressive agent**" refers to any agent (*e.g.*, therapeutic molecule) that slows or halts an immune response in a subject. Immunosuppressive agents can be given to a subject to prevent the subject's immune system from mounting an immune response after an organ transplant or for treating a disease that is caused by an overactive immune system. Examples of immunosuppressive agents include, but are not limited to, a calcineurin inhibitor, such as, but not limited to, cyclosporine, ISA(TX) 247, tacrolimus or calcineurin, a target of rapamycin, such as, but not limited to, sirolimus, everolimus, FK778 or Tafa-93, an interleukin-2  $\alpha$ -chain blocker, such as, but not limited to, basiliximab and daclizumab, an inhibitor of inosine monophosphate dehydrogenase, such as mycophenolate mofetil, an inhibitor of dihydrofolate reductase, such as, but not limited to, methotrexate, a corticosteroid, such as, but not limited to, prednisolone and methylprednisolone, or an immunosuppressive antimetabolite, such as, but not limited to, azathioprine. In certain aspects, an immunosuppressive agent comprises an antisense oligonucleotide. In some aspects, an EV disclosed herein (*e.g.*, exosome) can comprise both an antigen and an immunosuppressive agent. Not to be bound by any one theory, an EV (*e.g.*, exosome) comprising both an antigen and an immunosuppressive agent can be used to induce tolerance to the antigen.

**[0107]** As used herein, the term "**antibody**" encompasses an immunoglobulin whether natural or partly or wholly synthetically produced, and fragments thereof. The term also covers any protein having a binding domain that is homologous to an immunoglobulin binding domain. "**Antibody**" further includes a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. Use of the term antibody is meant to include whole antibodies, polyclonal, monoclonal and recombinant antibodies, fragments thereof, and further includes single-chain antibodies, humanized antibodies, murine antibodies, chimeric, mouse-human, mouse-primate, primate-human monoclonal antibodies, anti-idiotypic antibodies, antibody fragments, such as, *e.g.*, scFv, (scFv)<sub>2</sub>, Fab, Fab', and F(ab')<sub>2</sub>, F(ab)<sub>2</sub>, Fv, dAb, and Fd fragments, diabodies, and antibody-related polypeptides. Antibody includes bispecific antibodies and multispecific antibodies so long as they exhibit the desired biological activity or function. In some aspects, the antibody or

antigen-binding fragment thereof comprises a scFv, scFab, scFab-Fc, nanobody, or any combination thereof. In some aspects, the antibody or antigen-binding fragment thereof comprises an agonist antibody, a blocking antibody, a targeting antibody, a fragment thereof, or a combination thereof. In some aspects, the agonist antibody is a CD40L agonist. In some aspects, the blocking antibody binds a target protein selected from programmed death 1 (PD-1), programmed death ligand 1 (PD-L1), cytotoxic T-lymphocyte-associated protein 4, and any combination thereof.

**[0108]** As used herein, the term "**immune modulator**" refers to an agent that acts on a target (*e.g.*, a target cell) that is contacted with the extracellular vesicle, and regulates the immune system. Non-limiting examples of immune modulator that can be introduced into an EV (*e.g.*, exosome) and/or a producer cell include agents such as, modulators of checkpoint inhibitors, ligands of checkpoint inhibitors, cytokines, derivatives thereof, or any combination thereof. The immune modulator can also include an agonist, an antagonist, an antibody, an antigen-binding fragment, a polynucleotide, such as siRNA, antisense oligonucleotide, phosphorodiamidate morpholino oligomer (PMO), peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO), miRNA, lncRNA, mRNA, DNA, or a small molecule.

**[0109]** As used herein, "**a mammalian subject**" includes all mammals, including without limitation, humans, domestic animals (*e.g.*, dogs, cats and the like), farm animals (*e.g.*, cows, sheep, pigs, horses and the like) and laboratory animals (*e.g.*, monkey, rats, mice, rabbits, guinea pigs and the like).

**[0110]** The terms "**individual**," "**subject**," "**host**," and "**patient**," are used interchangeably herein and refer to any mammalian subject for whom diagnosis, treatment, or therapy is desired, particularly humans. The methods described herein are applicable to both human therapy and veterinary applications. In some embodiments, the subject is a mammal, and in other embodiments the subject is a human.

**[0111]** As used herein, the term "**substantially free**" means that the sample comprising EVs (*e.g.*, exosomes) comprise less than about 10% of macromolecules by mass/volume (m/v) percentage concentration. Some fractions can contain less than about 0.001%, less than about 0.01%, less than about 0.05%, less than about 0.1%, less than about 0.2%, less than about 0.3 %, less than about 0.4%, less than about 0.5%, less than about 0.6%, less than about 0.7%, less than about 0.8%, less than about 0.9%, less than about 1%, less than about 2%, less than about 3%,

less than about 4%, less than about 5%, less than about 6%, less than about 7%, less than about 8%, less than about 9%, or less than about 10% (m/v) of macromolecules.

[0112] As used herein, the term "**macromolecule**" means nucleic acids, contaminant proteins, lipids, carbohydrates, metabolites, or a combination thereof.

[0113] As used herein, the term "**conventional exosome protein**" means a protein previously known to be enriched in EVs (*e.g.*, exosomes), including but is not limited to CD9, CD63, CD81, PDGFR, GPI anchor proteins, lactadherin LAMP2, and LAMP2B, a fragment thereof, or a peptide that binds thereto.

[0114] As used herein, the term "**linker**" refers to any molecular structure that can conjugate a peptide or a protein to another molecule (*e.g.*, a different peptide or protein, a small molecule, *etc.*). Suitable linkers are well known to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, or peptide linkers (see, *e.g.*, Chen *et al.*, *Advanced Drug Delivery Reviews*, 2013, Vol. 65:10, pp. 1357-1369). The linkers can be joined to the carboxyl and amino terminal amino acids through their terminal carboxyl or amino groups or through their reactive side-chain groups. In addition, in some aspects, linkers can be classified as flexible or rigid, and they can be cleavable (*e.g.*, comprise one or more protease-cleavable sites, which can be located within the sequence of the linker or flanking the linker at either end of the linker sequence).

[0115] "**Administering**," as used herein, means to give a composition comprising an EV, *e.g.*, exosome, disclosed herein to a subject via a pharmaceutically acceptable route. Routes of administration can be intravenous, *e.g.*, intravenous injection and intravenous infusion. Additional routes of administration include, *e.g.*, subcutaneous, intramuscular, oral, nasal, and pulmonary administration. EVs, *e.g.*, exosomes can be administered as part of a pharmaceutical composition comprising at least one excipient.

[0116] An "**immune response**," as used herein, refers to a biological response within a vertebrate against foreign agents or abnormal, *e.g.*, cancerous cells, which response protects the organism against these agents and diseases caused by them. An immune response is mediated by the action of one or more cells of the immune system (for example, a T lymphocyte, B lymphocyte, natural killer (NK) cell, macrophage, eosinophil, mast cell, dendritic cell or neutrophil) and soluble macromolecules produced by any of these cells or the liver (including antibodies, cytokines, and complement) that results in selective targeting, binding to, damage to, destruction of, and/or elimination from the vertebrate's body of invading pathogens, cells or

tissues infected with pathogens, cancerous or other abnormal cells, or, in cases of autoimmunity or pathological inflammation, normal human cells or tissues. An immune reaction includes, *e.g.*, activation or inhibition of a T cell, *e.g.*, an effector T cell, a Th cell, a CD4<sup>+</sup> cell, a CD8<sup>+</sup> T cell, or a Treg cell, or activation or inhibition of any other cell of the immune system, *e.g.*, NK cell. Accordingly an immune response can comprise a humoral immune response (*e.g.*, mediated by B-cells), cellular immune response (*e.g.*, mediated by T cells), or both humoral and cellular immune responses. In some aspects, an immune response is an **"inhibitory"** immune response. An inhibitory immune response is an immune response that blocks or diminishes the effects of a stimulus (*e.g.*, antigen). In certain aspects, the inhibitory immune response comprises the production of inhibitory antibodies against the stimulus. In some aspects, an immune response is a **"stimulatory"** immune response. A stimulatory immune response is an immune response that results in the generation of effectors cells (*e.g.*, cytotoxic T lymphocytes) that can destroy and clear a target antigen (*e.g.*, tumor antigen or viruses).

**[0117]** "Treat," **"treatment,"** or **"treating,"** as used herein, refers to, *e.g.*, the reduction in severity of a disease or condition; the reduction in the duration of a disease course; the amelioration or elimination of one or more symptoms associated with a disease or condition; the provision of beneficial effects to a subject with a disease or condition, without necessarily curing the disease or condition. The term also include prophylaxis or prevention of a disease or condition or its symptoms thereof. In one aspect, the term **"treating"** or **"treatment"** means inducing an immune response in a subject against an antigen.

**[0118]** **"Prevent"** or **"preventing,"** as used herein, refers to decreasing or reducing the occurrence or severity of a particular outcome. In some aspects, preventing an outcome is achieved through prophylactic treatment.

## **7.2. Other interpretational conventions**

**[0119]** Ranges recited herein are understood to be shorthand for all of the values within the range, inclusive of the recited endpoints. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, and 50.

**[0120]** The term **"about"** indicates and encompasses an indicated value and a range above and below that value. In certain embodiments, the term **"about"** indicates the designated

value  $\pm 10\%$ ,  $\pm 5\%$ , or  $\pm 1\%$ . In certain embodiments, where applicable, the term "**about**" indicates the designated value(s)  $\pm$  one standard deviation of that value(s).

[0121] Throughout this disclosure, the term "**a**" or "**an**" entity refers to one or more of that entity; for example, "an antibody" is understood to represent one or more antibodies. As such, the terms "**a**" (or "**an**"), "**one or more**," and "**at least one**" can be used interchangeably herein.

[0122] Furthermore, "**and/or**" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. Thus, the term "**and/or**" as used in a phrase such as "A and/or B" herein is intended to include "A and B," "A or B," "A" (alone), and "B" (alone). Likewise, the term "**and/or**" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following aspects: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

### 7.3. EV (*e.g.*, Exosome) Proteins

[0123] An aspect of the present disclosure relates to identification, use and modification of heterologous exosomal vesicle proteins (HEVPs), which are highly enriched on EV (*e.g.*, exosome) membranes produced from certain producer cells. As described herein, different cells (*e.g.*, mesenchymal stem cells vs. HEK293) naturally express different proteins. EVs (*e.g.*, exosomes) produced from a cell express one or more proteins that are naturally expressed in the producer cell. Accordingly, EVs (*e.g.*, exosomes) produced from different producer cells can have different protein composition. HEVPs can be identified by analyzing highly purified EVs (*e.g.*, exosomes) with mass spectrometry or other methods known in the art.

[0124] The HEVPs of the present disclosure include various membrane proteins, such as transmembrane proteins, integral proteins and peripheral proteins, enriched on the EV (*e.g.*, exosome) membranes. They include various CD proteins, transporters, integrins, lectins and cadherins. Specifically, the proteins include, but are not limited to, CD13, MME, ENPP1, and NRP1. In some aspects, the HEVP is CD13 (or a fragment or variant thereof). In some aspects, the HEVP is MME (or a fragment or variant thereof). In some aspects, the HEVP is ENPP1 (or a fragment or variant thereof). In some aspects, the HEVP is NRP1 (or a fragment or variant thereof). In some aspects, the HEVP is a type II transmembrane protein. Non-limiting examples of type II transmembrane proteins that can be used with the present disclosure include CD252, CD154, CD178, CD70, CD153, CD137, CD253, CD254, CD256, CD257, CD258, TL1, GITRL, and combinations thereof.

[0125] The present disclosure shows that an exosomal vesicle protein (*e.g.*, HEVP) that is expressed only in the EVs derived from certain cell types (*e.g.*, EVs from MSCs) can be engineered to be expressed on an EV derived from other cell types that do not naturally express the exosomal vesicle protein (*e.g.*, EVs from HEK293). One or more HEVPs identified herein can be selectively used depending on a producer cell, production condition, purification methods, or intended application of the EVs (*e.g.*, exosomes). For example, HEVPs enriched on a specific population of EVs (*e.g.*, exosomes) can be used to purify the specific population of EVs (*e.g.*, exosomes). HEVPs enriched on the surface of certain EVs (*e.g.*, exosomes) with a specific size range, a targeting moiety, a charge density, a payload, *etc.* can be identified and used in some embodiments of the present disclosure. In some embodiments, more than one HEVP can be used, concurrently or subsequently, for the generation, purification, and isolation of therapeutic EVs (*e.g.*, exosomes).

#### **7.4. Surface-engineered and/or Lumen-engineered EVs (*e.g.*, Exosomes)**

[0126] Another aspect of the present disclosure relates to generation and use of surface-engineered EVs (*e.g.*, exosomes). In some aspects, present disclosure relates to generation and use of lumen-engineered EVs (*e.g.*, exosomes). Surface-engineered and/or lumen-engineered EVs (*e.g.*, exosomes) have a membrane modified in its compositions. For example, their membrane compositions can be modified by changing the protein, lipid or glycan content of the membrane.

[0127] In some embodiments, the surface-engineered and/or lumen engineered EVs (*e.g.*, exosomes) are generated by chemical and/or physical methods, such as PEG-induced fusion and/or ultrasonic fusion.

[0128] In other embodiments, the surface-engineered and/or lumen-engineered EVs (*e.g.*, exosomes) are generated by genetic engineering. EVs (*e.g.*, exosomes) produced from a genetically-modified producer cell or a progeny of the genetically-modified cell can contain modified membrane compositions. In some aspects, the genetically-modified producer cell or progeny of the genetically-modified cell comprises one or more exogenous proteins that are not naturally found in the cell. In certain aspects, the one or more exogenous proteins are scaffold moieties, such as the heterologous exosomal vesicle proteins (HEVP) disclosed herein. In some embodiments, surface-engineered and/or lumen-engineered EVs (*e.g.*, exosomes) have the HEVP at a higher or lower density (compared to the density of HEVP expression in a cell that naturally expresses the HEVP) or include a variant or a fragment of the HEVP.

**[0129]** For example, surface-engineered and/or lumen-engineered EVs (*e.g.*, exosomes) can be produced from a cell transformed with an exogenous sequence encoding the HEVP or a variant or a fragment of the HEVP. EVs (*e.g.*, exosomes) including proteins expressed from the exogenous sequence can include modified membrane protein compositions.

**[0130]** Various modifications or fragments of the HEVP can be used for the embodiments of the present disclosure. For example, proteins modified to have enhanced affinity to a binding agent can be used for generating surface-engineered and/or lumen-engineered EVs (*e.g.*, exosomes) that can be purified using the binding agent. Proteins modified to be more effectively targeted to EVs (*e.g.*, exosomes) and/or membranes can be used. Proteins modified to comprise a minimal fragment required for specific and effective targeting to EV (*e.g.*, exosome) membranes can be also used. In some aspects, HEVPs (including fragments and variants thereof) that are capable of anchoring a cargo or any other exogenously biologically active molecules (*e.g.*, those disclosed herein) can be used in constructing a surface-engineered and/or lumen-engineered EV (*e.g.*, exosome). In certain aspects, HEVPs (including fragments and variants thereof) that are capable of anchoring certain classes of proteins can be used.

**[0131]** For example, in some aspects, HEVPs are type I transmembrane proteins, and such HEVPs can be used to anchor the extracellular domain of a type I protein to the EVs (*e.g.*, exosomes). In certain aspects, the expression of the extracellular domain of a type I protein on the EV (*e.g.*, exosome) is increased when anchored to a type I transmembrane HEVP, compared to the corresponding expression when the protein is anchored to a different type of scaffold moiety (*e.g.*, a non-type I transmembrane HEVP) or to the corresponding expression when the protein is overexpressed in an EV (*e.g.*, exosome) producer cell that naturally expresses the protein.

**[0132]** In some aspects, HEVPs are type II transmembrane proteins, and such HEVPs can be used to anchor the extracellular domain of a type II protein to the EVs (*e.g.*, exosomes). In certain aspects, the expression of the extracellular domain of a type II protein on the EV (*e.g.*, exosome) is increased when anchored to a type II transmembrane HEVP, compared to the corresponding expression when the protein is anchored to a different type of scaffold moiety (*e.g.*, a non-type II transmembrane HEVP) or to the corresponding expression when the protein is overexpressed in an EV (*e.g.*, exosome) producer cell that naturally expresses the protein.

**[0133]** In some aspects, HEVPs are type III transmembrane proteins, and such HEVPs can be used to anchor the extracellular domains of a type III protein to the EVs (*e.g.*, exosomes).

In certain aspects, the expression of the extracellular domains of a type III protein on the EV (*e.g.*, exosome) is increased when anchored to a type III transmembrane HEVP, compared to the corresponding expression when the protein is anchored to a different type of scaffold moiety (*e.g.*, a non-type III transmembrane HEVP) or to the corresponding expression when the protein is overexpressed in an EV (*e.g.*, exosome) producer cell that naturally expresses the protein.

**[0134]** In some aspects, HEVPs are type IV transmembrane proteins, and such HEVPs can be used to anchor the extracellular domain of a type IV protein to the EVs (*e.g.*, exosome). In certain aspects, the expression of the extracellular domain of a type IV protein on the EV (*e.g.*, exosome) is increased when anchored to a type IV transmembrane HEVP, compared to the corresponding expression when the protein is anchored to a different type of scaffold moiety (*e.g.*, a non-type IV transmembrane HEVP) or to the corresponding expression when the protein is overexpressed in an EV (*e.g.*, exosome) producer cell that naturally expresses the protein.

**[0135]** Fusion proteins can be also used; for example, HEVPs or their fragments fused to an affinity tag (*e.g.*, His tag, GST tag, glutathione-S-transferase, S-peptide, HA, Myc, FLAG™ (Sigma-Aldrich Co.), MBP, SUMO, and Protein A) can be used for purification or removal of the surface-engineered EVs (*e.g.*, exosomes) with a binding agent specific to the affinity tag.

**[0136]** Fusion proteins having a therapeutic activity can be also used for generating surface-engineered EVs (*e.g.*, exosomes). Accordingly, in some aspects, an EV (*e.g.*, exosome) disclosed herein has been engineered or modified to express the fusion protein and can be used to deliver one or more (*e.g.*, two, three, four, five or more) therapeutic molecules to a target. For example, the fusion protein can comprise a HEVP (*e.g.*, CD13, MME, ENPP1, or NRP1, or a fragment or a variant thereof), and a therapeutic compound (*e.g.*, peptide). In some aspects, the fusion protein comprises CD13 (or a fragment or variant thereof) and a therapeutic compound. In some aspects, the fusion protein comprises MME (or a fragment or variant thereof) and a therapeutic compound. In some aspects, the fusion protein comprises ENPP1 (or a fragment or variant thereof) and a therapeutic compound. In some aspects, the fusion protein comprises NRP1 (or a fragment or variant thereof) and a therapeutic compound. In some aspects, the therapeutic compound is fused directly to the HEVP. In some aspects, the therapeutic compound is anchored to the HEVP via a linker (*e.g.*, those disclosed herein).

**[0137]** In some aspects, the linker is a peptide linker. In some aspects, the peptide linker can comprise at least about two, at least about three, at least about four, at least about five, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least

about 35, at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95, or at least about 100 amino acids.

**[0138]** In some aspects, the peptide linker is synthetic, *i.e.*, non-naturally occurring. In some aspects, a peptide linker includes peptides (or polypeptides) (*e.g.*, natural or non-naturally occurring peptides) which comprise an amino acid sequence that links or genetically fuses a first linear sequence of amino acids to a second linear sequence of amino acids to which it is not naturally linked or genetically fused in nature. For example, in some aspects the peptide linker can comprise non-naturally occurring polypeptides which are modified forms of naturally occurring polypeptides (*e.g.*, comprising a mutation such as an addition, substitution or deletion).

**[0139]** Linkers can be susceptible to cleavage ("cleavable linker") thereby facilitating release of the exogenous biologically active molecule (*e.g.*, targeting moiety, therapeutic molecule, adjuvant, or immune modulator).

**[0140]** In some aspects, the linker is a "reduction-sensitive linker." In some aspects, the reduction-sensitive linker contains a disulfide bond. In some aspects, the linker is an "acid labile linker." In some aspects, the acid labile linker contains hydrazone. Suitable acid labile linkers also include, for example, a cis-aconitic linker, a hydrazide linker, a thiocarbamoyl linker, or any combination thereof.

**[0141]** In some aspects, the linker comprises a non-cleavable linker.

**[0142]** In some aspects, the therapeutic peptide is selected from the group consisting of a natural peptide, a recombinant peptide, a synthetic peptide, or a linker to a therapeutic compound. The therapeutic compound can be nucleotides, amino acids, lipids, carbohydrates, or small molecules. The therapeutic peptide can be an antibody, an enzyme, a ligand, a receptor, an antimicrobial peptide or a fragment or a variant thereof. In some embodiments, the therapeutic peptide is a nucleic acid binding protein. The nucleic acid binding protein can be Dicer, an Argonaute protein, TRBP, or MS2 bacteriophage coat protein. In some embodiments, the nucleic acid binding protein additionally comprises one or more RNA or DNA molecules. The one or more RNA can be a miRNA, siRNA, antisense oligonucleotide, phosphorodiamidate morpholino oligomer (PMO), peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO), guide RNA, lincRNA, mRNA, antisense RNA, dsRNA, or combinations thereof.

**[0143]** In some embodiments, the therapeutic peptide is a part of a protein-protein interaction system. In some embodiments, the protein-protein interaction system comprises an

FRB-FKBP interaction system, *e.g.*, the FRB-FKBP interaction system as described in Banaszynski *et al.*, J Am Chem Soc. 2005 Apr 6;127(13):4715-21.

**[0144]** In some aspects, a therapeutic molecule which can be anchored to a HEVP and expressed on an EV (*e.g.*, exosome) comprises an antigen. In certain aspects, the antigen comprises a tumor antigen. Non-limiting examples of tumor antigens include: alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), epithelial tumor antigen (ETA), mucin 1 (MUC1), Tn-MUC1, mucin 16 (MUC16), tyrosinase, melanoma-associated antigen (MAGE), tumor protein p53 (p53), CD4, CD8, CD45, CD80, CD86, programmed death ligand 1 (PD-L1), programmed death ligand 2 (PD-L2), NY-ESO-1, PSMA, TAG-72, HER2, GD2, cMET, EGFR, Mesothelin, VEGFR, alpha-folate receptor, CE7R, IL-3, Cancer-testis antigen (CTA), MART-1 gp100, TNF-related apoptosis-inducing ligand, Brachyury (preferentially expressed antigen in melanoma (PRAME)), or combinations thereof. In further aspects, an antigen can comprise a neoantigen. As used herein, the term "**neoantigen**," refers to antigens encoded by tumor-specific mutated genes. In some aspects, the antigen is derived from a bacterium, a virus, fungus, protozoa, or any combination thereof. In some aspects, the antigen is derived from an oncogenic virus. In further aspects, the antigen is derived from a group comprising: a Human Gamma herpes virus 4 (Epstein Barr virus), influenza A virus, influenza B virus, cytomegalovirus, staphylococcus aureus, mycobacterium tuberculosis, chlamydia trachomatis, HIV-1, HIV-2, corona viruses (*e.g.*, MERS-CoV and SARS CoV), filoviruses (*e.g.*, Marburg and Ebola), Streptococcus pyogenes, Streptococcus pneumoniae, Plasmodia species (*e.g.*, vivax and falciparum), Chikungunya virus, Human Papilloma virus (HPV), Hepatitis B, Hepatitis C, human herpes virus 8, herpes simplex virus 2 (HSV2), Klebsiella sp., Pseudomonas aeruginosa, Enterococcus sp., Proteus sp., Enterobacter sp., Actinobacter sp., coagulase-negative staphylococci (CoNS), Mycoplasma sp., or combinations thereof.

**[0145]** In some aspects, a therapeutic molecule comprises an immunosuppressive agent. Accordingly, in certain aspects, an EV disclosed herein comprises a HEVP and an immunosuppressive agent.

**[0146]** Non-limiting examples of other suitable therapeutic molecules include pharmacologically active drugs and genetically active molecules, including antineoplastic agents, anti-inflammatory agents, hormones or hormone antagonists, ion channel modifiers, and neuroactive agents. Examples of suitable payloads of therapeutic agents include those described in, "The Pharmacological Basis of Therapeutics," Goodman and Gilman, McGraw-Hill, New

York, N.Y., (1996), Ninth edition, under the sections: Drugs Acting at Synaptic and Neuroeffector Junctional Sites; Drugs Acting on the Central Nervous System; Autacoids: Drug Therapy of Inflammation; Water, Salts and Ions; Drugs Affecting Renal Function and Electrolyte Metabolism; Cardiovascular Drugs; Drugs Affecting Gastrointestinal Function; Drugs Affecting Uterine Motility; Chemotherapy of Parasitic Infections; Chemotherapy of Microbial Diseases; Chemotherapy of Neoplastic Diseases; Drugs Used for Immunosuppression; Drugs Acting on Blood-Forming organs; Hormones and Hormone Antagonists; Vitamins, Dermatology; and Toxicology, all incorporated herein by reference. Suitable payloads further include toxins, and biological and chemical warfare agents, for example see Somani, S. M. (ed.), Chemical Warfare Agents, Academic Press, New York (1992)).

**[0147]** In some aspects, a therapeutic molecule comprises a self-antigen. As used herein, the term "**self-antigen**" refers to an antigen that is expressed by a host cell or tissue. Under normal healthy state, such antigens are recognized by the body as self and do not elicit an immune response. However, under certain diseased conditions, a body's own immune system can recognize self-antigens as foreign and mount an immune response against them, resulting in autoimmunity. In certain aspects, EVs, *e.g.*, exosomes, of the present disclosure can comprise a self-antigen (*i.e.*, the self (germline) protein to which T cell responses have been induced and resulted in autoimmunity). Such EVs, *e.g.*, exosomes, can be used to target the autoreactive T cells and suppress their activity. Non-limiting examples of self-antigens (including the associated disease or disorder) include: beta-cell proteins (type I diabetes), myelin oligodendrocyte glycoprotein (MOG, multiple sclerosis), synovial proteins (rheumatoid arthritis), or combinations thereof.

**[0148]** In some aspects, the therapeutic molecule comprises an antibody or antigen-binding fragment thereof. In some aspects, the therapeutic molecule comprises at least 2, at least 3, at least 4, or at least 5 antibodies or antigen-binding fragments thereof. In some aspects, the antibody or antigen-binding fragment thereof comprises a scFv, scFab, scFab-Fc, nanobody, or any combination thereof. In some aspects, the antibody or antigen-binding fragment thereof comprises an agonist antibody, blocking antibody, a targeting antibody, a fragment thereof, or a combination thereof. In some aspects, the agonist antibody is a CD40L agonist. In some aspects, the blocking antibody binds a target protein selected from programmed death 1 (PD-1), programmed death ligand 1 (PD-L1), cytotoxic T-lymphocyte-associated protein 4, and any combination thereof. In some aspects, the EV, *e.g.*, exosome, comprises an anti-IL12 antibody or

an antigen-binding fragment thereof and an anti-CD40L antibody or antigen-binding fragment thereof.

**[0149]** The fusion proteins can be targeted to the surface of EVs (*e.g.*, exosomes) and provide a therapeutic activity to the EV (*e.g.*, exosome). In some embodiments, the fusion protein does not comprise IGSF8 or a fragment or modification thereof.

**[0150]** In some embodiments, fusion proteins having a targeting moiety are used. For example, fusion proteins can comprise a HEVP (*e.g.*, CD13, MME, ENPP1, or NRP1, or a fragment or a variant thereof), and a targeting moiety. In some aspects, the fusion protein comprises CD13 and a targeting moiety. In some aspects, the fusion protein comprises MME and a targeting moiety. In some aspects, the fusion protein comprises ENPP1 and a targeting moiety. In some aspects, the fusion protein comprises NRP1 and a targeting moiety. The targeting moiety can be used for targeting the EV (*e.g.*, exosome) to a specific organ, tissue, or cell for a treatment using the EV (*e.g.*, exosome). In certain aspects, the targeting moiety binds to a marker (or target molecules) expressed on a cell or a population of cells. In certain aspects, the marker is expressed on multiple cell types, *e.g.*, all antigen-present cells (*e.g.*, dendritic cells, macrophages, and B lymphocytes). In some aspects, the marker is expressed only on a specific population of cells (*e.g.*, dendritic cells). Non-limiting examples of markers that are expressed on specific population of cells (*e.g.*, dendritic cells) include a C-type lectin domain family 9 member A (CLEC9A) protein, a dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), CD207, CD40, Clec6, dendritic cell immunoreceptor (DCIR), DEC-205, lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), MARCO, Clec12a, DC-asialoglycoprotein receptor (DC-ASGPR), DC immunoreceptor 2 (DCIR2), Dectin-1, macrophage mannose receptor (MMR), BDCA-1 (CD303, Clec4c), Dectin-2, Bst-2 (CD317), or any combination thereof.

**[0151]** In some embodiments, the targeting moiety is an antibody or antigen-binding fragment thereof. Antibodies and antigen-binding fragments thereof include whole antibodies, polyclonal, monoclonal and recombinant antibodies, fragments thereof, and further includes single-chain antibodies, humanized antibodies, murine antibodies, chimeric, mouse-human, mouse-primate, primate-human monoclonal antibodies, anti-idiotypic antibodies, antibody fragments, such as, *e.g.*, scFv, (scFv)<sub>2</sub>, Fab, Fab', and F(ab')<sub>2</sub>, F(ab)<sub>2</sub>, Fv, dAb, and Fd fragments, diabodies, and antibody-related polypeptides. Antibodies and antigen-binding

fragments thereof also includes bispecific antibodies and multispecific antibodies so long as they exhibit the desired biological activity or function.

**[0152]** In some aspects, an EV (*e.g.*, exosome) described herein can comprise a HEVP (*e.g.*, CD13, MME, ENPP1, or NRP1, or a fragment or a variant thereof) and one or more exogenous biologically active molecules. In certain aspects, an exogenous biologically active molecule that can be expressed in an EV (*e.g.*, exosome) is an adjuvant. Accordingly, in some aspects, an EV (*e.g.*, exosome) comprises CD13 (or a fragment or variant thereof) and an adjuvant. In some aspects, an EV (*e.g.*, exosome) comprises MME (or a fragment or variant thereof) and an adjuvant. In some aspects, an EV (*e.g.*, exosome) comprises ENPP1 (or a fragment or variant thereof) and an adjuvant. In some aspects, an EV (*e.g.*, exosome) comprises NRP1 (or a fragment or variant thereof) and an adjuvant. In some aspects, EVs (*e.g.*, exosome) disclosed herein comprises two, three, four, five or more different adjuvants. As used herein, the term "**adjuvant**" refers to any substance that enhances the therapeutic effect of a cargo (*e.g.*, increasing an immune response to an antigen).

**[0153]** In some aspects, an adjuvant useful for the present disclosure induces the activation of a cytosolic pattern recognition receptor. Non-limiting examples of cytosolic pattern recognition receptor includes: stimulator of interferon genes (STING), retinoic acid-inducible gene I (RIG-1), Melanoma Differentiation-Associated protein 5 (MDA5), Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing (NLRP), inflammasomes, or combinations thereof. In certain aspects, an adjuvant is a STING agonist. Stimulator of Interferon Genes (STING) is a cytosolic sensor of cyclic dinucleotides that is typically produced by bacteria. Upon activation, it leads to the production of type I interferons and initiates an immune response. In certain aspects, the STING agonist comprises a cyclic dinucleotide STING agonist or a non-cyclic dinucleotide STING agonist.

**[0154]** In some aspects, an adjuvant comprises a toll-like receptor (TLR) agonist. Non-limiting examples of TLR agonists include: TLR2 agonist (*e.g.*, lipoteichoic acid, atypical LPS, MALP-2 and MALP-404, OspA, porin, LcrV, lipomannan, GPI anchor, lysophosphatidylserine, lipophosphoglycan (LPG), glycerophosphatidylinositol (GPI), zymosan, hsp60, gH/gL glycoprotein, hemagglutinin), a TLR3 agonist (*e.g.*, double-stranded RNA, *e.g.*, poly(I:C)), a TLR4 agonist (*e.g.*, lipopolysaccharides (LPS), lipoteichoic acid,  $\beta$ -defensin 2, fibronectin EDA, HMGB1, snapin, tenascin C), a TLR5 agonist (*e.g.*, flagellin), a TLR6 agonist, a TLR7/8 agonist (*e.g.*, single-stranded RNA, CpG-A, Poly G10, Poly G3, Resiquimod), a TLR9 agonist (*e.g.*,

unmethylated CpG DNA), and combinations thereof. Non-limiting examples of TLR agonists can be found at WO2008115319A2, US20130202707A1, US20120219615A1, US20100029585A1, WO2009030996A1, WO2009088401A2, and WO2011044246A1, each of which is incorporated by reference in its entirety.

**[0155]** In some embodiments, the fusion protein does not comprise IGSF8 or a fragment or modification thereof.

**[0156]** In some aspects, an EV (*e.g.*, exosome) described herein can comprise a HEVP (*e.g.*, CD13, MME, ENPP1, or NRP1, or a fragment or a variant thereof) and one or more exogenous biologically active molecules, wherein the one or more exogenous biologically active molecules comprise one or more (*e.g.*, two, three, four, five or more) immune modulators. In some aspects, an EV (*e.g.*, exosome) comprises CD13 (or a fragment or variant thereof) and an immune modulator. In some aspects, an EV (*e.g.*, exosome) comprises MME (or a fragment or variant thereof) and an immune modulator. In some aspects, an EV (*e.g.*, exosome) comprises ENPP1 (or a fragment or variant thereof) and an immune modulator. In some aspects, an EV (*e.g.*, exosome) comprises NRP1 (or a fragment or variant thereof) and an immune modulator. In certain aspects, the one or more immune modulators are expressed in combination with other exogenous biologically active molecules disclosed herein (*e.g.*, targeting moiety, therapeutic molecule, and/or adjuvant).

**[0157]** In some aspects, an immune modulator comprises an inhibitor for a negative checkpoint regulator or an inhibitor for a binding partner of a negative checkpoint regulator. In certain aspects, the negative checkpoint regulator comprises cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), lymphocyte-activated gene 3 (LAG-3), T-cell immunoglobulin mucin-containing protein 3 (TIM-3), B and T lymphocyte attenuator (BTLA), T cell immunoreceptor with Ig and ITIM domains (TIGIT), V-domain Ig suppressor of T cell activation (VISTA), adenosine A2a receptor (A2aR), killer cell immunoglobulin like receptor (KIR), indoleamine 2,3-dioxygenase (IDO), CD20, CD39, CD73, or any combination thereof.

**[0158]** In some aspects, the immune modulator is an inhibitor of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). In certain aspects, the CTLA-4 inhibitor is a monoclonal antibody of CTLA-4 ("anti-CTLA-4 antibody"). In certain aspects, the inhibitor is a fragment of a monoclonal antibody of CTLA-4. In certain aspects, the antibody fragment is a scFv, (scFv)<sub>2</sub>, Fab, Fab', and F(ab')<sub>2</sub>, F(ab1)<sub>2</sub>, Fv, dAb, or Fd of a monoclonal antibody of CTLA-4. In certain

aspects, the inhibitor is a nanobody, a bispecific antibody, or a multispecific antibody against CTLA-4. In some aspects, the anti-CTLA-4 antibody is ipilimumab. In other aspects, the anti-CTLA-4 antibody is tremelimumab.

**[0159]** In some aspects, the immune modulator is an inhibitor of programmed cell death protein 1 (PD-1). In some aspects, the immune modulator is an inhibitor of programmed death-ligand 1 (PD-L1). In some aspects, the immune modulator is an inhibitor of programmed death-ligand 2 (PD-L2). In certain aspects, the inhibitor of PD-1, PD-L1, or PD-L2 is a monoclonal antibody of PD-1 ("anti-PD-1 antibody"), PD-L1 ("anti-PD-L1 antibody"), or PD-L2 ("anti-PD-L2 antibody"). In some aspects, the inhibitor is a fragment of an anti-PD-1 antibody, anti-PD-L1 antibody, or anti-PD-L2 antibody. In certain aspects, the antibody fragment is a scFv, (scFv)<sub>2</sub>, Fab, Fab', and F(ab')<sub>2</sub>, F(ab1)<sub>2</sub>, Fv, dAb, or Fd of a monoclonal antibody of PD-1, PD-L1, or PD-L2. In certain aspects, the inhibitor is a nanobody, a bispecific antibody, or a multispecific antibody against PD-1, PD-L1, or PD-L2. In some aspects, the anti-PD-1 antibody is nivolumab. In some aspects, the anti-PD-1 antibody is pembrolizumab. In some aspects, the anti-PD-1 antibody is pidilizumab. In some aspects, the anti-PD-L1 antibody is atezolizumab. In other aspects, the anti-PD-L1 antibody is avelumab.

**[0160]** In some aspects, the immune modulator is an inhibitor of lymphocyte-activated gene 3 (LAG3). In certain aspects, the inhibitor of LAG3 is a monoclonal antibody of LAG3 ("anti-LAG3 antibody"). In some aspects, the inhibitor is a fragment of an anti-LAG3 antibody, *e.g.*, scFv, (scFv)<sub>2</sub>, Fab, Fab', and F(ab')<sub>2</sub>, F(ab1)<sub>2</sub>, Fv, dAb, or Fd. In certain aspects, the inhibitor is a nanobody, a bispecific antibody, or a multispecific antibody against LAG3.

**[0161]** In some aspects, the immune modulator is an inhibitor of T-cell immunoglobulin mucin-containing protein 3 (TIM-3). In some aspects, the immune modulator is an inhibitor of B and T lymphocyte attenuator (BTLA). In some aspects, the immune modulator is an inhibitor of T cell immunoreceptor with Ig and ITIM domains (TIGIT). In some aspects, the immune modulator is an inhibitor of V-domain Ig suppressor of T cell activation (VISTA). In some aspects, the immune modulator is an inhibitor of adenosine A2a receptor (A2aR). In some aspects, the immune modulator is an inhibitor of killer cell immunoglobulin like receptor (KIR). In some aspects, the immune modulator is an inhibitor of indoleamine 2,3-dioxygenase (IDO). In some aspects, the immune modulator is an inhibitor of CD20, CD39, or CD73.

**[0162]** In some aspects, the immune modulator comprises an activator for a positive co-stimulatory molecule or an activator for a binding partner of a positive co-stimulatory molecule.

In certain aspects, the positive co-stimulatory molecule comprises a TNF receptor superfamily member (*e.g.*, CD120a, CD120b, CD18, OX40, CD40, Fas receptor, M68, CD27, CD30, 4-1BB, TRAILR1, TRAILR2, TRAILR3, TRAILR4, RANK, OCIF, TWEAK receptor, TACI, BAFF receptor, ATAR, CD271, CD269, AITR, TROY, CD358, TRAMP, and XEDAR). In some aspects, the activator for a positive co-stimulatory molecule is a TNF superfamily member (*e.g.*, TNF $\alpha$ , TNF-C, OX40L, CD40L, FasL, LIGHT, TL1A, CD27L, Siva, CD153, 4-1BB ligand, TRAIL, RANKL, TWEAK, APRIL, BAFF, CAMLG, NGF, BDNF, NT-3, NT-4, GITR ligand, and EDA-2).

**[0163]** In some aspects, the immune modulator is an activator of TNF Receptor Superfamily Member 4 (OX40). In certain aspects, the activator of OX40 is an agonistic anti-OX40 antibody. In further aspects, the activator of OX40 is a OX40 ligand (OX40L).

**[0164]** In some aspects, the immune modulator is an activator of CD27. In certain aspects, the activator of CD27 is an agonistic anti-CD27 antibody. In other aspects, the activator of CD27 is a CD27 ligand (CD27L).

**[0165]** In some aspects, the immune modulator is an activator of CD40. In certain aspects, the activator of CD40 is an agonistic anti-CD40 antibody. In some aspects, the activator of CD40 is a CD40 ligand (CD40L). In certain aspects, the CD40L is a monomeric CD40L. In other aspects, the CD40L is a trimeric CD40L.

**[0166]** In some aspects, the immune modulator is an activator of glucocorticoid-induced TNFR-related protein (GITR). In certain aspects, the activator of GITR is an agonistic anti-GITR antibody. In other aspects, the activator of GITR is a natural ligand of GITR.

**[0167]** In some aspects, the immune modulator is an activator of 4-1BB. In specific aspects, the activator of 4-1BB is an agonistic anti-4-1BB antibody. In certain aspects, the activator of 4-1BB is a natural ligand of 4-1BB.

**[0168]** In some aspects, the immune modulator is a Fas receptor (Fas). In such aspects, the Fas receptor is displayed on the surface of the EV, *e.g.*, exosome. In some aspects, the immune modulator is Fas ligand (FasL). In certain aspects, the Fas ligand is displayed on the surface of the EV, *e.g.*, exosome. In some aspects, the immune modulator is an anti-Fas antibody or an anti-FasL antibody.

**[0169]** In some aspects, the immune modulator is an activator of a CD28-superfamily co-stimulatory molecule. In certain aspects, the CD28-superfamily co-stimulatory molecule is ICOS or CD28. In certain aspects, the immune modulator is ICOSL, CD80, or CD86.

[0170] In some aspects, the immune modulator is an activator of inducible T cell co-stimulator (ICOS). In certain aspects, the activator of ICOS is an agonistic anti-ICOS antibody. In other aspects, the activator of ICOS is a ICOS ligand (ICOSL).

[0171] In some aspects, the immune modulator is an activator of CD28. In some aspects, the activator of CD28 is an agonistic anti-CD28 antibody. In other aspects, the activator of CD28 is a natural ligand of CD28. In certain aspects, the ligand of CD28 is CD80.

[0172] In some aspects, the immune modulator comprises a cytokine or a binding partner of a cytokine. In certain aspects, the cytokine comprises IL-2, IL-4, IL-7, IL-10, IL-12, IL-15, IL-21, or IFN- $\gamma$ . In some aspects, the immune modulator comprises FLT-3 (CD135).

[0173] In some aspects, the immune modulator comprises a protein that supports intracellular interactions required for germinal center responses. In certain aspects, such a protein comprises a signaling lymphocyte activation molecule (SLAM) family member or a SLAM-associated protein (SAP). In some aspects, a SLAM family members comprises SLAM, CD48, CD229 (Ly9), Ly108, 2B4, CD84, NTB-A, CRACC, BLAME, CD2F-10, or combinations thereof.

[0174] In some aspects, the immune modulator comprises a T-cell receptor (TCR) or a derivative thereof. In certain aspects, the immune modulator is a TCR  $\alpha$ -chain or a derivative thereof. In other aspects, the immune modulator is a TCR  $\beta$ -chain or a derivative thereof. In further aspects, the immune modulator is a co-receptor of the T-cell or a derivative thereof.

[0175] In some aspects, the immune modulator comprises a chimeric antigen receptor (CAR) or a derivative thereof. In certain aspects, the CAR binds to one or more of the therapeutic molecules disclosed herein (*e.g.*, tumor antigen, *e.g.*, alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), epithelial tumor antigen (ETA), mucin 1 (MUC1), Tn-MUC1, mucin 16 (MUC16), tyrosinase, melanoma-associated antigen (MAGE), tumor protein p53 (p53), CD4, CD8, CD45, CD80, CD86, programmed death ligand 1 (PD-L1), programmed death ligand 2 (PD-L2), NY-ESO-1, PSMA, TAG-72, HER2, GD2, cMET, EGFR, Mesothelin, VEGFR, alpha-folate receptor, CE7R, IL-3, Cancer-testis antigen, MART-1 gp100, and TNF-related apoptosis-inducing ligand).

[0176] In certain aspects, the immune modulator is an activator of CD28. In certain aspects, the activator is a fragment of a monoclonal antibody of CD28. In certain aspects, the antibody fragment is a scFv, (scFv)<sub>2</sub>, Fab, Fab', and F(ab')<sub>2</sub>, F(ab1)<sub>2</sub>, Fv, dAb, or Fd of a

monoclonal antibody of CD28. In certain aspects, the activator is a nanobody, a bispecific antibody, or a multispecific antibody against CD28.

**[0177]** In some aspects, the immune modulator comprises a NF- $\kappa$ B inhibitor. Non-limiting examples of NF- $\kappa$ B inhibitors that can be used with the present disclosure includes: IKK complex inhibitors (*e.g.*, TPCA-1, NF- $\kappa$ B Activation Inhibitor VI (BOT-64), BMS 345541, Amlexanox, SC-514 (GK 01140), IMD 0354, IKK-16), I $\kappa$ B degradation inhibitor (*e.g.*, BAY 11-7082, MG-115, MG-132, Lactacystin, Epoxomicin, Parthenolide, Carfilzomib, MLN-4924 (Pevonedistat)), NF- $\kappa$ B nuclear translocation inhibitor (*e.g.*, JSH-23, Rolipram), p65 acetylation inhibitor (*e.g.*, Gallic acid, Anacardic acid), NF- $\kappa$ B-DNA binding inhibitor (*e.g.*, GYY 4137, p-XSC, CV 3988, Prostaglandin E2 (PGE2)), NF- $\kappa$ B transactivation inhibitor (*e.g.*, LY 294002, Wortmannin, Mesalamine), or combinations thereof. *See also* Gupta, S.C., *et al.*, *Biochim Biophys Acta* 1799:775-787 (2010), which is herein incorporated by reference in its entirety. In further aspects, an immune modulator comprises a COX-2 inhibitor, mTOR inhibitor (*e.g.*, rapamycin and derivatives), prostaglandins, nonsteroidal anti-inflammatory agents (NSAIDS), antileukotriene, or combinations thereof.

**[0178]** In some aspects, the immune modulator is an agonist. In certain aspects, the agonist is an endogenous agonist, such as a hormone, or a neurotransmitter. In other aspects, the agonist is an exogenous agonist, such as a drug. In some aspects, the agonist is a physical agonist, which can create an agonist response without binding to the receptor. In some aspects, the agonist is a superagonist, which can produce a greater maximal response than the endogenous agonist. In certain aspects, the agonist is a full agonist with full efficacy at the receptor. In other aspects, the agonist is a partial agonist having only partial efficacy at the receptor relative to a full agonist. In some aspects, the agonist is an inverse agonist that can inhibit the constitutive activity of the receptor. In some aspects, the agonist is a co-agonist that works with other co-agonists to produce an effect on the receptor. In certain aspects, the agonist is an irreversible agonist that binds permanently to a receptor through formation of covalent bond. In certain aspects, the agonist is selective agonist for a specific type of receptor

**[0179]** In some aspects, the immune modulator is an antagonist. In specific aspects, the antagonist is a competitive antagonist, which reversibly binds to the receptor at the same binding site as the endogenous ligand or agonist without activating the receptor. Competitive antagonist can affect the amount of agonist necessary to achieve a maximal response. In other aspects, the antagonist is a non-competitive antagonist, which binds to an active site of the receptor or an

allosteric site of the receptor. Non-competitive antagonist can reduce the magnitude of the maximum response that can be attained by any amount of agonist. In further aspects, the antagonist is an uncompetitive antagonist, which requires receptor activation by an agonist before its binding to a separate allosteric binding site.

**[0180]** In some aspects, the immune modulator comprises an antibody or an antigen-binding fragment. The immune modulator can be a full length protein or a fragment thereof. The antibody or antigen-binding fragment can be derived from natural sources, or partly or wholly synthetically produced. In some aspects, the antibody is a monoclonal antibody. In some of these aspects, the monoclonal antibody is an IgG antibody. In certain aspects, the monoclonal antibody is an IgG1, IgG2, IgG3, or IgG4. In some other aspects, the antibody is a polyclonal antibody. In certain aspects, the antigen-binding fragment is selected from Fab, Fab', and F(ab')<sub>2</sub>, F(ab1)<sub>2</sub>, Fv, dAb, and Fd fragments. In certain aspects, the antigen-binding fragment is an scFv or (scFv)<sub>2</sub> fragment. In certain other aspects, the antibody or antigen-binding fragment is a NANOBODY<sup>®</sup> (single-domain antibody). In some aspects, the antibody or antigen-binding fragment is a bispecific or multispecific antibody.

**[0181]** In various aspects, the antibody or antigen-binding fragment is fully human. In some aspects, the antibody or antigen-binding fragment is humanized. In some aspects, the antibody or antigen-binding fragment is chimeric. In some of these aspects, the chimeric antibody has non-human V region domains and human C region domains. In some aspects, the antibody or antigen-binding fragment is non-human, such as murine or veterinary.

**[0182]** In certain aspects, the immune modulator is a polynucleotide. In some of these aspects, the polynucleotide includes, but is not limited to, an mRNA, a miRNA, an siRNA, antisense oligonucleotide, a phosphorodiamidate morpholino oligomer (PMO), a peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO), an antisense RNA, an shRNA, a lncRNA, and a dsDNA. In some aspects, the polynucleotide is an RNA (*e.g.*, an mRNA, a miRNA, an siRNA, an antisense RNA, an shRNA, or an lncRNA). In some of these aspects, when the polynucleotide is an mRNA, it can be translated into a desired polypeptide. In some aspects, the polynucleotide is a microRNA (miRNA) or pre-miRNA molecule. In some of these aspects, the miRNA is delivered to the cytoplasm of the target cell, such that the miRNA molecule can silence a native mRNA in the target cell. In some aspects, the polynucleotide is a small interfering RNA (siRNA) or a short hairpin RNA (shRNA) capable of interfering with the expression of an oncogene or other dysregulating polypeptides. In some of these aspects, the

siRNA is delivered to the cytoplasm of the target cell, such that the siRNA molecule can silence a native mRNA in the target cell. In some aspects, the polynucleotide is an antisense RNA that is complementary to an mRNA. In some aspects, the polynucleotide is a long non-coding RNA (lncRNA) capable of regulating gene expression and modulating diseases. In some aspects, the polynucleotide is a DNA that can be transcribed into an RNA. In some of these aspects, the transcribed RNA can be translated into a desired polypeptide.

**[0183]** In some aspects, the immune modulator is a protein, a peptide, a glycolipid, or a glycoprotein.

**[0184]** In some embodiments, the EVs (*e.g.*, surface-engineered exosomes) described herein demonstrate superior characteristics compared to EVs (*e.g.*, surface-engineered exosomes) known in the art. For example, EVs (*e.g.*, surface-engineered exosomes) produced by using the HEVPs provided herein contain modified proteins that are more highly enriched on their surface than EVs (*e.g.*, exosomes) in the prior art, *e.g.*, those produced using conventional exosome proteins. In some aspects, the expression level of the modified proteins is increased (*i.e.*, enriched) by at least about 5%, at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 100%, at least about 150%, at least about 200%, or at least about 300% or more, compared to the expression of the corresponding protein using conventional exosome proteins.

**[0185]** Moreover, in some aspects, the EVs (*e.g.*, surface-engineered exosomes) of the present disclosure can have greater, more specific, or more controlled biological activity compared to EVs (*e.g.*, surface-engineered exosomes) known in the art. For example, a surface engineered EV (*e.g.*, exosome) comprising a therapeutic or biologically relevant exogenous sequence fused to an HEVP protein or a fragment thereof described herein (*e.g.*, CD13, MME, ENPP1, or NRP1 or a fragment thereof) can have more of the desired engineered characteristics than fusion to scaffolds known in the art. Scaffold proteins known in the art include tetraspanin molecules (*e.g.*, CD63, CD81, CD9 and others), lysosome-associated membrane protein 2 (LAMP2 and LAMP2B), platelet-derived growth factor receptor (PDGFR), GPI anchor proteins, lactadherin and fragments thereof, and peptides that have affinity to any of these proteins or fragments thereof. Previously, overexpression of exogenous proteins relied on stochastic or random disposition of the exogenous proteins onto the EV (*e.g.*, exosome) for producing surface-engineered EVs (*e.g.*, exosomes). This resulted in low-level, unpredictable density of the

exogenous proteins on the EVs (*e.g.*, exosomes). Thus, the HEVP proteins and fragments thereof described herein provide important advancements in novel EV (*e.g.*, exosome) compositions and methods of making the same.

**[0186]** In some embodiments, the surface-engineered EV (*e.g.*, exosome) comprising a fusion protein containing an exogenous sequence (*e.g.*, encoding an exogenous biologically active molecule, *e.g.*, antigen, adjuvant, targeting moiety, and/or immune modulator) and an HEVP identified herein has a higher density of the fusion protein than similarly engineered EVs (*e.g.*, exosomes) comprising an exogenous sequence conjugated to a conventional EV (*e.g.*, exosome) protein known in the art (*e.g.*, CD9, CD63, CD81, PDGFR, GPI anchor proteins, lactadherin LAMP2, and LAMP2B, a fragment thereof, or a peptide that binds thereto). In some embodiments, the fusion protein containing an HEVP as described herein is present at about 2-, about 4-, about 8-, about 16-, about 32-, about 64-, about 100-, about 200-, about 400-, about 800-, about 1,000-fold or a higher density on the EV (*e.g.*, exosome) surface than fusion proteins on other EV (*e.g.*, exosome) surfaces similarly modified using a conventional EV (*e.g.*, exosome) protein. In some embodiments, the fusion protein containing an HEVP as described herein is present at about 2 to about 4-fold, about 4 to about 8-fold, about 8 to about 16-fold, about 16 to about 32-fold, about 32 to about 64-fold, about 64 to about 100-fold, about 100 to about 200-fold, about 200 to about 400-fold, about 400 to about 800-fold, about 800 to about 1,000-fold or to a higher density on the EV (*e.g.*, exosome) surface than fusion proteins on other EV (*e.g.*, exosome) surfaces similarly modified using a conventional EV (*e.g.*, exosome) protein.

**[0187]** In some embodiments, a fusion protein of CD13, MME, ENPP1, or NRP1, a variant, a fragment, a variant of a fragment or a modification thereof is present at about 2-, about 4-, about 8-, about 16-, about 32-, about 64-, about 100-, about 200-, about 400-, about 800-, about 1,000-fold or a higher density on the EV (*e.g.*, exosome) surface than fusion proteins on other EV (*e.g.*, exosome) surfaces similarly modified using CD9. In some embodiments, a fusion protein of CD13, MME, ENPP1, or NRP1, a variant, a fragment, a variant of a fragment or a modification thereof is present at about 2-, about 4-, about 8-, about 16-, about 32-, about 64-, about 100-, about 200-, about 400-, about 800-, about 1,000-fold or a higher density on the EV (*e.g.*, exosome) surface than fusion proteins on other EVs (*e.g.*, exosome) surfaces similarly modified using CD63. In some embodiments, a fusion protein of CD13, MME, ENPP1, or NRP1, a variant, a fragment, a variant of a fragment or a modification thereof is present at about 2-, about 4-, about 8-, about 16-, about 32-, about 64-, about 100-, about 200-, about 400-, about

800-, about 1,000-fold or a higher density on the EV (*e.g.*, exosome) surface than fusion proteins on other EV (*e.g.*, exosome) surfaces similarly modified using CD81. In some embodiments, a fusion protein of CD13, MME, ENPP1, or NRP1, a variant, a fragment, a variant of a fragment or a modification thereof is present at about 2-, about 4-, about 8-, about 16-, about 32-, about 64-, about 100-, about 200-, about 400-, about 800-, about 1,000-fold or a higher density on the EV (*e.g.*, exosome) surface than fusion proteins on other EV (*e.g.*, exosome) surfaces similarly modified using PDGFR. In some embodiments, a fusion protein of CD13, MME, ENPP1, or NRP1, a variant, a fragment, a variant of a fragment or a modification thereof is present at about 2-, about 4-, about 8-, about 16-, about 32-, about 64-, about 100-, about 200-, about 400-, about 800-, about 1,000-fold or a higher density on the EV (*e.g.*, exosome) surface than fusion proteins on other EV (*e.g.*, exosome) surfaces similarly modified using GPI anchor proteins. In some embodiments, a fusion protein of CD13, MME, ENPP1, or NRP1, a variant, a fragment, a variant of a fragment or a modification thereof is present at about 2-, about 4-, about 8-, about 16-, about 32-, about 64-, about 100-, about 200-, about 400-, about 800-, about 1,000-fold or a higher density on the EV (*e.g.*, exosome) surface than fusion proteins on other EV (*e.g.*, exosome) surfaces similarly modified using lactadherin. In some embodiments, a fusion protein of CD13, MME, ENPP1, or NRP1, a variant, a fragment, a variant of a fragment or a modification thereof is present at about 2-, about 4-, about 8-, about 16-, about 32-, about 64-, about 100-, about 200-, about 400-, about 800-, about 1,000-fold or a higher density on the EV (*e.g.*, exosome) surface than fusion proteins on other EV (*e.g.*, exosome) surfaces similarly modified using LAMP2. In some embodiments, a fusion protein of CD13, MME, ENPP1, or NRP1, a variant, a fragment, a variant of a fragment or a modification thereof is present at about 2-, about 4-, about 8-, about 16-, about 32-, about 64-, about 100-, about 200-, about 400-, about 800-, about 1,000-fold or a higher density on the EV (*e.g.*, exosome) surface than fusion proteins on other EV (*e.g.*, exosome) surfaces similarly modified using LAMP2B. In some embodiments, a fusion protein of CD13, MME, ENPP1, or NRP1, a variant, a fragment, a variant of a fragment or a modification thereof is present at about 2-, about 4-, about 8-, about 16-, about 32-, about 64-, about 100-, about 200-, about 400-, about 800-, about 1,000-fold or a higher density on the EV (*e.g.*, exosome) surface than fusion proteins on other EV (*e.g.*, exosome) surfaces similarly modified using a fragment of a conventional EV (*e.g.*, exosome) protein. In some embodiments, a fusion protein of CD13, MME, ENPP1, or NRP1, a variant, a fragment, a variant of a fragment or a modification thereof is present at about 2-, about 4-, about 8-, about 16-, about 32-, about 64-

, about 100-, about 200-, about 400-, about 800-, about 1,000-fold or a higher density on the EV (*e.g.*, exosome) surface than fusion proteins on other EV (*e.g.*, exosome) surfaces similarly modified using a variant of a conventional EV (*e.g.*, exosome) protein.

**[0188]** In particular embodiments, a fusion protein of CD13, MME, ENPP1, or NRP1, a variant, a fragment, a variant of a fragment or a modification thereof is present at about 2-, about 4-, about 8-, about 16-, about 32-, about 64-, about 100-, about 200-, about 400-, about 800-, about 1,000-fold or a higher density on the EV (*e.g.*, exosome) surface than fusion proteins on other EV (*e.g.*, exosome) surfaces similarly modified using a conventional EV (*e.g.*, exosome) protein (*e.g.*, a tetraspanin molecule, like CD63).

**[0189]** Fusion proteins provided herein can comprise a HEVP (*e.g.*, CD13, MME, ENPP1, or NRP1, or a fragment or a variant thereof), and an additional peptide (*e.g.*, exogenous biologically active molecules disclosed herein, such as antigen, targeting moiety, adjuvant, and/or immune modulator). The additional peptide can be attached to either the N terminus or the C terminus of the HEVP or a fragment or a variant thereof. The additional peptide can be located inside (in the luminal side) or outside of the EV (*e.g.*, exosome) attached to the HEVP.

**[0190]** In some embodiments, fusion proteins provided herein comprise a HEVP (*e.g.*, CD13, MME, ENPP1, or NRP1, or a fragment or a variant thereof), and two additional peptides (*e.g.*, exogenous biologically active molecules disclosed herein, such as antigen, targeting moiety, adjuvant, and/or immune modulator). For instance, in some aspects, a fusion protein comprises CD13 (or a fragment or variant thereof) and two additional peptides. In some aspects, a fusion protein comprises MME (or a fragment or variant thereof) and two additional peptides. In some aspects, a fusion protein comprises ENPP1 (or a fragment or variant thereof) and two additional peptides. In some aspects, a fusion protein comprises NRP1 (or a fragment or variant thereof) and two additional peptides. Both of the two additional peptides can be attached to either the N terminus or the C terminus of the HEVP or a fragment or a variant thereof. In some embodiments, one of the two additional peptides is attached to the N terminus and the other of the two additional peptides is attached to the C terminus of the HEVP or a fragment or a variant thereof. The additional peptides can be located inside (in the luminal side) or outside of the EV (*e.g.*, exosome) attached to the HEVP, or both.

#### **7.5. Producer Cell For Production Of Surface-Engineered EVs (*e.g.*, exosomes)**

**[0191]** EVs (*e.g.*, exosomes) of the present disclosure can be produced from a cell grown *in vitro* or a body fluid of a subject. When EVs (*e.g.*, exosomes) are produced from *in vitro* cell

culture, various producer cells, *e.g.*, HEK293 cells, Chinese hamster ovary (CHO) cells, mesenchymal stem cells (MSCs), HT-1080 cells, MB-231 cells, Raji cells, PER.C6 cells, and CAP cells can be used for the present disclosure. In some aspects, the producer cell is HEK293 cells. In some aspects, the producer is MSCs.

**[0192]** The producer cell can be genetically modified to comprise one or more exogenous sequences to produce surface-engineered EVs (*e.g.*, exosomes). In some aspects, the one or more exogenous sequences encode a HEVP disclosed herein. In some aspects, the one or more exogenous sequences encode an exogenous biologically active molecule disclosed herein (*e.g.*, antigen, targeting moiety, adjuvant, and/or immune modulator). In some aspects, the one or more exogenous sequences encode both a HEVP and an exogenous biologically active molecule disclosed herein. The genetically-modified producer cell can contain the exogenous sequence introduced by transient or stable transformation. The exogenous sequence can be introduced to the producer cell as a plasmid. The exogenous sequences can be stably integrated into a genomic sequence of the producer cell, at a targeted site or in a random site. In some embodiments, a stable cell line is generated for production of surface-engineered EVs (*e.g.*, exosomes).

**[0193]** In some aspects, a genetically modified producer cell disclosed herein expresses an endogenous level of HEVP. In such aspects, the exogenous sequences can be inserted into a genomic sequence of the producer cell, located within, upstream (5'-end) or downstream (3'-end) of an endogenous sequence encoding the HEVP. Various methods known in the art can be used for the introduction of the exogenous sequences into the producer cell. For example, cells modified using various gene editing methods (*e.g.*, methods using a homologous recombination, transposon-mediated system, loxP-Cre system, CRISPR/Cas9 or TALEN) are within the scope of the present disclosure.

**[0194]** The exogenous sequences can comprise a sequence encoding the HEVP or a variant or a fragment of the EV (*e.g.*, exosome) protein. An extra copy of the sequence encoding the HEVP can be introduced to produce a surface-engineered EV (*e.g.*, exosome) having the HEVP at a higher density. An exogenous sequence encoding a variant or a fragment of the HEVP can be introduced to produce a surface-engineered EV (*e.g.*, exosome) containing the modification or the fragment of the HEVP. An exogenous sequence encoding an affinity tag can be introduced to produce a surface-engineered EV (*e.g.*, exosome) containing a fusion protein comprising the affinity tag attached to the HEVP. As described herein, in some aspects, an exogenous sequence encoding an exogenous biologically active molecule (*e.g.*, antigen, targeting

moiety, adjuvant, and/or immune modulator) can be introduced to produce a surface-engineered EV (*e.g.*, exosome) containing a fusion protein comprising the exogenous biologically active molecule attached (*e.g.*, directly or via a linker) to the HEVP.

**[0195]** In some embodiments, a surface-engineered EV (*e.g.*, exosome) has a higher density of the HEVP than native EVs (*e.g.*, exosomes) isolated from the same or similar producer cell types. In some embodiments, the HEVP is present at about 2-, about 4-, about 8-, about 16-, about 32-, about 64-, about 100-, about 200-, about 400-, about 800-, about 1,000-fold or to a higher density on the surface-engineered EV (*e.g.*, exosome) than the native EV (*e.g.*, exosome). In some embodiments, the HEVP is present at about 2 to about 4-fold, about 4 to about 8-fold, about 8 to about 16-fold, about 16 to about 32-fold, about 32 to about 64-fold, about 64 to about 100-fold, about 100 to about 200-fold, about 200 to about 400-fold, about 400 to about 800-fold, about 800 to about 1,000-fold or to a higher density on the surface-engineered EV (*e.g.*, exosome) than the native EV (*e.g.*, exosome). In some embodiments, a fusion protein comprising the HEVP is present at about 2 to about 4-fold, about 4 to about 8-fold, about 8 to about 16-fold, about 16 to about 32-fold, about 32 to about 64-fold, about 64 to about 100-fold, about 100 to about 200-fold, about 200 to about 400-fold, about 400 to about 800-fold, about 800 to about 1,000-fold or to a higher density on the surface-engineered EV (*e.g.*, exosome) than the unmodified HEVP on the native EV (*e.g.*, exosome). In some embodiments, a fragment or a variant of the HEVP is present at about 2 to about 4-fold, about 4 to about 8-fold, about 8 to about 16-fold, about 16 to about 32-fold, about 32 to about 64-fold, about 64 to about 100-fold, about 100 to about 200-fold, about 200 to about 400-fold, about 400 to about 800-fold, about 800 to about 1,000-fold or to a higher density on the surface-engineered EV (*e.g.*, exosome) than the unmodified HEVP on the native EV (*e.g.*, exosome).

**[0196]** In particular embodiments, CD13, a fragment or variant of CD13, or a modification thereof is present at about 2 to about 4-fold, about 4 to about 8-fold, about 8 to about 16-fold, about 16 to about 32-fold, about 32 to about 64-fold, about 64 to about 100-fold, about 100 to about 200-fold, about 200 to about 400-fold, about 400 to about 800-fold, about 800 to about 1,000-fold or to a higher density on the surface-engineered EV (*e.g.*, exosome) than the unmodified CD13 on the native EV (*e.g.*, exosome). In particular embodiments, MME, a fragment or a variant of MME, or a modification thereof is present at about 2 to about 4-fold, about 4 to about 8-fold, about 8 to about 16-fold, about 16 to about 32-fold, about 32 to about 64-fold, about 64 to about 100-fold, about 100 to about 200-fold, about 200 to about 400-fold, about

400 to about 800-fold, about 800 to about 1,000-fold or to a higher density on the surface-engineered EV (*e.g.*, exosome) than the unmodified MME on the native EV (*e.g.*, exosome). In particular embodiments, ENPP1, a fragment or a variant of ENPP1, or a modification thereof is present at about 2 to about 4-fold, about 4 to about 8-fold, about 8 to about 16-fold, about 16 to about 32-fold, about 32 to about 64-fold, about 64 to about 100-fold, about 100 to about 200-fold, about 200 to about 400-fold, about 400 to about 800-fold, about 800 to about 1,000-fold or to a higher density on the surface-engineered EV (*e.g.*, exosome) than the unmodified ENPP1 on the native EV (*e.g.*, exosome). In particular embodiments, NRP1, a fragment or a variant of NRP1, or a modification thereof is present at about 2 to about 4-fold, about 4 to about 8-fold, about 8 to about 16-fold, about 16 to about 32-fold, about 32 to about 64-fold, about 64 to about 100-fold, about 100 to about 200-fold, about 200 to about 400-fold, about 400 to about 800-fold, about 800 to about 1,000-fold or to a higher density on the surface-engineered EV (*e.g.*, exosome) than the unmodified NRP1 on the native EV (*e.g.*, exosome).

**[0197]** In some embodiments, the producer cell is further modified to comprise an additional exogenous sequence. For example, an additional exogenous sequence can be introduced to modulate endogenous gene expression, or produce an EV (*e.g.*, exosome) including a certain polypeptide as a payload. In some embodiments, the producer cell is modified to comprise two exogenous sequences, one encoding the HEVP or a variant or a fragment of the HEVP, and the other encoding a payload. In some embodiments, the producer cell can be further modified to comprise an additional exogenous sequence conferring additional functionalities to EVs (*e.g.*, exosomes), for example, specific targeting capabilities, delivery functions, enzymatic functions, increased or decreased half-life *in vivo*, *etc.* In some embodiments, the producer cell is modified to comprise two exogenous sequences, one encoding the HEVP or a variant or a fragment of the EV (*e.g.*, exosome) protein, and the other encoding a protein conferring the additional functionalities to EVs (*e.g.*, exosomes).

**[0198]** In some embodiments, the producer cell is modified to comprise two exogenous sequences, each of the two exogenous sequences encoding a fusion protein on the EV (*e.g.*, exosome) surface. In some embodiments, a surface-engineered EV (*e.g.*, exosome) from the producer cell has a higher density of an HEVP compared to native EVs (*e.g.*, exosomes) isolated from an unmodified cell of the same or similar cell type. In some embodiments, surface-engineered EV (*e.g.*, exosome) contain an HEVP at a density about 2-, about 4-, about 8-, about 16-, about 32-, about 64-, about 100-, about 200-, about 400-, about 800-, about 1,000-fold or

higher than a native EV (*e.g.*, exosome) isolated from an unmodified cell of the same or similar cell type. In some embodiments, the producer cell is further modified to comprise one, two, three, four, five, six, seven, eight, nine, or ten or more additional exogenous sequences. As described herein, in some aspects, the exogenous sequences encode one or more HEVPs disclosed herein. In some aspects, the exogenous sequences encode one or more exogenous biologically active molecules disclosed herein (*e.g.*, antigen, targeting moiety, adjuvant, and/or immune modulator). In some aspects, the exogenous sequences encode both a HEVP and an exogenous biologically active molecule disclosed herein.

**[0199]** More specifically, surface-engineered EVs (*e.g.*, exosomes) can be produced from a cell transformed with a sequence encoding one or more HEVPs or a variant thereof including, but not limited to CD13, MME, ENPP1, and NRP1. Any of the one or more HEVPs described herein can be expressed in the producer cell from a plasmid, an exogenous sequence inserted into the genome or other exogenous nucleic acid such as a synthetic messenger RNA (mRNA).

**[0200]** In some embodiments, the one or more HEVPs is expressed in a cell transformed with an exogenous sequence encoding its full length, endogenous form. In some embodiments, such an exogenous sequence encodes CD13 protein of **SEQ ID NO: 47**. In some embodiments, such an exogenous sequence encodes MME protein of **SEQ ID NO: 48**. In some embodiments, such an exogenous sequence encodes ENPP1 protein of **SEQ ID NO: 49**. In some embodiments, such an exogenous sequence encodes NRP1 protein of **SEQ ID NO: 50**.

**[0201]** Surface-engineered EVs (*e.g.*, exosomes) can be produced from a cell transformed with a sequence encoding a fragment of one or more HEVPs including, but not limited to, CD13, MME, ENPP1, and NRP1. In some aspects, the EVs (*e.g.*, exosomes) are produced from a cell transformed with a sequence encoding a fragment of CD13. In some aspects, the EVs (*e.g.*, exosomes) are produced from a cell transformed with a sequence encoding a fragment of MME. In some aspects, the EVs (*e.g.*, exosomes) are produced from a cell transformed with a sequence encoding a fragment of ENPP1. In some aspects, the EVs (*e.g.*, exosomes) are produced from a cell transformed with a sequence encoding a fragment of NRP1. In some embodiments, the sequence encodes a fragment of the HEVP lacking at least about 5, about 10, about 50, about 100, about 200, about 300, about 400, about 500, about 600, about 700, or about 800 amino acids from the N-terminus of the native protein. In some embodiments, the sequence encodes a fragment of the HEVP lacking at least about 5, about 10, about 50, about 100, about 200, about 300, about 400, about 500, about 600, about 700, or about 800 amino acids from the C-terminus

of the native protein. In some embodiments, the sequence encodes a fragment of the HEVP lacking at least about 5, about 10, about 50, about 100, about 200, about 300, about 400, about 500, about 600, about 700, or about 800 amino acids from both the N-terminus and C-terminus of the native protein. In some embodiments, the sequence encodes a fragment of the HEVP lacking one or more functional or structural domains of the native protein.

**[0202]** In some embodiments, the fragment of the HEVP is fused to one or more heterologous proteins (*e.g.*, exogenous biologically active molecules, *e.g.*, antigen, targeting moiety, adjuvant, and/or immune modulator). In some embodiments, the one or more heterologous proteins are fused to the N-terminus of the fragment. In some embodiments, the one or more heterologous proteins are fused to the C-terminus of the fragment. In some embodiments, the one or more heterologous proteins are fused to the N-terminus and the C-terminus of the fragment. In some embodiments, the one or more heterologous proteins are mammalian proteins. In some embodiments, the one or more heterologous proteins are human proteins.

**[0203]** Surface engineered EVs (*e.g.*, exosomes) can be produced from a cell transformed with a sequence encoding fragments of CD13. In some embodiments, the fragments of CD13 lack one or more functional or structural domains. In some embodiments, the fragments of CD13 are fused to one or more heterologous proteins (*e.g.*, exogenous biologically active molecules, *e.g.*, antigen, targeting moiety, adjuvant, and/or immune modulator). The one or more heterologous proteins can be fused to the N-terminus of the CD13 fragments. The one or more heterologous proteins can be fused to the C-terminus of the CD13 fragments. In some embodiments, the one or more heterologous proteins are fused to both the N-terminus and the C-terminus of the CD13 fragments. In some embodiments, the heterologous protein is a mammalian protein. In some embodiments, the heterologous protein is a human protein. In some embodiments, the heterologous protein fused to the CD13 fragment additionally contains a signal sequence peptide.

**[0204]** Surface engineered EVs (*e.g.*, exosomes) can be produced from a cell transformed with a sequence encoding fragments of MME. In some embodiments, the fragments of MME lack one or more functional or structural domains. In some embodiments, the fragments of MME are fused to one or more heterologous proteins (*e.g.*, exogenous biologically active molecules, *e.g.*, antigen, targeting moiety, adjuvant, and/or immune modulator). In some embodiments, the one or more heterologous proteins are fused to the N-terminus of the MME fragments. In some

embodiments, the one or more heterologous proteins are fused to the C-terminus of the MME fragments. In some embodiments, the one or more heterologous proteins are fused to both the N-terminus and the C-terminus of the MME fragments. In some embodiments, the heterologous protein is a mammalian protein. In some embodiments, the heterologous protein is a human protein. In some embodiments, the heterologous protein fused to the MME fragment additionally contains a signal sequence peptide.

**[0205]** Surface engineered EVs (*e.g.*, exosomes) can be produced from a cell transformed with a sequence encoding fragments of ENPP1. In some embodiments, the fragments of ENPP1 lack one or more functional or structural domains. In some embodiments, the fragments of ENPP1 are fused to one or more heterologous proteins (*e.g.*, exogenous biologically active molecules, *e.g.*, antigen, targeting moiety, adjuvant, and/or immune modulator). In some embodiments, the one or more heterologous proteins are fused to the N-terminus of the ENPP1 fragments. In some embodiments, the one or more heterologous proteins are fused to the C-terminus of the ENPP1 fragments. In some embodiments, the one or more heterologous proteins are fused to both the N-terminus and the C-terminus of the ENPP1 fragments. In some embodiments, the heterologous protein is a mammalian protein. In some embodiments, the heterologous protein is a human protein. In some embodiments, the heterologous protein fused to the ENPP1 fragment additionally contains a signal sequence peptide.

**[0206]** Surface engineered EVs (*e.g.*, exosomes) can be produced from a cell transformed with a sequence encoding fragments of NRP1. In some embodiments, the fragments of NRP1 are fused to one or more heterologous proteins (*e.g.*, exogenous biologically active molecules, *e.g.*, antigen, targeting moiety, adjuvant, and/or immune modulator). In some embodiments, the one or more heterologous proteins are fused to the N-terminus of the NRP1 fragments. In some embodiments, the one or more heterologous proteins are fused to the C-terminus of the NRP1 fragments. In some embodiments, the one or more heterologous proteins are fused to both the N-terminus and the C-terminus of the NRP1 fragments. In some embodiments, the heterologous protein is a mammalian protein. In some embodiments, the heterologous protein is a human protein. In some embodiments, the heterologous protein fused to the NRP1 fragment additionally contains a signal sequence peptide.

**[0207]** In some embodiments, surface-engineered EVs (*e.g.*, exosomes) comprise a polypeptide of a sequence identical or similar to a full-length or a fragment of a HEVP protein including, but not limited to, CD13, MME, ENPP1, and NRP1. In some embodiments, the

peptide is at least about 50% identical to a full-length or a fragment of HEVP, *e.g.*, at least about 50% identical to SEQ ID NOs: 47-50 (*i.e.*, SEQ ID NOs: 47, 48, 49, or 50). In some embodiments, the polypeptide is at least about 60% identical to a full-length or a fragment of a HEVP, *e.g.*, at least about 60% identical to SEQ ID NOs: 47-50. In some embodiments, the polypeptide is at least about 70% identical to a full-length or a fragment of a HEVP, *e.g.*, at least about 70% identical to SEQ ID NOs: 47-50. In some embodiments, the polypeptide is at least about 80% identical to a full-length or a fragment of a HEVP, *e.g.*, at least about 80% identical to SEQ ID NOs: 47-50. In some embodiments, the polypeptide is at least about 90% identical to a full-length or a fragment of a HEVP, *e.g.*, at least about 90% identical to SEQ ID NOs: 47-50. In some embodiments, the polypeptide is at least about 95% identical to a full-length or a fragment of a HEVP, *e.g.*, at least about 95% identical to SEQ ID NOs: 47-50. In some aspects, the polypeptide is at least about 96% identical to a full-length or a fragment of a HEVP, *e.g.*, at least about 96% identical to SEQ ID NOs: 47-50. In some aspects, the polypeptide is at least about 97% identical to a full-length or a fragment of a HEVP, *e.g.*, at least about 97% identical to SEQ ID NOs: 47-50. In some aspects, the polypeptide is at least about 98% identical to a full-length or a fragment of a HEVP, *e.g.*, at least about 98% identical to SEQ ID NOs: 47-50. In some embodiments, the polypeptide is at least about 99% identical to a full-length or a fragment of a HEVP, *e.g.*, at least about 99% identical to SEQ ID NOs: 47-50. In some embodiments, the polypeptide is at least about 99.9% identical to a full-length or a fragment of a HEVP, *e.g.*, at least about 99.9% identical to SEQ ID NOs: 47-50.

#### **7.6. Affinity Purification**

**[0208]** Some embodiments of the present disclosure relate to isolation, purification and sub-fractionation of EVs (*e.g.*, exosomes) using a specific binding interaction between a protein enriched on the EV (*e.g.*, exosome) membrane and an immobilized binding agent. These methods generally comprise the steps of (1) applying or loading a sample comprising EVs (*e.g.*, exosomes), (2) optionally washing away unbound sample components using appropriate buffers that maintain the binding interaction between the target proteins of EVs (*e.g.*, exosomes) and binding agents, and (3) eluting (dissociating and recovering) the EVs (*e.g.*, exosomes) from the immobilized binding agents by altering the buffer conditions so that the binding interaction no longer occurs.

**[0209]** Some embodiments relate to a method of removing EVs (*e.g.*, exosomes) from a sample using a specific binding interaction between a protein enriched on the EV (*e.g.*, exosome)

membrane and an immobilized binding agent. In the cases, EVs (*e.g.*, exosomes) bound to the binding agent are not eluted from the binding agent and a fraction which does not bind to the binding agent can be collected. The method can be used to purify a sample comprising EVs (*e.g.*, exosomes) and a non-EV (*e.g.*, exosomal) material such as viruses (*e.g.*, lentivirus, retrovirus, adeno-associated virus, or any other enveloped or non-enveloped virus) or a recombinant protein (*e.g.*, antibodies, enzymes or other polypeptides), wherein the EVs (*e.g.*, exosomes) are contaminating particles. The bound EVs (*e.g.*, exosomes) can be retained bound to the binding agent and the non-EV (*e.g.*, exosomal) material is collected, substantially free of EVs (*e.g.*, exosomes).

**[0210]** The target protein, used for this isolation, purification, sub-fractionation or removal process, can be an endogenous protein produced from the genome of a producer cell, a protein introduced to the producer cell by a genetic modification, or a protein modified by chemical, physical or other biological methods. In some cases, the protein is a non-mutant protein or a mutant protein, *e.g.*, a variant or a fragment of an endogenous protein. In some cases, the protein is a fusion protein (*e.g.*, such as those described herein).

**[0211]** Various binding agents having affinity to the target protein can be used for the embodiments of the present disclosure. For example, proteins, peptides, and small molecules with specific affinities to the target protein can be used as a binding agent. In some embodiments, binding agents are obtained from organic or inorganic sources. Examples of binding agents from organic sources include serum proteins, lectins or antibodies. Examples of binding agents from inorganic sources include boronic acids, metal chelates, and triazine dyes.

**[0212]** The binding agents can be chemically immobilized or coupled to a solid support so that EVs (*e.g.*, exosomes) having specific affinity to the binding agent become bound. Various forms of solid support can be used, *e.g.*, a porous agarose bead, a microtiter plate, a magnetic bead, or a membrane. In some embodiments, the solid support forms a chromatography column and can be used for affinity chromatography of EVs (*e.g.*, exosomes).

**[0213]** In some cases, isolation, purification, sub-fractionation and removal of EVs (*e.g.*, exosomes) are done by column chromatography using a column where the binding agents and the solid support are packed. In some embodiments, a sample containing EVs (*e.g.*, exosomes) run through the column to allow setting, a wash buffer run through the column, and the elution buffer subsequently applied to the column and collected. These steps can be done at ambient pressure or with application of additional pressure.

[0214] In some cases, isolation, purification, sub-fractionation and removal of EVs (*e.g.*, exosomes) are done using a batch treatment. For example, a sample is added to the binding agent attached to a solid support in a vessel, mixing, separating the solid support, removing the liquid phase, washing, centrifuging, adding the elution buffer, re-centrifuging and removing the elute.

[0215] In some cases, a hybrid method can be employed. For example, a sample is added to the binding agent attached to a solid support in a vessel, the solid support bound to the EVs (*e.g.*, exosomes) is subsequently packed onto a column, and washing and elution are done on the column.

[0216] In some cases, isolation, purification, sub-fractionation and removal of EVs (*e.g.*, exosomes) are done using a binding agent attached to microtiter plates, magnetic beads, or membranes. In the cases, a sample is added to the binding agent attached to a solid support, followed by the steps of mixing, separating the solid support, removing the liquid phase, washing, removing the washing buffer, adding the elution buffer, and removing the elute.

[0217] The binding between the binding agent and a target protein on the EV (*e.g.*, exosome) is done in various physiological conditions optimal for specific interactions between the binding agent and the target protein on the EV (*e.g.*, exosome). Elution of the bound (*e.g.*, exosomes) can be achieved by changing salt concentrations, pH, pI, charge and ionic strength directly or through a gradient.

[0218] In some embodiments, a sample isolated, purified or sub-fractionated with one binding agent is subsequently processed with a different binding agent.

[0219] In some embodiments, more than one columns are used in series, where each of the multiple columns contains a different binding agent specific to a different target protein.

[0220] In some embodiments, a single column contains multiple binding agents, each specific to a different target protein.

[0221] In some cases, the binding agent and solid support are reused by introduction of a periodic sanitization step. For example, they can be sanitized with a combination of propylene glycol, isopropanol, high ionic strength, and/or sodium hydroxide.

#### **7.6.1. Sample Preparation**

[0222] The methods described herein can be used to purify, isolate, sub-fractionate or remove EVs (*e.g.*, exosomes) from various samples comprising EVs (*e.g.*, exosomes). In some embodiments, the sample is a clarified harvest material containing EVs (*e.g.*, exosomes). In some cases, the sample comprises EVs (*e.g.*, exosomes) partially purified by a purification

method well known in the art. For example, ultrafiltration/diafiltration, hydroxyl apatite chromatography, hydrophobic interaction chromatography, deep filtration, or ion exchange bind/elute chromatography can be used to partially purify EVs (*e.g.*, exosomes) before applying to a binding agent for affinity purification.

**[0223]** In some cases, the partially purified material is further processed to have certain physiological conditions (*e.g.*, pH, temperature, salt concentration, salt type, polarity) for desired interaction with the binding agent. A sample can be prepared by dilution or concentration to obtain certain EV (*e.g.*, exosome) concentrations, or by adding excipients to change structure of EVs (*e.g.*, exosomes). In some cases, the partially purified material is applied to the binding agent without any manipulation.

#### **7.6.2. Binding**

**[0224]** The methods described herein requires specific interaction between a target protein of an EV (*e.g.*, exosome) and a binding agent. High-throughput screening can be performed to identify buffer conditions ideal for the specific binding – through altering salt concentration, pH, and/or reducing polarity with an organic modifier, ethylene glycol, propylene glycol, or urea. The interaction between a target protein and a binding agent can also change depending on sample conditions (*e.g.*, sample amount loaded per volume of chromatographic resin, concentration of EVs (*e.g.*, exosomes), concentration of impurities), loading buffers (*e.g.*, pH, salt concentrations, salt types, polarity), and other physical conditions (*e.g.*, temperature). Furthermore, adding excipients that alter the structure of the EVs (*e.g.*, exosomes) can also change their interactions. In addition, residence time can be adjusted based on differential adsorption rates between impurities and EVs (*e.g.*, exosomes). Thus, various purification conditions described herein can be tested to identify ideal conditions for the step.

**[0225]** Similar approaches can be used to improve purity and yield, and aid in enriching, depleting, or isolating sub-populations of EVs (*e.g.*, exosomes). These properties, along with maximizing load challenge and applying more stringent elution conditions, could be employed to further enhance the concentration of EVs (*e.g.*, exosomes).

##### **7.6.2.1. Elution**

**[0226]** Elution of EVs (*e.g.*, exosomes) can be achieved through altering salt concentration, pH, and/or polarity with an organic modifier, ethylene glycol, propylene glycol, or urea.

**[0227]** Selective elution of EVs (*e.g.*, exosomes) can be achieved by increasing the concentration of a monovalent cationic halide salt (*e.g.*, sodium chloride, potassium chloride, sodium bromide, lithium chloride, sodium iodide, potassium bromide, lithium bromide, sodium fluoride, potassium fluoride, lithium fluoride, lithium iodide, sodium acetate, potassium acetate, lithium acetate, and potassium iodide), a divalent or trivalent salt (*e.g.*, calcium chloride, magnesium chloride, calcium sulfate, sodium sulfate, magnesium sulfate, chromium trichloride, chromium sulfate, sodium citrate, iron (III) chloride, yttrium (III) chloride, potassium phosphate, potassium sulfate, , sodium phosphate, ferrous chloride, calcium citrate, magnesium phosphate, and ferric chloride), or a combination thereof, in the elution buffer, through the use of an increasing gradient (step or linear) of a monovalent cationic halide salt (*e.g.*, sodium chloride, potassium chloride, sodium bromide, lithium chloride, sodium iodide, potassium bromide, lithium bromide, sodium fluoride, potassium fluoride, lithium fluoride, lithium iodide, sodium acetate, potassium acetate, lithium acetate, and potassium iodide), a divalent or trivalent salt (*e.g.*, calcium chloride, magnesium chloride, calcium sulfate, sodium sulfate, magnesium sulfate, chromium trichloride, chromium sulfate, sodium citrate, iron (III) chloride, yttrium (III) chloride, potassium phosphate, potassium sulfate, , sodium phosphate, ferrous chloride, calcium citrate, magnesium phosphate, and ferric chloride), or a combination thereof, at a fixed pH.

**[0228]** Substantial EV (*e.g.*, exosome) purity can be achieved by flowing through impurities during the column loading phase, eluting impurities during selective excipient washes and selectively eluting product during elution while leaving additional impurities bound to the column. Absorbance measured from column eluates can indicate the purity of EVs (*e.g.*, exosomes) obtained by the methods.

**[0229]** Elution can be also achieved by modulating the pH range, salts, organic solvents, small molecules, detergents, zwitterions, amino acids, polymers, temperature, and any combination of the above. Similar elution agents can be used to improve purity, improve yield, and/or isolate sub-populations of EVs (*e.g.*, exosomes).

**[0230]** Elution can be also done with multiple elution buffers having different properties, such as pH, salts, organic solvents, small molecules, detergents, zwitterions, amino acids, polymers, temperature, and any combination of the above. A plurality of eluted fractions can be collected, wherein EVs (*e.g.*, exosomes) collected in each fraction has different properties. For example, EVs (*e.g.*, exosomes) collected in one fraction has a higher purity, a smaller or larger average size, a preferred composition, *etc.* than EVs (*e.g.*, exosomes) in other fractions.

[0231] Elution buffers with different properties can be applied as a continuous flow, while a plurality of eluted fractions are collected. Eluted fractions can be collected during isocratic elution or gradient elution. Once at least one eluted fraction is collected, a composition of the eluted fraction can be analyzed. For example, the concentration of EVs (*e.g.*, exosomes), a host cell protein, a contaminant protein, DNA, carbohydrates, or lipids can be measured in each eluted fraction. Other properties of EVs (*e.g.*, exosomes) in each eluted fraction can be also measured. The properties include an average size, an average charge density, and other physiological properties related to bio-distribution, cellular uptake, half-life, pharmacodynamics, potency, dosing, immune response, loading efficiency, stability, or reactivity to other compounds.

#### 7.6.2.2. Washing

[0232] Optionally, purity of EVs (*e.g.*, exosomes) can be further improved by washing samples prior to elution. In some embodiments, excipient can be a washing buffer. The excipient can be a solution having specific pH ranges, salts, organic solvents, small molecules, detergents, zwitterions, amino acids, polymers, and any combination of the above.

[0233] More specifically, the excipient can comprise arginine, lysine, glycine, histidine, calcium, sodium, lithium, potassium, iodide, magnesium, iron, zinc, manganese, urea, propylene glycol, aluminum, ammonium, guanidinium polyethylene glycol, EDTA, EGTA, a detergent, chloride, sulfate, carboxylic acids, sialic acids, phosphate, acetate, glycine, borate, formate, perchlorate, bromine, nitrate, dithiothreitol, beta mercaptoethanol, or tri-n-butyl phosphate.

[0234] The excipient can also comprise a detergent, selected from the group consisting of cetyl trimethylammonium chloride, octoxynol-9, TRITON<sup>TM</sup> X-100 (*i.e.*, polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether) and TRITON<sup>TM</sup> CG-110 available from Sigma-Aldrich; sodium dodecyl sulfate; sodium lauryl sulfate; deoxycholic acid; Polysorbate 80 (*i.e.*, Polyoxyethylene (20) sorbitan monooleate); Polysorbate 20 (*i.e.*, Polyoxyethylene (20) sorbitan monolaurate); alcohol ethoxylate; alkyl polyethylene glycol ether; decyl glucoside; octoglucosides; SafeCare; ECOSURF<sup>TM</sup> EH9, ECOSURF<sup>TM</sup> EH6, ECOSURF<sup>TM</sup> EH3, ECOSURF<sup>TM</sup> SA7, and ECOSURF<sup>TM</sup> SA9 available from DOW Chemical; LUTENSOL<sup>TM</sup> M5, LUTENSOL<sup>TM</sup> XL, LUTENSOL<sup>TM</sup> XP and APG<sup>TM</sup> 325N available from BASF; TOMADOL<sup>TM</sup> 900 available from AIR PRODUCTS; NATSURF<sup>TM</sup> 265 available from CRODA; SAFECARE<sup>TM</sup> 1000 available from Bestchem, TERGITOL<sup>TM</sup> L64 available from DOW; caprylic acid; CHEMBETAINE<sup>TM</sup> LEC available from Lubrizol; and Mackol DG.

### 7.6.3. Other methods for improving outcome

[0235] The amount of EVs (*e.g.*, exosomes) that can be loaded per volume of chromatographic resin can be improved by modulating the feed material, for example, by increasing the concentration of EVs (*e.g.*, exosomes), decreasing the concentration of impurities, altering the pH, decreasing the salt concentrations, decreasing the ionic strength, or altering the specific sub-populations of EVs (*e.g.*, exosomes). Owing to mass transfer constraints and slow adsorption and desorption of EVs (*e.g.*, exosomes) on the resin, the amount of EVs (*e.g.*, exosomes) that can be loaded per volume of chromatographic resin can be increased by slowing the flow rate during column loading, employing longer columns to increase the residence time.

## 7.7. Applications

### 7.7.1. Purification of EVs (*e.g.*, exosomes)

[0236] The use of EVs (*e.g.*, exosomes) for medical purposes requires that the EVs (*e.g.*, exosomes) be free or mostly free of impurities including but not limited to macromolecules, such as nucleic acids, contaminant proteins, lipids, carbohydrates, metabolites, small molecules, metals, or a combination thereof. The present disclosure provides a method of purifying EVs (*e.g.*, exosomes) from contaminating macromolecules. In some embodiments, purified EVs (*e.g.*, exosomes) are substantially free of contaminating macromolecules.

### 7.7.2. Sub-fractionation of EVs (*e.g.*, exosomes)

[0237] Embodiments of the present disclosure further provide methods for sub-fractionating populations of EVs (*e.g.*, exosomes) based on their membrane protein, size, charge density, ligand type (*e.g.*, tetraspanins) and heparin or other sulfated carbohydrate binding sites. The choice of affinity tag, loading and elution buffer compositions and protocols can result in elution of different sub-populations of EVs (*e.g.*, exosomes).

[0238] For example, embodiments of the present disclosure provide methods of purifying a population of EVs (*e.g.*, exosomes) with a smaller or larger size. The size of EVs (*e.g.*, exosomes) can be determined by methods available in the field. For example, the size can be measured by nanoparticle tracking analysis, multi-angle light scattering, single angle light scattering, size exclusion chromatography, analytical ultracentrifugation, field flow fractionation, laser diffraction, tunable resistive pulse sensing, or dynamic light scattering.

[0239] Embodiments of the present disclosure further relate to methods of sub-fractionating EVs (*e.g.*, exosomes) based on their charge density. The charge density of EVs (*e.g.*, exosomes) can be determined by potentiometric titration, anion exchange, cation exchange,

isoelectric focusing, zeta potential, capillary electrophoresis, capillary zone electrophoresis, gel electrophoresis, or any combination thereof.

**[0240]** Embodiments of the present disclosure also relate to sub-fractionating EVs (*e.g.*, exosomes) based on other physiological properties, such as bio-distribution, cellular uptake, half-life, pharmacodynamics, potency, dosing, immune response, loading efficiency, stability, or reactivity to other compounds. The method enables isolation of a population of EVs (*e.g.*, exosomes) that are appropriate for a specific application.

### **7.7.3. Uses**

**[0241]** In some aspects, the present disclosure provides methods of preventing and/or treating a disease or disorder in a subject in need thereof, comprising administering an EV (*e.g.*, exosome) disclosed herein.

**[0242]** In some aspects, a disease or disorder that can be treated with the present methods comprises a cancer, hemophilia, diabetes, growth factor deficiency, eye diseases, graft-versus-host disease (GvHD), autoimmune diseases, gastrointestinal diseases, cardiovascular diseases, respiratory diseases, allergic diseases, degenerative diseases, infectious diseases, fibrotic diseases, or any combination thereof. In certain aspects, a disease or disorder that can be treated is associated with chronic inflammation. In some aspects, the treatment is prophylactic. In some aspects, the EVs (*e.g.*, exosomes) of the present disclosure are used to induce an immune response. In some aspects, the EVs of the present disclosure are used to vaccinate a subject.

**[0243]** In some aspects, the disease or disorder is a cancer. In some aspects, the disease or disorder is a graft-versus-host disease (GvHD). In some aspects, the disease or disorder is an infectious disease.

**[0244]** As described herein, cells (*e.g.*, producer cells) can be modified to express a protein (*e.g.* HEVP) that is not naturally expressed in the cells (*i.e.*, heterologous protein). In some aspects, such modification results in EVs (*e.g.*, exosomes) produced from the modified cell to express the heterologous protein. Accordingly, in some aspects, the present disclosure provides a method of expressing a non-naturally occurring protein (*i.e.*, heterologous protein) in an EV (*e.g.*, exosome) derived from a cell. In some aspects, the method comprises transfecting a cell with a nucleic acid encoding at least one heterologous protein (*e.g.*, HEVPs disclosed herein) (or a fragment or variant thereof) and isolating an EV (*e.g.*, exosome) produced from the cell, wherein the EV (*e.g.*, exosome) comprises the at least one heterologous protein and wherein the heterologous protein is not naturally expressed in an EV derived from the cell.

[0245] In some aspects, an EV (*e.g.*, exosome) produced from the above-described cell (*i.e.*, transfected with a nucleic acid encoding at least one heterologous protein) comprises at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 heterologous proteins. In certain aspects, the heterologous proteins are different (*e.g.*, an exosome is engineered to express both CD13 and MME proteins, wherein the exosomes are produced from a cell that does not naturally express CD13 and MME proteins). In some aspects, the heterologous proteins are the same, such that the total amount of heterologous proteins expressed in an EV (*e.g.*, exosome) is greater than an EV produced from a cell that naturally expresses the heterologous proteins.

[0246] To quantify the amount or level of heterologous proteins (*e.g.*, HEVPs) expressed on an EV (*e.g.*, exosome), any appropriate method known in the art can be used. In some aspects, the amount of proteins (*e.g.*, HEVPs) expressed on an EV (*e.g.*, exosome) can be assessed by measuring the number of peptide spectral matches in a given sample comprising an EV (*e.g.*, exosome) using liquid chromatography with tandem mass spectrometry (LC-MS/MS). *See* Examples 1 and 3. As used herein, "**peptide spectral match**" (PSM) refers to a system which assigns a numerical value to a peptide-spectrum pair ( $P, S$ ) expressing the likelihood that the fragmentation of a peptide with sequence  $P$  is recorded in the experimental mass spectrum  $S$ . Frank, A.M., *J Proteome Res*, 8(5): 2241-2252 (May 2009). PSMs correlate with protein abundance in a given sample.

[0247] In some aspects, the level (or amount) of heterologous proteins (*e.g.*, HEVPs) expressed in an EV (*e.g.*, exosome) produced from a cell disclosed herein (*e.g.*, transfected with a nucleic acid encoding at least one heterologous proteins) is at least about 10 peptide spectral matches (PSMs), at least about 20 PSMs, at least about 30 PSMs, at least about 40 PSMs, at least about 50 PSMs, at least about 60 PSMs, at least about 70 PSMs, at least about 80 PSMs, at least about 90 PSMs, at least about 100 PSMs, at least about 110 PSMs, at least about 120 PSMs, at least about 130 PSMs, at least about 140 PSMs, at least about 150 PSMs, at least about 160 PSMs, at least about 170 PSMs, at least about 180 PSMs, at least about 190 PSMs, at least about 200 PSMs, at least about 210 PSMs, at least about 220 PSMs, at least about 230 PSMs, at least about 240 PSMs, at least about 250 PSMs, at least about 260 PSMs, at least about 270 PSMs, at least about 280 PSMs, at least about 290 PSMs, at least about 300 PSMs, at least about 350 PSMs, at least about 400 PSMs, at least about 450 PSMs, at least about 500 PSMs, at least about 550 PSMs, at least about 600 PSMs, at least about 650 PSMs, at least about 700 PSMs, at least

about 750 PSMs, at least about 800 PSMs, at least about 850 PSMs, at least about 900 PSMs, at least about 950 PSMs, or at least about 1,000 or more PSMs, as measured using LC-MS/MS.

**[0248]** In certain aspects, the level of heterologous proteins (*e.g.*, HEVPs) expressed in an EV (*e.g.*, exosome) is equal to or greater than about 125 PSMs, as measured using LC-MS/MS. In certain aspects, the level of heterologous proteins (*e.g.*, HEVPs) expressed in an EV (*e.g.*, exosome) is equal to or greater than about 150 PSMs, as measured using LC-MS/MS. In some aspects, the level of heterologous proteins (*e.g.*, HEVPs) expressed in an EV (*e.g.*, exosome) is equal to or greater than about 200 PSMs. In certain aspects, the level of heterologous proteins (*e.g.*, HEVPs) expressed in an EV (*e.g.*, exosome) is equal to or greater than about 700 PSMs, as measured using LC-MS/MS. In some aspects, the level of heterologous proteins (*e.g.*, HEVPs, *e.g.*, MME) expressed in an EV (*e.g.*, exosome) is about 177 PSMs, as measured using LC-MS/MS. In some aspects, the level of heterologous proteins (*e.g.*, HEVPs, *e.g.*, CD13) expressed in an EV (*e.g.*, exosome) is about 742 PSMs, as measured using LC-MS/MS.

**[0249]** In some aspects, level of heterologous proteins (*e.g.*, HEVPs) expressed in an EV (*e.g.* exosome) is between about 20 and about 80 PSMs, as measured using LC-MS/MS. In some aspects, the level of heterologous proteins (*e.g.*, HEVPs) expressed in an EV (*e.g.* exosome) is between about 80 and about 200 PSMs, as measured using LC-MS/MS. In some aspects, the level of heterologous proteins (*e.g.*, HEVPs) expressed in an EV (*e.g.* exosome) is between about 150 PSMs and about 750 PSMs, as measured using LC-MS/MS.

## **7.8. Characterization of EVs (*e.g.*, exosomes)**

**[0250]** In some embodiments, the methods described herein further comprise the step of characterizing EVs (*e.g.*, exosomes) contained in each collected fraction. In some embodiments, contents of the EVs (*e.g.*, exosomes) can be extracted for study and characterization. In some embodiments, EVs (*e.g.*, exosomes) are isolated and characterized by metrics including, but not limited to, size, shape, morphology, or molecular compositions such as nucleic acids, proteins, metabolites, and lipids.

### **7.8.1. Measurement of the contents of EVs (*e.g.*, exosomes)**

**[0251]** EVs (*e.g.*, exosomes) can include proteins, peptides, RNA, DNA, and lipids. Total RNA can be extracted using acid-phenol:chloroform extraction. RNA can then be purified using a glass-fiber filter under conditions that recover small-RNA containing total RNA, or that separate small RNA species less than 200 nucleotides in length from longer RNA species such as

mRNA. Because the RNA is eluted in a small volume, in some aspects, no alcohol precipitation step can be required for isolation of the RNA.

**[0252]** EV (*e.g.*, exome) compositions can be assessed by methods known in the art including, but not limited to, transcriptomics, sequencing, proteomics, mass spectrometry, or HP-LC.

**[0253]** The composition of nucleotides associated with isolated EVs (*e.g.*, exosomes) (including RNAs and DNAs) can be measured using a variety of techniques that are well known to those of skill in the art (*e.g.*, quantitative or semi-quantitative RT-PCR, Northern blot analysis, solution hybridization detection). In a particular embodiment, the level of at least one RNA is measured by reverse transcribing RNA from the EV (*e.g.*, exosome) composition to provide a set of target oligodeoxynucleotides, hybridizing the target oligodeoxynucleotides to one or more RNA-specific probe oligonucleotides (*e.g.*, a microarray that comprises RNA-specific probe oligonucleotides) to provide a hybridization profile for the EV (*e.g.*, exosome) composition, and comparing the EV (*e.g.*, exosome) composition hybridization profile to a hybridization profile generated from a control sample. An alteration in the signal of at least one RNA in the test sample relative to the control sample is indicative of the RNA composition.

**[0254]** Also, a microarray can be prepared from gene-specific oligonucleotide probes generated from known RNA sequences. The array can contain two different oligonucleotide probes for each RNA, one containing the active, mature sequence and the other being specific for the precursor of the RNA (for example miRNA and pre-miRNAs). The array can also contain controls, such as one or more mouse sequences differing from human orthologs by only a few bases, which can serve as controls for hybridization stringency conditions. tRNAs and other RNAs (*e.g.*, rRNAs, mRNAs) from both species can also be printed on the microchip, providing an internal, relatively stable, positive control for specific hybridization. One or more appropriate controls for non-specific hybridization can also be included on the microchip. For this purpose, sequences are selected based upon the absence of any homology with any known RNAs.

**[0255]** The microarray can be fabricated using techniques known in the art. For example, probe oligonucleotides of an appropriate length, *e.g.*, 40 nucleotides, are 5'-amine modified at position C6 and printed on activated slides using commercially available microarray systems, *e.g.*, the GeneMachine OmniGrid.<sup>TM</sup>100 Microarrayer and Amersham CodeLink.<sup>TM</sup> Labeled cDNA oligomer corresponding to the target RNAs is prepared by reverse transcribing the target RNA with labeled primer. Following first strand synthesis, the RNA/DNA hybrids are denatured

to degrade the RNA templates. The labeled target cDNAs thus prepared are then hybridized to the microarray chip under hybridizing conditions, *e.g.*, 6.times. SSPE/30% formamide at 25° C. for 18 hours, followed by washing in 0.75.times. TNT at 37 °C. for 40 minutes. At positions on the array where the immobilized probe DNA recognizes a complementary target cDNA in the sample, hybridization occurs. The labeled target cDNA marks the exact position on the array where binding occurs, allowing automatic detection and quantification. The output consists of a list of hybridization events, indicating the relative abundance of specific cDNA sequences, and therefore the relative abundance of the corresponding complementary RNAs, in the EV (*e.g.*, exosome) preparation. According to one embodiment, the labeled cDNA oligomer is a biotin-labeled cDNA, prepared from a biotin-labeled primer. The microarray is then processed by direct detection of the biotin containing transcripts using, *e.g.*, Streptavidin-Alexa647 conjugate, and scanned utilizing conventional scanning methods. Image intensities of each spot on the array are proportional to the abundance of the corresponding RNA in the EV (*e.g.*, exosome).

**[0256]** Data mining work is completed by bioinformatics, including scanning chips, signal acquisition, image processing, normalization, statistic treatment and data comparison as well as pathway analysis. As such, microarray can profile hundreds and thousands of polynucleotides simultaneously with high throughput performance. Microarray profiling analysis of mRNA expression has successfully provided valuable data for gene expression studies in basic research. And the technique has been further put into practice in the pharmaceutical industry and in clinical diagnosis. With increasing amounts of miRNA data becoming available, and with accumulating evidence of the importance of miRNA in gene regulation, microarray becomes a useful technique for high through-put miRNA studies. The analysis of miRNA levels utilizing polynucleotide probes can be carried out in a variety of physical formats as well. For example, the use of microtiter plates or automation can be used to facilitate the processing of large numbers of test samples.

#### **7.8.2. Measurement of the size of EVs (*e.g.*, exosomes)**

**[0257]** In some embodiments, the methods described herein comprise measuring the size of EVs (*e.g.*, exosomes) and/or populations of EVs (*e.g.*, exosomes) included in the purified fractions. In some embodiments, EV (*e.g.*, exosome) size is measured as the longest measurable dimension. Generally, the longest general dimension of an EV (*e.g.*, exosome) is also referred to as its diameter.

**[0258]** EV (*e.g.*, exosome) size can be measured using various methods known in the art, for example, nanoparticle tracking analysis, multi-angle light scattering, single angle light scattering, size exclusion chromatography, analytical ultracentrifugation, field flow fractionation, laser diffraction, tunable resistive pulse sensing, or dynamic light scattering.

**[0259]** EV (*e.g.*, exosome) size can be measured using dynamic light scattering (DLS) and/or multiangle light scattering (MALS). Methods of using DLS and/or MALS to measure the size of EVs (*e.g.*, exosomes) are known to those of skill in the art, and include the nanoparticle tracking assay (NTA, *e.g.*, using a Malvern Nanosight NS300 nanoparticle tracking device). In a specific embodiment, the EV (*e.g.*, exosome) size is determined using a Malvern NanoSight NS300. In some embodiments, the EVs (*e.g.*, exosomes) described herein have a longest dimension of about 20-1000 nm as measured by NTA (*e.g.*, using a Malvern Nanosight NS300). In other embodiments, the EVs (*e.g.*, exosomes) described herein have a longest dimension of about 40-1000 nm as measured by NTA (*e.g.*, using a Malvern Nanosight NS300). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population, wherein 90% of the EVs (*e.g.*, exosomes) have a longest dimension of about 20-1000 nm as measured by NTA (*e.g.*, using a Malvern Nanosight NS300). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population, wherein 95% of the EVs (*e.g.*, exosomes) have a longest dimension of about 20-1000 nm as measured by NTA (*e.g.*, using a Malvern Nanosight NS300). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population, wherein 99% of the EVs (*e.g.*, exosomes) have a longest dimension of about 20-1000 nm as measured by NTA (*e.g.*, using a Malvern Nanosight NS300). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population, wherein 90% of the EVs (*e.g.*, exosomes) have a longest dimension of about 40-1000 nm as measured by NTA (*e.g.*, using a Malvern Nanosight NS300). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population, wherein 95% of the EVs (*e.g.*, exosomes) have a longest dimension of about 40-1000 nm as measured by NTA (*e.g.*, using a Malvern Nanosight NS300). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population, wherein 99% of the EVs (*e.g.*, exosomes) have a longest dimension of about 40-1000 nm as measured by NTA (*e.g.*, using a Malvern Nanosight NS300).

**[0260]** EV (*e.g.*, exosome) size can be measured using tunable resistive pulse sensing (TRPS). In a specific embodiment, EV (*e.g.*, exosome) size as measured by TRPS is determined using an iZON qNANO Gold. In some embodiments, the EVs (*e.g.*, exosomes) described herein

have a longest dimension of about 20-1000 nm as measured by TRPS (*e.g.*, using an iZON qNano Gold). In other embodiments, the EVs (*e.g.*, exosomes) described herein have a longest dimension of about 40-1000 nm as measured by TRPS (*e.g.*, an iZON qNano Gold). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population, wherein 90% of the EVs (*e.g.*, exosomes) have a longest dimension of about 20-1000 nm as measured by TRPS (*e.g.*, using an iZON qNano Gold). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population, wherein 95% of the EVs (*e.g.*, exosomes) have a longest dimension of about 20-1000 nm as measured by TRPS (*e.g.*, using an iZON qNano Gold). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population, wherein 99% of the EVs (*e.g.*, exosomes) have a longest dimension of about 20-1000 nm as measured by TRPS (*e.g.*, using an iZON qNano Gold). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population, wherein 90% of the EVs (*e.g.*, exosomes) have a longest dimension of about 40-1000 nm as measured by TRPS (*e.g.*, using an iZON qNano Gold). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population, wherein 95% of the EVs (*e.g.*, exosomes) have a longest dimension of about 40-1000 nm as measured by TRPS (*e.g.*, using an iZON qNano Gold). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population, wherein 99% of the EVs (*e.g.*, exosomes) have a longest dimension of about 40-1000 nm as measured by TRPS (*e.g.*, using an iZON qNano Gold).

**[0261]** EV (*e.g.*, exosome) size can be measured using electron microscopy. In some embodiments, the method of electron microscopy used to measure EV (*e.g.*, exosome) size is transmission electron microscopy. In a specific embodiment, the transmission electron microscope used to measure EV (*e.g.*, exosome) size is a Tecnai™ G2 Spirit BioTWIN. Methods of measuring EV (*e.g.*, exosome) size using an electron microscope are well-known to those of skill in the art, and any such method can be appropriate for measuring EV (*e.g.*, exosome) size. In some embodiments, the EVs (*e.g.*, exosomes) described herein have a longest dimension of about 20-1000 nm as measured by a scanning electron microscope (*e.g.*, a Tecnai™ G2 Spirit BioTWIN scanning electron microscope). In other embodiments, the EVs (*e.g.*, exosomes) described herein have a longest dimension of about 40-1000 nm as measured by a scanning electron microscope (*e.g.*, a Tecnai™ G2 Spirit BioTWIN scanning electron microscope). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population, wherein 90% of the EVs (*e.g.*, exosomes) have a longest dimension of

about 20-1000 nm as measured by a scanning electron microscope (*e.g.*, a Tecnai™ G2 Spirit BioTWIN scanning electron microscope). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population, wherein 95% of the EVs (*e.g.*, exosomes) have a longest dimension of about 20-1000 nm as measured by a scanning electron microscope (*e.g.*, a Tecnai™ G2 Spirit BioTWIN scanning electron microscope). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population, wherein 99% of the EVs (*e.g.*, exosomes) have a longest dimension of about 20-1000 nm as measured by a scanning electron microscope (*e.g.*, a Tecnai™ G2 Spirit BioTWIN scanning electron microscope). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population wherein 90% of the EVs (*e.g.*, exosomes) have a longest dimension of about 40-1000 nm as measured by a scanning electron microscope (*e.g.*, a Tecnai™ G2 Spirit BioTWIN scanning electron microscope). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population wherein 95% of the EVs (*e.g.*, exosomes) have a longest dimension of about 40- 1000 nm as measured by a scanning electron microscope (*e.g.*, a Tecnai™ G2 Spirit BioTWIN scanning electron microscope). In other embodiments, the EV (*e.g.*, exosome) populations described herein comprise a population wherein 99% of the EVs (*e.g.*, exosomes) have a longest dimension of about 40-1000 nm as measured by a scanning electron microscope (*e.g.*, a Tecnai™ G2 Spirit BioTWIN scanning electron microscope).

#### **7.8.3. Measurement of the charge density of EVs (*e.g.*, exosomes)**

**[0262]** In some embodiments, the methods described herein comprise measuring the charge density of EVs (*e.g.*, exosomes) and/or populations of EVs (*e.g.*, exosomes) included in the purified fractions. In some embodiments, the charge density is measured by potentiometric titration, anion exchange, cation exchange, isoelectric focusing, zeta potential, capillary electrophoresis, capillary zone electrophoresis, or gel electrophoresis.

#### **7.8.4. Measurement of density of EV (*e.g.*, exosome) proteins or HEVPs**

**[0263]** In some embodiments, the methods described herein comprise measuring the density of EV (*e.g.*, exosome) proteins or HEVPs on the EV (*e.g.*, exosome) surface. The surface density can be calculated or presented as the mass per unit area, the number of proteins per area, number of molecules or intensity of molecule signal per EV (*e.g.*, exosome), molar amount of the protein, *etc.* The surface density can be experimentally measured by methods known in the art, for example, by using bio-layer interferometry (BLI), FACS, Western blotting, fluorescence

(*e.g.*, GFP-fusion protein) detection, nano-flow cytometry, ELISA, alphaLISA, and/or densitometry by measuring bands on a protein gel.

#### **7.9. Pharmaceutical Compositions**

**[0264]** Provided herein are pharmaceutical compositions comprising an EV, *e.g.*, exosome, of the present disclosure having the desired degree of purity, and a pharmaceutically acceptable carrier or excipient, in a form suitable for administration to a subject. Pharmaceutically acceptable excipients or carriers can be determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions comprising a plurality of extracellular vesicles. (*See, e.g.*, Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa. 21st ed. (2005)). The pharmaceutical compositions are generally formulated sterile and in full compliance with all Good Manufacturing Practice (GMP) regulations of the U.S. Food and Drug Administration.

**[0265]** In some aspects, a pharmaceutical composition comprises one or more therapeutic agents and an EV, *e.g.*, exosome, described herein. In certain aspects, the EVs, *e.g.*, exosomes, are co-administered with one or more additional therapeutic agents, in a pharmaceutically acceptable carrier. In some aspects, the pharmaceutical composition comprising the EV, *e.g.*, exosome is administered prior to administration of the additional therapeutic agents. In other aspects, the pharmaceutical composition comprising the EV, *e.g.*, exosome is administered after the administration of the additional therapeutic agents. In further aspects, the pharmaceutical composition comprising the EV, *e.g.*, exosome is administered concurrently with the additional therapeutic agents.

#### **7.10. Kits**

**[0266]** Also provided herein are kits comprising one or more exosomes described herein. In some aspects, provided herein is a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions described herein, such as one or more exosomes provided herein, optional an instruction for use. In some aspects, the kits contain a pharmaceutical composition described herein and any prophylactic or therapeutic agent, such as those described herein.

#### **7.11. Examples**

**[0267]** The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present disclosure, and

are not intended to limit the scope of what the inventors regard as their present disclosure nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperature, *etc.*) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations can be used, *e.g.*, bp, base pair(s); kb, kilobase(s); pl, picoliter(s); s or sec, second(s); min, minute(s); h or hr, hour(s); aa, amino acid(s); nt, nucleotide(s); and the like.

**[0268]** The practice of the present disclosure will employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. *See, e.g.*, T.E. Creighton, *Proteins: Structures and Molecular Properties* (W.H. Freeman and Company, 1993); A.L. Lehninger, *Biochemistry* (Worth Publishers, Inc., current addition); Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); Remington's *Pharmaceutical Sciences*, 21th Edition (Easton, Pennsylvania: Mack Publishing Company, 2005); Carey and Sundberg *Advanced Organic Chemistry* 3rd Ed. (Plenum Press) Vols A and B (1992).

#### **7.11.1. Example 1: Identification of EV (*e.g.*, exosome) proteins**

##### **7.11.1.1. Collection of EVs (*e.g.*, exosomes)**

**[0269]** EVs (*e.g.*, exosomes) were collected from the supernatant of high-density suspension cultures of bone marrow-derived MSC cells after 4 days. The supernatant was filtered and concentrated by tangential flow filtration using 1,000 kDa MWCO membranes (PXC01MC50). The concentrated cell culture supernatant was pelleted by ultracentrifugation using 100 mL Quick-Seal Ultra-Clear tubes (345778). The crude EV (*e.g.*, exosome) pellets were further fractionated on an OPTIPREP™ (60% iodixanol w/v) density gradient by ultracentrifugation.

**[0270]** The pelleted material was resuspended in 1 mL PBS and 3 mL of OPTIPREP™, bringing the final iodixanol concentration to 45%. For the OPTIPREP™ gradient, a 4-tier sterile gradient was prepared with 4 mL of 45% iodixanol containing the resuspended material, 3 mL 30% iodixanol, 2 mL 22.5% iodixanol, 2 mL 17.5% iodixanol, and 1 mL PBS in a 12 mL Ultra-Clear (344059) tube for a SW 41 Ti rotor. The OPTIPREP™ gradient was ultracentrifuged at

150,000 x g for 16 hours at 4 °C to separate the EV (*e.g.*, exosome) fraction. The EV (*e.g.*, exosome) layer was then gently collected from the top ~3 mL of the tube.

[0271] The EV (*e.g.*, exosome) fraction was diluted in ~32 mL PBS in a 38.5 mL Ultra-Clear (344058) tube and ultracentrifuged at 133,900 x g for 3 hours at 4 °C to pellet the purified EVs (*e.g.*, exosomes). The pelleted EVs (*e.g.*, exosomes) were then resuspended in a minimal volume of PBS (~200 µL) and stored at 4 °C.

#### 7.11.1.2. Sample preparation for LC-MS/MS analysis

[0272] To determine proteins specific to EVs (*e.g.*, exosomes), the Top Fraction and Bottom Fraction of the Optiprep™ gradient were analyzed by liquid chromatography-tandem mass spectrometry. All samples were received in either phosphate-buffered saline (PBS) buffer or PBS and 5% sucrose. Prior to analysis, the total protein concentration of each sample was determined by bicinchoninic acid (BCA) assay, after which each sample was appropriately diluted to 125 µg/mL in PBS buffer. Next, 50.0 µL of each sample was added to a separate 1.5 mL microcentrifuge tube containing an equal volume of EV (*e.g.*, exosome) lysis buffer (60 mM Tris, 400 mM GdmCl, 100 mM EDTA, 20 mM TCEP, 1.0% Triton X-100) followed by the transfer of 2.0 µL 1.0% Triton X-100 solution. All samples were then incubated at 55°C for 60 minutes.

[0273] Protein precipitation was performed by adding 1250 µL of ethanol at -20°C. To improve efficiency, samples were vigorously vortexed for approximately 10 minutes and then incubated at -20°C for 60 minutes. After incubation, samples were sonicated in a water bath for 5 minutes. Precipitated material was pelleted by centrifuging for 5 minutes at 15,000 g at 4°C. The supernatant was decanted, and the pelleted material was thoroughly dried using nitrogen gas. Pellets were resuspended in 30.0 µL digestion buffer (30 mM Tris, 1.0 M GdmCl, 100 mM EDTA, 50 mM TCEP, pH 8.5) which also reduced disulfide bonds. Free cysteine residues were alkylated by adding 5.0 µL alkylation solution (375 mM iodoacetamide, 50 mM Tris, pH 8.5) and incubating the resulting solution at room temperature in the dark for at least 30 minutes.

[0274] After incubation, each sample was diluted using 30.0 µL 50 mM Tris pH 8.5, and proteolytic digestion was initiated by adding 2.0 µg trypsin. All samples were mixed and then incubated overnight at 37°C. After the incubation, trypsin activity was ceased by adding 5.0 µL 10% formic acid. Prior to analysis by LC-MS/MS, each sample was desalted using Pierce C18 spin columns. At the end of this process, each sample was dried down and reconstituted in 50.0 µL of water with 0.1% formic acid and transferred to an HPLC vial for analysis.

#### 7.11.1.3. LC-MS/MS analysis

[0275] Samples were injected into an UltiMate 3000 RSCLnano (Thermo Fisher Scientific) low flow chromatography system, and tryptic peptides were loaded onto an Acclaim PepMap 100 C18 trapping column (75  $\mu\text{m}$  x 2 cm, 3  $\mu\text{m}$  particle size, 100 Å pore size, Thermo Fisher Scientific) using loading mobile phase (MPL: water, 0.1% formic acid) at a flowrate of 1.000  $\mu\text{L}/\text{min}$ . Peptides were eluted and separated with a gradient of mobile phase A (MPA: water, 0.1% formic acid) and mobile phase B (MPB: acetonitrile, 0.1% formic acid) at a flowrate of 300 nL/min across an EASY-Spray C18 analytical column (75  $\mu\text{m}$  x 25 cm, 2  $\mu\text{m}$  particle size, 100 Å pore size, Thermo Fisher Scientific). The stepwise gradient used for elution began at 2% MPB, where it was held for 8 minutes during loading. The percentage MPB then increased from 2-17% over 35 minutes, again from 17-25% over 45 minutes, and finally from 25-40% over 10 minutes. The most hydrophobic species were removed by increasing to 98% MPB over 5 minutes, then holding there for 10 minutes. The total runtime for the method was 135 minutes and allowed sufficient time for column re-equilibration. Wash cycles were performed in between non-identical analytical injections to minimize carry-over.

[0276] Mass analyses were performed with a Q Exactive Basic (Thermo Fisher Scientific) mass spectrometer. Precursor ion mass spectra were measured across an  $m/z$  range of 400-1600 Da at a resolution of 70,000. The 10 most intense precursor ions were selected and fragmented in the HCD cell using a collision energy of 27, and MS/MS spectra were measured across an  $m/z$  range of 200-2000 Da at a resolution of 35,000. Ions with charge states from 2-4 were selected for fragmentation and the dynamic exclusion time was set to 30 seconds. An exclusion list containing 14 common polysiloxanes was utilized to minimize misidentification of known contaminants.

#### 7.11.1.4. Data processing

[0277] Proteins were first identified and quantified (label-free) using Proteome Discoverer software (version 2.1.1.21, Thermo Fisher Scientific) and the Sequest HT algorithm combined with the Target Decoy PSM Validator. Searches were performed against the full Swiss-Prot Homo sapiens (taxonomy 9606 version 2017-05-10: 42,153 entries) reference database, as well as a custom Uniprot database containing E1a proteins (7 entries). The following search parameters were used: enzyme, trypsin; maximum of 2 missed cleavages; minimum peptide length of 6 residues; 10 ppm precursor mass tolerance; and 0.02 Da fragment mass tolerance. The search also included specific dynamic modifications (oxidation of M; deamidation

of N or Q; phosphorylation of S, T, or Y; pyro-glutamation of peptide-terminal E; and acetylation of protein N terminus) and static modifications (carbamidomethylation of C).

[0278] In the Target Decoy PSM Validator, the maximum delta Cn and both strict and relaxed target false discovery rates (FDRs) were set to 1 because the data were searched again using Scaffold software (version 4.8.2, Proteome Software Inc.). In Scaffold, the data were also searched using the X! Tandem open source algorithm to identify proteins using a protein threshold of 99.0%, a minimum of 2 peptides, and a peptide threshold of 95%.

[0279] To determine the identity of EV (*e.g.*, exosome)-specific proteins, total peptide spectral matches (PSMs) were compared for proteins found in the top EV (*e.g.*, exosome) fraction of the Optiprep™ gradient versus those in the lower fraction. The results showed that there are a number of membrane-associated proteins highly enriched in the EVs (*e.g.*, exosomes) fraction. The EV (*e.g.*, exosome) proteins included CD13, MME, ENPP1, and NRP1.

#### 7.11.2. Example 2: Verification of Surface Protein Expression

[0280] To confirm that the EV (*e.g.*, exosome)-specific proteins identified in the mass spectrometry studies are highly enriched on the surface of EVs (*e.g.*, exosomes), protein blotting is carried out on total cell lysate and purified EV (*e.g.*, exosome) populations from bone marrow-derived MSC. The total protein pattern differs substantially between total cell lysate and EV (*e.g.*, exosome) lysate. Specifically, there is a strong band in the EV (*e.g.*, exosome) lysate that is absent in the total cell lysate. Western blotting for CD13 reveals that the strong band corresponds to CD13, indicating that CD13 is highly enriched in EVs (*e.g.*, exosomes) produced from bone marrow-derived MSC, and can be visually detectable in total EV (*e.g.*, exosome) lysate.

#### 7.11.3. Example 3: Specific expression of exosome proteins

[0281] To determine the specificity of exosome proteins, mass spectrometry studies and Western blot analysis were performed (as described in Example 1) to identify potential scaffold proteins expressed on exosomes purified (*see, e.g.*, FIG. 1) and to analyze expression of several novel exosome-associated membrane proteins on exosomes produced from various cell types. Specifically, exosomes derived from mesenchymal stem cells (MSCs) and HEK293 cells were analyzed. EV (*e.g.*, exosome) fractions from these cell types were purified on Optiprep™ gradients and analyzed by Western blotting.

[0282] To determine the identity of different exosome proteins, total peptide spectral matches (PSMs) were compared for proteins found in exosomes derived from MSC to those

found in exosomes from HEK293 cells. As shown in FIG. 1, when exosome samples from marrow-derived MSC were analyzed, CD13 was observed to be highly enriched in the exosome fraction. However, when samples from HEK293 cells were analyzed, CD13 was not detected in the exosome fraction or in any other fractions. Similar results were observed for MME (*see* FIG. 1).

**[0283]** Each of the novel surface marker proteins was similarly analyzed. The data show that certain exosome proteins (*e.g.*, CD13 and MME) are enriched on exosomes from certain cell types (*e.g.*, MSCs) but absent on exosomes produced from different cell types (*e.g.*, HEK293).

#### **7.11.4. Example 4: Overexpression of CD13 from producer cells**

**[0284]** The results in Example 3 demonstrated that CD13 and other exosome proteins (*e.g.*, MME) are not universally present on EVs (*e.g.*, exosomes) produced from various cell types. For example, CD13 was highly enriched on exosomes produced from bone marrow-derived MSC, but absent on exosomes produced from HEK293 cells.

**[0285]** To determine whether CD13 can be used as an exosome scaffold protein on a producer cell, which naturally does not produce exosomes with CD13, HEK293 cells were stably transfected with a plasmid expressing full-length CD13 fused to a FLAG tag ("the CD13-FLAG plasmid"). Exosomes were purified from wild-type HEK293SF cells and HEK293SF cells transfected with the CD13-FLAG plasmid. As a control, exosomes were also purified from HEK293SF cells engineered to overexpress the PTGFRN scaffold protein.

**[0286]** The SDS-Page analysis results provided in FIG. 2A show that CD13-FLAG was successfully overexpressed in HEK293SF cells transfected with the CD13-FLAG plasmid. Overexpression of CD13 resulted in similar enrichment on HEK293-derived exosomes compared to PTGFRN. And, as shown in FIG. 2B (right graph), exosomes produced from the CD13-FLAG plasmid transfected HEK293SF cells also expressed high levels of CD13 on their surface. The CD13 expressed on the EVs were biologically active with activity levels comparable to that of recombinant CD13 protein (*see* FIGs. 3A and 3B). These results demonstrate that a producer cell (*e.g.*, HEK293SF), which does not naturally express an exosomal protein (*e.g.*, CD13), can be modified to produce exosomes that overexpress the exosomal protein (*e.g.*, CD13). These results indicate that exosomal proteins such as CD13 can be used for generating engineered EVs (*e.g.*, exosomes) comprising an heterologous exosome protein, *i.e.*, CD13. Furthermore, EVs (*e.g.*, exosomes) purified from modified cells disclosed herein (*e.g.*, HEK293SF cells transfected with

the CD13-FLAG plasmid) show native catalytic activity of the heterologous exosome protein (*e.g.*, CD13).

[0287] Similar procedures can be carried out on CD13 and other HEVPs described herein using various cell types, *e.g.*, CD13 can be introduced into CHO cells (where it is not natively expressed); MME can be introduced into CHO cells and HEK cells (where it is not natively expressed); ENPP1 can be introduced into CHO cells (where it is not natively expressed); and NRP1 can be introduced into CHO cells and HEK cells (where it is not natively expressed).

#### 7.11.5. Example 5: Generation of modified exosome proteins

[0288] As described herein, the exosomal proteins disclosed herein can be modified (*e.g.*, truncated or attached to another moiety) to provide an EV (*e.g.*, exosome) expressing the modified exosomal proteins to exhibit certain properties (*e.g.*, increased targeting to certain cell types). A polynucleotide encoding a modified exosome protein is generated using a polynucleotide encoding a whole exosome protein or a truncated exosome protein. A specific truncated exosome protein is selected by screening various truncated exosome proteins and selecting a truncated protein having optimal capabilities to incorporate into exosome membranes and interact with a binding agent. Targeting of the truncated proteins to exosome membranes is tested by nano-flow cytometry.

[0289] A polynucleotide encoding a modified exosome protein is generated by adding a polynucleotide encoding an affinity tag (glutathione-S-transferase, S-peptide, FLAG tag, GFP, *etc.*) to the polynucleotide encoding a whole or truncated exosome protein (*e.g.*, CD13, MME, ENPP1, and NRP1). The modified polynucleotide expresses a fusion protein. The polynucleotide is further modified to improve their targeting into exosome membranes and/or their affinity to a binding agent.

[0290] A different type of polynucleotide encoding a modified exosome protein is generated by adding a polynucleotide encoding a therapeutic peptide (*e.g.*, an antibody, an enzyme, a ligand, a receptor, an antimicrobial peptide, a variant or a fragment thereof) to the polynucleotide encoding a whole or truncated exosome protein (*e.g.*, CD13, MME, ENPP1, and NRP1). The modified polynucleotide expresses a fusion protein presented on the surface of an exosome. The fusion protein maintains therapeutic activity of the therapeutic peptide.

[0291] A different type of polynucleotide encoding a modified exosome protein is generated by adding a polynucleotide encoding a targeting moiety (*e.g.*, a targeting moiety specific to a specific organ, tissue or cell) to the polynucleotide encoding a whole or truncated

exosome protein (*e.g.*, CD13, MME, ENPP1, and NRP1). The modified polynucleotide expresses a fusion protein presented on the surface of an exosome. The fusion protein allows the exosome to be targeted to a specific organ, tissue or cell.

[0292] Localization of modified exosome proteins on the exosome surface is also tested by nano flow cytometry.

#### **7.11.6. Example 6: Generation of surface-engineered exosomes**

[0293] A producer cell generating surface-engineered exosomes is made by introducing an exogenous sequence encoding an exosome protein or a variant or a fragment of the exosome protein. A plasmid encoding an exosome protein is transiently transfected to induce high-level expression of the exosome protein on the exosome surface. A plasmid encoding a modified exosome protein is transiently transfected to produce exosomes having the modified exosome protein on the surface.

[0294] A polynucleotide encoding an exosome protein, a variant or a fragment of an exosome protein, or an exogenous sequence encoding an affinity tag, a therapeutic peptide or a targeting moiety is stably transformed into a producer cell to produce surface-engineered exosomes. The exogenous sequence encoding an affinity tag, a therapeutic peptide or a targeting moiety is inserted into a genomic site encoding an exosome protein to generate a fusion protein comprising the affinity tag attached to the exosome protein. A polynucleotide encoding a modified exosome protein is knocked in to a genomic site encoding an exosome protein.

[0295] A producer cell line is generated by stably transfecting at least two polynucleotides, each encoding an exosome protein, a variant or a fragment of an exosome protein, or an exogenous peptide (*e.g.*, affinity tag, targeting moiety, therapeutic peptide). A different producer cell line is also generated by inserting two or more exogenous sequences (*e.g.*, exogenous sequences encoding an affinity tag, a marker, a targeting peptide, a therapeutic peptide, *etc.*) into multiple genomic sites, within or in a close proximity to the genomic sequence encoding an exosome protein, to generate a surface-engineered exosome comprising multiple modified exosome proteins. Each of the plurality of modified exosome proteins is targeted to the surface of exosomes. The exosomes have affinities to two different binding agents and are purified by either or both of the binding agents.

#### **7.11.7. Example 7: Isolation, purification and sub-fractionation of exosomes by affinity purification**

[0296] Binding agents for affinity purification of exosomes are developed by biopanning/directed evolution that elute under mild conditions.

[0297] The binding agent is attached to a solid support (*e.g.*, a porous agarose bead) and formed into a conventional chromatography system (*e.g.*, GE AKTA). A sample containing exosomes is applied to the column for affinity purification.

#### 7.11.8. Example 8: Analysis of Different Scaffold Moieties to Target Exosomes

[0298] Further to Example 6 described above, the ability of CD13 and MME proteins were tested for their ability to target proteins to the surface of exosomes derived from HEK293 cells. Briefly, plasmids expressing full-length CD13 fused to green fluorescent protein (GFP) or full-length MME fused to GFP were stably transfected into HEK293 cells. For comparison purposes, HEK293 cells engineered to overexpress PTGFRN fused to GFP were also generated.

[0299] As shown in FIG. 4A, HEK293 cells transfected with the CD13-GFP or MME-GFP plasmids expressed the particular exosomal protein of interest. The overall expression was similar to that of PTGFRN-GFP observed in the HEK293 cells engineered to overexpress PTGFRN. And, as shown in FIG. 4B, both the CD13-GFP and MME-GFP fusion proteins were properly folded and expressed in the EVs produced from the respective HEK293 producer cells.

[0300] Collectively, the above results demonstrate that certain exosomal proteins (*e.g.*, heterologous exosomal vesicle proteins disclosed herein, *e.g.*, CD13 and MME) can be exogenously introduced to a producer cell that does not naturally express the heterologous protein. The results further demonstrate that EVs (*e.g.*, exosomes) produced from such modified producer cells can express the heterologous exosomal proteins, which can be used to anchor various molecules (*e.g.*, antigen, targeting moiety, adjuvants, and/or immune modulators) to the EVs (*e.g.*, exosomes).

### 8. INCORPORATION BY REFERENCE

[0301] All publications, patents, patent applications and other documents cited in this application are hereby incorporated by reference in their entireties for all purposes to the same extent as if each individual publication, patent, patent application or other document were individually indicated to be incorporated by reference for all purposes.

### 9. EQUIVALENTS

[0302] The present disclosure provides, *inter alia*, compositions of cannabinoid and entourage compositions. The present disclosure also provides method of treating neurodegenerative diseases by administering the cannabinoid and entourage compositions. While various specific embodiments have been illustrated and described, the above specification is not restrictive. It will be appreciated that various changes can be made without departing from

the spirit and scope of the disclosure(s). Many variations will become apparent to those skilled in the art upon review of this specification.

## Informal Sequence Listing

## SEQ ID 47

CD13

>sp|P15144|AMPN\_HUMAN Aminopeptidase N OS=Homo sapiens OX=9606 GN=ANPEP PE=1 SV=4

MAKGFYISKSLGILGILLGVAAVCTIIALSVVYSQEKNKNANSSPVASTTPSASATTNPA  
SATTLDQSKAWNRYRLPNTLPKDSYRVTLRPYLTPNDRGLYVFKGSSTVRFTCKEATDVI  
IIHSSKKLNYTLTSQGHRVVLRGVGGSPDPIDKTELVEPTEYLVVHLKGS�VKDSQYEMDS  
EFEGELADDLAGFYRSEYMEGNVRKVATTQMQAADARKSFPCFDEPAMKAEFNITLIHP  
KDLTALSNMLPKGPSTPLPEDPNWNVTEFHTTPKMSTYLLAFIVSEFDYVEKQASNGVLI  
RIWARPSAIAAGHGDIYALNVTGPILNFFAGHYDTPYPLPKSDQIGLPDFNAGAMENWGLV  
TYRENSLLFDPLSSSSSNKERVVTVIAHELAHQWFGNLVTIEWWDLWLNNEGFASYVEYL  
GADYAEPTWNLKDLMLVNDVYRMAVDALASSHPLSTPASEINTPAQISELFDIAISYSG  
ASVLRMLSSFLSEDVFKQGLASYLHTFAYQNTIYLNLDHLQEAVERNRSIQLPPTVRDIM  
NRWTLQMGFPVITVDTSTGTLSEHFLDPDSNVTRPSEFNYYVWIVPITSIRDGRQQQDY  
WLLDVRAQNDLFSTSGNEWVLLNLNVTGYRVNYDEENWRKIQTQLQRDHSAPVINRAQ  
IINDAFNLASAHKVPVTLALNNTLFLIEERQYMPWEAALSSLSYFKLMFDRSEVYGPMMN  
YLKQVTPFLFIHRNNTNNWREIPENLMDQYSEVNAISTACSNVPECEEMVSGLFQWM  
ENPNNNPIHNLNRSTVYCNALAQGGEEWDFAWEQFRNATLVNEADKLRAALACSKELWI  
LNRYLSYTLNPDILRKQDATSTIISITNNVIGQGLVWDFVQSNWKKLFNDYGGGSFSFSN  
LIQAVTRRFSTEYELQQLQEQFKKDNEETGFGSGTRALEQALEKTKANIKWVKENKEVVLO  
WFTENSK

## SEQ ID 48

MME

>sp|P08473|NEP\_HUMAN Neprilysin OS=Homo sapiens OX=9606 GN=MME PE=1 SV=2

MGKSESQMDITDINTPKPKKKQRWTPLEISLSVLVLLLTIIAVTMIALYATYDDGICKSS  
DCIKSAARLIQNMDATEPCTDFKYACGGWLKRNVIPETSSRYGNFDILRDELEVVLKD  
VLQEPKTEDIVAVQKAKALYRSCINESAIDSRGGEPLKLLPDIYGPVATENWEQKYGA  
SWTAEKAIQNLNSKYGKKVLINLFGVTDKNSVNHVIHIDQPRGLPSRDYECTGIYKE  
ACTAYVDFMISVARLIRQEERLPIDENQLALEMNKVMLEKEIANATAKPEDRNDPMLLY  
NKMTLAQIQNNFSLINGKPFSLNFTNEIMSTVNISITNEEDVVVYAPEYLTCLKPILT  
KYSARDLQNLMSWRFIMDLVSSLSRTYKESRNAFRKALYGTTSATWRRCANVYNGNME  
NAVGRLYVEAAFAGESKHVVEDLIAQIREVFIQTLDDLTMWDAETKKRAEEKALAIKERI  
GYPDDIVSNDKNLNNEYLELNKYEDEFENIIQNLKFSQSKQLKKLREKVDKDEWISGAA  
VNAFYSSGRNQIVFPAGILQPPFFSAQQSNSLNYGGIGMVIGHEITHGFDDNGRNFNKD  
GDLVDWWTQQSASNFEQSQCVMYQYGNFSWDLAGGQHLNGINTLGENIADNGGLGQAYR  
AYQNYIKKNGEKLLPGLDLNHKQLFFLNFAQVWCGTYRPEYAVNSIKTDVHSPGNFRII  
GTLQNSAEFSEAFHCRKNSYMNPEKKCRVW

## SEQ ID 49

ENPP1

>sp|P22413|ENPP1\_HUMAN Ectonucleotide pyrophosphatase/phosphodiesterase family member 1 OS=Homo sapiens OX=9606 GN=ENPP1 PE=1 SV=2

MERDGCAGGSGRGEGGRAPREGPAGNGRDRGRSHAAEAPGDPQAAASLLAPMDVGEEPL  
EKAARARTAKDPNTYKVLVLSVLCVLTITLGCIFGLKPSCAKEVKCKGRCFERTFGNC  
RCDAACVELGNCCLDYQETCIEPEHIWTCNKFRGCKRLTRSLCACSDCKDKGDCCINY  
SSVCQGEKSWVEEPCESINEPQCPAGFETPPTLLFSLDGFRAEYLHTWGGLLPVISKLLK  
CGTYTKNMRPVYPTKTFPNHYSIVTGLYPESHGIIDNKMYDPKMNASFSLSKSEKFNPEW  
YKGEPIWVTAKYQGLKSGTFFWPGSDVEINGIFPDIYKMYNGSVPFEEILAVLQWLQLP  
KDERPHFYTYLLEEDSSGHSYGPVSSEVIKALQRVDGMVGMMDGLKELNLHRCLNLIL  
ISDHGMEQGSCKKIYLNKYLGDVKNIKVIYGPAAARLRPSDVPDKYYSFNYEGIARNLSC  
REPQNHFKPYLKHFLPKRLHFAKSDRIEPLTFYLDPQWQLALNPSEKCYGSGFHGSDNV  
FSNMQALFVGYPGPGFKHGIEADTFENIEVYNLMCDLLNLTPAPNNGTHGSLNHLKKNPVY  
TPKHPKEVHPLVQCPFTRNPRNLGCSCNPSILPIEDFQTQFNLTVAEEKIHKHETLPYG  
RPRVLQKENTICLLSQHQFMSGYSQDILMPLWTSYTVDRNDSFSTEDFSNCLYQDFRIPL

SPVHKCSFYKNNTKVSYGFLSPPQLNKNSSGIYSEALLTTNIVPMYQSFQVIWRYFHDTL  
LRKYAEERNGVNVVSGPVDFDYDGRCDSENLQRKRRVIRNQEILIPTHFFIVLTSCKD  
TSQTPHLCENLDTLAFILPHRTDNSESCVHGKHDSSWVEELMLHRARITDVEHITGLSF  
YQQRKEPVSDILKCLKTHLPTFSQED

**SEQ ID 50**

NRP1

>sp|014786|NRP1\_HUMAN Neuropilin-1 OS=Homo sapiens OX=9606 GN=NRP1 PE=1 SV=3  
MERGLPLLCAVLALVLAPAGAFRNDKCGDTIKIESPGYLTSPGYPHSYHPSEKCEWLIQA  
PDYPYQRIMINFNPHFDLEDRCCKYDYVEVFDGENENGHFRGKFCGKIAPPPVVSSGPFLF  
IKFVSDYETHGAGFSIRYEIFKRGPECSQNYTTPSGVIKSPGPFPEKYPNSLECTYIVFVP  
KMSEIILEFESFDLEPDSNPPGGMFRCYDRLEIWDGFPDVGPHIGRYCGQKTPGRIRSSS  
GILSMVFYTDSAIAKEGFSANYSVLQSSVSEDFKCMEALGMESGEIHSQITASSQYSTN  
WSAERSRLNYPENGWTPGEDSYREWIQVDLGLLRFTAVGTQGAISKETKKKYVVKTYKI  
DVSSNGEDWITIKENKPVLFQGNTNPTDVVVAVFPKPLITRFVRIKPATWETGISMRFE  
VYGCKITDYPCSGMLGMVSGLISDSQITSSNQGDRNWMPENIRLVTSRSGWALPPAPHSY  
INEWLQIDLGEEKIVRGIIQGGKHRENKVFMRKFKIGYSNNGSDWKIMDDSKRKAKSF  
EGNNNYDTPELRTFPALSTRFIRIYPERATHGGLGLRMELLGCEVEAPTAGPTTPNGNLV  
DECDDQANCHSGTGDDFQLTGGTTVLATEKPTVIDSTIQSEFPTYGFNCEFGWGSHKTF  
CHWEHDNHVQLKWSVLTSKTGPIQDHTGDGNFIYSQADENQKGKVARLVSPVVYSQNSAH  
CMTFWYHMSGSHVGTLRVKLRYQKPEEYDQLVWMAIGHQGDHWKEGRVLLHKSLKLYQVI  
FEGEIGKGNLGGIAVDDISINNHSQEDCAKPADLDKKNPEIKIDETGSTPGYEGEGEGD  
KNISRKPGNVLKTLDPILITIIAMSALGVLLGAVCGVLYCACWHNGMSERNLSALENYN  
FELVDGVKLLKDKLNTQSTYSEA

**WHAT IS CLAIMED IS:**

1. An extracellular vesicle (EV) comprising at least one heterologous extracellular vesicle protein (HEVP) or a fragment thereof, wherein the EV is produced from a producer cell which does not naturally express the HEVP, and wherein the HEVP is produced naturally by a donor cell.
2. The EV of claim 1, wherein the EV comprises 2, 3, 4, 5, 6, 7, 8, 9 or 10 heterologous extracellular vesicle proteins (HEVPs) or fragments thereof, wherein the EV is produced from a producer cell which does not naturally express the HEVPs, and wherein the HEVPs are produced naturally by a donor cell.
3. The EV of any one of the above claims, wherein the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of HEVP in the exosomes produced by the donor cell is equal to or greater than about 20 peptide spectral matches (PSM) measured using liquid chromatography with tandem mass spectrometry (LC-MS/MS).
4. The EV of any one of the above claims, wherein the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of HEVP in the exosomes produced by the donor cell is equal to or greater than about 80 peptide spectral matches (PSM) measured using LC-MS/MS.
5. The EV of any one of the above claims, wherein the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of HEVP in the exosomes produced by the donor cell is equal to or greater than about 125 peptide spectral matches (PSM) measuring using LC-MS/MS.
6. The EV of any one of the above claims, wherein the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of HEVP in the exosomes produced by the donor cell is equal to or greater than about 170 peptide spectral matches (PSM) measuring using LC-MS/MS.

7. The EV of any one of the above claims, wherein the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of HEVP in the exosomes produced by the donor cell is equal to or greater than about 200 peptide spectral matches (PSM) measuring using LC-MS/MS.

8. The EV of any one of the above claims, wherein the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of HEVP in the exosomes produced by the donor cell is equal to or greater than about 700 peptide spectral matches (PSM) measuring using LC-MS/MS.

9. The EV of any one of claims 1-6, wherein the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of HEVP in the exosomes produced by the donor cell is about 177 peptide spectral matches (PSM) measuring using LC-MS/MS.

10. The EV of any one of claims 1-8, wherein the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of HEVP in the exosomes produced by the donor cell is about 742 peptide spectral matches (PSM) measuring using LC-MS/MS.

11. The EV of any one of claims 1-3, wherein the level of the at least one HEVP in the exosomes produced by the donor cell is between about 20 and about 80 peptide spectral matches (PSM) measured using LC-MS/MS.

12. The EV of any one of claims 1-5, wherein the level of the at least one HEVP in the exosomes produced by the donor cell is between about 80 and about 200 peptide spectral matches (PSM) measured using LC-MS/MS.

13. The EV of any one of claims 1-8, wherein the level of the at least one HEVP in the exosomes produced by the donor cell is between about 150 and about 750 peptide spectral matches (PSM) measured using LC-MS/MS.

14. The EV of any one of the above claims, wherein the at least one HEVP is produced naturally by a donor cell which produces exosomes, and wherein the level of the at least one HEVP in the exosomes produced by the donor cell is equal to or greater than about 5% of the total protein content of the exosomes produced by the donor cell.
15. The EV of any one of the above claims, wherein the at least one HEVP or fragment thereof is a fusion protein.
16. The EV of any one of the above claims, wherein the at least one HEVP or fragment thereof is modified by the addition of a functional moiety.
17. The EV of claim 16, wherein the functional moiety has affinity to a binding agent.
18. The EV of claim 17, wherein the functional moiety is an affinity tag.
19. The EV of claim 18, wherein the affinity tag is a peptide.
20. The EV of claim 16, wherein the functional moiety is a therapeutic compound.
21. The EV of claim 20, wherein the therapeutic compound is selected from the group consisting of a natural peptide, a recombinant peptide, a synthetic peptide, and any combination thereof.
22. The EV of claim 20, wherein the therapeutic compound is selected from the group consisting of nucleotides, amino acids, lipids, carbohydrates, small molecules, and any combination thereof.
23. The EV of claim 20, wherein the therapeutic compound is an antibody or a fragment thereof.

24. The EV of claim 20, wherein the therapeutic compound is an enzyme, a ligand, a receptor, or a fragment thereof.
25. The EV of claim 16, wherein the functional moiety is a targeting moiety.
26. The EV of claim 25, wherein the targeting moiety is specific to an organ, a tissue, or a cell.
27. The EV of any one of claims 15-26, wherein the fusion protein comprises a linker.
28. The EV of any one of claims 16-27, further comprising a linker between the at least one HEVP or fragment thereof and the functional moiety.
29. The EV of claim 27 or 28, wherein the linker is a flexible linker or a rigid linker.
30. The EV of any one of claims 27-29, wherein the linker is a straight-chain carbon linker, a branched-chain carbon linker, a heterocyclic carbon linker, or a peptide linker.
31. The EV of any one of claims 27-30, wherein the linker is a cleavable linker.
32. The EV of any one of the above claims, wherein the producer cell is selected from the group consisting of HEK, CHO, MB-231, Raji, PER.C6, CAP, MSC cells, and any combination thereof.
33. The EV of any one of the above claims, wherein the at least one HEVP is selected from the group consisting of a lung fibroblast EVP, an aortic endothelium cell EVP, an acute myeloid leukemia cell EVP, a monocyte EVP, a B-cell lymphoma EVP, a macrophage EVP, a brain endothelium cell EVP, a mesenchymal cell EVP, and any combination thereof.
34. The EV of claim 33, wherein the at least one HEVP is a human EVP, mouse EVP, rat VEP, dog EVP, or monkey EVP.

35. The EV of any one of the above claims, wherein the at least one HEVP is selected from the group consisting of CD13, MME, ENPP1, and NRP1, or a fragment thereof.
36. The EV of any one of the above claims, wherein the at least one HEVP is selected from the group consisting of CD13, MME, ENPP1, and NRP1, or a fragment thereof and wherein the producer cell is a CHO cell.
37. The EV of any one of the above claims, wherein the at least one HEVP is selected from the group consisting of CD13, MME, and NRP1, or a fragment thereof and wherein the producer cell is a CHO cell or a HEK cell.
38. The EV of any one of claims 1-34, wherein the at least one HEVP is selected from the group consisting of PTGFRN, BSG, IGSF3, ITGB1, ITGA4, SLC3A2, ATP transporter, or a fragment thereof, and any combination thereof.
39. The EV of any one of claims 1-37, where the extracellular vesicle further comprises an EVP selected from the group consisting of PTGFRN, BSG, IGSF3, ITGB1, ITGA4, SLC3A2, aATP transporter, or a fragment thereof, and any combination thereof.
40. The EV of any one of the above claims, wherein the producer cell is a non-human cell and wherein the at least one HEVP is a human HEVP.
41. The EV of any one of claims 1-39, wherein the producer cell is a human cell and wherein the at least one HEVP is a non-human HEVP.
42. A pharmaceutical formulation comprising the EV of any one of the above claims.
43. A kit comprising an EV or pharmaceutical formulation of any one of the above claims, wherein the EV or pharmaceutical formulation is contained in a container or package, and wherein the kit further comprises instructions describing a recommended use of the EV or pharmaceutical formulation.

44. A method for treating a patient in need thereof, comprising administering to the patient an effective amount of the EV or pharmaceutical formulation of any one of claims 1-42.
45. A method of expressing a non-naturally occurring protein in an EV derived from a cell, comprising transfecting a nucleic acid encoding at least one heterologous extracellular vesicle protein (HEVP) or a fragment thereof in the cell and isolating an EV comprising the HEVP or a fragment thereof from the cell, wherein the HEVP is not naturally occurring in the EV derived from the cell.
46. The method of claim 45, wherein the EV comprises the EV of any one of claims 1 to 41.
47. The method of claim 45 or 46, further characterizing the HEVP of the EV.