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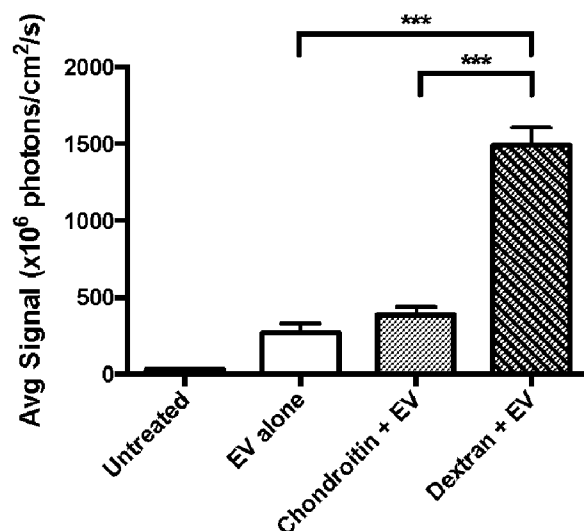
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[Continued on next page]

(54) Title: EXTRACELLULAR VESICLES, METHODS OF MAKING THEM, AND METHODS OF REDUCING LIVER UPTAKE OF EXTRACELLULAR VESICLES

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(57) Abstract: An isolated or purified extracellular vesicle comprising a membrane comprising a phospholipid bilayer and a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha is disclosed. Also provided are methods of preparing isolated or purified extracellular vesicles comprising a therapeutic protein, methods of reducing liver uptake of extracellular vesicles administered to a mammal, and methods of treating or preventing cancer in a mammal.



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EXTRACELLULAR VESICLES, METHODS OF MAKING THEM, AND METHODS OF REDUCING LIVER UPTAKE OF EXTRACELLULAR VESICLES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority benefit of U.S. provisional application no.62/316,276, filed March 31, 2016, which application is herein incorporated by reference for all purposes.

REFERENCE TO A SUBMISSION OF A SEQUENCE LISTING AS AN ASCII TEXT FILE

[0002] This application includes a Sequence Listing as a text file named "077867-1044125_ST25.txt created on March 29, 2017 and containing 97,160 bytes. The material contained in this text file is hereby incorporated by reference in its entirety for all purposes.

BACKGROUND OF THE INVENTION

[0003] Extracellular vesicles (EVs) may have therapeutic potential. However, obstacles to the successful use of EVs remain. For example, low production yield of EVs from cell culture may impede any one or more of basic extracellular vesicle (EV) research, EV therapeutics engineering, and preclinical/clinical *in vivo* studies. Alternatively or additionally, administered EVs may be rapidly cleared by the reticuloendothelial system (RES) such as, for example, monocytes and macrophages. The clearance of the EVs by the RES may, undesirably, decrease the delivery of the EVs to a desired target site (e.g., a tumor).

[0004] Accordingly, there is a need for improved EVs, methods of producing EVs, and methods of improving delivery of EVs to a target site.

BRIEF SUMMARY OF THE INVENTION

[0005] An embodiment of the invention provides an isolated or purified EV comprising a phospholipid bilayer membrane and a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha, wherein: the phospholipid bilayer comprises an exterior surface, an interior surface, and an interior region positioned between the exterior surface and

the interior surface, and at least a portion of the heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha is positioned in the interior region of the phospholipid bilayer.

[0006] Another embodiment of the invention provides an isolated or purified EV comprising a phospholipid bilayer, a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha, and a carrier, wherein: the phospholipid bilayer comprises an exterior surface, an interior surface, and an interior region positioned between the exterior surface and the interior surface, the EV comprises an exterior surface, the carrier is positioned on the exterior surface of the EV or at least a portion of the carrier is embedded in the phospholipid bilayer, and the heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha is positioned on the carrier.

[0007] Further embodiments of the invention provide compositions relating to the inventive isolated or purified EVs and methods of treating or preventing cancer comprising administering the isolated or purified EVs.

[0008] Still another embodiment of the invention provides a method of preparing isolated or purified EVs comprising a therapeutic protein, the method comprising: introducing a nucleotide sequence into cells, wherein the nucleotide sequence encodes the therapeutic protein; culturing the cells in medium in a hollow fiber bioreactor under conditions sufficient for the cells to express the therapeutic protein; secreting from the cells the EVs comprising the expressed therapeutic protein into the medium; and separating the EVs comprising the expressed therapeutic protein from the cells to produce isolated or purified EVs comprising the therapeutic protein.

[0009] Another embodiment of the invention provides isolated or purified EVs prepared according to the inventive methods and methods of treating or preventing cancer comprising administering the isolated or purified EVs.

[0010] Another embodiment of the invention provides a method of reducing liver uptake of EVs administered to a mammal, the method comprising: administering EVs comprising a therapeutic protein to the mammal; and administering a scavenger receptor antagonist, *e.g.*, a Scavenger Receptor A antagonist, to the mammal in an amount effective to reduce uptake of the EVs by the liver of the mammal.

[0011] Still another embodiment of the invention provides a method of treating or preventing cancer in a mammal, the method comprising: administering EVs comprising a therapeutic protein to the mammal in an amount effective to treat or prevent cancer in the

mammal; and administering a Scavenger Receptor A antagonist to the mammal in an amount effective to reduce uptake of the EVs by the liver of the mammal.

[0012] In a further aspect, provided herein is a mammalian host cell comprising a DNA construct encoding interleukin-15 and an interleukin-15 receptor alpha-lactadherin carrier polypeptide. In some embodiments, the DNA construct comprises the nucleic acid sequence of SEQ ID NO:13. In some embodiments, the DNA construct encodes a human interleukin-15 of SEQ ID NO:7 and a human IL-15 receptor alpha-human lactadherin fusion protein of SEQ ID NO:10. In some embodiments, the DNA construct comprises the region of SEQ ID NO:13 that encodes human IL-15 receptor alpha-human lactadherin fusion protein of SEQ ID NO:10 and the region of SEQ ID NO:13 that encodes the human interleukin-15 of SEQ ID NO:7. In some embodiments, a polypeptide of interest is fused to IL-15. In some embodiments, the polypeptide of interest is a cytokine, such as IL-12. The mammalian host cell may be any kind of host cell, including, for example human, non-human primate, murine, rat, hamster, or any other species. In some embodiments, the host cell is an HEK 293 cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] Figure 1A is a graph showing the yield of EV-associated protein in μg per milliliter of conditioned medium (mean $\pm$ SEM) obtained (i) using conventional flask culture (ultrafiltration to concentrate conditioned media, filtration, and differential centrifugation), (ii) using a bioreactor (ultrafiltration to concentrate conditioned media, filtration, and differential centrifugation), or (iii) a bioreactor and a modified protocol (filtration, dialysis, and differential centrifugation)). Yield was quantified in independent preparations.

[0014] Figure 1B is a graph showing the bioactivity quantified by mean MTT assay (O.D in 6 wells per sample (mean $\pm$ SEM)) of 3 independent EV preparations from (i) bioreactor supernatants of a HEK293 cell clone overexpressing hetIL-15 (circles) or (ii) protein standard (grey line lacking symbols). Incubation of cells with equal amount of control HEK293 cell EV resulted in MTT O.D. <0.25 .

[0015] Figure 2A is a graph showing the uptake of EV (normalized to untreated monocyte uptake) by monocytes, B cells, T cells, and NK cells treated with EV alone (unshaded bars) or pretreated with chondroitin sulfate (grey bars) or dextran sulfate (diagonal striped bars) followed by treatment with EV. Boxes denote minimum to maximum values, and line in box marks the group mean. Statistical analysis was with multiple t-tests, with

Holm-Sidak correction for multiple comparisons (Cumulative alpha-error <0.05). One (*), two (**), and three (***) asterisks denote $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

[0016] Figure 2B is a graph showing the amount of IL-15 (pg/mL) measured in the plasma 2.5 hours following injection with 15 μ g of EV or the combination of 0.6 mg dextran sulfate and EV. Uninjected mice served as a control.

[0017] Figure 2C is a graph showing the quantitation of EV accumulation (measured as the average signal ($\times 10^6$ photons/cm²/s) in excised tumors of untreated mice or mice treated with EV alone, the combination of chondroitin and EV, or the combination of dextran sulfate and EV, 24h after EV administration. Bars display group mean $\pm$ SEM.

[0018] Figure 3 is a graph showing the yield of mcg EV-associated protein (IL-15) per mg of EV protein obtained, wherein the EV comprises (i) a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha with lactadherin as a carrier (encoded by an AG304 vector comprising SEQ ID NO: 13), (ii) a natural heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha (encoded by an AG152 vector comprising SEQ ID NO: 16), or (iii) control EV with no heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha.

[0019] Figure 4 is a graph showing the bioactivity quantified by mean MTT assay (O.D in 6 wells per sample (mean $\pm$ SEM)) of 3 independent EV preparations from (i) a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha with lactadherin as a carrier (encoded by an AG304 vector comprising SEQ ID NO: 13) (circles) or (ii) a natural heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha protein standard (grey line lacking symbols).

[0020] Figure 5 is a graph showing the purity of EV prepared in a conventional flask and in a bioreactor, as estimated by the ratio of particles (10^7) per μ g EV.

[0021] Figure 6 provides a schematic showing EV purification protocols.

[0022] Figure 7 shows the results of size exclusion chromatography (SEC) monitoring to identify EV-rich chromatography fractions. EV-containing conditioned medium supernatant was subjected to size exclusion chromatography. Supernatant was applied to a 60 cm SEC column and UV-light absorbance was monitored at 260 nm (A260) and 280 nm (A280). Particle concentration in a subset of collected fraction was assessed by nanoparticle tracking analysis. The first peak, which would include relatively large molecules, had a high A260/A280 ratio, suggesting the presence of nucleic acids. As EVs carry significant amount of RNA molecules, we analyzed the early fraction by nanoparticle tracking, which

demonstrated that they were EV-rich. Later fractions were observed (after 60 minutes of elution) that had a lower A260/A280 and were likely correspond to EV-free protein fractions.

[0023] Figure 8 provides data illustrating that the yield of EV particles using SEC was similar to that achieved with using ultracentrifugation. Statistical analysis was performed by 2-way ANOVA. Three independent bioreactor supernatants were divided and processed to compare ultracentrifugation to size-exclusion chromatography. Purified EV preparations from these two methodologies were characterized by nanoparticle tracking analysis to estimate particle concentration. The results demonstrated that there were no differences in the particle yield, indicating that SEC did not reduce EV yield. There was also no difference in the overall EV protein yield when comparing the two methods (data not shown).

[0024] Figure 9 provides data illustrating that EV-associated hetIL-15/lactaderin fusion protein is not lost during SEC purification. Three independent bioreactor harvests were divided and processed either by U/C or SEC. IL-15 content was then assayed on intact EV by ELISA. The amounts of IL-15 associated with the EV preparations were equivalent for the two purification methods. (Analysis by paired t-test)

[0025] Figure 10 provides data illustrating that SEC separates EV from non-EV macromolecules better than ultracentrifugation. Three independent bioreactor harvests were divided and processed either by U/C or SEC. Ferritin content was assayed on intact EV by ELISA. Ferritin is a large, 8nm protein complex that co-purifies with ultracentrifuge-pelleted EV, as previous analyses of U/C preparations of EV showed that ferritin was a major contaminant of material prepared with this methodology. SEC decreased the presence of ferritin in EV preparations by 100-fold. This data indicated that SEC is better able to separate large protein complexes from EV, thereby increasing purity. (Analysis by paired t-test)

[0026] Figure 11 provides data illustrating that SEC EV display a more uniform size distribution. Bioreactor harvest was divided and purified by either SEC or U/C. Size distribution of EV in purified preparations was analyzed by nanoparticle tracking analysis (5 technical replicates per sample). Solid black line denotes mean +/- SEM of SEC EV measurements. Dotted line and gray shaded area represent mean +/- SEM of U/C measurements. The results indicated that EV purified by SEC have a very sharp size distribution, with a major population peak at 90nm and 150nm. The size distribution of EV purified by U/C had a broader size distribution, suggesting changes in the qualities of the EV, *e.g.*, aggregation with protein complexes, including ferritin.

[0027] Figure 12 provides data illustrating that EV-rich SEC fractions have a high CD63 content. Bioreactor supernatant was processed either by ultracentrifugation of SEC in 60 cm column for EV purification. 10 µg protein from matching cell lysate, ultracentrifuge (UC) EV, and EV-rich SEC-EV fractions were analyzed by Western blot. CD63 is an EV surface marker and was highly enriched in EV preparations vs. cell lysate. SEC fractions F29 and F30 contained more CD63 than UC-EV, while F31/F32 had slightly lower levels of this protein. Calnexin is a cell-associated protein, which was detectable only in the cell lysate, suggesting lack of contamination by cell debris or apoptotic bodies in our EV preps. The calnexin blot was overexposed (360 sec) to confirm absence of low-level protein expression in EV preps.

[0028] Figure 13 provides data illustrating that tangential-flow filtration (TFF) alone removes much of the non-EV components of conditioned media supernatant. Bioreactor harvest was divided and processed in 24 cm SEC column following: no TFF (top panel), 750kDa TFF (middle panel), or 50 nm TFF (bottom panel). Column eluate was monitored by UV-light absorbance at two different wavelengths: EV-rich fractions were confirmed by nanoparticle tracking analysis. As shown in Figure 7, EV-rich fractions coincided with the first light absorbance peak, characterized by a high A260/A280 ratio. TFF reduced later peaks (beyond 15min). However, 50 nm TFF resulted in an even narrower first peak, and further reduced a minor peak (at 10 min elution). This narrowing of the peak is likely associated with the removal of additional macromolecular complexes between 750kDa and 50nm. One of these complexes is ferritin (8nm). Removal of non-EV proteins allowed for significant concentration of bioreactor conditioned media, which can be followed by an SEC chromatography step to achieve maximum purity.

[0029] Figure 14 provides illustrative data demonstrating that TFF concentrates EV. The ability to concentrate large volumes of bioreactor-conditioned media is an important step in achieving large-scale production of EV for therapeutic use. This experiment demonstrated that EV can be concentrated with TFF, a fully scalable methodology. Bioreactor conditioned medium was divided, and processed as follows: No TFF; TFF in micro-scale apparatus (750 kDa molecular-weight cut-off [MWCO]); and TFF in micro-scale apparatus (0.05 µm pore size). TFF samples were first subjected to “active dialysis” within the filtration apparatus. This was performed by adding PBS to the sample as it was circulated through the TFF apparatus, meanwhile maintaining a stable volume (1x concentration). The result is a gradual removal of small molecular weight components that may aggregate and limit concentration of

bioreactor conditioned media. After addition of 5 volumes of PBS, a small aliquot of unconcentrated/ultrafiltered conditioned medium was obtained (1x sample). The remaining sample was concentrate, approximately 5-fold using the same TFF apparatus (5x sample). Particle concentration in each sample obtained was estimated by nanoparticle tracking analysis. Both 750 kDa and 0.05 μ m apparatuses were able to concentrate EV.

[0030] Figure 15 provides data illustrating that TFF with a 0.05 μ m filter removes ferritin. The samples prepared in the TFF pilot experiment were assayed for ferritin, a macromolecular protein complex 8 nm in diameter. This was a contaminant of EV prepared by ultracentrifugation. The results showed that the absolute concentration (mcg/mL) of ferritin increased when conditioned media were concentrated in a 750 kDa filter, suggesting retention of this contaminant. In contrast, the concentration decreased using the 0.05 μ m filter. The rate of ferritin depletion was similar to other EV-free protein, as the proportion of ferritin (% of total protein) didn't change significantly during 0.05 μ m filtration. EV (~100 nm) are thus retained and concentrated by 0.05 μ m TFF, while contaminating non-EV proteins (including ferritin) were significantly depleted.

[0031] Figures 16(a) and 16(b) provide data illustrating that hetIL-15/lactadherin fusion increases IL-15 loading of EVs.

[0032] Figure 17 provides schematics of mouse IL-15:IL-12p40 chimera-expressing DNA constructs.

[0033] Figure 18 provides data showing hetIL-15 determination.

[0034] Figure 19 provides data illustrating the expression of IL-15:IL-12p40 (L) and presence in supernatant. Transfection 100 ng DNA EF, harvested at 48 hours. Anti-mouse IL-12, 1:1000; anti-goat, 1:5000

[0035] Figure 20 provides data illustrating that IL-15:IL-12 fusion protein maintains the ability to interact with IL-12p35 and IL-15sR α . HEK293 were transiently transfected with plasmids encoding for IL-12p35 together with the fusion protein (cloned with FLAG-tagged IL-12p40) or with IL-15sR α and harvested at 48 hours. Band shift indicates an interaction between IL-15:IL-12, IL-12p35, and IL-15sR α . Primary antibody: Anti-FLAG

[0036] Figure 21 provides data illustrating IFN- γ production by NK-92 cells stimulated by IL-15:IL-12 fusion protein or by IL-12p70.

[0037] Figure 22 provides data illustrating that co-delivery of IL-12 and IL-15 into BALB/c mice has synergistic effects on IFN- γ production and CD8+ T cell proliferation.

[0038] Figure 23 provides data illustrating that co-delivery of fusion protein and IL-12p35 promotes CD8+ T cell proliferation similarly to combination of heterodimeric IL-15 and IL-12.

DETAILED DESCRIPTION OF THE INVENTION

[0039] An embodiment of the invention provides an isolated or purified EV comprising a phospholipid bilayer membrane and a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha, wherein: the phospholipid bilayer comprises an exterior surface, an interior surface, and an interior region positioned between the exterior surface and the interior surface, and at least a portion of the heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha is positioned in the interior region of the phospholipid bilayer.

[0040] The interleukin-15 (IL-15) and interleukin-15 receptor alpha (IL-15Ra) may be from any suitable mammalian species. In a preferred embodiment, the interleukin-15 and interleukin-15 receptor alpha are human interleukin-15 and human interleukin-15 receptor alpha. Examples of suitable interleukin-15 and interleukin-15 receptor alpha sequences include, but are not limited to, those described in US Patent Application Publication Nos. 2015/0359853 and 2011/0081311. In an embodiment of the invention, human IL-15 comprises the amino acid sequence of SEQ ID NO: 7 and human IL-15Ra comprises the amino acid sequence of SEQ ID NO: 8. In another embodiment of the invention, human IL-15 comprises the amino acid sequence of SEQ ID NO: 7 and human IL-15Ra comprises the amino acid sequence of SEQ ID NO: 14 (the natural form of human heterodimeric IL-15).

[0041] In an embodiment of the invention, the four amino acids surrounding the cleavage site of IL-15Ra, QGHS (SEQ ID NO: 18), are deleted or changed in different combinations to derive hetIL-15 forms with altered processing (e.g., non-cleaved). Accordingly, in an embodiment of the invention, the IL-15Ra comprises the amino acid sequence of SEQ ID NO: 19, wherein X at position 199 is any naturally occurring amino acid residue or is absent; X at position 200 is any naturally occurring amino acid residue or is absent; X at position 201 is any naturally occurring amino acid residue or is absent; X at position 202 is any naturally occurring amino acid residue or is absent, and wherein SEQ ID NO: 19 does not comprise the wild-type cleavage site amino acid sequence of QGHS (SEQ ID NO: 18). In an embodiment of the invention, the IL-15Ra comprises the amino acid sequence of SEQ ID NO: 19, wherein X at positions 199-202 are all absent. In an embodiment of the invention, the heterodimeric

complex of interleukin-15 and interleukin-15 receptor alpha comprises the amino acid sequences of 7 and 19.

[0042] Another embodiment of the invention provides an isolated or purified extracellular vesicle comprising a membrane comprising a phospholipid bilayer, a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha, and a carrier, wherein: the phospholipid bilayer comprises an exterior surface, an interior surface, and an interior region positioned between the exterior surface and the interior surface, the extracellular vesicle comprises an exterior surface, the carrier is positioned on the exterior surface of the extracellular vesicle or at least a portion of the carrier is embedded in the phospholipid bilayer, and the heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha is positioned on the carrier. In another embodiment of the invention, the carrier is positioned on the exterior surface of the extracellular vesicle or at least a portion of the carrier is positioned in the interior region of the phospholipid bilayer, and the heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha is positioned on the carrier. The interleukin-15 and interleukin-15 receptor alpha may be as described herein with respect to other aspects of the invention.

[0043] The carrier may be any carrier suitable for attaching the interleukin-15 and interleukin-15 receptor alpha to the exterior surface of the EV. Suitable carriers may include any protein that binds to the surface of EV, proteins that are naturally found in complex with EV, or any other EV-associated protein. Examples of carriers that may be useful in the present invention include, but are not limited to, lactadherin (*e.g.*, Delcayre *et al.*, *Blood Cells, Molecules, and Diseases* 35:158-168, 2005; US Patent Application Publication 20040197314), annexin, tetraspanins (*e.g.*, CD63, CD9, and CD81), and lysosome-associated proteins (*e.g.*, Lamp-1 and Lamp-2). In an embodiment of the invention, the carrier is human lactadherin comprising the amino acid sequence of SEQ ID NO: 9. In this regard, the EV may comprise a human IL-15Ra-human lactadherin fusion protein comprising the amino acid sequence of SEQ ID NO: 10. In an embodiment of the invention, the carrier comprises lactadherin C1/C2 domains. The carrier may provide any one or more advantages including, for example, an increased association of the heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha with EV as compared to the EV without the carrier. Without being bound to a particular theory or mechanism, it is believed that the ability of lactadherin to bind to lipids that are enriched specifically on EV (for example, phosphatidylserine) may increase the amount of the heterodimeric complex that is associated with EV.

[0044] Another embodiment of the invention provides a heterodimeric complex of lactadherin, interleukin-15 and interleukin-15 receptor alpha comprising the amino acid sequences of SEQ ID NOs:7-9 or SEQ ID NOs:7 and 10.

[0045] In other embodiments, the lactadherin, interleukin-15, and interleukin-15 receptor alpha comprises, consists essentially of, or consists of an amino acid sequence which is at least about 75%, e.g., at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identical to any of the lactadherin, interleukin-15, and interleukin-15 receptor alpha amino acid sequences, respectively, described herein.

[0046] Still another embodiment of the invention provides nucleic acids encoding the heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha. The nucleic acids may encode any of the lactadherin, interleukin-15 and interleukin-15 receptor alpha described herein with respect to other aspects of the invention. Examples of suitable nucleic acids include, but are not limited to, SEQ ID NO: 11 (human IL-15Ra-human lactadherin fusion protein), SEQ ID NO: 12 (IL-15), and SEQ ID NO: 15 (natural form of human IL-15Ra). In other embodiments, the nucleic acid comprises, consists essentially of, or consists of a nucleic acid sequence which is at least about 75%, e.g., at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identical to any of the nucleotide sequences described herein.

[0047] In some embodiments, a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha incorporated on an EV may comprise a further polypeptide of interest. Thus, in some embodiments, a heterodimeric complex of IL-15 and IL-15Ra may comprise an IL-15 fusion polypeptide in which an IL-15 as described herein is recombinantly joined to the further polypeptide of interest. In some embodiments the IL-15 fusion polypeptide may comprise a polypeptide sequence that targets EVs comprising the interleukin-15 and interleukin-15 receptor alpha heterodimer to a desired cell type, e.g., the second polypeptide may be a ligand that binds to a receptor present on a desired cell type. In some embodiments, the IL-15 fusion polypeptide may comprise a second cytokine, such as IL-12 or GM-CSF, e.g., human IL-12 or GM-CSF. In further embodiments, chemokines, soluble receptors, antibodies, or antibody fragments may be fused to IL-15.

[0048] In some embodiments, IL-12 is fused to IL-15. Thus, for example, IL-12 may be fused at the carboxyl end of IL-15, either with or without a linker sequence (*e.g.*, Figure 17 and illustrative fusion polypeptides SEQ ID NOs:23 and 25). In some embodiments, an IL-15-IL-12 fusion polypeptide in accordance with the invention comprises human IL-15 and IL-12 sequences. An illustrative human IL-12p40 protein sequence is available under accession number P29460. An illustrative human IL-12p40 nucleic acid sequence is available under accession number NM_002187.

[0049] In certain preferred embodiments, the nucleic acid encoding the heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha is carried in a recombinant expression vector. Accordingly, an embodiment of the invention provides a vector comprising (i) a nucleic acid encoding any of the heterodimeric complexes of interleukin-15 and interleukin-15 receptor alpha described herein and (ii) a heterologous nucleic acid sequence. An example of a vector encoding human IL-15 and a human lactadherin-human IL-15Ra fusion protein comprises the nucleotide sequence of SEQ ID NO: 13. An example of a vector encoding the natural form of human heterodimeric IL-15 and human IL-15Ra comprises the nucleotide sequence of SEQ ID NO: 16. An example of a vector encoding human heterodimeric IL-15 and human IL-15Ra, wherein the human IL-15Ra includes an altered cleavage site, comprises the nucleotide sequence of SEQ ID NO: 17. In some embodiments, the IL-15 may be fused to another polypeptide. Illustrative constructs encoding murine IL-15-IL-12 fusion polypeptides are provided in SEQ ID NOS:22 and 24.

[0050] Another embodiment of the invention provides a method of preparing isolated or purified EVs comprising a therapeutic protein. The inventive methods of preparing isolated or purified EVs may provide many advantages. For example, culturing cells in a hollow fiber bioreactor may yield more EVs as compared to culturing the cells in conventional flasks. The inventive methods may improve the yield of EVs by any amount such as, for example, about 5-fold, about 10-fold, about 20-fold, about 50-fold, or more, as compared to culturing the cells in conventional flasks.

[0051] The method of producing EVs may comprise introducing a nucleotide sequence into cells, wherein the nucleotide sequence encodes the therapeutic protein. The nucleotide sequence is introduced into the cells under conditions to express the therapeutic protein by the cells. The nucleotide sequence may be introduced into the cells using any suitable method such as, for example, transfection, transduction, or electroporation. For example, cells can be transduced with viral vectors using viruses (*e.g.*, retrovirus or lentivirus) and

cells can be transduced with transposon vectors using electroporation. The nucleotide sequence may be DNA or RNA.

[0052] The cells may be any cells capable of producing EVs comprising the therapeutic protein. Examples of cell types which may be employed in the inventive methods include, but are not limited to, mammalian cells, bacterial cells, yeast cells, primary cells, immortalized cells, and insect cells. In an embodiment of the invention, the cells are a mammalian cell line. The cells may be primary non-human animal cells or human cells, for example, cells obtained from the peripheral blood of a human. Examples of mammalian cell lines which may be employed in the inventive methods include, but are not limited to, COS, CHO, HeLa, NIH3T3, HepG2, MCF7, RD, PC12, hybridomas, pre-B cells, K562, SkBr3, BT474, A204, M07Sb, TF.beta.1, Raji, Jurkat, MOLT-4, CTLL-2, MC-IXC, SK-N-MC, SK-N-MC, SK-N-DZ, SH-SY5Y, C127, N0, HEK293, and BE(2)-C cells. Other mammalian cell lines are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC). In a preferred embodiment, the cells are HEK293 cells.

[0053] The therapeutic protein is not limited and may include any protein that is capable of being included in an EV and which may have therapeutic value. Types of therapeutic proteins which may be employed in the inventive methods and products include, but are not limited to, cytokines, hormones, enzymes, growth factors, thrombolytics, anticoagulants, blood factors, bone morphogenetic proteins, mammalian immune-acting proteins (e.g., chemokines, alarmins, defensins, and heat-shock proteins), endogenous or foreign antigens (neo-antigens, i.e., mutated proteins expressed by a human tumor), non-natural (synthetic) therapeutic proteins, and antibodies that bind to and neutralize soluble factors, such as other cytokines. Specific examples of therapeutic proteins may include, but are not limited to, interleukin (IL)-1, IL-2, IL-3, IL4, IL5-, IL-6, IL-, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, receptors for any of the foregoing interleukins, and heterodimeric complexes of any of the foregoing interleukins and their respective receptors. Further examples of therapeutic proteins include, but are not limited to, insulin, human growth hormone, mecasemin, erythropoietin, granulocyte colony stimulating factor, follicle-stimulating hormone, human chorionic gonadotropin, lutropin-alpha, botulinum toxin types A and B, collagenase, human deoxy-ribonuclease I, hyaluronidase, papain, glucagon, growth hormone releasing hormone, secretin, and thyroid stimulating hormone. In a preferred embodiment, the therapeutic protein is a heterodimeric complex of interleukin-15 and

interleukin-15 receptor alpha. The interleukin-15 and interleukin-15 receptor alpha may be as described herein with respect to other aspects of the invention.

[0054] The method may comprise culturing the cells in medium in a hollow fiber bioreactor under conditions sufficient for the cells to express the therapeutic protein. The hollow fiber bioreactor may comprise, for example, a cartridge comprising at least one hollow fiber, preferably a plurality of hollow fibers. The cartridge may further comprise an intracapillary (IC) space within the hollow fiber(s) and an extracapillary (EC) space surrounding the hollow fiber(s). After seeding the cells into the EC space, the cells may multiply and adhere to the hollow fiber surface or the cells may remain in suspension without adhering to the hollow fibers. Culturing the cells may further comprise circulating cell culture medium through the IC space of the hollow fibers. Cell culture medium may be supplemented with additional components such as growth factors or other molecules to support growth of the cells. Examples of hollow fiber bioreactors which may be employed in the inventive methods include, for example, a FIBERCELL hollow fiber bioreactor (available from FiberCell Systems, Frederick, MD) and the hollow fiber bioreactors described, for example, in U.S. Patent No. 6,933,144, which is incorporated herein by reference.

[0055] The cells may be cultured in any suitable cell culture medium. Some cell culture media may contain protein (e.g., fetal bovine serum), which may contain any of a variety of contaminants such as, for example, endogenous protein, which may contaminate the desired EV product. Accordingly, while the cell culture medium may contain protein, in a preferred embodiment of the invention, the method comprises culturing the cells in protein-free medium. Protein-free medium may, advantageously, reduce the amount of contaminants in the desired EV product and, therefore, promote efficient purification of the EVs. The protein-free medium may comprise, for example, chemically defined medium for high density cell culture (CDM-HD) available from FiberCell Systems, Frederick, MD.

[0056] The method may comprise secreting from the cells the EVs comprising the expressed therapeutic protein into the medium. In an embodiment of the invention, the EVs accumulate in the medium in the EC space of the hollow fiber bioreactor. In an embodiment of the invention, each EV comprises a membrane, and the therapeutic protein is embedded in the membrane of the EV. In another embodiment of the invention, each EV comprises a membrane with an external surface, and the therapeutic protein is positioned on the external surface of the membrane. In still another embodiment of the invention, each EV comprises a membrane with an internal surface that encapsulates an interior space, and the therapeutic

protein is positioned in the interior space. The EV may be as described herein with respect to other aspects of the invention.

[0057] The method may comprise separating the EVs comprising the expressed therapeutic protein from the cells to produce isolated or purified EVs comprising the therapeutic protein. The method may comprise harvesting the medium comprising the EVs from the hollow fiber bioreactor, e.g., from the EC space of the hollow fiber bioreactor. The harvested medium may comprise whole cells and other contaminants in addition to the EVs.

[0058] Separating the EVs from the cells may comprise centrifugating the harvested medium comprising the EVs, whole cells, and other contaminants to produce a supernatant comprising the EVs and a pellet comprising cell debris. Centrifugation may remove one or more of live cells, dead cells, cell fragments, and large EVs (>350 nm in diameter).

[0059] The method may further comprise filtering the supernatant comprising the EVs to produce a filtered supernatant comprising EVs. Filtration may remove one or more of larger EVs, microvesicles, and large apoptotic bodies (>220 nm in diameter).

[0060] The method may further comprise dialyzing the filtered supernatant to produce a dialyzed supernatant comprising EVs. Dialysis may remove one or more of salts, metal ions (e.g., iron), proteins, and other components with a small molecular weight. In an embodiment of the invention, dialysis may remove contaminants with a molecular weight <10 kDa. Because EVs may be ~1,000 kDa, it may be possible to carry out dialysis using a dialysis membrane with a higher molecular weight cut off, for example, to remove contaminants < 500kDa.

[0061] The method may further comprise further centrifugating the dialyzed supernatant to produce isolated or purified EVs. The final centrifugation may pellet the remaining EVs. The final centrifugation may remove contaminants such as, for example, free proteins (i.e., proteins that are not in complex with EV), and which are not removed by dialysis, e.g., proteins that are >10 kDa or greater than the molecular weight cut-off of the dialysis membrane used for dialysis.

[0062] In an embodiment of the invention, separating the EVs from the cells optionally comprises concentrating the supernatant prior to centrifuging the dialyzed supernatant. The supernatant may be concentrated in any suitable manner, e.g., by ultrafiltration. However, in some embodiments of the invention, the method does not comprise concentrating the supernatant by “normal flow filtration”, i.e., where the filtration feed stream moves

perpendicular to the filter membrane, as this can result in partial EV losses (lower yield) due to EV adhering to the membrane.

[0063] In some embodiments, methods of purifying EVs comprises a step of tangential flow filtration (TFF). In TFF, the filtration feed stream is circulated and moves parallel with the filter membrane, which prevents substantial EV loss. Clarified conditioned medium (i.e., conditioned medium from which cells, debris, etc. are removed, *e.g.*, a “supernatant” obtained by centrifugation as described in the preceding paragraphs) can be circulated through a TFF filter device such that molecules smaller than a desired pore size, *e.g.*, 50 nm pores, exit the main tubing circuit as filtrate, which concentrates desired particle sizes, *e.g.*, >50nm and thus provides a concentrated conditioned medium that comprises the EVs. This concentrated conditioned medium may be further dialyzed (by addition of a dialysis buffer, *e.g.*, saline, to the main tubing circuit) and circulated through the filter device to further concentrate the population of EVs present in the conditioned medium. The conditioned medium can then be subjected to size-exclusion chromatography (SEC) (*e.g.*, gel filtration chromatography) and the fractions containing EV's can be collected.

[0064] The inventive methods advantageously produce isolated or purified EVs. The term “isolated,” as used herein, means having been removed from the hollow fiber bioreactor medium environment. The term “purified,” as used herein, means having been increased in purity, wherein “purity” is a relative term, and not to be necessarily construed as absolute purity. A “purified” EV refers to an EV which has been separated from other components of the medium in the hollow fiber bioreactor, such as whole cells, cell membrane fragments, cell organelles, proteins other than the therapeutic protein, etc.

[0065] Another embodiment of the invention provides isolated or purified EVs prepared according to any of the inventive methods described herein. The EVs may be as described herein with respect to other aspects of the invention. In an embodiment of the invention, the therapeutic protein is a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha, as described herein with respect to other aspects of the invention.

[0066] An embodiment of the invention provides a composition comprising a plurality of any of the isolated or purified extracellular vesicles (EVs) described herein. The inventive EVs and compositions can be formulated into a composition, such as a pharmaceutical composition. In this regard, the invention provides a pharmaceutical composition comprising any of the inventive EVs and compositions described herein and a pharmaceutically acceptable carrier. The inventive pharmaceutical compositions can comprise more than one

inventive EV, e.g., two or more different EVs. Alternatively, the pharmaceutical composition can comprise an inventive EV or composition in combination with additional pharmaceutically active agents or drugs, such as a chemotherapeutic agent, e.g., asparaginase, busulfan, carboplatin, cisplatin, daunorubicin, doxorubicin, fluorouracil, gemcitabine, hydroxyurea, methotrexate, paclitaxel, rituximab, vinblastine, vincristine, etc.

[0067] Preferably, the carrier is a pharmaceutically acceptable carrier. With respect to pharmaceutical compositions, the carrier can be any of those conventionally used and is limited only by chemico-physical considerations, such as solubility and lack of reactivity with the inventive EVs, and by the route of administration. The pharmaceutically acceptable carriers described herein, for example, vehicles, adjuvants, excipients, and diluents, are well-known to those skilled in the art and are readily available to the public. It is preferred that the pharmaceutically acceptable carrier be one which is chemically inert to the inventive EVs and one which has no detrimental side effects or toxicity under the conditions of use.

[0068] The choice of carrier will be determined in part by the particular inventive EV, as well as by the particular method used to administer the inventive EVs. Accordingly, there are a variety of suitable formulations of the pharmaceutical composition of the invention. The following formulations for topical, oral, aerosol, parenteral, subcutaneous, intravenous, intramuscular, intratumoral, intraarterial, intrathecal, intranasal, and interperitoneal, administration are exemplary and are in no way limiting. More than one route can be used to administer the inventive EVs, and in certain instances, a particular route can provide a more immediate and more effective response than another route.

[0069] Topical formulations are well-known to those of skill in the art. Such formulations are particularly suitable in the context of the invention for application to the skin.

[0070] Formulations suitable for oral administration can include (a) liquid solutions; (b) capsules, sachets, tablets, lozenges, and troches, each containing a predetermined amount of the inventive EV, as solids or granules; (c) powders; (d) suspensions in an appropriate liquid; and (e) suitable emulsions.

[0071] The inventive EVs, alone or in combination with other suitable components, can be made into aerosol formulations to be administered via inhalation. These aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like. They also may be formulated as

pharmaceuticals for non-pressured preparations, such as in a nebulizer or an atomizer. Such spray formulations also may be used to spray mucosa.

[0072] Preferably, the composition is formulated for a parenteral route of administration. An exemplary pharmaceutically acceptable carrier for EVs for injection may include any isotonic carrier such as, for example, normal saline (about 0.90% w/v of NaCl in water, about 300 mOsm/L NaCl in water, or about 9.0 g NaCl per liter of water), NORMOSOL R electrolyte solution (Abbott, Chicago, IL), PLASMA-LYTE A (Baxter, Deerfield, IL), about 5% dextrose in water, or Ringer's lactate. Another example of a pharmaceutically acceptable carrier may be pH-buffered saline solution, such as phosphate buffered saline. In an embodiment, the pharmaceutically acceptable carrier is supplemented with one, two, or all three of sucrose, trehalose, and human serum albumin.

[0073] The EVs and compositions described herein may be used for any purpose, e.g., the treatment or prevention of a condition, especially cancer. In this regard, an embodiment of the invention provides a method of treating or preventing cancer in a mammal, the method comprising administering to the mammal any of the EVs or compositions described herein to the mammal in an amount effective to treat or prevent cancer in the mammal.

[0074] It has also been discovered that blocking the class A scavenger receptor family ("Scavenger Receptor A") may, advantageously, decrease the uptake of the EV by monocytes and/or macrophages, decrease the uptake of EV by the liver, and/or increase the delivery of the EVs to a target site. Scavenger receptors are a large family of receptors used to recognize and internalize unopsonized pathogens, apoptotic host cells, and modified lipoproteins. Scavenger receptors are transmembrane proteins belonging to at least eight different subclasses (A–H) based on their tertiary structure. The class A scavenger receptors are widely expressed on macrophages. There are three isoforms of encoded by Scavenger Receptor A gene *MSRI*: isoform I (SR-A1), isoform II (SR-A2), and isoform III (SR-A3). The SR-A1, SR-A2, and SR-A3 isoforms are derived from the same gene (*MSRI*), with alternative splicing producing the different proteins. Of these splice variants, only SR-A1 and SR-A2 are phagocytic, whereas SR-A3 is unable to bind extracellular ligands because it is retained within the endoplasmic reticulum (Thelen et al., *J. Immunol.*, 185: 4328-35 (2010)). Another Scavenger Receptor A is MARCO (Macrophage Receptor With Collagenous Structure), which is encoded by the *MARCO* gene. Hereinafter, the term "SRA" collectively refers to MARCO and all three *MSRI* SRA isoforms, unless specified otherwise.

[0075] An embodiment of the invention provides method of reducing liver uptake of EVs administered to a mammal. The method may comprise administering EVs comprising a therapeutic protein to the mammal. The EVs and the therapeutic protein may each be as described herein with respect to other aspects of the invention. The EVs may be administered to the mammal by any route of administration described herein with respect to other aspects of the invention. In a preferred embodiment, the method comprises administering the EVs to the mammal parenterally.

[0076] The method may further comprise administering a Scavenger Receptor A (SRA) antagonist to the mammal in an amount effective to reduce uptake of the EVs by the liver of the mammal. The SRA antagonist may be administered to the mammal by any route of administration described herein with respect to other aspects of the invention. In a preferred embodiment, the method comprises administering the SRA antagonist to the mammal parenterally.

[0077] The inventive methods may provide any of a variety of advantages. For example, in an embodiment of the invention, administering the SRA antagonist increases delivery of the EVs to a target site as compared to administering the EVs to the mammal without administering the Scavenger Receptor A antagonist, wherein the target site is a tumor or an extra-hepatic organ. In this regard, the inventive methods may, advantageously, augment the ability of the EVs to treat or prevent the condition being treated. The inventive methods may, advantageously, reduce or prevent clearance of the EVs by macrophages and/or monocytes and/or increase the quantity of EVs delivered to a target site.

[0078] The SRA antagonist may be an antagonist of any SRA, including any one or more of MARCO, SRA-A1, SRA-A2, and SRA-A3. The SRA antagonist may be a non-specific antagonist that blocks the biological activity of more than one SRA, e.g., all four SRAs, or any two SRAs (e.g., SRA-A1 and SRA-A2). Alternatively, the SRA antagonist is a specific antagonist that blocks the activity of only one of the four SRAs, e.g., a specific antagonist of MARCO, a specific antagonist of SRA-A1, a specific antagonist of SRA-A2, or a specific antagonist of SRA-A3. In a preferred embodiment, the SRA antagonist is a specific or non-specific antagonist of SRA-A1 and/or SRA-A2. In an embodiment, the SRA antagonist is a specific or non-specific antagonist of SRA-A1.

[0079] The SRA antagonist can be any agent that inhibits the biological activity of SRA. The biological activity of SRA may be inhibited in any manner, e.g., by inhibiting the expression of any one or more of MARCO protein, *MARCO* mRNA, *MSRI* mRNA and SRA

protein; and/or by inhibiting the binding of EVs to SRA, as compared to that which is observed in the absence of the SRA antagonist. The biological activity may be inhibited to any degree that realizes a beneficial therapeutic effect. For example, in some embodiments, the biological activity may be completely inhibited (i.e., prevented), while in other embodiments, the biological activity may be partially inhibited (i.e., reduced).

[0080] In an embodiment, the SRA antagonist is an agent that inhibits the binding of an EV to SRA. In this regard, the SRA antagonist may be an agent that binds to the SRA protein, thereby reducing or preventing the binding of the EV to the SRA and inhibiting SRA function, as well as agents that compete with EV for the native EV binding site of the SRA. The SRA antagonist may be an agent that binds to EV, thereby reducing or preventing the binding of the EV to the SRA and inhibiting SRA function, as well as agents that compete with SRA for the native SRA binding site of the EV. By way of illustration, the agent that inhibits the binding of EV to the SRA can be any of the antibodies or antibody fragments, antisense nucleic acids, or chemical inhibitors (e.g., small molecule or peptide inhibitor) described herein.

[0081] In an embodiment of the invention, the SRA antagonist is an antibody or antibody fragment that specifically binds to SRA. Anti-SRA antibodies and antibody fragments can be monoclonal or polyclonal. Anti-SRA antibodies and antibody fragments can be prepared using the SRA proteins disclosed herein and routine techniques. Examples of such antibodies or antibody fragments include those specific to the native EV binding site of SRA.

[0082] Chemical inhibitors of SRA include polymeric molecules, small molecules, and peptides or polypeptides that bind to SRA or compete with EV for its native binding site of the SRA. Suitable inhibitors can include, for example, a non-active fragment of EV or a functional fragment of SRA. Chemical SRA antagonists can be identified using routine techniques. For example, chemical inhibitors can be tested in binding assays to identify molecules and peptides (or polypeptides) that bind to SRA with sufficient affinity to inhibit SRA biological activity (e.g., binding of SRA to EV). Also, competition assays can be performed to identify small-molecules and peptides (or polypeptides) that compete with EV for binding to its native binding site of SRA. Such techniques could be used in conjunction with high-throughput screens of known chemical inhibitors. Examples of SRA antagonists include, but are not limited to, fucoidan, polyinosinic acid, PLGA (poly(lactic-co-glycolic acid)), sennoside B, rhein, and dextran sulfate. In a preferred embodiment, the SRA

antagonist is dextran sulfate. In an embodiment of the invention, the dextran sulfate is parenterally administered to the mammal.

[0083] The functional fragment of the SRA protein can comprise any contiguous part of the SRA protein that retains a relevant biological activity of the SRA protein, e.g., binds to EV. Any given fragment of an SRA protein can be tested for such biological activity using methods known in the art. For example, the functional fragment can comprise, consist essentially of, or consist of the EV binding portion of the SRA protein. In reference to the parent SRA protein, the functional fragment preferably comprises, for instance, about 10% or more, 25% or more, 30% or more, 50% or more, 60% or more, 80% or more, 90% or more, or even 95% or more of the parent SRA protein.

[0084] In an embodiment of the invention, the SRA antagonist is any suitable agent that inhibits the expression of one or more of MARCO protein, *MARCO* mRNA, *MSRI* mRNA and SRA protein. The SRA antagonist can be a nucleic acid at least about 10 nucleotides in length that specifically binds to and is complementary to a target nucleic acid encoding one or more of MARCO protein, *MARCO* mRNA, *MSRI* mRNA and SRA protein or a complement thereof. The SRA antagonist may be introduced into a host cell, wherein the cell is capable of expressing one or more of MARCO protein, *MARCO* mRNA, *MSRI* mRNA and SRA protein, in an effective amount for a time and under conditions sufficient to interfere with expression of one or more of MARCO protein, *MARCO* mRNA, *MSRI* mRNA and SRA protein, respectively. In some embodiments, RNA interference (RNAi) is employed. In this regard, the SRA antagonist may comprise an RNAi agent. In an embodiment, the RNAi agent may comprise a small interfering RNA (siRNA), a microRNA (miRNA), or an antisense nucleic acid. The RNAi agent, e.g., siRNA, miRNA, and/or antisense nucleic acid can comprise overhangs. That is, not all nucleotides need bind to the target sequence. RNA interference nucleic acids employed can be at least about 19, at least about 40, at least about 60, at least about 80, at least about 100, at least about 120, at least about 140, at least about 160, at least about 180, at least about 200, at least about 220, at least about 240, from about 19 to about 250, from about 40 to about 240, from about 60 to about 220, from about 80 to about 200, from about 60 to about 180, from about 80 to about 160, and/or from about 100 to about 140 nucleotides in length.

[0085] The RNAi agent, e.g., siRNA or shRNA, can be encoded by a nucleotide sequence included in a cassette, e.g., a larger nucleic acid construct such as an appropriate vector. Examples of such vectors include lentiviral and adenoviral vectors, as well as other vectors

described herein with respect to other aspects of the invention. An example of a suitable vector is described in Aagaard et al. *Mol. Ther.*, 15(5): 938–45 (2007). When present as part of a larger nucleic acid construct, the resulting nucleic acid can be longer than the comprised RNAi nucleic acid, e.g., greater than about 70 nucleotides in length. In some embodiments, the RNAi agent employed cleaves the target mRNA. In other embodiments, the RNAi agent employed does not cleave the target mRNA.

[0086] Any type of suitable siRNA, miRNA, and/or antisense nucleic acid can be employed. In an embodiment, the antisense nucleic acid comprises a nucleotide sequence complementary to at least about 8, at least about 15, at least about 19, or from about 19 to about 22 nucleotides of a nucleic acid encoding one or more of MARCO protein, *MARCO* mRNA, *MSRI* mRNA and SRA protein or a complement thereof. In an embodiment, the siRNA may comprise, e.g., trans-acting siRNAs (tasiRNAs) and/or repeat-associated siRNAs (rasiRNAs). In another embodiment, the miRNA may comprise, e.g., a short hairpin miRNA (shMIR).

[0087] The SRA antagonist may inhibit or downregulate to some degree the expression of the protein encoded by a *MARCO* gene or *MSRI* gene, e.g., at the DNA, RNA, or other level of regulation. In this regard, a host cell comprising an SRA antagonist expresses none of one or more of MARCO protein, *MARCO* mRNA, *MSRI* mRNA and SRA protein or lower levels of one or more of MARCO protein, *MARCO* mRNA, *MSRI* mRNA and SRA protein as compared to a host cell that lacks an SRA antagonist. In accordance with an embodiment of the invention, the SRA antagonist, such as an RNAi agent, such as a shMIR, can target a nucleotide sequence of a *MARCO* gene or *MSRI* gene or mRNA encoded by the same.

[0088] In an embodiment, the *MSRI* sequence is a human *MSRI* sequence. For example, human *MSRI* is assigned Gene NCBI Entrez Gene ID No. 4481, and an Online Mendelian Inheritance in Man (OMIM) No. 153622. The human *MSRI* gene is found on chromosome 8 at 8p22. Three transcriptional variants, and the proteins they encode, are set forth in Table A below.

[0089] In an embodiment, the *MARCO* sequence is a human *MARCO* sequence. For example, human *MARCO* is assigned Gene NCBI Entrez Gene ID No. 8685, and an Online Mendelian Inheritance in Man (OMIM) No. 604870. The human *MARCO* gene is found on chromosome 2 at 2q14.2. The *MARCO* transcript, and the protein that it encodes, are set forth in Table A below.

TABLE A

Name	Transcript variant	Protein
SRA-A1	NM_138715.2 (SEQ ID NO: 1)	NP_619729.1 (SEQ ID NO: 2)
SRA-A2	NM_002445.3 (SEQ ID NO: 3)	NP_002436.1 (SEQ ID NO: 4)
SRA-A3	NM_138716.2 (SEQ ID NO: 5)	NP_619730.1 (SEQ ID NO: 6)
MARCO	NM_006770.3 (SEQ ID NO: 20)	NP_006761.1 (SEQ ID NO: 21)

[0090] Human genomic *MSRI* sequences include GenBank Accession Nos: NC_000008.11, NC_018919.2, AC023396.4, CH471080.2, DQ144993.1, and JA885108.1. Human *MSRI* mRNA sequences also include Genbank Accession Nos: AF037351.1, AK293217.1, AK293409.1, BC063878.1, D13264.1, D13265.1, D90187.1, D90188.1, and DA995755.1. Human SRA amino acid sequences include Genbank Accession Nos: EAW63830.1, EAW63832.1, EAW63834.1, AAZ38715.1, CCQ77679.1, AAC09251.1, BAG56756.1, BAG56916.1, BAA14208.1, and ADQ31955.1. Other human sequences, as well as other SRA/*MSRI* species can be employed in accordance with the invention.

[0091] In accordance with an embodiment of the invention, the SRA antagonist, such as an RNAi agent, such as a shMIR, can target a nucleotide sequence selected from the group consisting of the 5' untranslated region (5' UTR), the 3' untranslated region (3' UTR), and the coding sequence of *MARCO* or *MSRI*, complements thereof, and any combination thereof. Any suitable *MARCO* or *MSRI* target sequence can be employed. In an embodiment of the invention, the sequences of the SRA antagonist can be designed against human *MSRI* with any one of Accession Nos: NM_138715.2 (SEQ ID NO: 1), NM_002445.3 (SEQ ID NO: 3), and NM_138716.2 (SEQ ID NO: 5), but also recognize either of the other two sequences. In an embodiment of the invention, the sequences of the SRA antagonist can be designed against human *MARCO* with Accession No: NM_006770.3 (SEQ ID NO: 20). RNAi agents can be designed against any appropriate *MARCO* or *MSRI* mRNA sequence.

[0092] The SRA antagonist can be obtained by methods known in the art. For example, SRA antagonists that are peptides or polypeptides can be obtained by *de novo* synthesis as described in references, such as Jensen et al. (Eds.), *Peptide Synthesis and Applications*, 2nd

Ed., Humana Press (2013). Also, SRA antagonists can be recombinantly produced using standard recombinant methods. See, for instance, Green and Sambrook, *Molecular Cloning: A Laboratory Manual*, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2012). Further, the SRA antagonist can be isolated and/or purified from a natural source, e.g., a human. Methods of isolation and purification are well-known in the art. In this respect, the SRA antagonists may be exogenous and can be synthetic, recombinant, or of natural origin.

[0093] The SRA antagonists may be glycosylated, amidated, carboxylated, phosphorylated, esterified, N-acylated, cyclized via, e.g., a disulfide bridge, or converted into an acid addition salt and/or optionally dimerized or polymerized, or conjugated. Suitable pharmaceutically acceptable acid addition salts include those derived from mineral acids, such as hydrochloric, hydrobromic, phosphoric, metaphosphoric, nitric, and sulphuric acids, and organic acids, such as tartaric, acetic, citric, malic, lactic, fumaric, benzoic, glycolic, gluconic, succinic, and arylsulphonic acids, for example, p-toluenesulphonic acid.

[0094] Of course, the method of the invention can comprise administering two or more SRA antagonists, any of which may be the same or different from one another. Furthermore, the SRA antagonist can be provided as part of a larger polypeptide construct. For instance, the SRA antagonist can be provided as a fusion protein comprising an SRA antagonist along with other amino acid sequences or a nucleic acid encoding same. The SRA antagonist also can be provided as part of a conjugate or nucleic acid encoding same.

[0095] The SRA antagonist can be administered to the mammal by administering a nucleic acid encoding the SRA antagonist to the mammal. "Nucleic acid" as used herein includes "polynucleotide," "oligonucleotide," and "nucleic acid molecule," and generally means a polymer of DNA or RNA, which can be single-stranded or double-stranded, synthesized or obtained (e.g., isolated and/or purified) from natural sources, which can contain natural, non-natural or altered nucleotides, and which can contain a natural, non-natural or altered internucleotide linkage, such as a phosphoroamidate linkage or a phosphorothioate linkage, instead of the phosphodiester found between the nucleotides of an unmodified oligonucleotide.

[0096] Nucleic acids encoding the SRA antagonist (and degenerate nucleic acid sequences encoding the same amino acid sequences), can be constructed based on chemical synthesis and/or enzymatic ligation reactions using procedures known in the art. See, for example, Green and Sambrook, *supra*. For example, a nucleic acid can be chemically

synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed upon hybridization (e.g., phosphorothioate derivatives and acridine substituted nucleotides).

[0097] The nucleic acids can be incorporated into a recombinant expression vector. For purposes herein, the term "recombinant expression vector" means a genetically-modified oligonucleotide or polynucleotide construct that permits the expression of an mRNA or polypeptide by a host cell, when the construct comprises a nucleotide sequence encoding the mRNA or polypeptide, and the vector is contacted with the cell under conditions sufficient to have the mRNA or polypeptide expressed within the cell. The vectors are not naturally-occurring as a whole. However, parts of the vectors can be naturally-occurring. The recombinant expression vectors can comprise any type of nucleotides, including, but not limited to DNA and RNA, which can be single-stranded or double-stranded, synthesized or obtained in part from natural sources, and which can contain natural, non-natural or altered nucleotides. The recombinant expression vectors can comprise naturally-occurring or non-naturally-occurring internucleotide linkages, or both types of linkages. Preferably, the non-naturally occurring or altered nucleotides or internucleotide linkages do not hinder the transcription or replication of the vector.

[0098] The methods and compositions described herein may be used for any purpose, e.g., the treatment or prevention of a condition, especially cancer. In this regard, an embodiment of the invention provides a method of treating or preventing cancer in a mammal, the method comprising: administering any of the EVs comprising a therapeutic protein described herein to the mammal in an amount effective to treat or prevent cancer in the mammal; and administering any of the SRA antagonists described herein to the mammal in an amount effective to reduce uptake of the EVs by the liver of the mammal. The EVs and SRA antagonists may be administered to the mammal by any route of administration described herein with respect to other aspects of the invention.

[0099] The terms "treat," and "prevent" as well as words stemming therefrom, as used herein, do not necessarily imply 100% or complete treatment or prevention. Rather, there are varying degrees of treatment or prevention of which one of ordinary skill in the art recognizes as having a potential benefit or therapeutic effect. In this respect, the inventive methods can provide any amount of any level of treatment or prevention of a condition in a mammal. Furthermore, the treatment or prevention provided by the inventive methods can include

treatment or prevention of one or more conditions or symptoms of the condition, e.g., cancer, being treated or prevented. Also, for purposes herein, "prevention" can encompass delaying the onset of the condition, or a symptom or sign thereof.

[00100] With respect to the inventive methods, the cancer can be any cancer. The cancer may be any cancer, including any of acute lymphocytic cancer, acute myeloid leukemia, alveolar rhabdomyosarcoma, bone cancer, brain cancer, breast cancer, cancer of the anus, anal canal, or anorectum, cancer of the eye, cancer of the intrahepatic bile duct, cancer of the joints, cancer of the neck, gallbladder, or pleura, cancer of the nose, nasal cavity, or middle ear, cancer of the oral cavity, cancer of the vulva, chronic lymphocytic leukemia, chronic myeloid cancer, colon cancer, esophageal cancer, cervical cancer, gastrointestinal carcinoid tumor, Hodgkin lymphoma, hypopharynx cancer, kidney cancer, larynx cancer, liver cancer, lung cancer, malignant mesothelioma, melanoma, multiple myeloma, nasopharynx cancer, non-Hodgkin lymphoma, ovarian cancer, pancreatic cancer, peritoneum, omentum, and mesentery cancer, pharynx cancer, prostate cancer, rectal cancer, renal cancer (e.g., renal cell carcinoma (RCC)), small intestine cancer, soft tissue cancer, stomach cancer, testicular cancer, thyroid cancer, ureter cancer, and urinary bladder cancer.

[00101] With respect to the inventive methods, the amount or dose of one or both of the EVs and SRA antagonist administered should be sufficient to effect the desired biological response, e.g., a therapeutic or prophylactic response, in the mammal over a reasonable time frame. The dose may be determined by the efficacy of the particular EV, particular SRA antagonist, and the condition of the mammal (e.g., human), as well as the body weight of the mammal (e.g., human) to be treated. The dose of one or both of the EV and SRA antagonist also will be determined by the existence, nature and extent of any adverse side effects that might accompany the administration of a particular EV and/or SRA antagonist. Typically, the attending physician will decide the dosage of the EV and/or SRA antagonist with which to treat each individual patient, taking into consideration a variety of factors, such as age, body weight, general health, diet, sex, EV to be administered, SRA antagonist to be administered, route of administration, and the severity of the condition being treated.

[00102] The term "mammal," as used herein, refers to any mammal, including, but not limited to, mice, hamsters, rats, rabbits, cats, dogs, cows, pigs, horses, monkeys, apes, and humans. Preferably, the mammal is a human.

[00103] The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLES

[00104] The following materials and methods were employed in the experiments described in Example 1-8.

Cells

[00105] HEK293 and all mouse cell lines (RAW264.7, 4T1, B16, LLC1, MC38 and EG.7) were obtained from ATCC (American Type Culture Collection). HEK293 cells expressing high levels of hetIL-15 were previously described (Chertova et al., *J. Biol. Chem.*, 288: 18093-18103 (2013); Bergamaschi et al., *J. Biol. Chem.*, 283: 4189-4199 (2008)). Blood samples from healthy blood donors were collected in ACD (Acid Citrate Dextrose) tubes, under approved protocols for human subjects' research by the National Cancer Institute Investigational Review Board. Peripheral blood mononuclear cells (PBMC) were purified by gradient centrifugation over HISTOPAQUE medium (Sigma-Aldrich), according to the manufacturer's protocol.

[00106] 4T1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. RAW264.7 and E.G7 cells were cultured in RPMI 1640 medium, supplemented with 5% fetal calf serum. The remaining cell lines were cultured in DMEM medium, supplemented with 10% fetal calf serum.

[00107] Human PBMC were cultured overnight in RPMI medium supplemented with 10% fetal calf serum and 100 U/mL penicillin/streptomycin. The cells were used within 24 hours (h) of purification from whole blood.

Cell culture method for EV production

[00108] To obtain conditioned medium from conventional cultures, 3 million cells were seeded in 175 cm² tissue culture flasks in DMEM supplemented with 10% fetal calf serum and 100 U/mL penicillin/streptomycin. After an overnight incubation and cell wash, 15 ml of fresh EV/protein aggregate-depleted medium was added to each flask. 48 h later, the conditioned medium was harvested and pooled for immediate EV purification. Cells were collected in PBS (phosphate buffered saline) and pelleted by centrifugation at 300 x g. Cell lysates were prepared by the addition of N1 lysis buffer to cell pellet, incubation on ice for 1h, and two rounds of sonication for 6 seconds (sec.)

[00109] EV/protein aggregate depleted medium was obtained by ultrafiltration of complete medium through a 500 kDa commercial hollow fiber ultrafiltration module (mPES

MIDIKROS 500 kDa filter module, Spectrum Laboratories; Rancho Dominguez, CA), as previously described (Lamparski et al., *J. Immunol. Meth.*, 270: 211-226 (2002); Heinemann et al., *J. Chromatogr. A*, 1371C: 125-135 (2014)). Specifically, a peristaltic pump was used to slowly circulate culture medium through the filter module, and filtrate collected to be used as EV/protein aggregate depleted medium. The entire procedure was carried out using sterile materials within a biosafety cabinet. Ultrafiltered supernatant was filtered a second time through a 0.22 μ m filter device to ensure sterility.

FIBERCELL hollow-fiber bioreactor culture

[00110] The HEK293 cell clone stably expressing hetIL-15 (clone 19.7) was expanded in conventional culture flasks and used to seed a medium-sized, hollow-fiber culture cartridge, with a 20 kDa molecular weight cut-off (Fibercell Systems; Frederick, MD). Cells were adapted over two weeks to bioreactor culture conditions by gradually increasing the proportion of protein-free medium (DMEM + 10% Fibercell Systems CDMHD protein-free supplement + 100 U/mL penicillin/streptomycin). Bioreactor conditioned medium (20 ml) was collected for each harvest three times per week. Harvests were cleared of cells by 300 x g centrifugation, and supernatants stored at -80°C for further purification.

[00111] The cells were cultured in a FIBERCELL hollow fiber bioreactor. Cells adhered to the hollow fiber surface. The FIBERCELL DuetPump pumped fresh medium through the cell culture cartridge cylinder. Medium circulated within the hollow fibers of the culture cartridge, and nutrient/waste exchange occurred through the hollow-fiber wall pores (20 kDa MWCO). Larger molecular weight cellular products (including EV) accumulated within the extracellular space of the cell culture cartridge cylinder. The extracellular space medium containing large amounts of EV was harvested every 1-3 days.

EV purification

[00112] After removing large cell debris by centrifugation at 3,000 g for 15 min, the supernatants were carefully moved to polycarbonate tubes, and spun for 45 min. at 20,000g in a type 45Ti rotor (Beckman-Coulter; Brea, CA). Supernatants were then filtered through 0.22 μ m Stericup device (EMD Millipore; Billerica, MA), moved to Snakeskin 10 kDa MWCO dialysis tubing (ThermoScientific; Grand Island, NY), and dialyzed overnight in >30 volumes Tris-buffered saline (TBS). Dialyzed supernatants were centrifuged for 2 h at 110,000g in a type 70.1Ti rotor (Beckman-Coulter) to pellet EV. Pellets were resuspended to

the original volume in TBS, by passing through a 27G needle approximately 5 times (until aggregates were no longer visible), and centrifuged again at 110,000 g to wash away contaminating soluble proteins. EV pellets were resuspended in 1/50 original volume of TBS following the procedure described above. Finally, EV were additionally cleared of residual aggregation by a 3 min. centrifugation at 20,000g in a microfuge, and the supernatants containing the EV were transferred to a clean Lobind protein tube (Eppendorf; Hauppauge, NY), and stored at -80°C for downstream applications. All purification steps were conducted at 4°C.

[00113] For experiments directly comparing conventional culture to bioreactor EV, conditioned cell culture media were concentrated by ultrafiltration (CENTRICON-70 centrifugal filter unit, 100 kDa MWCO; EMD Millipore) prior to 110,000 g centrifugation. This allowed for a larger volume of conventional flask-derived supernatants to be pooled prior to ultracentrifugation. Furthermore, the initial EV pellet was not washed, but rather immediately resuspended in 100 µl TBS. These protocol modifications were implemented to allow for sufficient EV yields from conventional flask supernatants to allow for downstream comparison analyses.

Biophysical characterization and imaging

[00114] NTA was performed in triplicate using a NANOSIGHT LM10 instrument (Malvern Instruments; Malvern, United Kingdom), diluting samples to 1-2 µg/ml in PBS, and acquiring 3 videos of 30 seconds for each triplicate. DLS was performed on a nanoparticle analyzer SZ-100 (Horiba Scientific) at the same dilution.

[00115] For TEM sample preparation and imaging, EV suspensions were fixed with 2% glutaraldehyde (Electron Microscopy Sciences, Cat#16020) and then adsorbed to formvar/carbon coated TEM copper grids (SPI, Cat#3420C-MB). Samples were then negative stained with 1% Uranyl Acetate (Electron Microscopy Sciences, Cat# 22400) for 10 seconds. The samples were evaluated on a JEOL 1011 transmission electron microscope at 80kV, and digital images were acquired using an AMT camera system.

EV protein composition determination

[00116] Purified EV preparations were monitored for protein content by the Bradford assay, using bovine gamma-globulin as a standard. Specifically, 5 µl of EV preparation was added to 250 µl QUICKSTART Bradford reagent (Bio-Rad; Hercules, CA), and incubated

for 15 min. at room temperature. Protein concentration was quantified by measuring absorbance at 595 nm on a microplate reader.

[00117] For Western blots, EV were lysed by adding 5x RIPA buffer and incubating on ice for 45 min. Antibodies used to probe the blots included: α CD63 (System Biosciences; Mountain View, CA), α Alix (clone 3A9, LifeSpan Biosciences; Seattle, WA), α IL-15 (AF315, R&D Systems) and α IL-15R α (AF247, R&D Systems). All blots were probed overnight at 4°C.

[00118] Detection of EV-associated IL-15 was performed by flow cytometry using the EXOFLOW kit (System Biosciences) according to manufacturer's protocol. Briefly, 9.1 μ m streptavidin-coated magnetic beads were loaded with biotinylated anti-CD63 exosome capture antibody. Next, 100 μ g of purified EV were incubated overnight at 4°C with antibody-loaded beads in a rotating microtube holder. EV-loaded magnetic beads were then washed using a magnetic tube stand, and divided for staining with individual fluorophore-conjugated antibodies: FITC-lectin (provided in kit) and PE-conjugated anti-IL-15 (clone 34559; R&D Systems, Minneapolis, MN). Incubation with antibodies was for 2 h on ice, while gently flicking the tube to resuspend the beads every 30 min. Finally, unbound antibody was washed from the beads using a magnetic tube stand, and EV-loaded beads were acquired on a flow cytometer (LSR-II; BD Biosciences, Franklin Lakes, New Jersey).

Bioactivity assay

[00119] NK92 cells used for the IL-15 bioactivity assay were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin/streptomycin, 200 U/mL recombinant IL-2 (National Cancer Institute), and 10 ng/mL *het*IL-15 (Admune Therapeutics, Danvers, MA). Cell density was adjusted three times a week to a concentration of $3\text{--}4 \times 10^5$ cells/ml.

[00120] Purified EV were mixed with an equal volume of lysis buffer (1% Triton-X 100/200mM Tris-HCl, pH7.4) and lysed by five freeze/thaw cycles. The amount of EV-associated IL-15 was assessed by ELISA (Human IL-15 QUANTIKINE ELISA kit; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

[00121] The bioactivity of EV-associated *het*IL-15 was assessed on the human NK-92 cell line, which responds to IL-15 treatment by proliferating in a dose-dependent manner. NK-92 cells were cultured overnight at a concentration of 4×10^5 cells/mL in cytokine-free media prior to assay use. 50 μ l of cytokine-starved NK-92 cells (4×10^5 cells/mL) were seeded in

each assay well of 96-well plates. Protein standard and purified EV samples were used to prepare solutions containing 0.05 – 2 ng/mL IL-15 in complete cell culture medium; 50 μ L of each IL-15 protein or EV solution was then added to the corresponding cell culture wells. Equal amounts of EV lacking IL-15 were used as negative control. After 72 h incubation, 25 μ L MTT labeling reagent (Roche, Indianapolis, IN) was added to each well, and plates were incubated for 5 h at 37°C. Next, 100 μ L solubilization buffer (10% SDS in 0.01 M HCl; Roche) was added to each well, and plates were incubated for 24 h at 37 °C. Optical density of samples was measured at 570/690 nm, using a SPECTRAMAX Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA).

EV labeling for in vitro and in vivo tracking

[00122] Two different labeling dyes were used to monitor EV uptake: a green RNA dye (Syto RNASelect Green; Invitrogen, Grand Island, NY), and a near-infrared lipid dye (DiOC18(7) or DiR; Invitrogen). For Syto RNASelect Green staining, 100 μ g EV in 100 μ L TBS were incubated with the dye at a final concentration of 10 μ M for 30 min. at 37°C. For DiR staining, 62.5 μ g EV in 100 μ L TBS were incubated with the dye at a final concentration of 100 ng per microgram of EV for 1h at 37°C. After incubation, unincorporated dye was removed by gel filtration using PBS-hydrated Exosome Spin Columns (3 kDa MWCO, Invitrogen).

In vitro SRA blockade and EV uptake assessment

[0100] For EV uptake blocking experiments, mouse cells were first plated at a density of 250,000 cells per well in a 12-well plate and cultured overnight. E.G7 is a suspension cell line and 250,000 cells used for each experimental sample. Cells were pre-treated for 30 min. with fresh RPMI supplemented with 5% fetal calf serum medium, containing 100 μ g/ml chondroitin sulfate or dextran sulfate. Cells were washed once and incubated with fluorescently labeled EV for 2 hrs. The cells were harvested, washed, and resuspended in PBS+2% bovine serum albumin for the analysis of EV uptake by flow cytometry. The EV concentrations used in these experiments were adjusted to obtain a 2-4 fold increase in the fluorescent signal above the autofluorescence from untreated cells (RAW 3.2 μ g/ml; 4T1, MC38 and EG7: 1.6 μ g/ml; LLC1 and B16: 0.8 μ g/ml).

[0101] PBMC were cultured at 2×10^6 cells/mL and pretreated for 30 min. with 500 μ g/ml dextran sulfate (Sigma-Aldrich, St. Louis, MO), 500 μ g/ml chondroitin sulfate (Sigma-

Aldrich), or left untreated. After washing, the cells were resuspended at a density of 4×10^6 cells/ml and incubated for 2 hrs. with Syto RNASelect Green-labeled EV at a final concentration of 800 ng EV per mL, in a final volume of 0.5 mL. Finally, the cells were washed in PBS+0.2% AB+ human serum and stained with fluorophore-conjugated antibodies before flow cytometric analysis.

[0102] The following antibodies were used: α CD3-APC-Cy7 (clone SK7; BD Biosciences), α CD4-V500 (clone RPA-T4; BD Biosciences), α CD8-Alexa Fluor 405 (clone 3B5, Invitrogen), α CD14-PE (clone M5E2; BD Biosciences), α CD14-BV421 (clone HCD14; Biolegend; San Diego, CA), α CD19-Alexa Fluor 700 (clone HIB19; BD Biosciences), α CD19-APC (clone HIB19; BD Biosciences), α CD19-FITC (clone HIB19; Biolegend), α CD56-APC (clone B159; BD Biosciences), α CD56-PE (clone 5.1H11; Biolegend), and α CD204-PE (clone REA460; Miltenyi Biotec; San Diego, CA).

In vivo EV biodistribution in tumor-free and tumor-bearing mice after SRA blockade

[0103] All animal experiments were conducted in compliance with the guidelines for the care and use of research animals established by the Animal Studies Committee of the National Institutes of Health. FVB or Balb/c mice were treated with the SRA inhibitor dextran sulfate or chondroitin sulfate (negative control) at a dose of 30 mg/kg delivered in 100 μ L PBS by tail vein injection. Control mice received 100 μ L of PBS intraperitoneally. Two hours later, the mice received 100 μ L of PBS containing 15 μ g of DiR stained EV via the tail vein. Imaging of live mice was performed at various time points, between 1-24 h post injection. Some mice were euthanized at different time points and their organs imaged *ex vivo*. Imaging was done on a MAESTRO 2 imaging system (Perkin Elmer; Waltham, MA).

[0104] For tumor inoculation studies, 1×10^6 4T1 cells in 100 μ L of serum free medium were implanted subcutaneously on the right front flank of female BALB/c mice. Once the tumor size reached a volume of 90-100 mm³, the animals were used for *in vivo* imaging experiments.

Statistical analyses

[0105] EV preparation yields, monocyte uptake of EV *in vitro*, and liver and plasma uptake of EV (imaged *ex vivo*) were compared with multiple t-tests, with Holm-Sidak multiple comparison correction (overall alpha-error < 0.05 per comparison). Organ biodistribution of EV at 2 hrs. (imaged *ex vivo*) and time course of *in vivo* EV uptake by

tumor and liver were analyzed with 2-way ANOVA. Tumor uptake (imaged *ex vivo*) was analyzed by 1-way ANOVA. All statistical analyses were performed in PRISM 6 software (GraphPad Software, La Jolla, CA).

EXAMPLE 1

[0106] This example demonstrates that the yield of EV preparations is about 20-fold higher in a hollow fiber bioreactor as compared to a conventional flask.

[0107] In an effort to increase EV yield, a lab-scale hollow-fiber bioreactor with protein-free culture medium was used to obtain conditioned cell culture supernatants as a source of EV. HEK293 cells were adapted for growth in a hollow-fiber bioreactor using serum-free medium. EV from the bioreactor or conventional tissue culture flask supernatants were purified by differential centrifugation, and the protein concentration of the purified materials was determined using the Bradford assay. Using a similar protocol of EV purification, bioreactor-harvested conditioned media yielded approximately 10-fold more EV compared to conventional flasks (Fig. 1A). Further modification of the EV purification method, by omitting the supernatant concentration step, increased the EV yield about 2-fold, resulting in a total 20x improved EV yield from cell culture conditioned media (Fig. 1A).

[0108] The purity of the EV prepared in a conventional flask and in the bioreactor was also measured. The particle number was estimated by nanoparticle tracking analysis. The protein amount was estimated by Bradford assay. The results are shown in Fig. 5.

[0109] A similar experiment was performed using EV comprising (i) a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha with lactadherin as a carrier (encoded by an AG304 vector comprising SEQ ID NO: 13), (ii) a natural heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha (encoded by an AG152 vector comprising SEQ ID NO: 16), or (iii) control EV with no heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha. The results are shown in Figure 3.

EXAMPLE 2

[0110] This example demonstrates that the quantity of EV-associated markers is enriched in a hollow fiber bioreactor as compared to a conventional flask.

[0111] EV were prepared using a hollow fiber bioreactor or a conventional flask as described in Example 1. EV were purified by differential centrifugation and filtration and analyzed by Western blot. Each lane was loaded with 20 µg total protein. Band intensity

quantification was performed by densitometry. It was observed that the EV markers CD63 and Alix were enriched in preparations from bioreactor harvests as compared to conventional flasks (Table 1). These results suggest that the bioreactor preparations provided an increased purity as compared to the conventional flask preparations.

TABLE 1

	Relative increase in bioreactor EV preparation as compared to the conventional flask preparation
CD63	7.6-fold
Alix	2.1-fold

[0112] In the bioreactor EV preparations, differentially processed forms of associated proteins were also observed. Without being bound to a particular theory or mechanism, it is believed that the lower molecular weight bands observed for CD63 may reflect a differential glycosylation pattern, as this protein has several glycosylation modifications. It is also believed that the apparent secondary band observed for Alix may be explained by the differential status of the several known phosphorylation sites for the Alix protein. It is also believed that the detection of differentially processed proteins may be the result of an increased amount of the EV markers and/or an altered biocomposition of EV produced by cells grown under bioreactor conditions.

EXAMPLE 3

[0113] This example demonstrates the characterization of the EV prepared using the bioreactor as described in Example 1.

[0114] Dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), and transmission electron microscopy (TEM) were used to further characterize purified EV. Each sample for DLS/NTA was run in triplicate at 1-2 $\mu\text{g}/\text{ml}$ concentration. DLS showed that the majority of EV ranged in size from 40-200 nm. Transmission electron microscopy (TEM) showed well-defined particles of comparable size.

[0115] NTA was used to estimate the particle to protein amount ratio, an indicator of EV purity (El-Andaloussi et al., *Nat. Protoc.*, 7: 2112-2126 (2012); Webber et al., *J. Extracell. Vesicles*, 2: (2013)). A ratio of 1.7×10^9 ($\pm 4 \times 10^8$ particles)/ μg of protein was found, which is >3-fold greater than previously reported for EV obtained from HEK293 cells (El-

Andaloussi et al., *Nat. Protoc.*, 7: 2112-2126 (2012)). NTA was able to distinguish three main subpopulations based on size corresponding to peaks of approximately 120, 190, and 280 nm in diameter.

[0116] Together with the results of Examples 1-2, these data suggest that the use of hollow-fiber bioreactor, serum-free media, and a modified purification protocol represent a superior method to achieve high yield of purified EV (>3 mg/week from a single lab-scale culture cartridge).

EXAMPLE 4

[0117] This example demonstrates that EV-associated hetIL-15 is positioned on the vesicle surface.

[0118] A HEK293 cell clone that expresses the human membrane-associated heterodimeric interleukin-15 complex IL-15:IL-15R α (hetIL-15) (Chertova et al., *J. Biol. Chem.*, 288: 18093-18103 (2013); Bergamaschi et al., *J. Biol. Chem.*, 283: 4189-4199 (2008)) was used to interrogate whether purified EV maintain functional hetIL-15 on their surface. The presence of EV-associated hetIL-15 was confirmed by Western blot using antibodies against IL-15 and IL-15R α , whereas hetIL-15 was not detected in EV purified from the parental HEK293 cells (control cell lysate). Western blot showed the presence of IL-15 cytokine in cell lysate and in bioreactor-derived EV from a HEK293 cell clone that stably overexpresses the IL-15 heterodimer complex (IL-15:IL-15R α).

[0119] Upon surface expression, the full length ~59 kDa IL-15R α is cleaved and secreted as a soluble ~42 kDa molecule (Bergamaschi et al., *J. Biol. Chem.*, 283: 4189-4199 (2008)). Indeed, EV-associated IL-15R α was mostly of the larger membrane-associated species, which was also detected in association with cell lysates. The apparent molecular weight of the EV-associated protein was compared to that detected in the cell lysate (transmembrane protein) and in purified secreted hetIL-15 (truncated, secreted protein). The majority of EV-associated IL-15R α was the same size as that of the cell-associated (membrane-anchored) cytokine.

[0120] Flow cytometric analysis verified that hetIL-15 was associated with the EV surface. EV were first captured on α CD63 antibody-coated beads and probed with fluorophore-conjugated antibody to detect presence of surface cytokine. Control EV were derived from parental HEK293 cells and hetIL-15 EV came from a HEK293 cell clone stably overexpressing the cytokine.

EXAMPLE 5

[0121] This example demonstrates that the EV-associated IL-15 produced in Example 4 is bioactive *in vitro*.

[0122] The bioactivity of EV-associated hetIL-15 was confirmed *in vitro* by using an established MTT-based proliferation assay on the human NK-92 cell line. Upon stimulation by either form of the cytokine (soluble or EV-associated), NK-92 cells proliferated to a similar degree (Fig. 1B), indicating that purified EV maintain bioactive properties. A similar experiment was performed using EV comprising (i) a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha with lactadherin as a carrier (encoded by anAG304 vector comprising SEQ ID NO: 13) and (ii) a natural heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha protein standard. The results are shown in Figure 4. These results also demonstrate that non-processed, membrane bound heterodimeric IL-15 is associated with EV and can be an additional form of bioactive IL-15 in the body.

EXAMPLE 6

[0123] This example demonstrates that EV uptake by monocytes can be abrogated by SRA blocking.

[0124] One of the major challenges for EV therapeutics is to avoid rapid clearance by the reticuloendothelial system (RES) upon *in vivo* EV administration (Wiklander et al., *J. Extracell. Vesicles*, 4: 26316 (2015); Smyth et al., *J. Control Release*, 199: 145-155 (2015); Bala et al., *Sci. Rep.*, 5: 10721 (2015); Morishita et al., *J. Pharm. Sci.*, 104: 705-713 (2015)). The contribution of Scavenger Receptor Class A family (SRA) to EV uptake *in vitro* was assessed by using dextran sulfate. Chondroitin sulfate was used as a control for any non-specific activity of dextran sulfate (Thelen et al., *J. Immunol.*, 185: 4328-4335 (2010); Limmon et al., *FASEB J.*, 22: 159-167 (2008)), as both molecules are negatively charged sugar-multimers. By flow cytometry, it was confirmed that the murine macrophage cell line RAW 264.7 expresses the SR-A1 receptor isoform, while a panel of tumor cell lines (4T1, B16.FO, LLC1, MC38, and EG.7) lacks the expression of this receptor.

[0125] The cell lines (RAW 264.7, 4T1, B16.FO, LLC1, MC38, and EG.7) were pretreated with 100 µg/ml dextran sulfate (SRA blocker) or chondroitin sulfate (dextran control) for 30 min. After washing away inhibitor, stained EV were added for 2h. Flow cytometry was used to quantify EV uptake by fluorescent intensity of cells. In agreement with the SR-A1 expression levels, dextran pre-treatment of RAW 264.7 cells resulted in a 30-

50% decrease in EV uptake when incubated with fluorescently-labeled EV, whereas this effect was not observed in the tumor cell lines that lacked SR-A1 expression. To ensure that any differences in uptake of EV among cell lines tested was not due to decreased cell viability following treatment with dextran sulfate or chondroitin sulfate, viability was assessed using Live/Dead staining for flow cytometry. Cell viability was unaffected in all cell lines tested.

[0126] The contribution of SRA on EV uptake by human peripheral blood mononuclear cells (PBMC), a heterogeneous population of primary human cells, was also assessed. PBMC, pretreated with dextran sulfate, were exposed to labeled EV, and EV uptake in different primary cell subsets was monitored by flow cytometry, using a combination of fluorescently labeled monoclonal antibodies. Briefly, in two independent experiments (total n=4), cultured human PBMC were pretreated for 30 min. with 500 µg/ml dextran sulfate (SRA blocker) or chondroitin sulfate (dextran control). After washing away inhibitors, 800 ng/ml stained EV were added to the cells for 2h. EV uptake was quantified by mean fluorescent intensity above background, and normalized to EV uptake by untreated monocytes of each donor sample. In a separate experiment, it was determined that increasing the concentration of stained EV in the PBMC culture resulted in increased uptake by CD14+ primary human peripheral monocytes. These experiments demonstrated that most EV were bound to monocytes and that 30-60% of this uptake could be abrogated by SRA blocking (Fig. 2A).

EXAMPLE 7

[0127] This example demonstrates that SRA blockade increases circulating EV and changes the biodistribution of EV.

[0128] It was hypothesized that blocking SRA would impact EV biodistribution. Mice, pretreated with dextran sulfate, received a tail vein injection of DiR-labeled EV. EV biodistribution was monitored by fluorescence imaging of live mice as well as excised organs (Table 2). FVB mice were pre-treated with 0.6 mg dextran sulfate, chondroitin sulfate, or PBS. 15 µg DiR-stained EV were then injected intravenously. Mice were imaged 2 hrs later or the organs and plasma were excised 2h after EV administration and imaged. The results showed that liver uptake of EV dramatically decreased with SRA blocker pre-treatment. These imaging studies performed at 2 h post-injection of EV showed that SRA blockade decreased liver uptake of EV by ~50% (Table 2). At the same time, the amount of EV

circulating in the plasma significantly increased (Fig. 2B Table 2). Thus, blocking the EV receptor SRA led to significant changes in EV biodistribution.

TABLE 2

Organ	Fluorescently labelled EV average signal ($\times 10^6$ photons/cm ² /s); Mean $\pm$ SD				
	Uninjected mouse	EV alone injection	Chondroitin sulfate (0.6mg) + EV	Dextran sulfate (0.3mg) + EV	Dextran sulfate (0.6mg) + EV
Liver	53	4893 $\pm$ 366	5103 $\pm$ 1264	2607 $\pm$ 218	1333 $\pm$ 111 *
Spleen	440	1483 $\pm$ 162	5837 $\pm$ 2226	6253 $\pm$ 2467	3152 $\pm$ 614
Lungs	425	536 $\pm$ 8	798 $\pm$ 241	921 $\pm$ 75	849 $\pm$ 51
Heart	44	165 $\pm$ 9	289 $\pm$ 41	354 $\pm$ 35	326 $\pm$ 18
Kidneys	106	118 $\pm$ 15	103 $\pm$ 25	128 $\pm$ 48	76 $\pm$ 35
Plasma	22	157 $\pm$ 23	69 $\pm$ 35	241 $\pm$ 11 *	302 $\pm$ 56 *

Table 2:

[0129] Fluorescently stained EV were injected in FVB mice (n = 3 per group) after pretreatment with chondroitin sulfate or dextran sulfate, and were imaged 2 hrs later. Biodistribution of EV was monitored with live animal fluorescent imaging. A decrease of liver uptake and increase in circulating (plasma) EV were found at 2h in mice pre-treated with dextran sulfate. Statistical analysis was with two-way ANOVA. Asterisk (*) denotes statistically significant difference ($p < 0.05$) from both EV alone and Chondroitin + EV group.

EXAMPLE 8

[0130] This example demonstrates that SR blockade results in enhanced tumor accumulation of EV in the absence of targeting ligands.

[0131] It was assessed whether blocking monocyte/macrophage uptake results in enhanced tumor accumulation of EV, in the absence of targeting ligands. Immunocompetent mice bearing subcutaneous 4T1 breast cancer cell tumors were pretreated with the SRA blocker of Example 7, followed by systemic delivery of labeled EV. Imaging of live mice

over a period of 24 hours showed that significant intratumoral EV accumulation could only be observed in animals pretreated with dextran sulfate (Table 4), even though SRA blockade of the liver diminished over 24 h (Table 3). *Ex vivo* measurements also showed that EV tumor uptake increased by ~3-fold over the 24-h period (Fig. 2C).

TABLE 3

	Fluorescently labeled EV average signal over liver ($\times 10^6$ photons/cm ² /s); Mean $\pm$ SD		
Time after EV injection	EV alone	Chondroitin sulfate + EV	Dextran sulfate + EV *
1h	7222 $\pm$ 1325	6339 $\pm$ 591	1544 $\pm$ 113
2h	6805 $\pm$ 1235	7309 $\pm$ 1437	2671 $\pm$ 251
4h	6460 $\pm$ 723	7333 $\pm$ 1403	3071 $\pm$ 254
8h	5798 $\pm$ 586	5865 $\pm$ 1277	3933 $\pm$ 468
24h	5761 $\pm$ 746	5488 $\pm$ 1132	5452 $\pm$ 1090

Table 3

[0132] Fluorescently stained EV were injected in Balb/c mice, after pretreatment with either chondroitin sulfate or dextra sulfate. Biodistribution of EV was monitored with live animal fluorescent imaging. Time course of EV uptake over liver of 4T1 tumor-bearing Balb/c mice (n=4 per group) is shown, which was analyzed by 2-way ANOVA. Asterisk (*) denotes group is significantly different ($p < 0.05$) from both EV alone and Chondroitin + EV group.

TABLE 4

	Fluorescently labelled EV average signal over Tumor ($\times 10^6$ photons/cm ² /s); Mean $\pm$ SD		
Time after EV injection	EV alone	Chondroitin sulfate + EV	Dextran sulfate + EV *
1h	119 $\pm$ 200	97 $\pm$ 98	84 $\pm$ 104
2h	162 $\pm$ 226	253 $\pm$ 178	256 $\pm$ 126

4h	211 ± 309	194 ± 215	305 ± 114
8h	233 ± 302	152 ± 198	376 ± 216
24h	253 ± 211	241 ± 159	891 ± 298

Table 4

[0133] Fluorescently stained EV were injected in Balb/c mice, after pretreatment with either chondroitin sulfate or dextra sulfate. Biodistribution of EV was monitored with live animal fluorescent imaging. Time course of EV uptake over tumor of 4T1 tumor-bearing Balb/c mice (n=4 per group) is shown, which was analyzed by 2-way ANOVA. Asterisk (*) denotes group is significantly different ($p < 0.05$) from both EV alone and Chondroitin + EV group.

EXAMPLE 9

[0134] A method of purification of EV's that includes tangential-flow filtration and size-exclusion chromatography instead of ultracentrifugation that provides high EV yields from bioreactor cell culture was evaluated (see, the schematic presented in Figures 6 and the data provided in Figures 7-15). Clarified conditioned medium was prepared in the same manner as the clarified medium described in the methodology section that was subjected to ultracentrifugation (110,000 x g). Specifically, cells expressing desired EV-associated construct (e.g. hetIL-15 / lactadherin) were cultured in a hollow-fiber bioreactor. Conditioned medium was harvested every 1-3 days, centrifuged at 300 x g for 10 min, and 3,000 x g for 15 min (to remove cells and cell debris), and then stored at -80°C. Frozen condition medium was thawed, centrifuged at 20,000 x g for 45 min, and filtered through a 0.22 µm filter to remove vesicles greater than 200 nm.

[0135] Clarified conditioned medium was processed by tangential flow filtration (TFF). A commercial TFF filter device was connected to a pump-driven system that circulated clarified conditioned medium through a series of tubings. As the medium entered the TFF device, molecules smaller than the pore size (e.g., 50 nm pores) exited the main tubing circuit as filtrate, which was collected in a waste reservoir. This process leads to concentration of particles >50nm, which comprise the EVs. By connecting an additional dialysis buffer reservoir, a stable volume was maintained in the main tubing circuit, because the concentration of clarified conditioned medium leads to negative pressure developing in the main tubing circuit, thereby leading to an influx of dialysis buffer. Up to 5 volumes of buffer

exchange was performed by continuously circulating the conditioned medium while it was connected to the dialysis buffer reservoir. This allowed the majority of non-EV macromolecules to be filtered into the waste reservoir. The dialysis reservoir was then disconnected, and the conditioned medium was circulated through the filter device until the desired concentration was achieved. The buffer exchange step was important because the presence of a large amount of non-EV proteins limits the concentration of conditioned medium.

[0136] After TFF buffer exchange and concentration, conditioned medium was injected into a size-exclusion chromatography (SEC) column, in this case a pre-packed commercial column: Superdex 200 increase 10/300 GL or HiLoad 16/600 200pg), and eluted by constant injection of an elution buffer (e.g. PBS) using an HPLC apparatus. With SEC, larger molecules are eluted in earlier fractions. By monitoring UV-light absorbance (e.g. at 260nm and 280nm wavelengths), one can predict the fractions that are EV-rich. These coincide with the first peak of absorbance at these wavelengths. The fractions were collected and provided a highly purified preparation.

EXAMPLE 10

[0137] Figures 16(a) and (b) provides further data illustrating that hetIL-15/lactadherin fusion increases IL-15 loading of EVs. HEK293 cells expressing hetIL-15, hetIL-15/Lactadherin or not expressing IL-15 were cultured in hollow-fiber bioreactors. EVs were purified by SEC from bioreactor harvests. Figures 16(a): ELISA was used to quantitate EV-associated IL-15, and showed that fusion of hetIL-15 with Lactadherin increased cytokine loading by 50-fold. Figure 16(b): EV were incubated with 9 um latex beads, coated with EV anti-CD63 antibody, which detects the CD63 EV surface antigen. Following binding of beads to EV, fluorescently-labelled antibody specific for human IL-15 was added. Flow cytometric analysis of EV-bead complexes showed that hetIL-15/Lactadherin EV had significantly more cytokine on their surface. Surface association of IL-15 on EV was confirmed by immunoelectron microscopy (data not shown).

EXAMPLE 11

[0138] This example demonstrates the productions of EVs comprising functional cytokine fusions (IL-15-IL-12).

[0139] In addition to EVs having surface functional IL-15, additional cytokines and other molecules can be associated with EVs and EVs can be produced with these molecules. In the following example, a fusion of two heterodimeric cytokines, IL-15 and IL-12, has been produced and tested and can be incorporated on EVs following the protocols described above.

[0140] A DNA encoding a chimeric protein containing single-chain IL-15 fused to the p40 subunit of IL-12, referred to here as IL-15:IL-12p40, was generated. The IL-12p40 subunit was linked to the C terminus of IL-15 either directly (Plasmid code: AG282) or via a flexible linker of 4 amino acids (GAGA) (Plasmid Code: AG283) (Figure 17).

[0141] The ability of the chimera IL-15:IL-12p40 to bind soluble IL-15 receptor alpha (sIL-15R α) and IL-12p35 subunit (to form heterodimeric IL-15 and heterodimeric IL-12, respectively) was evaluated. AG282 and AG283 DNAs were used to transfect human HEK293 cells in combination with DNA plasmids expressing sIL-15R α and IL-12p35, and the expression level of hetIL-15 and IL-12 in the fusion configuration were determined by ELISA and Western immunoblot. A commercially available ELISA kit that specifically detects IL-15/IL-15Ra complexes, but not the single chain molecules, was employed for the analysis. Upon transfection of IL-15:IL-12p40 DNA alone or in combination with IL-12p35, no complexes were detected in the supernatant. In contrast, the addition of the DNA expressing sIL-15Ra resulted in formation of hetIL-15 that was detected by ELISA, indicating that IL-15:IL-12p40 maintained the ability to interact with sIL-15R α to form hetIL-15 (Figure 18). Similar results were obtained using both AG282 and AG283 DNAs.

[0142] Supernatants were also evaluated by Western immunoblot using polyclonal antibodies for IL-12, which recognize both IL-12p40 and IL-12p35, under denaturing and reducing conditions. These studies showed that co-expression of IL-15:IL-12p40 in the same cells with either IL-12p35 or sIL-15R α promoted the secretion of the chimeric IL-15:IL-12p40 protein, indicating that IL-15:IL-12p40 maintained the ability to interact with IL-12p35 to form IL-12 (Figure 19). Similar results were obtained using both AG282 and AG283. In addition, native Western immunoblot analysis further confirmed that the IL-15:IL-12p40 fusion protein maintained its ability to interact with IL-12p35 and soluble IL-15R α (IL-15sRa) as revealed by band shift (Figure 20).

[0143] *In vitro* bioactivity of the chimeric IL-15:IL-12 protein was also analyzed. Overnight stimulation of human NK-92 cells with supernatant containing either mouse IL-12p70 or IL-15:IL-12 fusion protein with IL-12p35 (ranging from 0.07 ng/ml-10 ng/ml of IL-

12) resulted in comparable levels of secreted IFN γ , indicating that the IL-15:IL-12 fusion protein maintained its ability to stimulate NK cells as well as IL-12p70 alone (Figure 21).

[0144] *In vivo* studies in mice were performed to evaluate synergistic effects of IL-12 and IL-15. Upon hydrodynamic injection of plasmids expressing mouse IL-12 and IL-15, levels of circulating IFN γ were ~1 log higher, in comparison to levels obtained when mice received DNAs encoding for the individual cytokines. Similarly, the combination of IL-12 and IL-15 resulted in synergistic effects on CD8+T cell proliferation in spleen (Figure 22). The ability of the IL-12:IL-15 fusion protein to perform similarly to the combination of IL-12 and IL-15 was then evaluated. Different co-injection regimens, involving increasing the amount of DNA expressing IL-15:IL-12 while maintaining a fixed amount of IL-12p35 DNA, were tested. A dose of 200 ng IL-15:IL-12 fusion protein-encoding DNA together with 20 ng IL-12p35-encoding DNA induced similar CD8+T cell proliferation as the combination of IL-12 and IL-15-expressing DNAs at doses of 20 ng and 50 ng, respectively (Figure 23). These results indicate that administration of the IL-15:IL-12 fusion protein *in vivo* promoted CD8+T cell proliferation to a degree similar to the combination of heterodimeric IL-15 and IL-12, and that the effects were dose-responsive. The IL-15:IL-12p40 protein thus maintained the ability to interact with IL-12p35 and sIL-15R α to form both a functional IL-12 and a functional IL-15.

[0145] This example thus supports that incorporation of the fusion polypeptide into EVs will direct the activity of the fusion cytokine to the areas of EV targeting and will increase the local activity of the combination IL-15 and IL-12.

[0146] All references, including publications, accession numbers, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference.

[0147] The use of the terms “a” and “an” and “the” and “at least one” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The use of the term “at least one” followed by a list of one or more items (for example, “at least one of A and B”) is to be construed to mean one item selected from the listed items (A or B) or any combination of two or more of the listed items (A and B), unless otherwise indicated herein or clearly

contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0148] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

Illustrative sequences

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SEQ ID NO:7 Human IL-15 (GM-CSF signal peptide fused to mature IL-15)

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SEQ ID NO:8 Human IL-15Ra 170 aa

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SEQ ID NO:9 Human lactadherin

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SEQ ID NO:10 Human IL-15Ra-human lactadherin fusion protein (mature, 507 AA)

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SEQ ID NO:11 Human IL-15Ra-human lactadherin fusion protein (mature, 507 AA)

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SEQ ID NO:12 IL-15

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SEQ ID NO:13 AG304 hIL-15Ra-blactadherin+hGM-CSF-hIL-15 vector

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SEQ ID NO:14 Human IL-15Ra natural form of human heterodimeric IL-15

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SEQ ID NO:15 Human IL-15Ra natural form of human heterodimeric IL-15

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SEQ ID NO16 AG152 DP hIL-15Ra+hGM-hIL-15 (5858 bp); Plasmid expressing the natural form of human heterodimeric IL-15

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SEQ ID NO:17 Plasmid expressing a synthetic protein, in which 1-4 amino acids at the cleavage site have been removed from the natural form of human heterodimeric IL-15 or changed.

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AGGCCTGAATCGCCCCATCATCCAGCCAGAAAGTGAGGGAGCCACGGTTGATGA
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ACGGTCTGCGTTGTGCGGAAGATGCGTGATCTGATCCTTCAACTCAGCAAAAGTT
CGATTTATTCAACAAAGCCGCCGTCCCGTCAAGTCAGCGTAATGCTCTGCCAGTG

TTACAACCAATTAACCAATTCTGATTAGAAAACTCATCGAGCATCAAATGAAAC
TGCAATTTATTCATATCAGGATTATCAATACCATATTTTTGAAAAAGCCGTTTCTG
TAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTA
TCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCCCCTCGT
CAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTG
AGAATGGCAAAAGCTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATT
ACGCTCGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGC
GCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGACAATTACAAACAGGA
ATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCATCAACAATATTTTCACCT
GAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTCCCGGGGATCGCAGTGG
TGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTTCGGAAGAG
GCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATTGGC
AACGCTACCTTTGCCATGTTTCAGAAACAACCTCTGGCGCATCGGGCTTCCCATAC
AATCGATAGATTGTGCGACCTGATTGCCCCGACATTATCGCGAGCCCATTATACC
CATATAAATCAGCATCCATGTTGGAATTTAATCGCGGCCTCGAGCAAGACGTTTC
CCGTTGAATATGGCTCATAACACCCCTTGTATTACTGTTTATGTAAGCAGACAGT
TTTATTGTTTCATGATGATATATTTTTATCTTGTGCAATGTAACATCAGAGATTTTG
AGACACAACGTGGATCATCCAGACATGATAAGATACATTGATGAGTTTGGACAA
ACCACAACCTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTA
TTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTG
CATTCATTTTATGTTTCAGGTTTCAGGGGGAGGTGTGGGAGGTTTTTTAAAGCAAG
TAAAACCTCTACAAATGTGGTATGGCTGATTATGATCGTCGAGGATCTGGATCCG
TTAACCGATATCCGCGAATTCGGCGCGCCGGGCCCTCACGACGTGTTGATGAACA
TCTGGACGATGTGCACGAACGACTGCAGGAACTCCTTGATGTTCTTCTCCTCCAG
CTCCTCGCACTCCTTGCAGCCCGACTCCGTGACGTTCCCGTTTCGACGACAGCGAG
TTGTTCGCCAGGATGATCAGGTTCTCCACCGTGTCTGATCGACGCGTCCCCCG
ACTCGAGCGAGATGACTTGAGCTCCAGGAGGAAGCACTTCATCGCCGTGACCT
TGCACGACGGGTGGACGTCCGACTCCGTGTACAGCGTCGCGTCGATGTGCATCGA
CTGGATGAGGTCTTCGATCTTCTTCAGGTCCGAGATCACGTTACCCAGTTCGAG
ATGCTGCAGGCCACCGTCCCCAGGAGTAGCAGGCTCTGGAGCCACATTTCTTGTC
GACAGATCCAAACGCTCCTCCGACGTCCCCAGGCAGAATGGCGGTTCCCTAAAC
GAGCATTGCTTATATAGACCTCCCATAGGCACGCCTACCGCCATTTACGTCAA
TGGAACGCCCATTGCGTCATTGCCCCCTCCCCATTGACGTCAATGGGGATGTACT

TGGCAGCCATCGCGGGCCATTTACCGCCATTGACGTCAATGGGAGTACTGCCAAT
 GTACCCTGGCGTACTTCCAATAGTAATGTACTTGCCAAGTTACTATTAATAGATA
 TTGATGTACTGCCAAGTGGGGCCATTTACCGTCATTGACGTCAATAGGGGGCGTGA
 GAACGGATATGAATGGGCAATGAGCCATCCCATTGACGTCAATGGTGGGTGGTC
 CTATTGACGTCAATGGGCATTGAGCCAGGCGGGCCATTTACCGTAATTGACGTCA
 ATGGGGGAGGCGCCATATACGTCAATAGGACCGCCCATATGACGTCAATAGGTA
 AGACCATGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTG
 ACACATGCAGCTCCCGGAGACGGTCACAGCTTGCTGTGTAAGCGGATGCCGGGAG
 CAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCT
 TAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGA
 AATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGATTGGCTATTGG

SEQ ID NO:18 IL-15Ra cleavage site
 QGHS

SEQ ID NO:19 Human IL-15Ra; the four amino acids surrounding the cleavage site
 (lower-case letters) are deleted or changed in different combinations to derive hetIL-15
 forms with altered processing (i. e., non-cleaved).

MAPRRARGCRTLGLPALLLLLLLRPPATRGITCPPPMSVEHADIWVKSYSLYSRERYI
 CNSGFKRKAGTSSLTECVLNKATNVAHWTTPSLKCIRDPALVHQRPAAPPSTVTTAGV
 TPQPESLSPSGKEPAASSPSSNNTAATTAAIVPGSQLMPSKSPSTGTTEISSHESSHGTP
 SQTAKNWELTASASHQPPGVYP_{xxxx}DTTVAISTSTVLLCGLSAVSLLACYLKSRQTP
 PLASVEMEAMEALPVTWGTSSRDEDLNCSHHL

X at position 199 is any naturally occurring amino acid residue or is absent;

X at position 200 is any naturally occurring amino acid residue or is absent;

X at position 201 is any naturally occurring amino acid residue or is absent;

X at position 202 is any naturally occurring amino acid residue or is absent.

SEQ ID NO:20 MARCO

gccaaagggaagtgtcgaggtttacaaccagctgcagtggttcgatgggaaggatctttccaagtgttctcttgaggggagc
 atttctgtggtccaggactttggccatctataagcttgcaatgagaaataagaaaattctcaaggaggacgagctcttgagtgaga
 cccaacaagctgcttttaccaaattgcaatggagcctttcgaaatcaatgttccaaagccaagaggagaaatggggtgaacttctcc

ctagctgtggtggtcatctacctgatcctgctcaccgctggcgctgggctgctggtggtccaagttctgaatctgcaggcgcggtccg
 ggtcctggagatgtatttctcaatgacactctggcggctgaggacagcccgtccttctccttctgctgcagtcagcacacctggagaac
 acctggctcagggtgcatcgaggctgcaagtcctgcaggcccaactcacctgggtccgctcagccatgagcacttgcctgcagcgg
 gtagacaacttactcagaaccagggtatgttcagaatcaaggtgaacaaggcgccccaggtctcaaggtcacagggggcccacat
 gggcatgcctggtgccccctggccccgggaccacctgctgagaaggagccaagggggctatggacgagatggagcaacag
 gccccctgggagcccccaaggccccaccgggagtcaggggagaggcgggcctccaaggacccccagggtgctccagggaagcaagg
 agccactggcacccccaggacccccaggagagaaggcgagcaaaaggcgatgggggtctcattggcccaaaaggggaaactggaa
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 cctggagcccaggggagtaagggtgacttcgggaggccaggcccaccagggttggctggtttctgagctaaaggagatcaagg
 acaacctggactgcagggtgttcggggccctcctggtgcagtgggacaccagggtccaagggtgagcctggcagtgcctggtccc
 ctggcgagcaggacttcaggggagccccgggagtcaggagccacaggcctgaaaggaaagcaaggggacacaggacttcaa
 ggacagcaagggaagaaaaggagaatcaggagttccaggccctgcagggtgaaggagaaacaggggagcccagggtgagcag
 gtccaaggggagccccctggacaagctggccagaaggagaccagggtgaaaggatctctggggagcaaggagtaaaaggga
 gaaaaagggtgaagagggtgaaaactcagtgtccgtcaggattgtcggcagtagtaaccgaggccgggctgaagtttactacagtgt
 acctgggggacaatttgcgatgacgagtgcaaaattctgatgccattgtcttctgccgcatgctgggttactcaaagggaagggcct
 gtacaaagtgggagctggcactgggcagatctggctggataatgttcagtgctggggcacggagagtacctgtggagctgcaccaa
 gaatagctggggccatcatgactgcagccacgaggaggacgcaggcgtggagtgacgctctgacctggaaacctttcacttctct
 gctcccaggtgtcctcgggctcatatgtgggaaggcagaggatctctgaggagttccctggggacaactgagcagcctctggagag
 gggccattaataaagctcaacatcattggcaaaaaaaaaaaaaaaaaa

SEQ ID NO:21 MARCO

mrnkilkedellsetqqaafhqiamepfeinvpkpkrngvnfslavvviylilltagagllvvqvlqlarlrivlemyflndtlaac
 dspsfslqsahpgehlqagrarlqlvqlaqltwrvrshelllqrndnftqnpqgmfrkgeqgagplqghkgamgmpgagppp
 ppaekgakgamgrdgatgpspqpqpvgkgeaglgpqpqagpkqgatgtpqpqgeksgkdggllgpkgetgtkgekddl
 glpgskgdrmkgdagvmgppgaqgskgdfgrpppplagfpgakgdqgqplqgvpppgavghpgakgepgsagsp
 graglpqspgspgatlgksgkdtglqqqgrkgesgvpgpagvkgeqgspglagpkgapgqagqkdqgvkgssgeqgvk
 gekgergensvsrvivgssnrgraevyysgtwtgicddewqnsdaivfcmlygskgralykvagtgqiwdnvqcrgtstl
 wscitknswhhdesheedagvecsv

SEQ ID NO:22 AG282: Murine IL-15 – murine IL-12p40 fusion sequence (5363 bp)

IL-15 sequence is bold. IL-12p40 sequence is bold and underlined.

CCTGGCCATTGCATACGTTGTATCCATATCATAATATGTACATTTATATTGGCTCATGTCCAACATTAC
 CGCCATGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGC

CCATATATGGAGTTCGCGGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACC
CCCCCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGT
CAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTA
CGCCCCCTATTGACGTCAATGATGGTAAATGGCCCGCCTGGCATTATGCCAGTACATGACCTTATG
GGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGC
AGTACATCAATGGGCGTGATAGCGGTTTGA CTACGGGGATTTCCAAGTCTCCACCCCATTGACGT
CAATGGGAGTTTTGTTTTGGCACAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCA
TTGACGCAAATGGGCGGTAGGCGGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAAC
CGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACGGGACCGATCCA
GCCTCCGCGGGCGCGCGTCGAGGAATTCTCTAGAAAGAAATGTGGCTCCAGAACCTGCTATTCTG
GGGATCGTGGTCTACAGCCTCTCGAACTGGATCGATGTGCGCTACGACCTGGAGAAGATCGAGTC
CCTCATCCAGTCGATCCACATCGACACCACGCTGTACACGGACTCGGACTTCCACCCGTCGTGCAAG
GTCACGGCGATGAACTGCTTCTCTGGAGCTGCAAGTCATCCTGCACGAGTACTCGAACATGACC
CTAACGAGACGGTGCGCAACGTGCTGTACCTGGCGAACTCGACCCTGTGTCGAACAAGAACGT
CGCCGAGTCGGGCTGCAAGGAGTGCGAGGAGCTGGAGGAGAAGACCTTCACGGAGTTCTGTCAG
TCGTTTCATCCGCATCGTCCAGATGTTTCATCAACACGTCGATGTGGGAGCTGGAGAAGGACGCTAC
GTCGTGGAGGTGGACTGGACGCCCCGACGCCCCGGGAGACGGTGAACCTCACCTGCGACACGCC
GGAGGAGGACGACATCACCTGGACCAGCGACCAGCGGCACGGCGTCATCGGCAGCGGCAAGACC
CTGACGATCACCGTCAAGGAGTTCCTCGACGCGCGCCAGTACACCTGCCACAAGGGCGGCGAGAC
GCTGAGCCACAGCCACCTGCTGCTCCACAAGAAGGAGAACGGCATCTGGTCCACCGAGATCCTCA
AGAACTTCAAGAACAGACCTTCTGAAGTGCGAGGCGCCCACTACTCCGGCCGGTTCACGTGCT
CCTGGCTGGTGACGCGCAACATGGACCTCAAGTTCAACATCAAGTCGTCCAGCAGTTCCCCGACT
CCCCGGCGGTGACCTGCGGCATGGCGTCCCTGTGCGCGGAGAAGGTACGCTGGACCAGAGGGA
CTACGAGAAGTACTCGGTGTCTGCCAGGAGGACGTACCTGCCCCACGGCCGAGGAGACCCTGC
CCATCGAGCTGGCGCTGGAGGCCCGGACGAGAACAAGTACGAGAATAACAGCACCAGCTTCTTC
ATCCGGGACATCATCAAGCCCCGACCCGCCAAGAACCTGCAGATGAAGCCCCTGAAGAACAGCCA
GGTGGAGGTGAGCTGGGAGTACCCGGACTCCTGGAGCACGCCCCACTCCTACTTCAGCCTGAAGTT
CTTCGTCAGAAATCCAGCGCAAGAAAGAGAAGATGAAGGAGACGGAGGAGGGGTGCAACCAGAA
GGGCGGTTCTCGTCGAGAAGACCTCCACCGAGGTCCAGTGCAAGGGCGGGAACGTCTGCGTGC
AGGCTCAGGACCGCTACTACAACCTCCAGCTGCAGCAAGTGGGCCTGCGTCCCCTGCAGGGTCCGG
TCCTAGTAAAGATCTGATATCGGATCTGCTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTGCCCTC
CCCCGTGCCCTTCTTGACCTGGAAGGTGCCACTCCCACTGTCCTTTCTAATAAAATGAGGAAATTG
CATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGG
GAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGGTACCCAGGTGCT
GAAGAATTGACCCGTTCTCTCTGGGCCAGAAAGAAGCAGGCACATCCCCTTCTGTGACACACCC
TGTCACGCCCCCTGGTTCTTAGTTCCAGCCCCACTCATAGGACACTCATAGCTCAGGAGGGCTCCGCC
TTCAATCCCACCCGCTAAAGTACTTGGAGCGGTCTCTCCCTCCCTCATCAGCCCACCAAACCAACCT
AGCCTCCAAGAGTGGGAAGAAATTAAAGCAAGATAGGCTATTAAGTGACAGAGGGAGAGAAAATGC
CTCAACATGTGAGGAAGTAATGAGAGAAATCATAGAATTTCTCCGCTTCTCGCTCACTGACTCGC
TGCGCTCGGTGTTGCGGTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCA
CAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGT
AAAAAGGCCGCGTTGCTGGCGTTTTTTCATAGGCTCCGCCCCCTGACGAGCATCACAAAATCGAC
GCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCT
CCCTCGTGCGCTCTCTGTTCCGACCCTGCCGTTACCGGATACCTGTCCGCTTTCTCCCTTCGGGAA
GCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTGTTGCTCCAAGCT
GGGCTGTGTGCACGAACCCCCCGTTAGCCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAG
TCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCG

AGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACA
 GTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCG
 GCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAA
 AAGGATCTCAAGAAGATCCTTTGATCTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACG
 TTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAATAATGAA
 GTTTTAAATCAATCTAAAGTATATATGAGTAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAG
 GCACCTATCTCAGCGATCTGTCTATTTCTGTTTCATCCATAGTTGCCTGACTCGGGGGGGGGGGGGCGCT
 GAGGTCTGCCTCGTGAAGAAGGTGTTGCTGACTCATACCAGGCCTGAATCGCCCCATCATCCAGCCA
 GAAAGTGAGGGAGCCACGGTTGATGAGAGCTTTGTTGTAGGTGGACCAGTTGGTGATTTTGAACCTT
 TTGCTTTGCCACGGAACGGTCTGCGTTGTCGGGAAGATGCGTGATCTGATCCTTCAACTCAGCAAAA
 GTTCGATTTATTCAACAAAGCCGCCGTCCCGTCAAGTCAGCGTAATGCTCTGCCAGTGTTACAACCAA
 TTAACCAATTCTGATTAGAAAACTCATCGAGCATCAAATGAACTGCAATTTATTATATCAGGATT
 ATCAATACCATATTTTGA AAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCAT
 AGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTT
 CCCCTCGTCAAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAA
 TGGCAAAAGCTTATGCATTTCTTCCAGACTTGTCAACAGGCCAGCCATTACGCTCGTCATCAAAT
 CACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCGAGACGAAATACGCGATCGCT
 GTTAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCATCAAC
 AATATTTTACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTCCCGGGGATCGCAGTGG
 TGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAAGAGGCATAAATCCG
 TCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCTTTGCCATGTTTCAGA
 AACAACTCTGGCGCATCGGGCTTCCCATACAATCGATAGATTGTCGCACCTGATTGCCCGACATTATC
 GCGAGCCCATTTATACCATATAAATCAGCATCCATGTTGGAATTTAATCGCGGCCTCGAGCAAGAC
 GTTTCCCGTTGAATATGGCTCATAACACCCCTTGTATTACTGTTTATGTAAGCAGACAGTTTTATTGTT
 CATGATGATATATTTTATCTTGCAATGTAACATCAGAGATTTTGAGACACAACGTGGCTTTCCCC
 CCCCCCATTTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTT
 AGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAA
 CCATTATTATCATGACATTAACCTATAAAAAATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTC
 GGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCG
 GATGCCGGGAGCAGACAAGCCGTCAGGGCGCGTCAGCGGGTGTGGCGGGTGTGGGGGCTGGCT
 TAACTATGCGGCATCAGAGCAGATTGACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAG
 ATGCGTAAGGAGAAAAATACCGCATCAGATTGGCTATTGG

SEQ ID NO:23 Murine IL-15 – murine IL-12p40 fusion protein (444 AA)

Mouse GM-CSF signal peptide is single underlined. IL-15 is bold. IL-12p40 is double-underlined.

MWLQNLLFLGIVVYSL**NWIDVRYDLEKIESLIQSIHIDTTLYTDSDFHP**SCKVTAMNCFLLELQVILHEYS
NMTLNETVRNVLYLANSTLSSNKNVAESGCKECEELEKTFTEFLQSFIRIVQMFINTS**MWELEKDVYV**
VEVDWTPDAPGETVNLCTDTP**EEDDITWTS**DQRHGVIGSGKTLTITVKEFLDAGQYTCHKGGETLSHSH
LLHKKENGWSTEILKNFNKTFKCEAPNYSGRFTCSWLVQRNMDLKFNIKSSSSSPDSRAVTCGMASL
SAEKVTLDORDYEKYSVSCQEDVTCPTAEETLPIELALEARQONKYENYSTSFFIRDIIKPDPPKNLQMKPL
KNSQVEVSWEYPDSWSTPHSYFSLKFFVRIQRKKEKMKETEEGCNQKGAFLVEKTSTEVQCKGGNVCVO
AQDRYYNSSCSKWACVPCRVRS

SEQ ID NO:24 AG283: Murine IL-15 – murine IL-12p40 fusion sequence, with linker (5375 bp)

IL-15 sequence is bold and single underlined. Linker is bold lowercase. IL-12p40 is bold and double underlined.

CCTGGCCATTGCATACGTTGTATCCATATCATAATATGTACATTTATATTGGCTCATGTCCAACATTAC
CGCCATGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGC
CCATATATGGAGTTCGCGCTTACATAACTTACGGTAAATGGCCCCGCTGGCTGACCGCCCAACGACC
CCCCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCATTGACGT
CAATGGGTGGAGTATTTACGGTAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTA
CGCCCCCTATTGACGTCAATGATGGTAAATGGCCCCGCTGGCATTATGCCAGTACATGACCTTATG
GGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGTTTTGGC
AGTACATCAATGGGCGTGGATAGCGTTTGACTCACGGGGATTTCGAAGTCTCCACCCCATGACGT
CAATGGGAGTTTGTGGTGGCACCAAAATCAACGGGACTTCCAAAATGTCGTAACAACCTCCGCCCCA
TTGACGCAAATGGGCGGTAGGCGGTGACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAAC
CGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACGGGGACCGATCCA
GCCTCCGCGGGCGCGCGTCGAGGAATTCTCTAGAAAGAAATGTGGCTCCAGAACCTGCTATTCTG
GGGATCGTGGTCTACAGCCTCTCGAACTGGATCGATGTGCGCTACGACCTGGAGAAGATCGAGTC
CCTCATCCAGTCGATCCACATCGACACCACGCTGTACACGGACTCGGACTTCCACCCGTCGTGCAAG
GTCACGGCGATGAAGTCTTCTCTGGAGCTGCAAGTCATCTGCACGAGTACTCGAACATGACC
CTCAACGAGACGGTGCGCAACGTGCTGTACCTGGCGAACTCGACCCTGTCGTCGAACAAGAAGCT
CGCCGAGTCGGGCTGCAAGGAGTGCGAGGAGCTGGAGGAGAAGACCTTCACGGAGTTCCTGCAG
TCGTTTCATCCGCATCGTCCAGATGTTTCATCAACACGTCGggagcaggtgctATGTGGGAGCTGGAGAA
GGACGTCTACGTCGTGGAGGTGGACTGGACGCCCCGACGCCCCGGGGAGACGGTGAACCTCACCT
GCGACACGCCGGAGGAGGACGACATCACCTGGACCAGCGACCAGCGGCACGGCGTCATCGGCAG
CGGCAAGACCCTGACGATCACCGTCAAGGAGTTCCTCGACGCCGGCCAGTACACCTGCCACAAGG
GCGGCGAGACGCTGAGCCACAGCCACCTGCTGCTCCACAAGAAGGAGAACGGCATCTGGTCCACC
GAGATCCTCAAGAACTTCAAGAACAAGACCTTCTGAAGTGCGAGGCGCCCAACTACTCCGGCCG
GTTACAGTGCTCCTGGCTGGTGACGCGCAACATGGACCTCAAGTTCAACATCAAGTCGTCCAGCAG
TTCCCCCGACTCCCCGGCGGTGACCTGCGGCATGGCGTCCCTGTCGGCGGAGAAGGTCACGCTGG
ACCAGAGGGACTACGAGAAGTACTCGGTGCTGCTGCCAGGAGGACGTCACCTGCCCCACGGCCGAG
GAGACCCTGCCATCGAGCTGGCGCTGGAGGCCCCGCGAGCAGAACAAGTACGAGAATAACAGCA
CCAGCTTCTTCATCCGGGACATCATCAAGCCCCGACCCGCCAAGAACCTGCAGATGAAGCCCCTGA
AGAACAGCCAGGTGGAGGTCAGCTGGGAGTACCCGGACTCCTGGAGCACGCCCCACTCCTACTTC
AGCCTGAAGTTCTTCGTCAGAATCCAGCGCAAGAAAGAGAAGATGAAGGAGACGGAGGAGGGG
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ACGTCTGCGTGCAAGGCTCAGGACCGCTACTACAACCTCCAGCTGCAGCAAGTGGGCCTGCGTCCCCCT
GCAGGGTCCGGTCTAGTAAAGATCTGATATCGGATCTGCTGTGCCTTCTAGTTGCCAGCCATCTGT
TGTTTGCCCCCTCCCCCGTGCTTCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTCTTAATAAAA
TGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCTATTCTGTTGGGGTGGGGTGGGGCAGGA
CAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGGT
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ACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGG
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 CAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCC
 CCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCTTTCT
 CCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTT
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 TCTTGATCCGGCAAAACAAACCACCGCTGGTAGCGGTGGTTTTTTGTTTGCAAGCAGCAGATTACGC
 GCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGA
 AAACCTCACGTAAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATT
 AAAAAATGAAGTTTAAATCAATCTAAAGTATATATGAGTAACTTGGTCTGACAGTTACCAATGCTTA
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 GGGGGCGCTGAGGTCTGCCTCGTGAAGAAGGTGTTGCTGACTCATACCAGGCCTGAATCGCCCCAT
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 TTACAACCAATTAACCAATTCTGATTAGAAAACTCATCGAGCATCAAATGAACTGCAATTTATTCA
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 CCTATTAATTTCCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATC
 CGGTGAGAATGGCAAAAGCTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCTCG
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 GCGATCGCTGTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAG
 CGCATCAACAATATTTTACCTGAATCAGGATATTCTTCTAATACTGGAATGCTGTTTTCCCGGGGA
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 GGCTGGCTTAATATGCGGCATCAGAGCAGATTGTAAGTGAAGTGCACCATATGCGGTGTGAAATA
 CCGCACAGATGCGTAAGGAGAAAAATACCGCATCAGATTGGCTATTGG

SEQ ID NO:25 Murine IL-15 – murine IL-12p40 fusion protein, with linker (448 AA)

Mouse GM-CSF signal peptide is single underlined. IL-15 is bold. Linker is lowercase. IL-12p40 is double-underlined.

MWLQNLLFLGIVVYSLSNWIDVRYDLEKIESLIQSIHIDTTLYTDSDFHPSCKVTAMNCFLELQVILHEYS
NMTLNETVRNVLYLANSTLSSNKNVAESGCKECEELEKTFTEFLQSFIRIVQMFINTSgagaMWELEKD
VYVVEVDWTPDAPGETVNLTCDTPEEDDITWTSDQRHGVIGSGKTLTITVKEFLDAGQYTCHKGGETLS
HSLLLLHKKENGIWSTEILKNFKNKTFLKCEAPNYSGRFTCSWLVRNMDLKFNKSSSSSPDSRAVTCG
MASLSAEKVTLDORDYEKYSVSCQEDVTCPTAEETLPIELALEARQQNKYENYSTSFFIRDIKPDPPKNLQ
MKPLKNSQVEVSWEYPDSWSTPHSYFSLKFFVRIORKKEKMKETEEGCNOKGAFLVEKTSTEVOCKGGN
VCVQAQDRYYNSSCSKWACVPCRVR

CLAIM(S):

1. An isolated or purified extracellular vesicle comprising a phospholipid bilayer membrane and a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha, wherein:

the phospholipid bilayer comprises an exterior surface, an interior surface, and an interior region positioned between the exterior surface and the interior surface, and

at least a portion of the heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha is positioned in the interior region of the phospholipid bilayer.

2. An isolated or purified extracellular vesicle comprising a membrane comprising a phospholipid bilayer, a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha, and a carrier, wherein:

the phospholipid bilayer comprises an exterior surface, an interior surface, and an interior region positioned between the exterior surface and the interior surface, the extracellular vesicle comprises an exterior surface, the carrier is positioned on the exterior surface of the extracellular vesicle or at least a portion of the carrier is embedded in the phospholipid bilayer, and the heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha is positioned on the carrier.

3. The isolated or purified extracellular vesicle according to claim 2, wherein the carrier comprises lactadherin C1/C2 domains.

4. The isolated or purified extracellular vesicle according to claim 1, 2, or 3, wherein a polypeptide of interest is recombinantly fused to interleukin-15.

5. The isolated or purified extracellular vesicle according to claim 4, wherein the polypeptide of interest is a cytokine.

6. The isolated or purified extracellular vesicle according to claim 4, wherein the cytokine is interleukin-12.

7. A composition comprising a plurality of isolated or purified extracellular vesicles according to any one of claims 1-6.

8. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and the extracellular vesicle according to any one of claims 1-6 or the composition according to claim 7.

9. A method of preparing isolated or purified extracellular vesicles comprising a therapeutic protein, the method comprising:

introducing a nucleotide sequence into cells, wherein the nucleotide sequence encodes the therapeutic protein;

culturing the cells in medium in a hollow fiber bioreactor under conditions sufficient for the cells to express the therapeutic protein;

secreting from the cells the extracellular vesicles comprising the expressed therapeutic protein into the medium; and

separating the extracellular vesicles comprising the expressed therapeutic protein from the cells to produce isolated or purified extracellular vesicles comprising the therapeutic protein.

10. The method according to claim 9, wherein the cells are HEK293 cells.

11. The method according to claim 9 or 10, wherein culturing the cells in a hollow fiber bioreactor comprises culturing the cells in protein-free medium.

12. The method according to any one of claims 9 to 11, wherein the therapeutic protein is a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha.

13. The method according to claim 12, wherein a polypeptide of interest is recombinantly fused to interleukin-15.

14. The method according to claim 13, when the polypeptide of interest is a cytokine.

15. The method according to claim 14, wherein the cytokine is interleukin-12.

16. The method according to any one of claims 9 to 15, wherein each extracellular vesicle comprises a membrane, and the therapeutic protein is embedded in the membrane of the extracellular vesicle.

17. The method according to any one of claims 12 to 15, wherein the extracellular vesicles comprise a membrane comprising a phospholipid bilayer and a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha, wherein:

the phospholipid bilayer comprises an exterior surface, an interior surface, and an interior region positioned between the exterior surface and the interior surface, and

at least a portion of the heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha is positioned in the interior region of the phospholipid bilayer.

18. The method according to any one of claims 9 to 15, wherein each extracellular vesicle comprises a membrane with an external surface, and the therapeutic protein is positioned on the external surface of the membrane.

19. The method according to any one of claims 9 to 11, wherein the isolated or purified extracellular vesicles comprise a membrane comprising a phospholipid bilayer, a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha, and a carrier, wherein:

the phospholipid bilayer comprises an exterior surface, an interior surface, and an interior region positioned between the exterior surface and the interior surface, the extracellular vesicle comprises an exterior surface, the carrier is positioned on the exterior surface of the extracellular vesicle or at least a portion of the carrier is positioned in the interior region of the phospholipid bilayer, and the heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha is positioned on the carrier.

20. The method according to claim 19, wherein the carrier comprises lactadherin C1/C2 domains.

21. The method according to claim 19 or 20, wherein a polypeptide of interest is recombinantly fused to interleukin-15.

22. The method according to claim 21, when the polypeptide of interest is a cytokine.

23. The method according to claim 11, wherein the cytokine is interleukin-12.

24. The method according to any one of claims 9 to 15, wherein each extracellular vesicle comprises a membrane with an internal surface that encapsulates an interior space, and the therapeutic protein is positioned in the interior space.

25. The method according to any one of claims 9 to 24 wherein separating the extracellular vesicles from the cells to produce isolated or purified extracellular vesicles comprises:

- harvesting the medium comprising the extracellular vesicles from the hollow fiber bioreactor;

- centrifugating the medium to produce a supernatant comprising the extracellular vesicles and a pellet comprising cell debris;

- filtering the supernatant;

- dialyzing the filtered supernatant;

- centrifugating the dialyzed supernatant to produce isolated or purified extracellular vesicles; and

- optionally concentrating the supernatant.

26. The method according to claim 25, wherein the method does not comprise concentrating the supernatant by normal flow filtration.

27. The method according to any one of claims 9 to 24, wherein separating the extracellular vesicles from the cells to produce isolated or purified extracellular vesicles comprises:

- filtering the supernatant using tangential-flow filtration,

- dialyzing filtered supernatant;

- subjecting dialyzed filtered supernatant to a step of size exclusion chromatography;

- and

- collecting fractions eluted from the column that comprises extracellular vesicles.

28. Isolated or purified extracellular vesicles prepared according to the method of any one of claims 9-27.

29. A method of reducing liver uptake of extracellular vesicles administered to a mammal, the method comprising:

administering extracellular vesicles comprising a therapeutic protein to the mammal;
and

administering a scavenger receptor antagonist to the mammal in an amount effective to reduce uptake of the extracellular vesicles by the liver of the mammal.

30. The method of claim 29, wherein the scavenger receptor antagonist is a Scavenger Receptor A antagonist.

31. The method according to claim 30, wherein administering the Scavenger Receptor A antagonist increases delivery of the extracellular vesicles to a target site as compared to administering the extracellular vesicles to the mammal without administering the Scavenger Receptor A antagonist, wherein the target site is a tumor or an extra-hepatic organ.

32. A method of treating or preventing cancer in a mammal, the method comprising:

administering extracellular vesicles comprising a therapeutic protein to the mammal in an amount effective to treat or prevent cancer in the mammal; and

administering a scavenger receptor antagonist to the mammal in an amount effective to reduce uptake of the extracellular vesicles by the liver of the mammal.

33. The method of claim 32, wherein the scavenger receptor antagonist is a Scavenger Receptor A antagonist.

34. The method according to any one of claims 30, 31, or 33, wherein the Scavenger Receptor A antagonist is dextran sulfate.

35. The method according to any one of claims 29 to 34, wherein the therapeutic protein is a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha.

36. The method of claim 35, wherein the interleukin-15 is fused to a polypeptide of interest.

37. The method of claim 36, wherein the polypeptide of interest is a cytokine.

38. The method of claim 37, wherein the cytokine is interleukin-12.

39. The method according to any one of claims 29 to 38, wherein each extracellular vesicle comprises a membrane, and the therapeutic protein is embedded in the membrane of the extracellular vesicle.

40. The method according to any one of claims 35 to 38, wherein the extracellular vesicles comprise a membrane comprising a phospholipid bilayer and a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha, wherein:

the phospholipid bilayer comprises an exterior surface, an interior surface, and an interior region positioned between the exterior surface and the interior surface, and

at least a portion of the heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha is positioned in the interior region of the phospholipid bilayer.

41. The method according to any one of claims 29 to 38, wherein each extracellular vesicle comprises a membrane with an external surface, and the therapeutic protein is positioned on the external surface of the membrane.

42. The method according to any one of claims 35 to 38, wherein the isolated or purified extracellular vesicles comprise a membrane comprising a phospholipid bilayer, a heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha, and a carrier, wherein:

the phospholipid bilayer comprises an exterior surface, an interior surface, and an interior region positioned between the exterior surface and the interior surface, the extracellular vesicle comprises an exterior surface, the carrier is positioned on the exterior surface of the extracellular vesicle or at least a portion of the carrier is positioned in the interior region of the phospholipid bilayer, and the heterodimeric complex of interleukin-15 and interleukin-15 receptor alpha is positioned on the carrier.

43. The method according to claim 42, wherein the carrier comprises lactadherin C1/C2 domains.

44. The method according to any one of claims 29 to 38, wherein each extracellular vesicle comprises a membrane with an internal surface, the interior surface of the membrane encapsulating an interior space, and the therapeutic protein is positioned in the interior space.

45. The method according to any one of claims 29 to 44, comprising administering the scavenger receptor antagonist parenterally.

46. A method of treating or preventing cancer in a mammal, the method comprising administering to the mammal the extracellular vesicle of any one of claims 1 to 6 and 28, the composition of claim 7, or the pharmaceutical composition of claim 8, in an amount effective to treat or prevent cancer in the mammal.

47. A mammalian host cell comprising a DNA construct encoding interleukin-15 and an interleukin-15 receptor alpha-lactadherin carrier polypeptide.

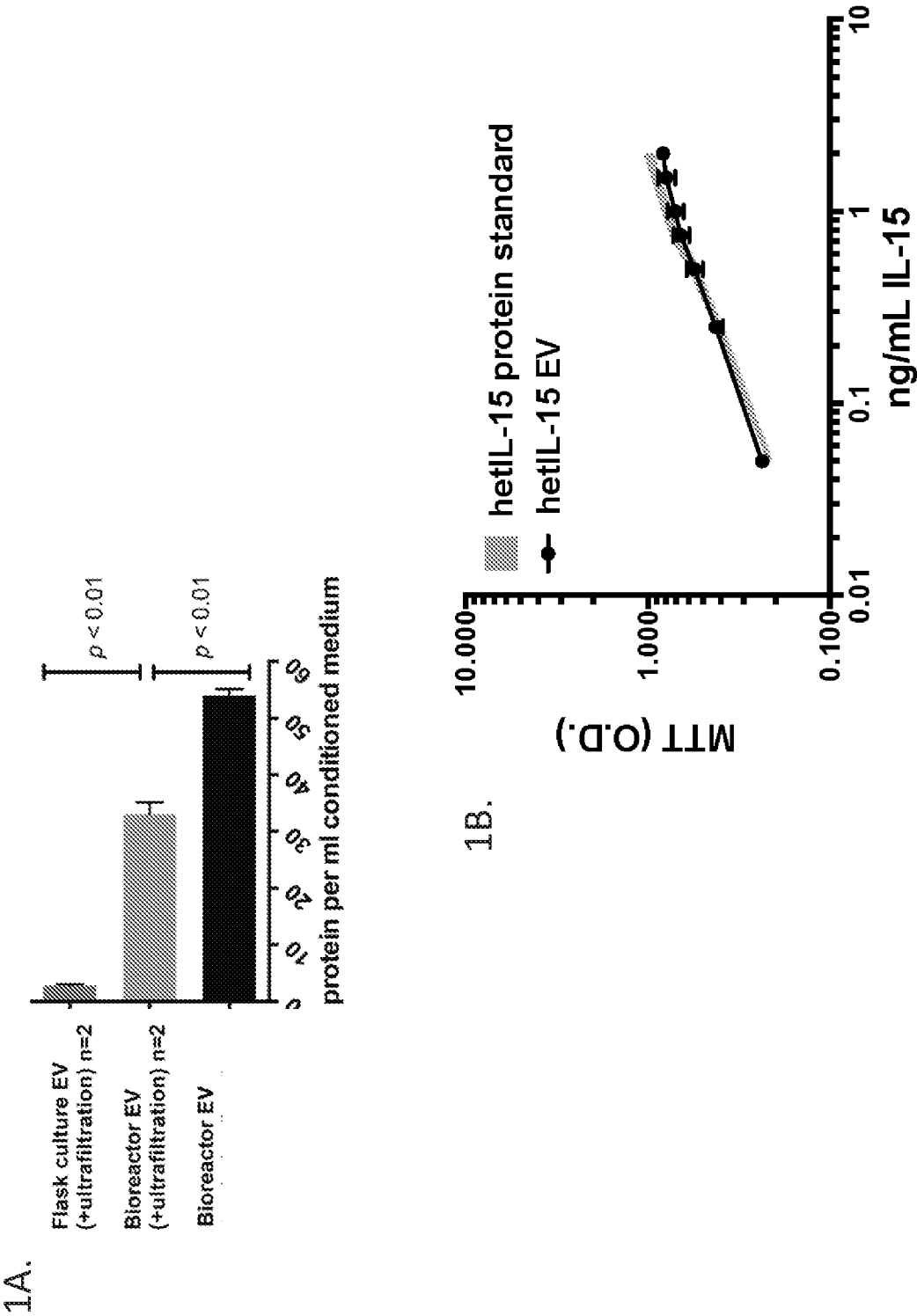
48. The mammalian host cell of claim 47, wherein the DNA construct encodes a human interleukin-15 of SEQ ID NO:7 and a human IL-15 receptor alpha-human lactadherin fusion protein of SEQ ID NO:10.

49. The mammalian host cell of claim 48, wherein the DNA constructs comprises the region of SEQ ID NO:13 that encodes human IL-15 receptor alpha-human lactadherin fusion protein of SEQ ID NO:10 and the region of SEQ ID NO:13 that encodes the human interleukin-15 of SEQ ID NO:7.

50. The mammalian host cell of claim 47, wherein the DNA construct comprises the nucleic acid sequence of SEQ ID NO:13.

51. The mammalian host cell of any one of claims 47 to 50, wherein the host cell is an HEK 293 cell.

Figure 1A-1B



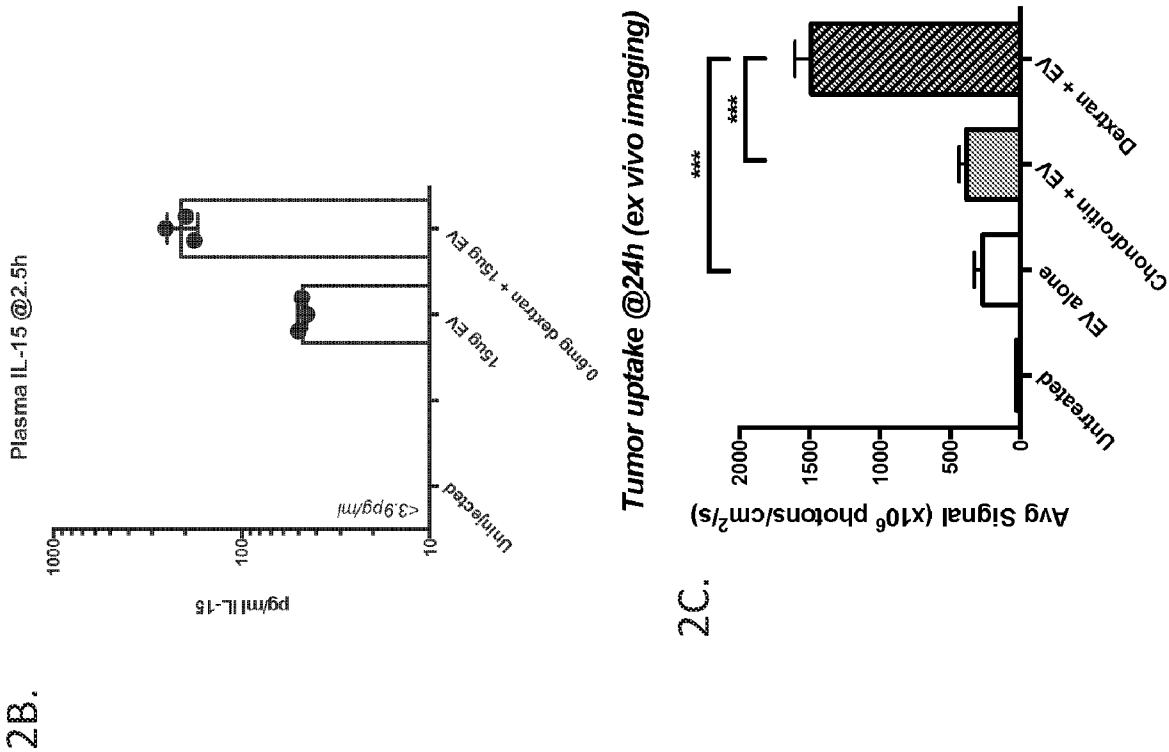


Figure 2A-2C

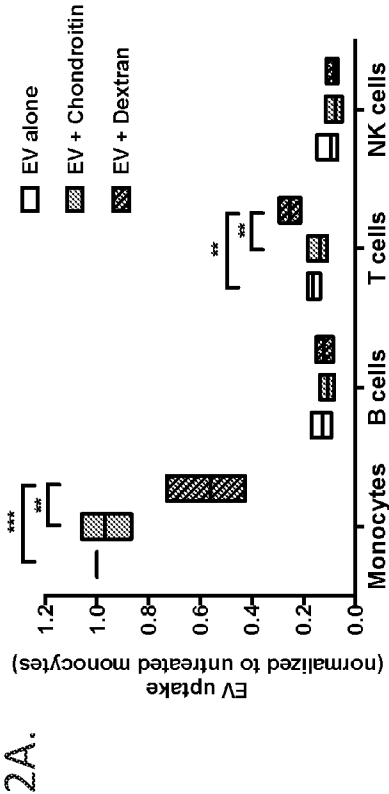


Figure 3

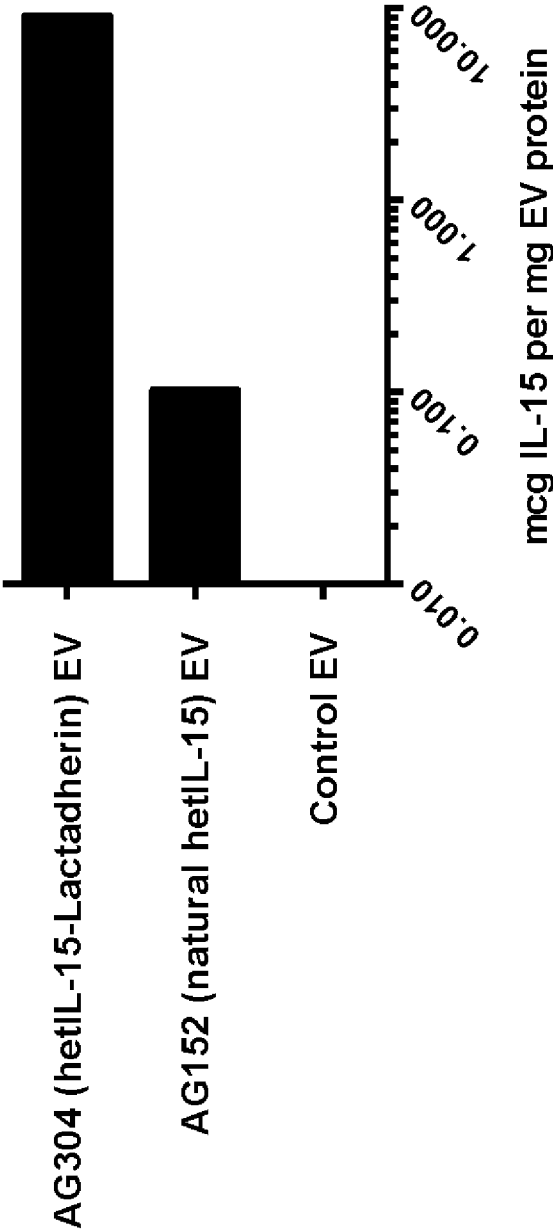


Figure 4

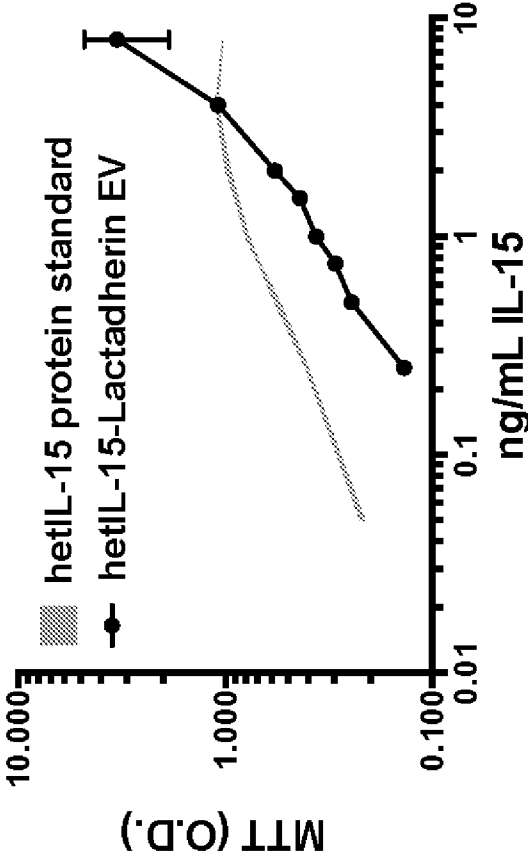


Figure 5

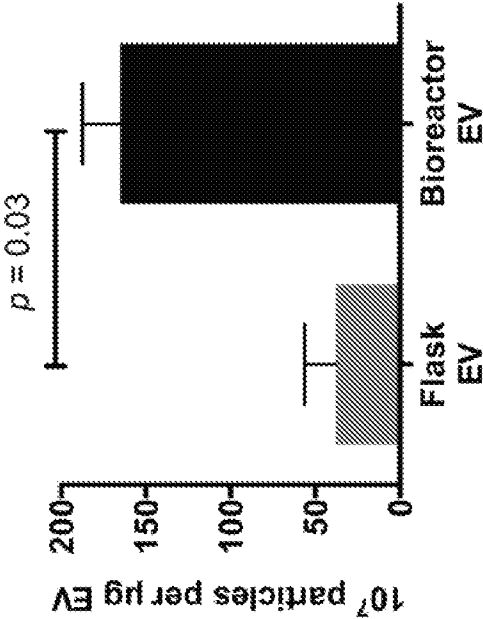


Figure 6

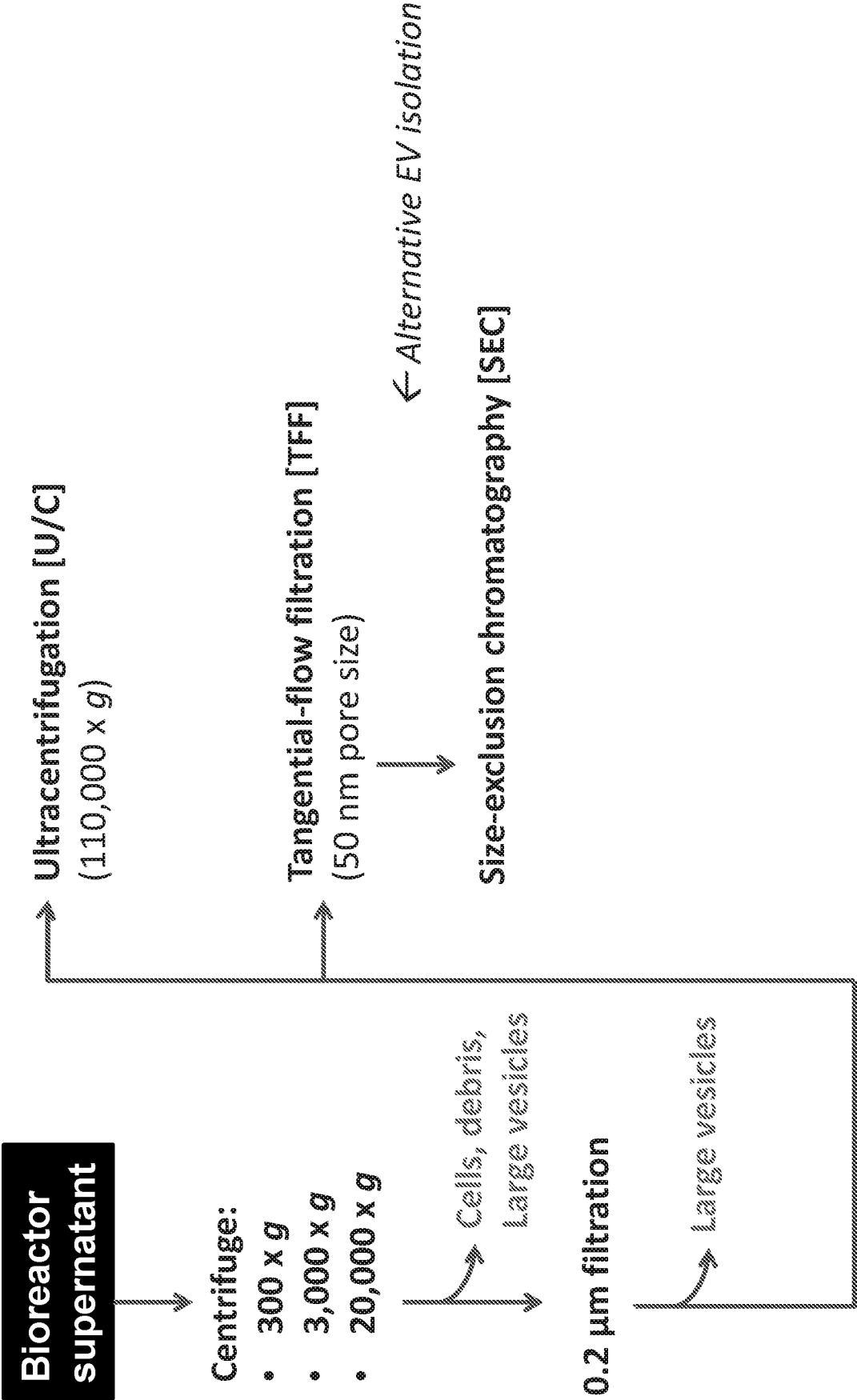


Figure 7

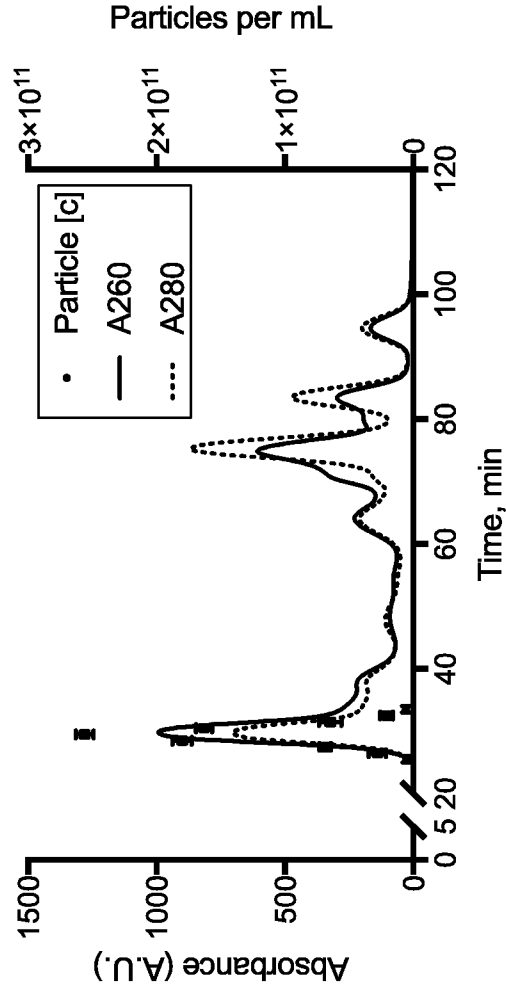


Figure 8

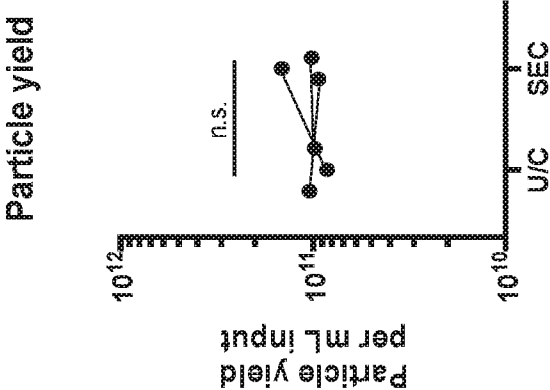


Figure 9

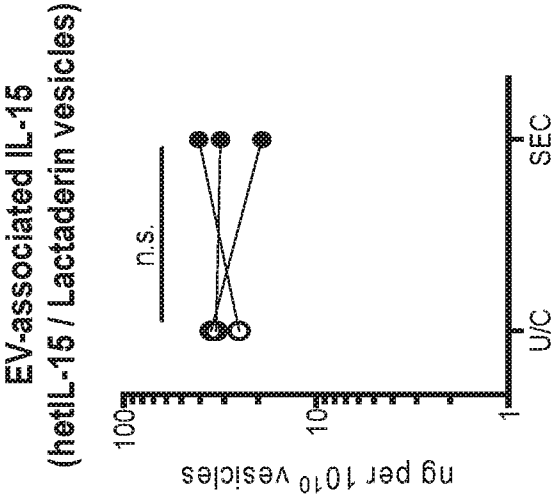


Figure 10

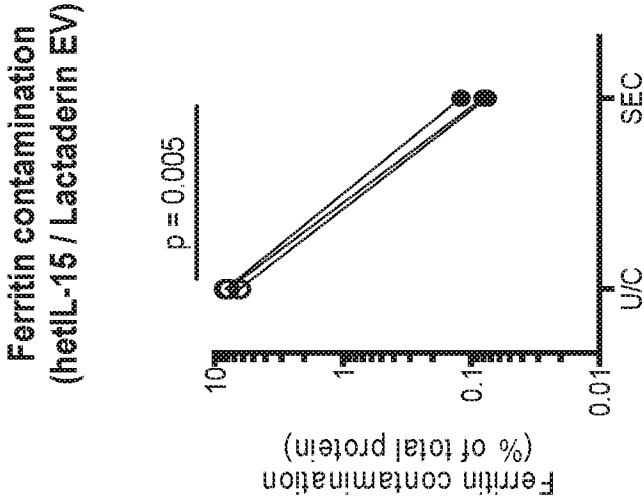


Figure 11

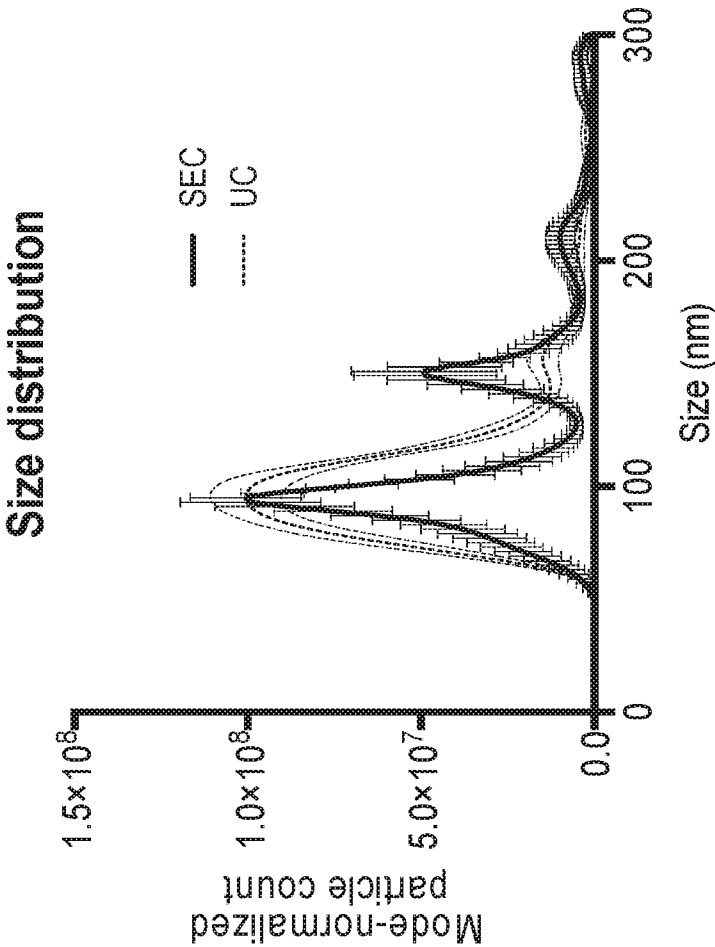


Figure 12

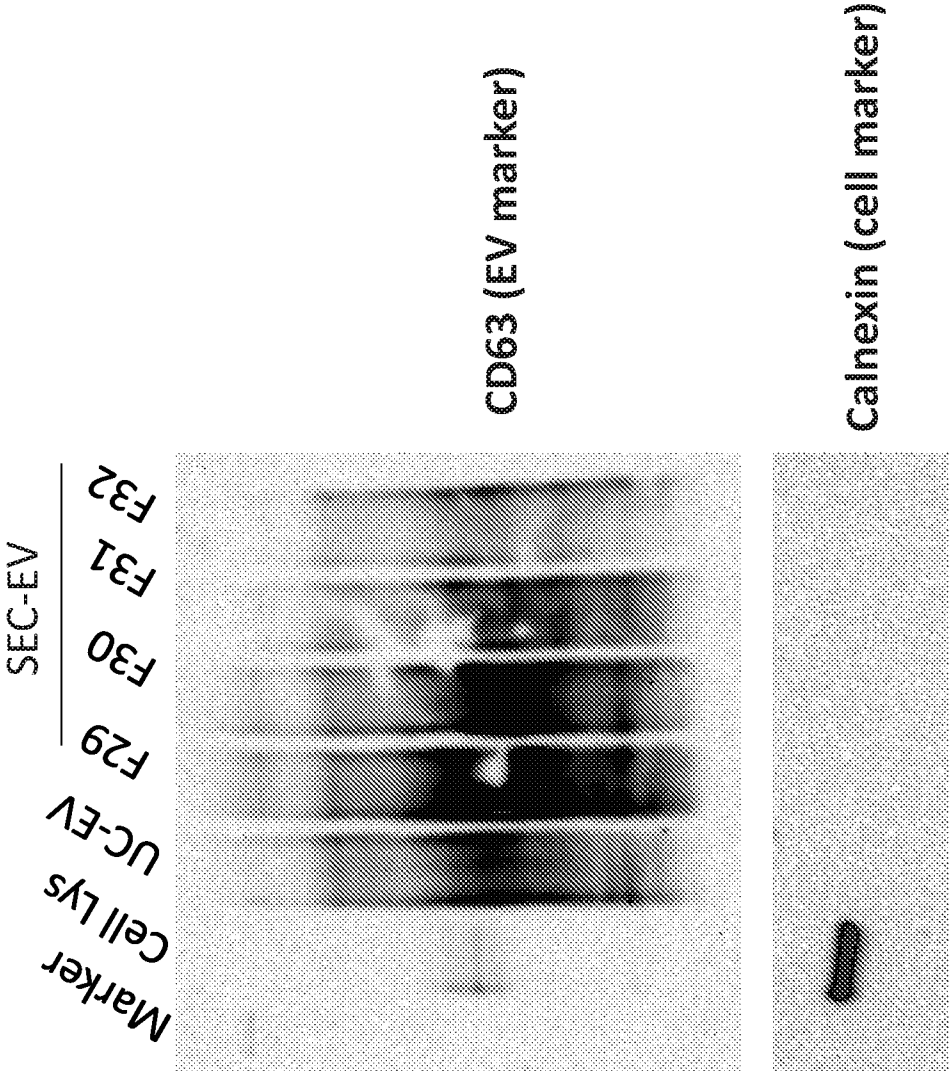


Figure 13

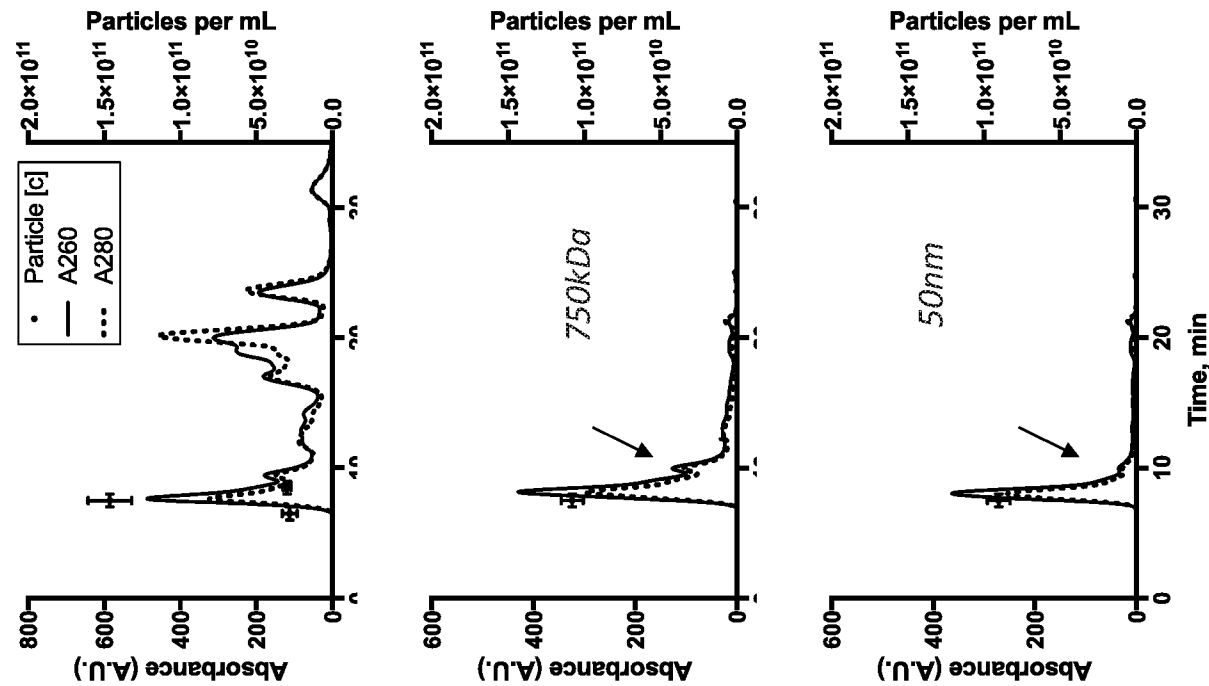


Figure 14

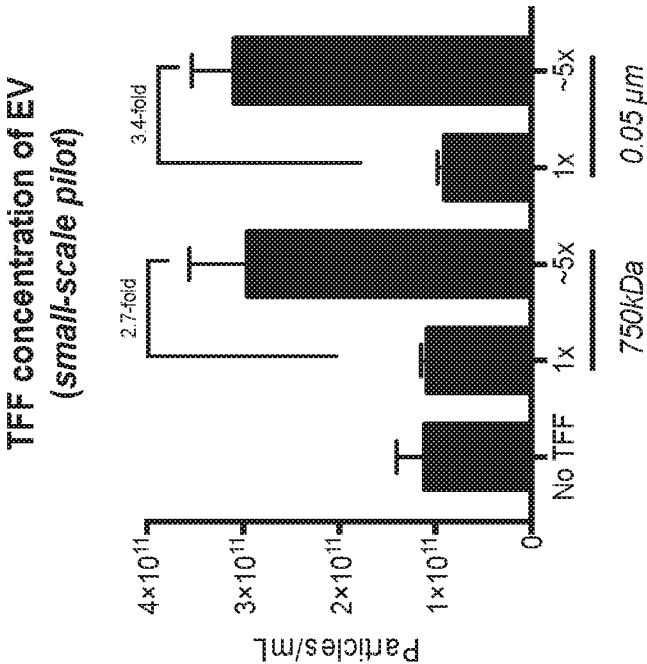


Figure 15

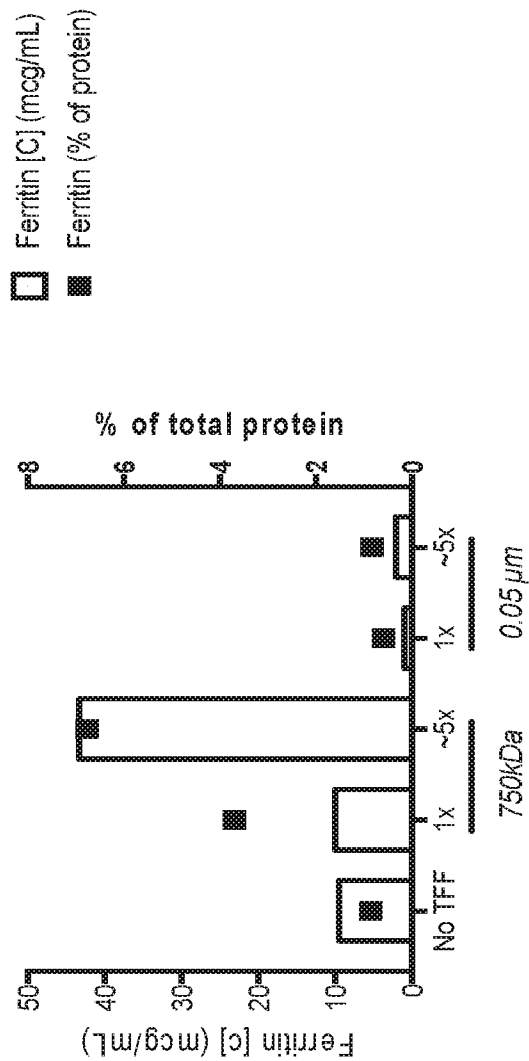


Figure 16a-b

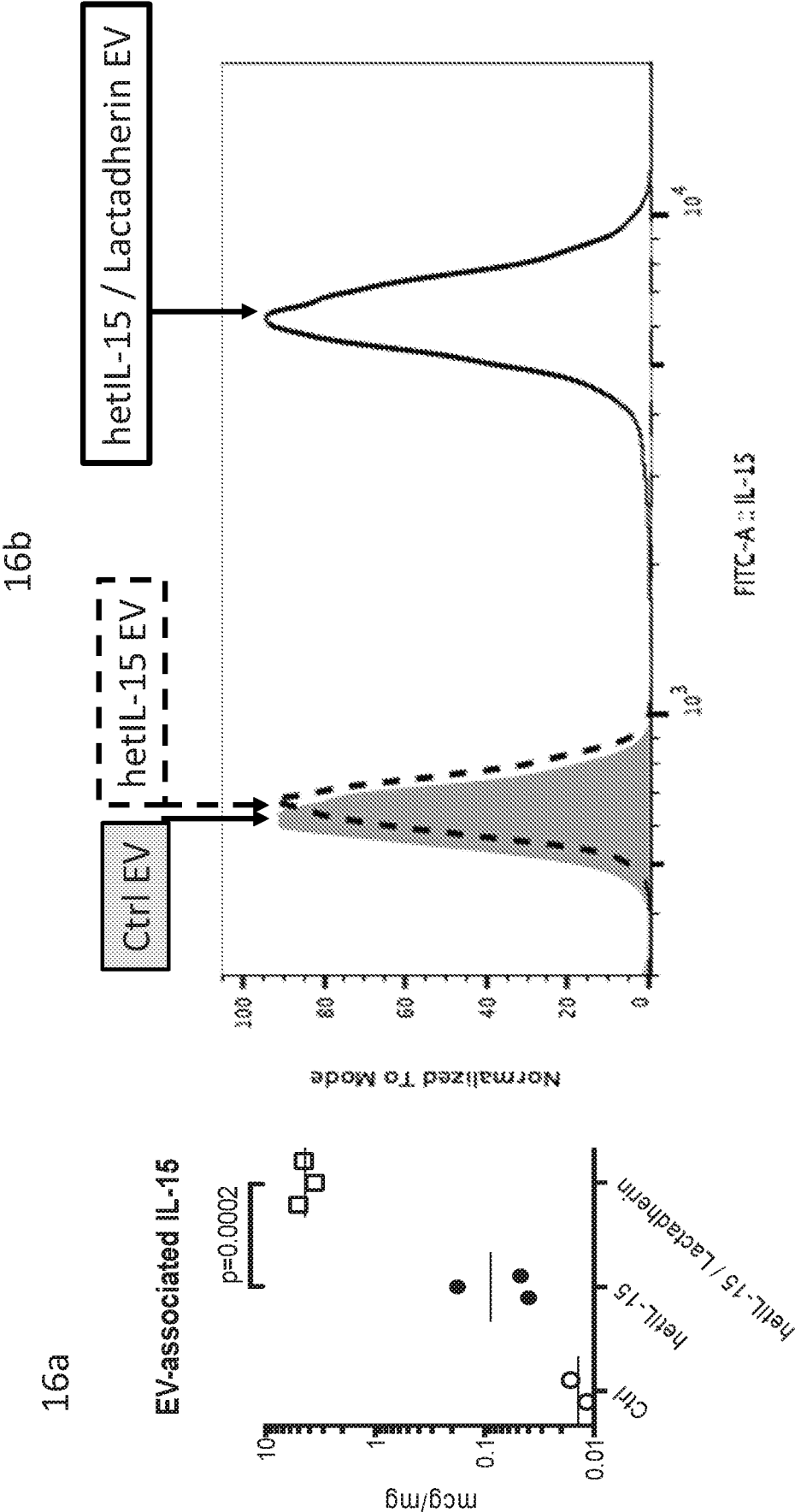


Figure 17

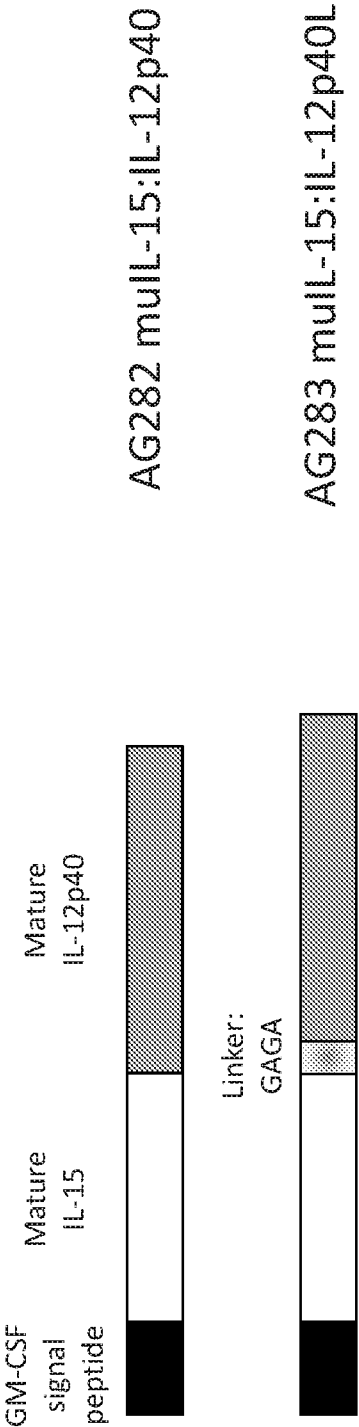


Figure 18

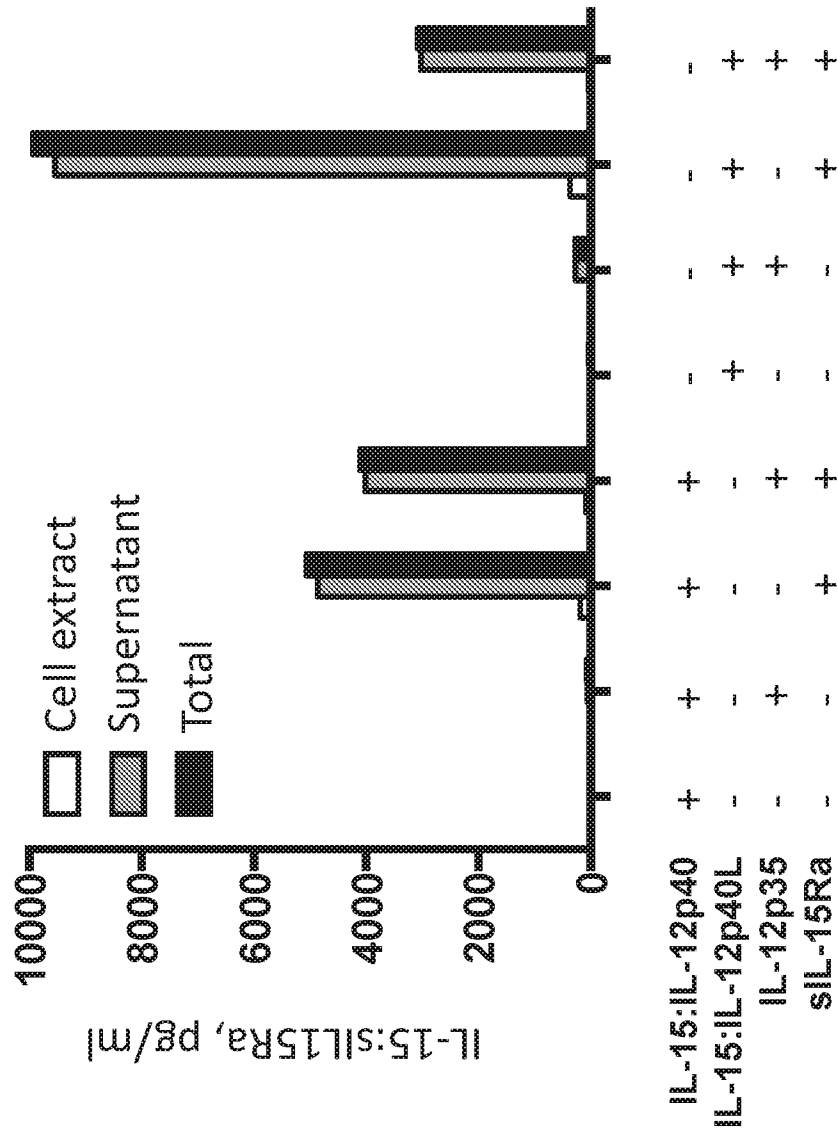


Figure 19

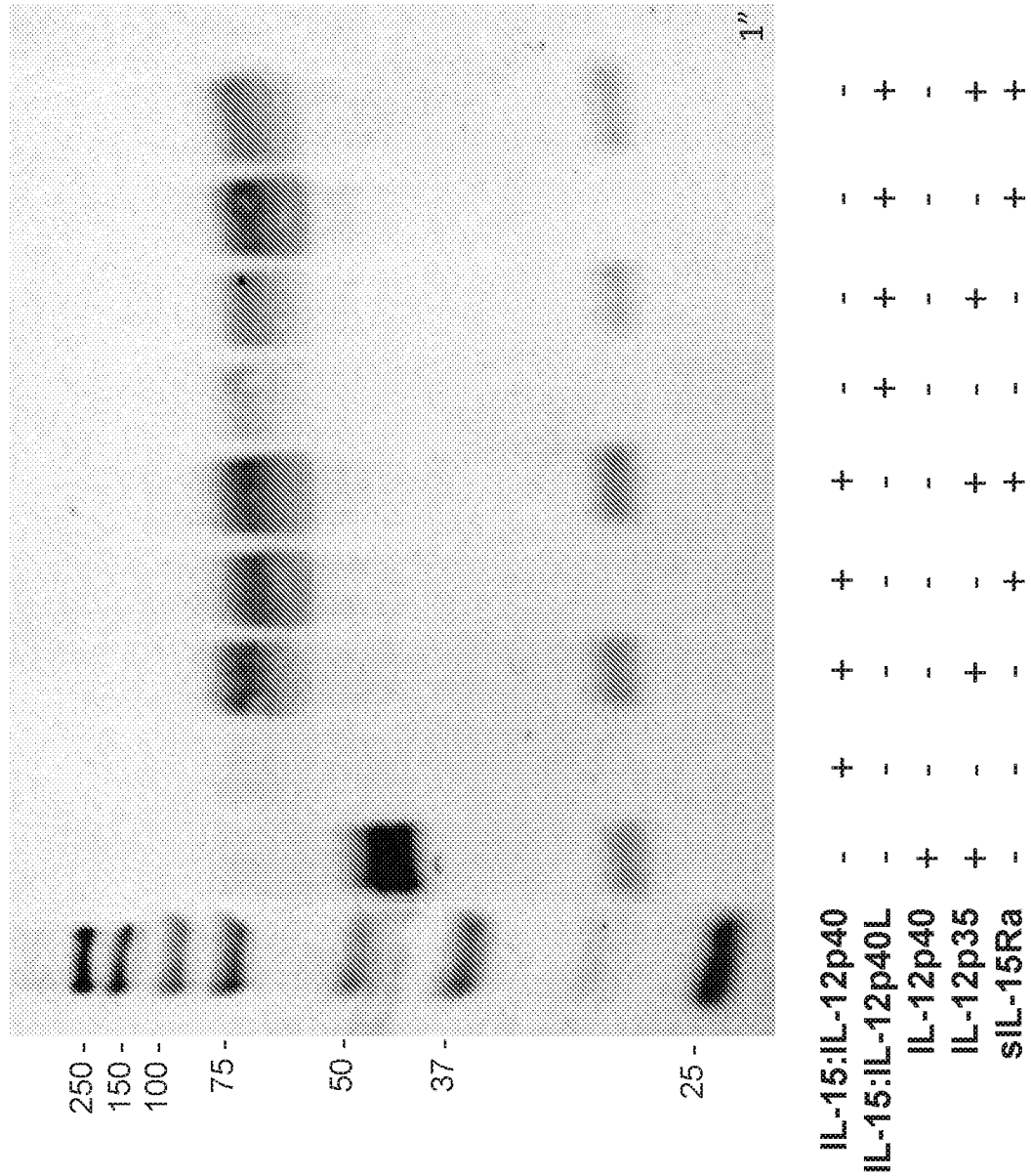


Figure 20

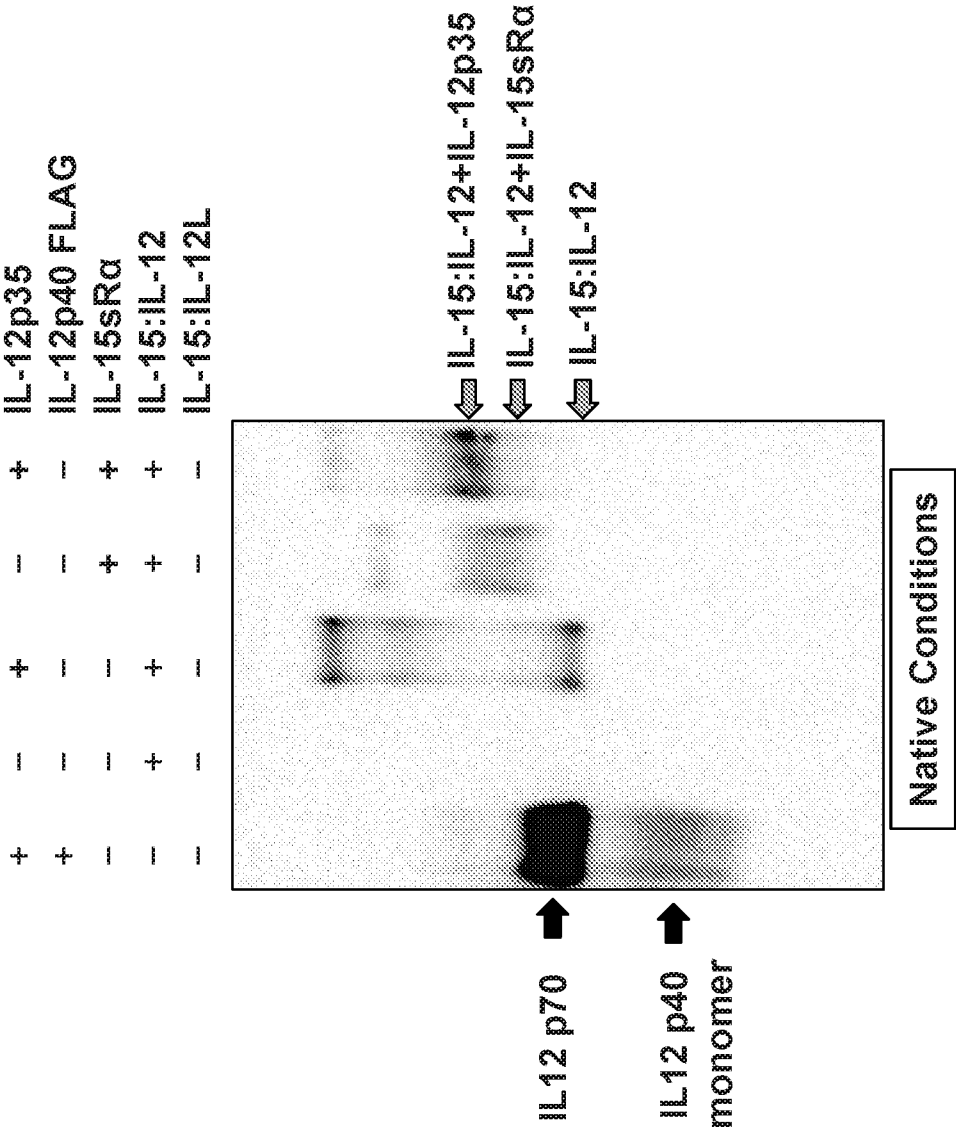


Figure 21

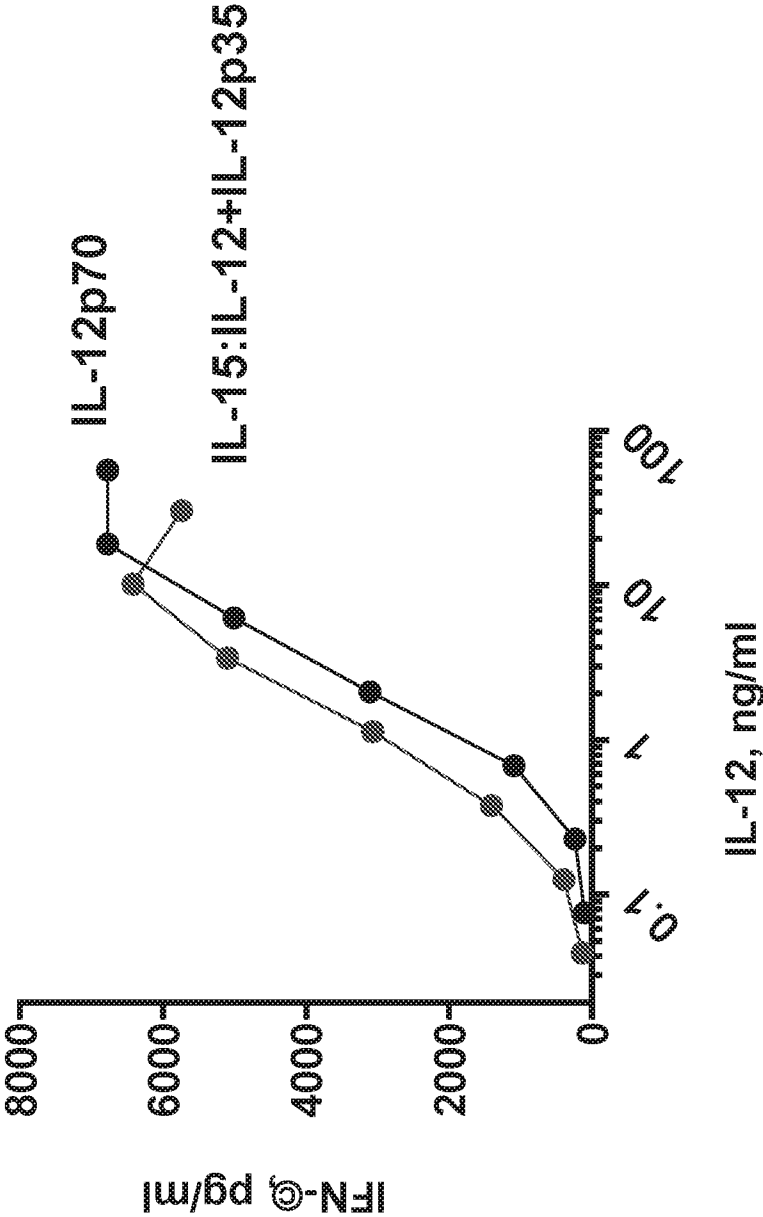
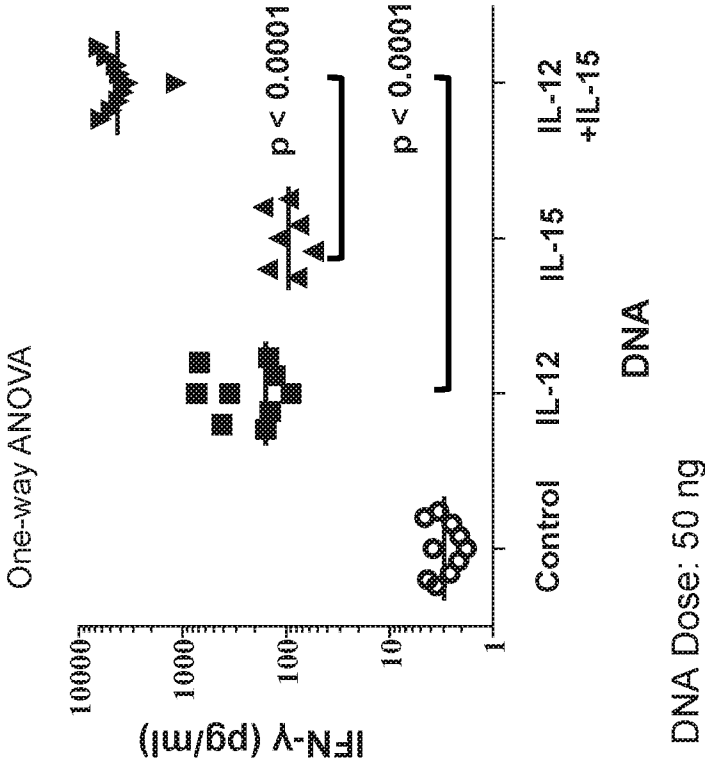


Figure 22

Plasma IFN- γ Concentration (Day 1)



CD8+T Cell Proliferation in Spleen (Day 4)

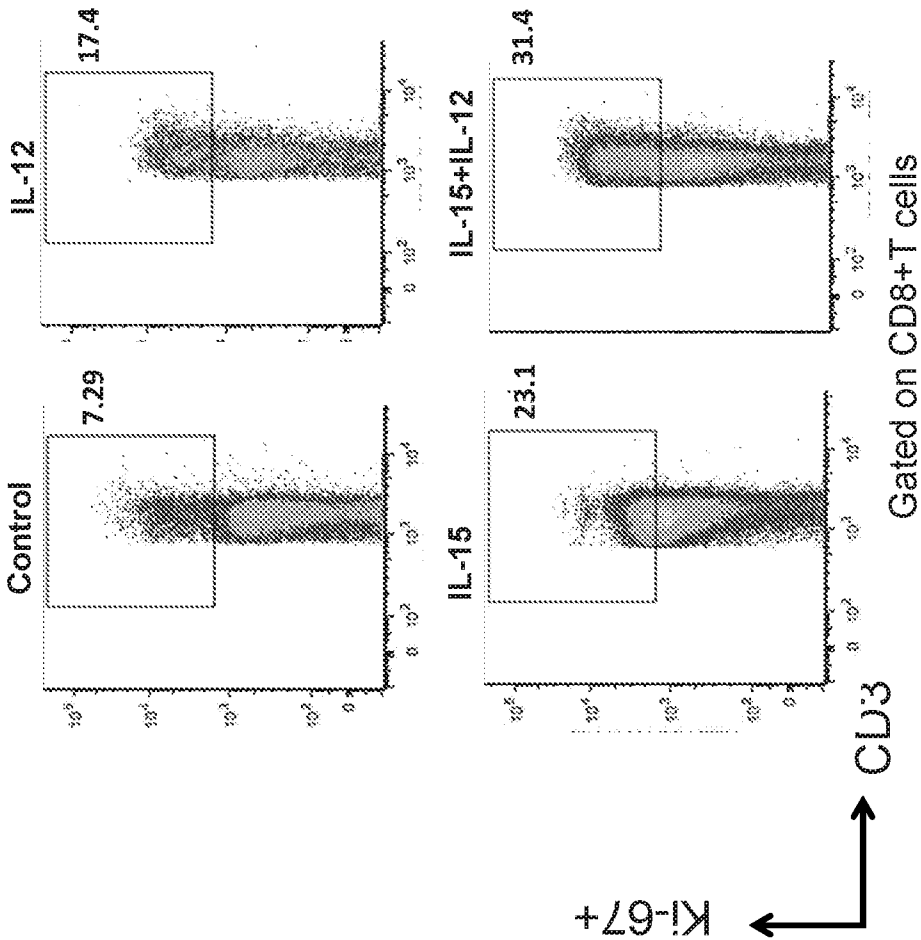


Figure 23

