MICROALGAE EXTRACELLULAR VESICLE BASED GENE THERAPY VECTORS (MEV-GTVS), THEIR PREPARATION, AND USES THEREOF

RELATED APPLICATIONS

Benefit of priority is claimed to U.S. provisional application Serial No. 63/663,030, filed June 21, 2024, entitled "MICROALGAE EXTRACELLULAR VESICLE BASED GENE THERAPY VECTORS (MEV-GTVS), THEIR PREPARATION, AND USES THEREOF," to inventors Lila Drittanti and Manuel Vega, and to Applicant AGS Therapeutics SAS.

Benefit of priority is claimed to U.S. provisional application Serial No. 63/556,325, filed February 21, 2024, entitled "OCULAR DELIVERY OF ACTIVE AGENTS VIA MICROALGAE EXTRACELLULAR VESICLES," to inventors Lila Drittanti and Manuel Vega, and to Applicant AGS Therapeutics SAS.

Benefit of priority is claimed to U.S. provisional application Serial No. 63/562,941, filed March 08, 2024, entitled "OCULAR DELIVERY OF ACTIVE AGENTS VIA MICROALGAE EXTRACELLULAR VESICLES," to inventors Lila Drittanti and Manuel Vega, and to applicant AGS Therapeutics SAS.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING PROVIDED ELECTRONICALLY

An electronic version of the Sequence Listing is filed herewith, the contents of which are incorporated by reference in their entirety. The electronic file was created on February 18, 2025, is 256,557 bytes in size, and is titled 5510SEQPC01.xml.

FIELD

Provided are gene therapy vectors designated MEV-GTVs, which are MEVs that contain an ITR-containing plasmid (also referred to as a minigene plasmid) that comprises Inverted Terminal Repeats (ITRs), such as viral ITRs, and nucleic acid encoding a product of interest, and optionally regulatory sequences. The ITRs serve to circularize the ITR plasmid. The plasmids do not contain additional viral components (other than regulatory sequences that are part of the transgene) so that resulting DNA is not encapsulated in a viral capsid or envelop and is not replicated by viral genes. The plasmids can be inserted into bacterial plasmids for propagation.

BACKGROUND

Gene therapy is an approach that has been under development since the 1980s whose goal is to replace, substitute or correct defective genes responsible for disease development and/or to treat diseases by delivery of gene products. Diseases, such as severe combined immunodeficiency, cystic fibrosis, hemophilia A, that stem from mutations in the DNA sequence can be targeted by introducing functional genes. Genetic treatment requires appropriate vectors to deliver genetic material into cells. The selection of suitable vectors is crucial for the safety and efficacy of gene therapies, as they serve as vehicles for transporting therapeutic genes (or gene-editing tools) to specific cellular locations. The choice and further development of these vectors involves optimization of their toxicity, immunogenicity, precision, and reliability in delivering genetic interventions.

Several vector types have been developed to address the challenges and requirements of gene therapy. Non-viral vectors, such as lipid nanoparticles and polymer-based vectors, are considered safe. Lipid nanoparticles, composed of lipids or lipid-like materials, encapsulate genetic material, and facilitate its delivery into cells. Polymer-based vectors, on the other hand, use synthetic polymers to form complexes with genetic cargo for gene delivery. These have failed to deliver satisfactory solutions, and continue to provide challenges related to low transduction efficiency and no targeting, and concerns related to toxicity and biological barriers for delivery.

Viral vectors, such as adeno-associated viruses (AAV), adenoviruses, traditional (Moloney Murine leukemia virus-derived) retroviruses, and lentiviruses, have gained prominence due to their natural ability to efficiently enter host cells and transfer their genetic cargo. Adenoviruses, although associated with immune responses, have been of interest due to their high transduction efficiency in various cell types. Lentiviruses, derived from the human immunodeficiency virus (HIV), can infect dividing and non-dividing cells and are known for their capacity to integrate genetic material into the host genome, providing stable and long-term gene expression.

The immune response to viruses poses a significant hurdle, hindering the efficient delivery of genes to target cells and potentially leading to severe

complications, as demonstrated by treatment-related patient death in 1999 during an adenoviral vector clinical trial. The choice of viral vectors introduces complexities. Some vectors, like retroviruses, integrate their genomes seemingly randomly into host chromosomes, potentially disrupting the function of cellular genes and contributing to cancer development. A 2002 trial for severe combined immunodeficiency using retroviral gene therapy resulted in development of leukemia in some of the trial participants.

Hence there remains a need for more versatile, convenient, and safe vectors for gene therapy and related applications. It is an object herein to provide such vectors.

SUMMARY

Provided are gene therapy vectors (GTVs) that comprise an ITR plasmid in a nanoparticle, such as an extracellular vesicle (EV) particularly a microalgae EV (MEV). The ITR plasmids contain one or more ITRs from a viral source, and a transgene encoding a gene product and any needed regulatory sequences. There is no viral capsid or other gene produces; the nanoparticle, such the EV, which encapsulates the ITR plasmid. Exemplary of the vectors are MEV-GTVs detailed herein.

MEV-GTVs contain two differentiated parts: the MEV moiety and the cargo or payload moiety, which is the ITR plasmid. The plasmid carries the following three genetic elements combined in a proper functional array. (1) ITRs of viral origin, such as AAV, origin. The ITRs assure the survival of the plasmid inside a recipient cell as a replicative, and non-integrative episome. The plasmid can carry either one or two ITRs. (2) Genetic elements for proper expression, processing, and eventually other events, of a transgene component. These elements are promoters, enhancers, Kozak consensus sequences, IRES, polyA, and any other regulatory sequences for transcription and translation of encoded product. (3) The transgene, which encodes a biologically active product, such as a therapeutic product. The transgene encodes or comprise one of such products, including, but not limited to, mRNA, genes, exons, proteins, peptides, siRNAs, miRNAs, mRNA, including RNA components of gene editing complexes, among a large diversity of genetic and/or biologically active elements.

As evident from the disclosure herein, provided are MEV-based nanoparticles that deliver their payload to the cell nucleus. This is in contrast to other available

delivery systems, such as lipid nanoparticles (LNPs), mammalian EVs, and AAV-based vectors. MEVs deliver their plasmids into the cell nucleus. The plasmids, as detailed herein do not integrate into the genome, nor do they have the undesirable side effects associated with AAVs or other viral vectors. Once in the nucleus, the expression cassette in the plasmid is transcribed into mRNA, and the resulting mRNA is translated into a biologically active protein. The ITR-plasmids provide for long-lasting expression of the transgene, which includes the expression cassette. Thus, the MEVs loaded with ITR-plasmids constitute an efficient, and non-viral alternative to viral vectors, such as AAV and other vectors, for gene therapy.

MEV-GTVs are produced as follows. The MEV-moiety as detailed herein by growing microalgae in photobioreactors, followed by purifying the produced MEVs from the supernatants of those cultures. The product of such production, clarification, plus purification process provides purified MEVs ready to use, characterized, stored and eventually loaded with custom cargo or payloads. The plasmid (payload) moiety is produced as described herein, using standard and well-established methods for plasmid production, including the transfection of the plasmid construct (carrying the three genetic elements described above) into suitable bacteria, the cloning or multiplication of the plasmid by growing the transformed bacteria, and the purification of the plasmid from the bacteria cultures following standard, well-known and well established methods for plasmid production and purification. Once the two moieties (purified MEVs and purified plasmid-payload) have been separately obtained, the plasmid is loaded into the MEVs using the protocols for exo-loading previously described and described herein.

Thus, the production or manufacturing of MEV-GTVs is simple and straightforward, high-yield, safe, and inexpensive, particularly compared to other gene therapy vectors, such as AAV vectors. Production and manufacturing of AAV vectors is complex, low-yield, not-necessarily safe, and costly. Manufacturing of MEV-GTVs is highly advantageous over the manufacturing of AAV vectors. This advantage or superiority of MEV-GTVs over AAVs, as far as manufacturing is concerned, adds to the many advantages of MEV-GTVs compared to AAVs as detailed herein.

Among the products detailed herein are gene therapy vectors that have two components: (1) an external envelop that is an extracellular vesicle from an EV, such as microalgae EV (MEV), which is a nano-sized lipid nanoparticle, and (2) an internal payload, that is an expression plasmid that carries at least three genetic elements: (i) AAV-ITR sequences, to confer stability, long-lasting live and episomal behavior to the plasmid, (ii) controlling genetic elements, such as promoters, enhancers, introns, IREs, Kozak sequences, among other possible, and (iii) a transgene, made of a simple or multiple coding sequences that codes for either mRNA, siRNA, miRNA, or any other kind of coding or no coding genetically active molecules.

ITRs sequences include viral ITRs, such as ITRs of any of known strain of adeno-associated virus (AAV), such as strains AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AA10, and other viruses, such as poxviruses and adenovirus.

As detailed herein, and in commonly owned publications, MEVs, and hence the MEV-GTVs can be formulated for and administered by various routes of administration: topical (on the ocular globe), topical of the body surface, orally, intranasally, intravenously, intramuscularly, respiratory, and other such routes. Different organs and tissues are targeted depending on the route of administration. Targeted organs and include, for example, the choroid and the retina of the eye by topical administration, endothelial cells, enterocytes, GALT cells, spleen cells, neurons, brain cells other than neurons, lung cells, skin cells, immune cells, blood cells, and mucosal cells. They also target intracellular endosomal Toll-like Receptors (TLR) that are buried inside different kinds of cells. Any gene product of interest can be encoded in the ITR plasmids. The MEV-GTVs can be used to treat, prevent (reduce the risk of) and/or modulate diseases, disorders, and conditions, such a metabolic diseases, genetic diseases, lung diseases, bowel diseases, ophthalmic diseases, brain diseases, and immunomodulatory diseases.

Provided are ITR plasmids that comprise an ITR and a transgene. The ITR is a eukaryotic viral ITR, such as from an adeno-associated virus (AAV), and, except for the promoter and regulatory sequences that are part of the transgene, the ITR is the only element of the plasmid from a eukaryotic virus. The transgene comprises nucleic acid encoding a gene product operably linked to a promoter recognized in a

eukaryotic cell and can comprise optional additional eukaryotic transcriptional and/or translational regulatory sequences. Exemplary plasmids generally can contain a transgene up to about 20 kB in size; this varies depending on the ITRs and/or the transgene .

In some embodiments provide are bacterial plasmids for propagation of ITR plasmid in a bacterial host. The bacterial plasmid comprise an ITR and a transgene, and a bacterial cloning vector for propagation in a bacterium. The cloning vector for propagation of the ITR plasmid is selected from plasmids, bacteriophages, cosmids, and bacterial artificial chromosomes (BACs), or, alternatively, the cloning vector can be a bacterial plasmid that has a pUC or pBR322 backbone.

The ITRs include eukaryotic virus ITRs, such as, for example, from a poxvirus, adenovirus, or adeno-associated virus (AAV), or the ITR can be a synthetic ITR. Exemplary of the ITRs are the ITRs listed in the table below:

Organism	**GenBank Accession No.**	**Nucleotide annotation**
Vaccinia virus (Copenhagen)	N/A	Source: 1..175860 ITR: 167703 and 175860
Vaccinia virus (Wyeth)	N/A	Source: 1..182664 ITR: 166359 and 182664
Vaccinia virus (Western Reserve)	N/A	Source: 1..181419 ITR: 174482 and 181419
Vaccinia virus (MVA)	N/A	Source: 1..163444 ITR: 159457 and 163444
Rabbitpox virus (Utrecht)	N/A	Source: 1..186491 ITR: 183030 and 186491
Cowpox (Brighton)	N/A	Source: 1..212521 ITR: 206015 and 212521
Sheeppox virus	JN118986.1	Source: 1..290 ITR: 1..290
Sheeppox virus V104	MW020571.1	Source: 1..150211 ITRs: 88..2460; 147752..150124
Goatpox virus V103	MW020570.1	Source: 1..150578 ITR: 90..2480; 148080..150470
Variola virus	U23793.1	Source: 1..515 ITRs: 72..140; 141..194
Variola virus	U22363.1	Source: 1..724 ITR: 72..400
Deer adenovirus A	OP405339.1	Source: 1..30666 ITRs: 1..29; 30638..30666
Skunk adenovirus 1	KP238322.1	Source: 1..31848 ITR: 1..181; 31668..31848
Guinea pig adenovirus strain AUS96	NC_075973.1	Source: 1..37031 ITRs: 1..166; 36866..37031

Organism	GenBank Accession No.	Nucleotide annotation
Bovine adenovirus 1	M17112.1	Source: 1..159 ITR: 1..159
bovine adenovirus 5	AF238881.1	Source: 1..67 ITR: 1..67
bovine adenovirus 4	AF238883.1	Source: 1..59 ITR: 1..59
Bovine adenovirus 10	AF238882.1	Source: 1..368 ITR: 1..368
Equine adenovirus 1	AH002313.2	Source: 1..320 5' ITR 1..110; 3' ITR 211..320
Fowl adenovirus 1	AH003428.2	Source: 1..236 5' ITR 1..68; 3' ITR 169..236
Simian adenovirus (SA7P)	X01027.1	Source: 1..2338 ITR: 1..183
Simian adenovirus 8	AH002309.2	Source: 1..506 5' ITR 1..185; 3' ITR 322..506
Duck adenovirus 1	M17114.1	Source: 1..52 ITR: 1..52
Canine adenovirus 2	M30882.1	Source: 1..80 ITR: 1..80
Canine adenovirus 2	M17111.1	Source: 1..196 ITR: 1..196
Human adenovirus 19	M17110.1	Source: 1..154 ITR: 1..154
Human adenovirus 19a	AF271991.1	Source: 1..134 ITR: 1..134
Human adenovirus 19p	AF271990.1	Source: 1..125 ITR: 1..125
Human adenovirus 40	M16619.1	Source: 1..163 ITR: 1..163
Human adenovirus D37	AF271992.1	Source: 1..135 ITR: 1..135
Human adenovirus B3	M17113.1	Source: 1..195 ITR: 1..195
Human adenovirus sp.	KY062623.1	Source: 1..208 ITR: 1..208
Canine mastadenovirus A	AH002312.2	Source: 1..434 5' ITR: 1..434
Canine mastadenovirus A	M16831.1	Source: 1..273 ITRs: 17..56; 57..96; 97..136; 137..176; 177..216; 217..256; 257..273
Human parvovirus B19	M29711.1	Source: 1..479 3' ITR: 97..479
Human parvovirus B19	M29710.1	Source: 1..479 5' ITR: 1..383
Goose parvovirus	NC_001701.1	Source: 1..5106 ITRs: 1..444; 4663..5106
Muscovy duck parvovirus	KY069274.1	Source: 1..5061 ITRs: 1..381; 4681..5061

Organism	GenBank Accession No.	Nucleotide annotation
Duck parvovirus strain M8	KR029614.1	Source: 1..5073 ITRs: 1..387; 4687..5073
Streptococcus phage CP-7	AH001311.2	Source: 1..3422 ITRs: 1..347; 2170..2516
Enterobacteria phage PRD1	AH003497.2	Source: 1..338 5' ITR 1..110; 3'ITR 230..329
Enterobacteria phage PR722	AH003496.2	Source: 1..340 5' ITR 1..111; 3' ITR 230..340
Enterobacteria phage PR4	AH003495	Source: 1..340 5' ITR 1..111; 3' ITR 230..340
Enterobacteria phage PR5	AH003526.2	Source: 1..338 5' ITR 1..110; 3' ITR 229..338
Enterobacteria phage L17	AH003524.2	Source: 1..340 5' ITR 1..111; 3' ITR 230..340
Equine herpesvirus 4 (strain NS80567)	AF030027.1	Source: 1..145597 ITRs: 1..27; 112426..112452; 112453..122630; 135420..145597
Rhodococcus sp. strain: MR2253	L14443.1	Source: 1..2235 5' ITR: 1..583
Rhodococcus sp. strain: MR2253	L14442.1	Source: 1..2349 3' ITR 1..560
African swine fever virus	L28109.1	Source: 1..4027 ITRs: 41..54; 45..58; 69..82; 143..156; 157..174; 173..187; 207..220; 221..238; 244..258; 302..1633; 302..335; 1894..2027; 1894..1920
Kluyveromyces lactis	AH001394.2	Source: 1..580 ITRs: 1..240; 341..580
Streptomyces rochei	AH000936.2	Source: 1..1295 ITRs: 1..614; 939..1295
Gallid alphaherpesvirus 1	U27244.1	Source: 1..800 ITR: 1..800
Dictyostelium discoideum (slime mold)	K03072.1	Source: 1..390 ITR: 1..347
Sterkiella nova (Oxytricha nova)	M17509.1	Source: 1..79 ITR: 1..79
Rabbit fibroma virus	M14899.1	Source: 1..2886 ITR: 1..2886
Bythograea thermydron	AJ511262.1	Source: 1..1006 ITR: 971..1006
Lachancea kluyveri	M35319.1	Source: 1..598 ITR: 1..598

Organism	GenBank Accession No.	Nucleotide annotation
Yersinia enterocolitica	Y13309.1	Source: 1..3702 ITR: 252..289
Halobacterium salinarum	X59158.1	Source: 1..932 ITRs: 1..16; 917..932
Claviceps purpurea (ergot fungus)	X68490.1	Source: 1..7113 ITRs: 1..574; 6540..7113
Bombyx mori transposon-like element	M26070.1	Source: 1..1219 ITRs: 1..25; 1194..1219
Artificial sequence (Saccharomyces cerevisiae DNA, plasmid pTLU1 left ITR)	AB036802.1	Source: 1..108 left ITR: 1..15
Artificial sequence (Saccharomyces cerevisiae DNA, plasmid pTLU1 right ITR)	AB036803.1	Source: 1..115 right ITR: 1..15
Artificial Sequence (Transposon Tn3)	M13165.1	Source: 1..58 ITR: 1..58
Artificial Sequence (Plasmid RSF1010)	AH000926.2	Source: 1..485 5' ITR 11..56; 3' ITR 430..475
Artificial sequence (Erwinia amylovora plasmid pEa34)	M96392.1	Source: 1..6705 ITRs: 1..81; 3660..3686; 4865..4891; 6625..6705
Artificial sequence (Mus musculus 5' and 3' LTR repeat regions)	AH002036.2	Source: 1..2017 ITRs: 29..32; 578..581; 1135..1138; 1684..1687
Artificial sequence (Bos taurus 5' LTR repeat region)	AH001143.2	Source: 1..1320 ITRs: 46..51; 571..576; 747..752; 1272..1277
Artificial sequence (Transposon Tn10 clone pNK)	AH003348.2	Source: 1..2195 ITRs: 1..23; 731..753; 867..889; 2173..2195
Artificial sequence	AX328971.1	Source: 1..58 ITR: 1..58
Artificial sequence	AX328972.1	Source: 1..62 ITR: 1..62
Artificial sequence	AX328973.1	Source: 1..54 ITR: 1..54
Artificial sequence	AX328974.1	Source: 1..54 ITR: 1..54
Artificial sequence	AX328975.1	Source: 1..27 ITR: 1..27
Artificial sequence	AX328976.1	Source: 1..27 ITR: 1..27
Artificial sequence	AX418199.1	Source: 1..27 ITR: 1..27
Artificial sequence	CS260167.1	Source: 1..198 ITR: 1..198
Artificial sequence	LQ585715.1	Source: 1..7671 ITR: 17..163; 4674..4819
Artificial sequence	LQ585716.1	Source: 1..7671 ITR: 17..163; 4674..4819

Organism	GenBank Accession No.	Nucleotide annotation
Artificial sequence	LQ585717.1	Source: 1..7671 ITR: 17..163; 4674..4819
Artificial sequence	LV542024.1	Source: 1..7671 ITR: 17..163; 4674..4819
Artificial sequence	LV542025.1	Source: 1..7505 ITR: 17..163; 4508..4653
Artificial sequence	LV542026.1	Source: 1..7671 ITR: 17..163; 4674..4819
Artificial sequence	LY417217.1	Source: 1..7782 ITR: 1845..4974
Artificial sequence	MA132305.1	Source: 1..7671 ITR: 17..163; 4674..4819
Artificial sequence	LQ841017.1	Source: 1..7862 ITR: 17..163; 4868..5003
Artificial sequence	LQ841018.1	Source: 1..5157 ITR: 17..163; 2171..2316

Provided are delivery vehicles that contain the ITR plasmids and/or vectors that comprise an ITR and a transgene. In particular, the ITR vectors are provide in delivery vehicles. Delivery vehicles include but are not limited to, for example, an extracellular vesicle, an exosome, or a nanoparticle. Extracellular vesicles include microalgae extracellular vesicles (MEVs). The MEVs containing the ITR vectors can serve as a microalgae extracellular vesicle gene therapy vector (MEV-GTV). The MEV-GTV can include, an MEV and an ITR plasmid containing a transgene operably linked to an ITR. The MEV-GTVs can be used for any application that a gene therapy vector, such as an AAV gene therapy vector is used. The MEV-GTVs have numerous advantages that prior gene therapy vectors, such as AAV vectors, do not possess. These advantages are apparent from the description herein.

The transgene can encode a therapeutic product or a plurality of therapeutic products, and regulatory elements for controlling/directing expression of the encoded product(s) in a eukaryotic cell. The encoded therapeutic product or products include, but are not limited to, a nucleic acid molecule, a polypeptide, a protein, a plasmid, an aptamer, or an antisense oligonucleotide (ASO) peptide, and can be for use for treating or preventing or modulating a disease, disorder, or condition. The plurality can be encoded under separate regulatory sequences and/or can encoded as a polycistronic message that is processed into separate products, such as a polycistronic message in which the nucleic acid encoding the produces is separated, for example, by nucleic acid encoding a 2A peptide for production of a polycistronic message in

which separate products are translated. Thus, the plurality of nucleic acid encoding separate products can comprise a separate promoter, single or promoter, and/or combinations of such structures.

The ITR plasmid can contain regulatory sequences or elements to control the expression of the encoded product or products. Such regulatory sequences or elements include promoter sequences, transcription and translation sequences, enhancers, introns, IRES, 2A peptides, and Kozak sequences. Promoters include, but are not limited to, promoters that are recognized by eukaryotic polymerases, including RNA polymerase II or RNA polymerase III. The promoters can be selected from among eukaryotic promoters, such as plant or animal promoters, and/or eukaryotic virus promoters, and synthetic promoters. Promoters include, but are not limited to, for example, a cytomegalovirus promoter, a simian virus 40 promoter, a herpes simplex promoter, an Epstein Barr virus promoter, an adenovirus promoter, a synthetic promoter, an actin promoter, and a synthetic chimeric promoter.

Encoded therapeutic products include, but are not limited to, to RNAi and mRNA, such as, for example, silencing RNA (siRNA), short-hairpin RNA (shRNA), micro-RNA (miRNA), short activating RNA (saRNA), or long non-coding RNA (lncRNA), an ASO, an oligonucleotide, dsRNA, or a ribozyme; and/or can be a polypeptide or peptide; and/or a complex or biochemical pathway or system, such as e a gene editing system, such as, for example, a CRISPR-associated or CRISPR-like system(s).

The encoded therapeutic products include, but are not limited to, for example, immune modulators or immunomodulator products, for example, an immunomodulatory agent to increase or decrease production of one or more cytokines; up-or down-regulators of self-antigen presentation; mask MHC antigens; and/or promote the proliferation, differentiation, migration, or activation state of one or more types of immune cells; and/or an antibody or antigen-binding fragment thereof, such as, for example, an scFv, a bi-specific antibody, an antigen-binding fragment thereof, a checkpoint inhibitor antibody or antigen-binding fragment thereof, or a tumor antigen-specific antibody or antigen-binding fragment thereof, or an anti-oncogene specific antibody or antigen-binding fragment thereof, or is a tumor-specific receptor, or a signaling molecule antibody or antigen-binding fragment thereof; and

the antibody or antigen-binding fragment can specifically bind to and inhibit one or more of CTLA-4, PD-1, PD-L1, PD-L2, the PD-1/PDL1 pathway, the PD-1/PDL2 pathway, HER2, EGFR, VEGFR, TIM-3, LAG-3, BTLA-4, HHLA-2, CD28, and other checkpoints or immune suppressors, or tumor antigens; and/or a vaccine for treating or preventing, or reducing the risk of a disease, disorder, or condition, the vaccine can be a DNA, RNA, or protein vaccine

Hormones, cytokines, and chemokines are among encoded therapeutic products. Exemplary molecules include, for example, human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-alpha and -beta; Müllerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors, transforming growth factors (TGFs); insulin-like growth factor-I and-II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF), granulocyte-macrophage-CSF (GM-CSF), and granulocyte-CSF (G-CSF); or an interleukin (IL); and/or

Encoded therapeutic include agents for treatment of an infection, such as, for example, an anti-fungal agent, an anti-bacterial agent, an anti-viral agent, or an anti-parasitic agent.

The encoded therapeutic product include prodrugs, and enzymes that convert prodrugs into drugs for treating preventing, or modulating a disease, disorder, or condition, such as a disease, disorder, or condition of the brain or involving the brain. The encoded product can be for treatment of cancer or tumor in the brain, or for treatment of an infectious disease in the brain, or for treatment of a neurodegenerative disease or other central nervous system (CNS) disorder, or for treating dementia. The transgene can additionally or alternatively encode a product that is psychoactive or treats a psychiatric disorder, or is an immunomodulatory product, or is a detectable product, or treats brain injury or trauma, or treats cancer, or treats neurological brain

disorders, or treats CNS disorders, or treats genetic brain disorders, or treats brain cancer, or has anti-aging activity, or has brain regenerative activity.

The MEV-GTVs can be used for any purpose a viral vector or other delivery system is used, such as to treat, prevent (reduce the risk of) and/or modulate diseases, disorders, and conditions, such a metabolic diseases, genetic diseases, lung diseases, bowel diseases, ophthalmic diseases, brain diseases, and immunomodulatory diseases.

The MEV-GTVs can be used to treat diseases, disorders, and conditions that involve or affect the eye, including the posterior eye or back of the eye, such as, for example, the choroid-retina region, retinal pigment epithelium (RPE) cells, and/or photoreceptor cells. Exemplary diseases, disorders, and conditions include, but are not limited to, one or more of an age-related macular degeneration (AMD), diabetic retinopathy (DR), retinal vein occlusion (RVO), macular edema, diabetic macular edema (DME), optical neuropathy glaucoma disease, geographic atrophy, retinitis pigmentosa, ocular hypertension, or glaucoma. Diseases, disorders, and conditions include, but are not limited to, one or more of an age-related macular degeneration (AMD), a retinal degenerative disease, disorder, or condition, or a disease, disorder, or condition involving choroidal neovascularization, an inheritable disease, disorder, or condition involving the retina, and an ocular hypertension. For example, the disease, disorder, or condition is selected from one or more of an age-related macular degeneration (AMD), diabetic retinopathy, retinal vein occlusion, diabetic macular edema (DME), retinal vein occlusion (RVO), and optical neuropathy glaucoma disease, such as, for example, wet AMD, geographic atrophy, diabetic retinopathy, macular edema, retinitis pigmentosa, glaucoma, and/or ocular hypertension. Diseases, disorders, and conditions include a disease of the retina that leads to blindness, including inheritable diseases that can be treated by gene therapy or a gene editor or a polypeptide.

Diseases, disorders, and conditions that can be treated by administering a plasmid, delivery vehicle, or MEV-GTV composition provided herein include, but are not limited to, neurodegenerative diseases, disorders, and conditions (such as Parkinson's, or Alzheimer's, Huntington's, Creutzfeldt-Jakob disease, or other neurodegenerative diseases); or a cognitive disorder (such as dementia, or amnesia, or delirium, or other cognitive disorder); or a brain disorder (such as encephalitis, or

seizures, or tumors, or other brain disorder); or a nervous system disorder (such as pain, or seizures, or infections, or other nervous system disorder); or a genetic disease (such as cystic fibrosis, thalassemia, sickle cell anemia, Huntington's Disease, Duchenne's muscular dystrophy, Tay-Sachs disease, Rett syndrome, or other genetic disease); or brain tumor; or Niemann-Pick disease; or prion disease; or Parkinson's disease; or auto-immune disease; or multiple sclerosis; or amyotrophic lateral sclerosis (ALS); or muscular dystrophy; or other disease of the brain or involving the brain, or cancers, such as those treated by polypeptides, including antibodies, such as checkpoint inhibitors.

The plasmid, delivery vehicle, or MEV-GTV can be formulated for administration by a particular route, such as for inhalation, oral, intranasal administration. They can be administered topically or locally or systemically. For example, the MEV-GTVs can be formulated for intranasal administration to deliver encoded product to one or more of neurons, astrocytes, oligodendrocytes, microglial cells, ependymal cells, and/or neural stem cells of the brain and can be for use for, emotional, behavioral, psychiatric, neurologic, degenerative, genetic, malignant (cancer), and/or traumatic brain diseases, disorders, or conditions.

The transgene can encode a product for treating, preventing, or modulating a disease, disorder, or condition that is caused by or involves a pathogen or is an inflammatory disease or involves a signaling pathway activated by a TLR or intracellular receptor or endosomal receptor, or is an autoimmune disease, or any disease, disorder, or condition treated by immunomodulation. Such transgenes may include those that encode a product that elicits or contributes to a protective humoral response that comprises serum IgG and IgA and/or mucosal IgG and IgA, such as, for example, a vaccine, or an antigen or immunogenic portion thereof, or epitope, or an immunomodulator to reduce or eliminate immune-tolerance to previous immunotherapies or vaccines. Exemplary products that can be encoded by transgenes are TLR antagonists or agonists. The TLR and agonist thereof can be one or more of:

TLR Member	**Ligand(s)/Agonists**
TLR1	Triacyl lipopeptides (Pam3CSK4)
TLR2	Zymosan, Porin, Modulin, Lipoproteins, Lipoteichoic acid, Diacyl lipopeptides, Atypical LPS, Peptidoglycan, Triacyl lipopeptides
TLR3	dsRNA
TLR4	Mannans, Taxol, LPS

TLR Member	Ligand(s)/Agonists
TLR5	bacterial flagellin, profilin, HMGB1, Small molecule agonists (CBLB502)
TLR6	Zymosan, Porin, Modulin, Lipoproteins, Lipoteichoic acid, Diacyl lipopeptides (Pam2CSK4), Atypical LPS, Peptidoglycan
TLR7	imidazoquinoline, loxoribine, ssRNA, bropirimine, resiquimod
TLR8	ssRNA, small synthetic compounds
TLR9	CpG DNA
TLR10	Diacyl and Triacyl lipopeptides
TLR11	Profilin-like protein, unpathogenic bacteria.

The TLR and antagonist can be one or more of:

TLR Member	Ligand(s)/Antagonists
TLR1	Small molecule antagonists (CU-T12-9, MMG-11)
TLR2	Small Molecule Antagonists (AT1-AT8, CU-CPT22, CU-T12-9, MMG-11, NPT1220-312), Phloretin, Sulfoglycolipids
TLR3	Small Molecule Antagonists (CU-CPT4a), Monoclonal antibodies (CNTO4685, CNTO5429)
TLR4	Small Molecule Antagonists (Norbinaltorphimine, T4Ics, T5342126, Simvastatin)
TLR5	Small Molecule Antagonist (TH1020)
TLR6	Simvastatin
TLR7	Chloroquine, hydroxychloroquine, quinacrine
TLR8	Small Molecule Antagonist (CU-CPT8m, CU-CPT9a)
TLR9	Small Molecule Antagonists (NPT1220-312), chloroquine, hydroxychloroquine, quinacrine; Suppressive or inhibitory oligonucleotides.

The MEV-GTVs can be for use for treating a disease, disorder, or condition that is cancer, or that is an inflammatory or autoimmune disease, disorder, or condition, or a disease, disorder, or condition in which inflammation plays a role in the etiology of the disease, disorder, or condition. Exemplary of such conditions include septic shock, autoimmunity, atherosclerosis, metabolic syndrome and gastric cancer, inflammatory bowel diseases, rheumatoid arthritis, sepsis, allergies, Alzheimer's Disease, Parkinson's disease, ulcerative colitis and Crohn's disease.

The MEVs are from microalgae, from a division of microalgae selected from among Euglenophyta (Euglenoids), Chrysophyta (Golden-brown algae and Diatoms), Pyrrophyta (Fire algae), Chlorophyta (Green algae), Rhodophyta (Red algae), Phaeophyta (Brown algae), and Xanthophyta (Yellow-green algae). Unicellular green algae include those that belong to the order Chlorellales, in particular the Chlorellaceae family, and in particular those that belong to the *Chlorella* or

Parachlorella genus, such as *Chlorella ellipsoidea*, *Chlorella pyrenoidosa*, *Chlorella sorokiniana*, *Chlorella vulgaris*, and *Chlorella variabili, or Parachlorella kessleri*, *Parachlorella beijerinckii*, and *Parachlorella hussii*.

The compositions containing the MEV-GTVs can be formulated for administration by any route of administration. The route of administration of MEVs are a function of the target organ or tissue. Routes include, but are not limited to, local, systemic, topical, parenteral, enteral, mucosal, subcutaneous, intramuscular, oral or nasal inhalation into the lung, intranasal, vaginal, rectal, aural, oral, and other routes of administration. Exemplary routes of administration and target:

Target organ/tissue	**Exemplary indication**	**Exemplary route of administration**
Lung	Cystic Fibrosis	Inhalation
Lung	Idiopathic Pulmonary Fibrosis	Inhalation
Lung	Primary Ciliary Dyskinesia	Inhalation
Lung	Pulmonary Arterial Hypertension	Inhalation
Liver	Inborn error of metabolism	Intravenous or direct injection into the liver
Lung	Covid-19 (preventive or therapeutic)	Intranasal or Inhalation
Lymphatic	Covid-19 (vaccine)	Intravenous or intramuscular
Lung	Influenza (preventive or therapeutic)	Intranasal or Inhalation
Lymphatic	Influenza (vaccine)	Intravenous or intramuscular
Lymphatic	Viral pathogen	Intravenous or intramuscular
Lymphatic	Bacterial pathogen	Intravenous or intramuscular

The MEVs can be formulated in any form, including as a tablet; as a liquid, such as an emulsion, including micro and nano emulsions; as a powder; as a pill; as a liquid solution or suspension (*e.g.*, including injectable; ingestible and topical formulations, for example, eye drops, gels, pastes, creams, or ointments); aerosols (*e.g.*, nasal sprays and inhalers); suppositories; pessaries; injectable and infusible solutions; and sustained release forms; the form and formulation respective to the route of administration including for oral administration, for nebulization, or for inhalation. For vaccination, routes include oral and IM. For administrations to the eye, the MEV-GTV compositions can be formulated for drop instillation on the eye surface, or intraocular injection, intravitreal injection, supra-choroidal, or subretinal injection. Compositions can be formulated for oral administration for treatment of a disease, disorder, or condition that involves the gastrointestinal tract or the immune system or the white spleen. Compositions also can be formulated for intranasal

administration for treatment of a disease, disorder, or condition of or involving the brain.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the AAV genome flanked by ITRs, encoding Cap and Rep AAV genes [adapted from: Asaad *et al.* (2023). AAV genome modification for efficient AAV production. Heliyon, 9(4), e15071].

Figure 2 shows the secondary structure of the ITR for AAV serotype 2. The characteristic 'T' shape of the ITR is formed by two adjacent inverted repeats (BB' and CC') separated by a single unpaired nucleotide. A third inverted repeat is the central AA', forming a three-way junction. The D sequences do not participate in the T secondary structure. The replicase binding element (RBE) is formed by specific tetranucleotide repeats. The 'flip' and 'flop' sequences exist in equal proportion on either end of AAV genomes [adapted from: Wilmott *et al.* (2019). A User's Guide to the Inverted Terminal Repeats of Adeno-Associated Virus. Human Gene Therapy Methods, 30(6), 206-213].

Figure 3 shows the sequence and structures of ITRs originating from AAV serotypes 1, 2, 3, 4, 6, and 7. Bold letters denote nucleotides that are not conserved among the ITR sequences [adapted from Earley *et al* (2020). Adeno-Associated Virus serotype-specific Inverted Terminal Repeat sequence role in vector transgene expression. Human gene therapy, 31(3-4), 151–162].

Figure 4 shows the comparison between the composition of MRV-GTVs and the composition of other (ITR)AAV-based delivery or gene therapy systems. (A) AAV genome: an ITR-flanked linear ssDNA molecule. (B) AAV or AAV-based vector carrying the native or engineered ITR-flanked linear ssDNA molecule inside the capsid. The engineered genome can be up to 5 kb in size. (C) The AAVs or AAV-vectors, approx. 20 nm in diameter, loaded into exosomes of 200-250 nm in size (the chimeric vectors of evoxtherapeutics.com). (D) MEV-GTV containing AAV-ITR-flanked dsDNA expression plasmids (up to 10 kb) loaded into MEVs.

Figure 5 depicts an exemplary elution profile for higher purity of MEV preparations, where MEVs previously concentrated by TFF and purified by ultracentrifugation and formulated in PBS at concentration of 1011 to 1013 per mL are seeded in a pre-packed column qEV1 from IZON. The MEVs are eluted using

PBS solution. The elution fractions of 0.5 mL are collected. MEVs are recovered in the first fractions as shown in the figure. The most concentrated fractions (4-5) are pooled and stored at 4°C before use.

Figure 6 provides exemplary images of MEVs obtained using Transmission Electron Microscopy (TEM). See Example 2.

Figures 7A-7B show exemplary images obtained with MEVs labelled with lipophilic dyes by confocal microscopy. Figure 7A shows MEVs labelled with pKH26. Figure 7B shows MEVs labelled with DiD. See Example 2.

Figures 8A-8C show MEVs uptake analysis by confocal microscopy. Uptake of MEVs labelled with pKH26 into cells after 16hs of incubation. **Figure 8A** layout of cells (2D image). **Figure 8B** one cell (2D image). **Figure 8C** one cell (3D image). See Example 2.

Figure 9 shows the distribution of labelled MEVs with lipophilic dyes by cytometry. See Example 2.

Figures 10A-10B show MEV uptake analysis by cytometry. Uptake of MEVs (labelled with lipophilic dyes) in different cell types as percentage of fluorescent cells. **Figure 10A** shows epithelial cells. **Figure 10B** shows monocytes. See Example 2.

Figure 11 shows MEVs loaded with fluorescent payload quantification by cytometry. Determination of percentage of loaded MEVs. See Example 4.

Figure 12 shows representative patterns of biodistribution according to the route of administration, for the Intravenous (IV), Intratracheal (IT) and Per os (PO) routes. See Example 5A

Figure 13 depicts the kinetics of accumulation in liver, lungs, and spleen (average of 6 animals) after intravenous administration. See Example 5A.

Figure 14 depicts the kinetics of accumulation in lungs, spleen, and intestine (average of 6 animals) Per os administration. See Example 5A.

Figure 15 depicts the kinetics of accumulation lungs and kidneys (average of 4 animals) after intranasal administration. See Example 5A.

Figure 16 depicts the kinetics of accumulation in lungs, spleen, and intestine (average of 3 animals) after intratracheal administration. See Example 5A.

Figure 17 shows representative pattern of biodistribution at different time points (0h-72h) for the Intravenous administration (IV). See Example 5A

Figure 18 shows representative pattern of biodistribution at different time points (0h-72h) for the Per os administration (PO). See Example 5A.

Figure 19 shows representative pattern of biodistribution at different time points (0h-72h) for the intranasal administration (IN). See Example 5A.

Figure 20 shows representative pattern of biodistribution at different time points (0h-72h) for the intratracheal administration (IT). See Example 5A.

Figure 21A depicts the accumulation in liver tissues after intravenous (IV), intranasal (IN), intratracheal (IT) and Per os (PO) administration (average of 6 animals), as described in Example 5A.

Figure 21B depicts the accumulation in spleen tissues intravenous (IV), intranasal (IN), intratracheal (IT) and Per os (PO) administration (average of 6 animals), as described in Example 5A.

Figure 21C depicts the accumulation in lung tissues after intravenous (IV), intranasal (IN), intratracheal (IT) and Per os (PO) administration (average of 6 animals), as described in Example 5A.

Figure 21D depicts the accumulation in brain tissues after intravenous (IV), intranasal (IN), intratracheal (IT) and Per os (PO) administration (average of 6 animals), as described in Example 5A.

Figure 22 shows a microscopic image of mouse intestinal epithelium 8 hours after PKH26-labeled MEV administration by Per os route. See Example 5B.

F**igures 23A-B** show a microscopy image of mouse GALT tissue 8 hours after PKH26-labeled MEV administration by Per os route. **Figure 23A** depicts Hematoxylin and Eosin staining of intestine (G = GALT tissue). **Figure 23B** depicts DAPI (nuclei) staining, and MEV-PKH26 fluorescence (for example portion labeled "ro"). See Example 5B.

Figure 24 shows a microscopy image of mouse spleen 24 h after PKH26-labeled MEV administration by Per Os route. The figure depicts spleen pulp with DAPI (nuclei) staining, and MEV-PKH26 is indicated by fluorescence (lighter gray staining/ puncta). See Example 5B.

Figure 25 is a diagram showing the migration of MEVs from the GALT to the spleen. See Example 5B.

Figure 26A shows whole-body bioluminescence imaging of a representative animal treated with MEVs loaded with luciferase mRNA. See Example 5C.

Figure 26B depicts whole-body bioluminescence imaging of a representative animal treated with MEVs loaded with luciferase protein enzyme. See Example 5C.

Figures 27A-27I show non-hematological toxicity response in mice after administered orally or intratracheally with MEVs. **Figures 27A-27D** depict evaluation of MEVs toxicity by chemistry parameters: ALAT, ASAT, urea and creatine. **Figures 27 E-27I** depict evaluation of MEV's toxicity by hematology parameters: RBCs, hemoglobin, hematocrit, MCV and Eosinophils. Group 1: mice received 100 µl of PBS (White bars) by PO delivery. Group 2: mice received 100 µl of $4*10^{11}$ MEV/ mouse by PO delivery (bar with black and white tiles). Group 3: mice received 100 µl of $4*10^{12}$ MEV/ mouse by PO delivery (bars with vertical lines). Group 4: mice were administered 100 µl of $4*10^{11}$ MEV/ mouse by IT delivery (squared bars). Data were obtained for 6 mice per group for each parameter. ALAT: Alanine Aminotransferase. ASAT: Aspartate Aminotransferase. MCV: Mean Corpuscular Volume. PO: per os (oral delivery). IT: Intratracheal. See Example 6.

Figures 28A-28C show responses to antigen (ovalbumin (OVA)) administered orally or intramuscularly with adjuvant compared to the responses following administration of MEVs loaded with OVA by the same routes. Example 7.

Figure 29 shows the expression of mRNA for GFP at increasing time points in cells cultured *in vitro* upon the MEV-GTV-mediated delivery of an ITR-carrying plasmid, in comparison with the MEV-mediated delivery of a mRNA for GFP and with the MEV-mediated delivery of a control plasmid which does not carry ITRs. See Example 8.

Figures 30A and **30B** show anti-anti-angiogenic activity of MEVs loaded with mRNA encoded Aflibercept (SEQ ID NO:73); the MEVs enter cells and the mRNA is translated. **Figure 30A** shows the biological activity (inhibition of the angiogenic activity of VEGF) of Aflibercept (SEQ ID NO:94) expressed from an ITR-carrying plasmid delivered by a MEV-GTV in a surrogate *in vitro* model for angiogenesis. Images from the Matrigel® tube formation assay showing the extent of tube formation (induced by VEGF) by HUVEC (primary human umbilical vascular endothelial cells). **Panel 1** Negative control: without VEFG, without MEV-GTV.

Panel 2 Positive control: 1ng/ml VEGF, without MEV-GTV. **Panel 3** 1ng/ml VEGF, MEV-GTV loaded with ITR-plasmid encoding Aflibercept. **Panel 4** 1ng/ml VEGF, MEV-GTV loaded with an ITR-plasmid encoding a different sequence of Aflibercept. See Example 9. **Figure 30B** shows the images of nodes and networks obtained in the tube formation assay; it shows the results of analysis using Angiogenesis plugin ImageJ: **5-6** from cells pre-treated with VEGF; **7-8** from cells non-pre-treated with VEGF; **9-10** from cells pre-treated with VEGF and with non-loaded MEVs; and **11-12** from cells pre-treated with VEGF and with MEVs loaded with mRNA encoding Aflibercept.

Figure 31 is a bar graph that shows the quantified biological activity (inhibition of the angiogenic activity of VEGF) of Aflibercept expressed from an ITR-carrying plasmid delivered by a MEV-GTV in a surrogate *in vitro* model for angiogenesis. Size of the mesh area calculated from tube formation assay on HUVEC (primary human umbilical vascular endothelial cells). **(A)** Negative control: without VEFG, without MEV-GTV. **(B)** Positive control: 1ng/ml VEGF, without MEV-GTV. **(C)** 1ng/ml VEGF, MEV-GTV loaded with ITR-plasmid encoding Aflibercept. **(D)** 1ng/ml VEGF, MEV-GTV loaded with an ITR-plasmid encoding a different sequence of Aflibercept. See Example 9.

Figure 32 shows the position of the 5 brain sections studied in the experiments on delivery, and gene expression of MEV-GTVs and their payload.

Figure 33 shows the penetration of MEVs in ARPE-19 cells. A time response study (data not shown) demonstrated that penetration is maximal at 48 hours of treatment. **Panel 1** — T=0h, ARPE-19 cells treated with MEVs labelled with PKH26 (merge DAPI/ Actin (Alexa 488) / MEVs labelled with PKH26 (Alexa 546)); **Panel 2** — T=24h, ARPE-19 cells treated with MEVs labelled with PKH26 (merge DAPI/ Actin (Alexa 488) / MEVs labelled with PKH26 (Alexa 546)). The expression of GFP from the mRNA delivered by the MEVs into ARPE-19 cells is maximal at 72h (data non shown). The results obtained demonstrate the ability of MEVs to deliver their payloads to retinal pigmented epithelial (RPE) cells and the escape of the encoded payload (mRNA) from the endosomal vesicles, which is translated into protein.

Figure 34 shows the long-lasting expression of the GFP protein in ARPE-19 cells treated with MEVs loaded with a plasmid carrying ITR plasmids and encoding

GFP protein, at one week after the treatment: **Panel 1**— T=0h, ARPE-19 cells treated with MEVs loaded with a plasmid carrying AAV's ITRs and encoding GFP (merge DAPI/ GFP (Alexa 488)); **Panel 2** —T=72h, ARPE-19 cells treated with MEVs loaded with a plasmid carrying AAV's ITRs and encoding GFP (merge DAPI / GFP (Alexa 488)) ; **Panel 3**— T= 96h, ARPE-19 cells treated with MEVs loaded with a plasmid carrying AAV's ITRs and encoding GFP (merge DAPI / GFP (Alexa 488)) ; **Panel 4** — T=144h , ARPE-19 cells treated with MEVs loaded with a plasmid carrying AAV's ITRs and encoding GFP (merge DAPI / GFP (Alexa 488)). The Figure shows the increasing expression of GFP protein from 72h to 144h, the longest time point studied. The results demonstrate the ability of MEVs to deliver their payloads into RPE cells and the endosomal escape of the payload (plasmid) to reach the nucleus, where it is transcribed into transcribed to mRNA, which then is translated into protein.

Figure 35 shows images of nodes and networks obtained from the tube formation assay and shows the anti-angiogenic effect of MEVs loaded the anti-angiogenesis product Aflibercept protein, or mRNAs encoding Aflibercept protein. The figure illustrates anti-angiogenic activity following the exposure of HUVEC cells to MEVs loaded either with Aflibercept protein or with mRNAs encoding Aflibercept. It presents images from the tube formation assay: **A-** Cells (Negative control (w/o VEGFa)); **B-** Cells (Positive control (1ng/mL VEGFa)); **C-** Cells (Positive control (2ng/mL VEGFa)); **D-** Cells treated with Aflibercept protein 0.5 ng/mL + VEGFa 1ng/mL; **E-** Cells treated with MEVs loaded with mRNA (encoding Aflibercept) +VEGFa 1ng/mL**; F-** Cells treated with MEVs loaded with Aflibercept protein + VEGFa 1ng/mL.

Figures 36A-36B show the efficacy of treatment against neo-angiogenesis, using HUVEC primary cells and the tube formation assay, using MEVs loaded with an ITR-plasmid (pITR) coding for aflibercept. **Figure 36A** shows the images obtained 4 hours after the scratch to demonstrate the anti-angiogenic activity (cell migration inhibition) following the exposure of HUVEC cells to MEVs loaded either with the Aflibercept protein, with mRNA encoding Aflibercept, or with ITR plasmids encoding Aflibercept (a.k.a. VEGF Trap-Eye). **Figure 36B** is a bar graph showing the quantified results of the wound healing assay.

DETAILED DESCRIPTION

A. DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention(s) belong. All patents, patent applications, published applications and publications, GenBank sequences, databases, websites, and other published materials referred to throughout the entire disclosure herein, unless noted otherwise, are incorporated by reference in their entirety. In the event, that there are a plurality of definitions for terms herein, those in this section prevail. Where reference is made to a URL or other such identifier or address, it is understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference thereto evidences the availability and public dissemination of such information.

As used herein, inverted terminal repeats (ITRs) are terminal hairpin structures that occur in viruses.

As used herein, an ITR plasmid (also referred to as a minigene plasmid) refers to a circular plasmid that contains one or more ITRs, generally two complementary ITRs, such as ITRS from the same source, flanking a transgene, and optionally regulatory sequences. Reference to minigene does not refer to size, but refers the constructs that comprise a gene or polycistronic gene and any needed regulatory sequences. The gene(s) can include introns or cannot have any introns, such as cDNA. As described herein, the amount of genetic material in the minigene ITR plasmid, including the ITR(s), is up to any amount that can be loaded into the MEVs. Based on data (see, discussion below), the amount is approximately 20 kB, but depending on sequences, could be more. An ITR plasmid is a plasmid that contains one or more viral ITRs and a transgene, whereby a plasmid that contains only the ITR(s) and transgene circularizes to form minicircles. Generally, the plasmid comprises two ITRs flanking the transgene. The resulting ITR plasmid minicircles can contain one or a plurality of copies of the ITR and transgene. ITR plasmids can be isolated by size, particularly those that contain one copy of the ITR and transgene. The ITR plasmid does not contain genes for replication or for a capsid; it can contain viral promoters

and transcriptional and translational regulatory sequences for expression of encoded products.

As used herein, a bacterial ITR plasmid is plasmid that contains the ITR plasmid and can replicate or be propagated in a bacterial cell. The bacterial ITR plasmid can contain sequences flanking the ITR plasmid sequences for excision of the ITR plasmid, such as by recombination. The bacterial plasmid is a bacterial cloning plasmid that contains the ITR plasmid, generally flanked by sequences for recombination or other sequences so that the ITR plasmid is excised.

As used herein, a transgene refers to nucleic acid encoding one or plurality of products and optionally transcriptional and translational regulatory sequences. The transgene generally includes a promoter operably linked to nucleic acid encoding a product and any other needed regulatory sequences to produce the product(s) in a eukaryotic cell.

As used herein, bacterial plasmid refers to a plasmid that can be propagated in bacteria. For purposes herein, the bacterial plasmid contains a minigene ITR plasmid, which can then be excised or removed from the bacterial plasmid by including sequences that allow and/or induce an internal recombination. The bacterial plasmid can comprise any suitable plasmid that replicates in bacteria and into which the selected ITR(s), transgene and any regulatory sequences, and the optional sequences for recombination to extract the minigene plasmid, can be inserted.

As used herein, bacterial cloning vectors (also generically referred to as a bacterial plasmids) include plasmids, bacteriophages, cosmids, and bacterial artificial chromosomes (BACs), and any such construct in which the ITR plasmids can be inserted and replicated intact in bacterial and remain episomal.

As used herein, gene therapy is the delivery of genetic material into cells. For purposes herein, genetic material includes DNA, to repair or replace a disease-related mutation or gene, and RNA, including mRNA, miRNA, siRNA, hybrids, and complexes of molecules of different nature, including gene editing complexes and moieties, such as editing complexes, made of pieces of RNA and of DNA and aimed at the correction of mutations on diseases genes (and thus to the cure of the disease gene) is considered to be a gene therapy approach.

As used herein, cargo refers to exogenous molecules, such as bioactive molecules, including biomolecules, and small molecules, and also the ITR plasmid and bacterial ITR plasmids provided herein, that are loaded into the microalgae extracellular vesicles (MEVs) provided herein after the MEVs have been isolated. This includes cargo that is heterologous to the MEVs. In particular, for purposes herein, the cargo comprises plasmids that are introduced into the MEVs to produce the MEV genetic therapy vectors (MEV-GTVs) as detailed and exemplified herein.

As used herein, in general, heterologous with respect to cargo in an MEV refers to cargo in the MEVs that does not naturally-occur in the MEVs but is loaded exogenously, as discussed above. It also refers to cargo in MEVs that have been loaded endogenously in the MEVs by genetically-modified microalgae. MEVs with heterologous cargo comprise cargo that does not occur naturally in the MEVs. Cargo that is heterologous to the microalgae and/or the MEVs is cargo that, in nature, does not occur in the microalgae nor is naturally packaged in the MEVS, and/or is cargo that does not occur in the MEVs unless the microalgae is modified to produce it or the cargo is exogenously loaded.

As used herein, a bioactive molecule or bioactive agent refers to any molecule or agent that can have a biological activity, such as therapeutic activity, or as a detectable marker, or that can act *in vivo* on a subject. Bioactive agents and molecules include biomolecules, such as DNA, RNA, proteins, other biopolymers, and small molecules, such as small molecule drugs and pharmaceuticals, immunogens, and any molecules that would be delivered to a subject, such as a human or other animal or a plant or a microorganism (bacteria or other), in connection with a therapy, a diagnostic application, or other such uses, such as a cosmetic. The bioactive agent or molecule can function as or have an activity as, for example, a therapeutic, an immunogen, a diagnostic, a detectable marker, or a cosmetic. The bioactive molecules for use herein are any that can be loaded into a microalgae extracellular vesicle (MEV). Bioactive molecules include therapeutics and also diagnostics, such as reporter molecules and detectable molecules, whereby a disease, disorder, or condition can be monitored or diagnosed.

As used herein, a biomolecule refers to any biologically active biopolymer or molecule that occurs, or can occur, in a living organism or virus or that is a modified

form of such biopolymer or molecule. Biomolecules, thus, include modified naturally-occurring biomolecules, such as, for example proteins that include a modified primary sequence, such as by deletions, insertions, and/or replacements of amino acids to alter the primary sequence, and or by modification, such as post-translational modifications of the protein.

As used herein, the recitation of therapeutic refers to any molecule that has any effect *in vivo* to ameliorate the symptoms of a disease, disorder, or condition or affects the progress of a disease, disorder, or condition, or prevents or reduces the risk of a disease, disorder, or condition or side effects of a treatment. Therapeutics includes, but are not limited to, nucleic acids encoding mRNA or protein, where the mRNA or protein is the therapeutic product, DNA that is the therapeutic product, aptamers, proteins, small molecules, and any such product as understood by those of skill in the art.

As used herein, when it is stated that MEVs have the same or substantially the same loaded cargo or amount thereof, it is understood that this refers to an average among the population of MEVs in a composition. It is understood, that when MEVs are loaded exogenously the ratio of cargo/MEV can be selected so that each MEV has, on average, a pre-determined amount of cargo. As a simple example, to load an average of one molecule of cargo/MEV, the skilled person could calculate the amount of cargo to load into a composition of MEVs, and understands that in the composition of MEVs, some would have more than one molecule of cargo/MEV, and others would have none. On average, the MEVs would have one molecule of cargo/MEV. The skilled person understands, that, in general, the amount of cargo/MEV will be more than the one molecule/MEV, and that the amount of cargo depends upon a variety of parameters, including the cargo, the target tissues and/or cells, the disease, disorder, or condition treated, and the subject treated. Generally, more than one molecule of cargo per MEV, on the average, such as at least 10 or about 10 molecules/MEV are loaded. Substantially more cargo, 100, 500, 1000, 10^4 molecules/MEV and more, also can be loaded. The amount loaded depends upon the target, disease, disorder, or condition, the subject, the cargo, and the capacity of the MEV. It is within the skill in the art to select the amount.

As used herein, a subject is any organism, generally an animal or plant, into which or on which the composition containing the MEV is introduced. Subjects include, but are not limited to, humans, plants, particularly crop plants, and animals, including farm animals and pets, such as dogs and cats, and zoo animals.

As used herein, disease or disorder or condition refers to a pathological or undesirable or undesired condition in an organism resulting from a cause or condition including, but not limited to, infections, acquired conditions, and genetic conditions, and those characterized by identifiable symptoms.

As used herein, treating a subject with a disease, disorder, or condition means that the subject's symptoms or manifestations of the disease or conditions are partially or totally alleviated, or remain static following treatment.

As used herein, a patient refers to a human subject.

As used herein, a subject includes an animal; an animal includes any animal, such as, but not limited to, primates including humans, gorillas, and monkeys; rodents, such as mice and rats; fowl, such as chickens; ruminants, such as goats, cows, deer, and sheep; and pigs and other animals. Non-human animals exclude humans as the contemplated animal.

As used herein, treatment refers to any effects that ameliorate symptoms of a disease or disorder. Treatment encompasses prophylaxis, therapy and/or cure. Treatment also encompasses any pharmaceutical use of any MEV or composition provided herein. Treatment refers to any effects that ameliorate or prevent or other reduce or eliminate any symptom or manifestation of a disease or disorder. Treatment also encompasses any pharmaceutical use of any MEV or composition provided herein.

As used herein, prophylaxis refers to prevention of a potential disease and/or a prevention of worsening of symptoms or progression of a disease. Prevention or prophylaxis, and grammatically equivalent forms thereof, refer to methods in which the risk or probability of developing a disease or condition is reduced or eliminated and products that reduce or eliminate the risk or probability of developing a disease or condition.

As used herein, a disease, disorder, or condition of the eye or involving the eye is a disease, disorder, or condition in which the etiology of a disease, disorder, or

condition involves the eye such that delivery of a therapeutic to the eye can effect treatment, which includes amelioration of or relief from symptoms, and/or treating cause or a manifestation of the disease, disorder, or condition, or delivery of a diagnostic molecule, such as a labeled molecule, a reporter, or an enzyme, that can be used to aid in or to effect diagnosis, or that can be used to monitor the progress of or effectiveness of treatment.

As used herein, a modification with reference to modification of a sequence of amino acids of a polypeptide or a sequence of nucleotides in a nucleic acid molecule refers to and includes deletions, insertions, and replacements of amino acids or nucleotides, respectively. These include modifications of the primary sequence of a polypeptide or protein. Methods of modifying a polypeptide and nucleic acid molecule are routine to those of skill in the art, such as by using recombinant DNA methodologies. Modifications, when referring to polypeptide or protein, not to a sequence, refer to post-translational or post-purification changes, such as conjugation or linkage of moieties that alter properties of polypeptide or protein, such as half-life extending moieties, glycosylation, purification tags, detectable reporters, and other such moieties.

As used herein, a modification of a genome or a plasmid or gene includes deletions, replacements, insertions, and translocations of nucleic acid. These include any changes to the native or naturally-occurring nucleic acid sequence.

As used herein, RNA interference (RNAi) is a biological process in which RNA molecules inhibit gene expression or translation, by neutralizing targeted mRNA molecules to inhibit translation and thereby expression of a targeted gene.

As used herein, RNA molecules that act via RNAi are referred to as inhibitory by virtue of their silencing of expression of a targeted gene. Silencing expression means that expression of the targeted gene is reduced or suppressed or inhibited.

As used herein, gene silencing via RNAi is said to inhibit, suppress, disrupt, or silence expression of a targeted gene. A targeted gene contains sequences of nucleotides that correspond to the sequences in the inhibitory RNA, whereby the inhibitory RNA silences expression of mRNA. Small interfering RNAs (siRNAs) are small pieces of double-stranded (ds) RNA, usually about 21 nucleotides long, with 3' overhangs (2 nucleotides) at each end that can be used to interfere with the translation

of proteins by binding to and promoting the degradation of messenger RNA (mRNA) at specific sequences. In doing so, siRNAs prevent the production of specific proteins based on the nucleotide sequences of their corresponding mRNAs. The process is called RNA interference (RNAi), and also is referred to as siRNA silencing or siRNA knockdown. A short-hairpin RNA or small-hairpin RNA (shRNA) is an artificial RNA molecule with a tight hairpin turn that can be used to silence target gene expression via RNA interference (RNAi). Expression of shRNA in cells is typically accomplished by delivery of plasmids or through viral or bacterial vectors.

As used herein, non-coding RNAs are RNAs that do not encode a protein. Classes of non-coding RNA, include, but are not limited to, small interfering RNAs (siRNAs) and microRNAs (miRNAs). As used herein, inhibiting, suppressing, disrupting, or silencing a targeted gene refers to processes that alter expression, such as translation, of the targeted gene, whereby activity or expression of the product encoded by the targeted gene is reduced. Reduction includes a complete knock-out or a partial knockout, whereby, with reference to the MEVs provided herein and administration herein, treatment is effected.

As used herein, a tumor microenvironment (TME) is the cellular environment in which the tumor exists, including surrounding blood vessels, immune cells, fibroblasts, bone marrow-derived inflammatory cells, lymphocytes, signaling molecules and the extracellular matrix (ECM). Conditions that exist include, but are not limited to, increased vascularization, hypoxia, low pH, increased lactate concentration, increased pyruvate concentration, increased interstitial fluid pressure and altered metabolites or metabolism, such as higher levels of adenosine, indicative of a tumor.

As used herein, recitation that a nucleic acid or encoded RNA targets a gene means that it inhibits or suppresses or silences expression of the gene by any mechanism. Generally, such nucleic acid includes at least a portion complementary to the targeted gene, where the portion is sufficient to form a hybrid with the complementary portion.

As used herein, deletion, when referring to a nucleic acid or polypeptide sequence, refers to the deletion of one or more nucleotides or amino acids compared

to a sequence, such as a target polynucleotide or polypeptide or a native or wild-type sequence.

As used herein, insertion, when referring to a nucleic acid or amino acid sequence, describes the inclusion of one or more additional nucleotides or amino acids, within a target, native, wild-type or other related sequence. Thus, a nucleic acid molecule that contains one or more insertions compared to a wild-type sequence, contains one or more additional nucleotides within the linear length of the sequence.

As used herein, additions to nucleic acid and amino acid sequences describe addition of nucleotides or amino acids onto either terminus compared to another sequence.

As used herein, substitution or replacement refers to the replacing of one or more nucleotides or amino acids in a native, target, wild-type or other nucleic acid or polypeptide sequence with an alternative nucleotide or amino acid, without changing the length (as described in numbers of residues) of the molecule. Thus, one or more substitutions in a molecule does not change the number of amino acid residues or nucleotides of the molecule. Amino acid replacements compared to a particular polypeptide can be expressed in terms of the number of the amino acid residue along the length of the polypeptide sequence.

As used herein, at a position corresponding to, or a recitation that nucleotides or amino acid positions correspond to nucleotides or amino acid positions in a disclosed sequence, such as set forth in the Sequence Listing, refers to nucleotides or amino acid positions identified upon alignment with the disclosed sequence to maximize identity using a standard alignment algorithm, such as the GAP algorithm. By aligning the sequences, one skilled in the art can identify corresponding residues, for example, using conserved and identical amino acid residues as guides. In general, to identify corresponding positions, the sequences of amino acids are aligned so that the highest order match is obtained (see, *e.g.*, *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D. W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A.M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; *Sequence Analysis Primer*, Gribskov, M. and

Devereux, J., eds., M Stockton Press, New York, 1991; and Carrillo *et al.* (1988) *SIAM J Applied Math* 48:1073).

As used herein, alignment of a sequence refers to the use of homology to align two or more sequences of nucleotides or amino acids. Typically, two or more sequences that are related by 50% or more identity are aligned. An aligned set of sequences refers to 2 or more sequences that are aligned at corresponding positions and can include aligning sequences derived from RNAs, such as ESTs and other cDNAs, aligned with genomic DNA sequence. Related or variant polypeptides or nucleic acid molecules can be aligned by any method known to those of skill in the art. Such methods typically maximize matches, and include methods, such as using manual alignments and by using the numerous alignment programs available (*e.g.*, BLASTP) and others known to those of skill in the art. By aligning the sequences of polypeptides or nucleic acids, one skilled in the art can identify analogous portions or positions, using conserved and identical amino acid residues as guides. Further, one skilled in the art also can employ conserved amino acid or nucleotide residues as guides to find corresponding amino acid or nucleotide residues between and among human and non-human sequences. Corresponding positions also can be based on structural alignments, for example by using computer simulated alignments of protein structure. In other instances, corresponding regions can be identified. One skilled in the art also can employ conserved amino acid residues as guides to find corresponding amino acid residues between and among human and non-human sequences.

As used herein, a property of a polypeptide, such as an antibody, refers to any property exhibited by a polypeptide, including, but not limited to, binding specificity, structural configuration or conformation, protein stability, resistance to proteolysis, conformational stability, thermal tolerance, and tolerance to pH conditions. Changes in properties can alter an activity of the polypeptide. For example, a change in the binding specificity of the antibody polypeptide can alter the ability to bind an antigen, and/or various binding activities, such as affinity or avidity, or *in vivo* activities of the polypeptide.

As used herein, an activity or a functional activity of a polypeptide, such as an antibody, refers to any activity exhibited by the polypeptide. Such activities can be empirically determined. Exemplary activities include, but are not limited to, ability to

interact with a biomolecule, for example, through antigen-binding, DNA binding, ligand binding, or dimerization, or enzymatic activity, for example, kinase activity or proteolytic activity. For an antibody (including antibody fragments), activities include, but are not limited to, the ability to specifically bind a particular antigen, affinity of antigen-binding (*e.g.*, high or low affinity), avidity of antigen-binding (*e.g.*, high or low avidity), on-rate, off-rate, effector functions, such as the ability to promote antigen neutralization or clearance, virus neutralization, and *in vivo* activities, such as the ability to prevent infection or invasion of a pathogen, or to promote clearance, or to penetrate a particular tissue or fluid or cell in the body. Activity can be assessed *in vitro* or *in vivo* using recognized assays, such as ELISA, flow cytometry, surface plasmon resonance or equivalent assays to measure on- or off-rate, immunohistochemistry and immunofluorescence histology and microscopy, cell-based assays, flow cytometry and binding assays (*e.g.*, panning assays).

As used herein, bind, bound, and grammatical variations thereof refer to the participation of a molecule in any interaction with another molecule or among molecules, resulting in a stable association in which the molecules are in close proximity to one another. Binding includes, but is not limited to, non-covalent bonds, covalent bonds (such as reversible and irreversible covalent bonds), and includes interactions between molecules such as, but not limited to, proteins, nucleic acids, carbohydrates, lipids, and small molecules, such as chemical compounds including drugs.

As used herein, antibody refers to immunoglobulins and immunoglobulin fragments, whether natural or partially or wholly synthetically, such as recombinantly produced, including any fragment thereof containing at least a portion of the variable heavy chain and light region of the immunoglobulin molecule that is sufficient to form an antigen binding site and, when assembled, to specifically bind an antigen. Hence, an antibody includes any protein having a binding domain that is homologous or substantially homologous to an immunoglobulin antigen-binding domain (antibody combining site). For example, an antibody refers to an antibody that contains two heavy chains (which can be denoted H and H') and two light chains (which can be denoted L and L'), where each heavy chain can be a full-length immunoglobulin heavy chain or a portion thereof sufficient to form an antigen binding site (*e.g.*, heavy

chains include, but are not limited to, VH chains, VH-CH1 chains and VH-CH1-CH2-CH3 chains), and each light chain can be a full-length light chain or a portion thereof sufficient to form an antigen binding site (*e.g.*, light chains include, but are not limited to, VL chains and VL-CL chains). Each heavy chain (H and H') pairs with one light chain (L and L', respectively). Typically, antibodies minimally include all or at least a portion of the variable heavy (VH) chain and/or the variable light (VL) chain. The antibody also can include all or a portion of the constant region.

For purposes herein, the term antibody includes full-length antibodies and portions thereof including antibody fragments, such as anti-tumor antibody or anti-pathogen or gene silencing fragments. Antibody fragments, include, but are not limited to, Fab fragments, Fab' fragments, $F(ab')_2$ fragments, Fv fragments, disulfide-linked Fvs (dsFv), Fd fragments, Fd' fragments, single-chain Fvs (scFv), single-chain Fabs (scFab), diabodies, anti-idiotypic (anti-Id) antibodies, or antigen-binding fragments of any of the above. Antibody also includes synthetic antibodies, recombinantly produced antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), human antibodies, non-human antibodies, humanized antibodies, chimeric antibodies, and intrabodies. Antibodies provided herein include members of any immunoglobulin class (*e.g.*, IgG, IgM, IgD, IgE, IgA and IgY), any subclass (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or sub-subclass (*e.g.*, IgG2a and IgG2b).

As used herein, nucleic acid refers to at least two linked nucleotides or nucleotide derivatives, including a deoxyribonucleic acid (DNA) and a ribonucleic acid (RNA), joined together, typically by phosphodiester linkages. Also included in the term nucleic acid are analogs of nucleic acids such as peptide nucleic acid (PNA), phosphorothioate DNA, and other such analogs and derivatives or combinations thereof. Nucleic acids also include DNA and RNA derivatives containing, for example, a nucleotide analog or a backbone bond other than a phosphodiester bond, for example, a phosphotriester bond, a phosphoramidate bond, a phosphorothioate bond, a thioester bond, or a peptide bond (peptide nucleic acid). The term also includes, as equivalents, derivatives, variants, and analogs of either RNA or DNA made from nucleotide analogs, single (sense or antisense) and double-stranded nucleic acids. Deoxyribonucleotides include deoxyadenosine, deoxycytidine, deoxyguanosine and deoxythymidine. For RNA, the uracil base is uridine.

As used herein, an isolated nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid molecule. An isolated nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Exemplary isolated nucleic acid molecules provided herein include isolated nucleic acid molecules encoding RNAi or a therapeutic protein.

As used herein, operably linked with reference to nucleic acid sequences, regions, elements, or domains means that the nucleic acid regions are functionally related to each other. For example, a nucleic acid encoding a leader peptide can be operably linked to a nucleic acid encoding a polypeptide, whereby the nucleic acids can be transcribed and translated to express a functional fusion protein, wherein the leader peptide effects secretion of the fusion polypeptide. In some instances, the nucleic acid encoding a first polypeptide (*e.g.*, a leader peptide) is operably linked to a nucleic acid encoding a second polypeptide and the nucleic acids are transcribed as a single mRNA transcript, but translation of the mRNA transcript can result in one of two polypeptides being expressed. For example, an amber stop codon can be located between the nucleic acid encoding the first polypeptide and the nucleic acid encoding the second polypeptide, such that, when introduced into a partial amber suppressor cell, the resulting single mRNA transcript can be translated to produce either a fusion protein containing the first and second polypeptides, or can be translated to produce only the first polypeptide. In another example, a promoter can be operably linked to nucleic acid encoding a polypeptide, whereby the promoter regulates or mediates the transcription of the nucleic acid.

As used herein, a eukaryotic virus is a virus that contain a gene or genes that is expressed in and by eukaryotic host cell transcription/translational regulatory components. Exemplary eukaryotic viruses, include, for example adenovirus, adeno-associated virus, herpes viruses, vaccinia viruses, and other such viruses known to those of skill in the art.

As used herein, the reference to a promoter and/or other regulatory sequences that is/are part of the transgene refer to such sequences that control or participate in

production of a product encoded by the transgene. The ITR plasmids provided herein contain at least one ITR 3' or 5' a transgene, generally two flanking the transgene, and not other genes encoded by a virus, unless the gene or other such sequence is part of the transgene.

As used herein, synthetic, with reference to, for example, a synthetic nucleic acid molecule or a synthetic gene or a synthetic peptide refers to a nucleic acid molecule or polypeptide molecule that is produced by recombinant methods and/or by chemical synthesis methods.

As used herein, the residues of naturally occurring α-amino acids are the residues of those 20 α-amino acids found in nature which are incorporated into protein by the specific recognition of the charged tRNA molecule with its cognate mRNA codon in humans.

As used herein, polypeptide refers to two or more amino acids covalently joined. The terms polypeptide and protein are used interchangeably herein.

As used herein, a peptide refers to a polypeptide that is from 2 to about or 40 amino acids in length.

As used herein, reference to proteins, unless otherwise specified, includes all forms of peptides, polypeptides, small peptides, and proteins.

As used herein, an amino acid is an organic compound containing an amino group and a carboxylic acid group. A polypeptide contains two or more amino acids. For purposes herein, amino acids contained in the antibodies provided include the twenty naturally-occurring amino acids (see Table below), non-natural amino acids, and amino acid analogs (*e.g.*, amino acids wherein the α-carbon has a side chain). As used herein, the amino acids, which occur in the various amino acid sequences of polypeptides appearing herein, are identified according to their well-known, three-letter or one-letter abbreviations (see Table below). The nucleotides, which occur in the various nucleic acid molecules and fragments, are designated with the standard single-letter designations used routinely in the art.

As used herein, amino acid residue refers to an amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are generally in the L isomeric form. Residues in the D isomeric form can be substituted for any L-amino acid residue, as long as the desired

functional property is retained by the polypeptide. NH_2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxyl terminus of a polypeptide. In keeping with standard polypeptide nomenclature described in *J. Biol. Chem.*, 243:3557-59 (1968) and adapted at 37 C.F.R. §§ 1.821 - 1.822, abbreviations for amino acid residues are shown in the following Table:

Table of Correspondence

SYMBOL		
1-Letter	**3-Letter**	**AMINO ACID**
Y	Tyr	Tyrosine
G	Gly	Glycine
F	Phe	Phenylalanine
M	Met	Methionine
A	Ala	Alanine
S	Ser	Serine
I	Ile	Isoleucine
L	Leu	Leucine
T	Thr	Threonine
V	Val	Valine
P	Pro	Proline
K	Lys	Lysine
H	His	Histidine
Q	Gln	Glutamine
E	Glu	Glutamic acid
Z	Glx	Glutamic Acid and/or Glutamine
W	Trp	Tryptophan
R	Arg	Arginine
D	Asp	Aspartic acid
N	Asn	Asparagine
B	Asx	Aspartic Acid and/or Asparagine
C	Cys	Cysteine
X	Xaa	Unknown or other

All sequences of amino acid residues represented herein by a formula have a left to right orientation in the conventional direction of amino-terminus to carboxyl-terminus. The phrase amino acid residue is defined to include the amino acids listed in the above Table of Correspondence, modified, non-natural and unusual amino acids. A dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues or to an amino-terminal group such as NH_2 or to a carboxyl-terminal group such as COOH.

In a peptide or protein, suitable conservative substitutions of amino acids are known to those of skill in the art and generally can be made without altering a biological activity of a resulting molecule. Those of skill in the art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, *e.g.*, Watson *et al.*, *Molecular Biology of the Gene*, 4th Edition, 1987, The Benjamin/Cummings Pub. Co., p. 224).

Such substitutions can be made in accordance with the exemplary substitutions set forth in the following Table:

Exemplary conservative amino acid substitutions

Original residue	**Exemplary Conservative substitution(s)**
Ala (A)	Gly; Ser
Arg (R)	Lys
Asn (N)	Gln; His
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
Gly (G)	Ala; Pro
His (H)	Asn; Gln
Ile (I)	Leu; Val
Leu (L)	Ile; Val
Lys (K)	Arg; Gln; Glu
Met (M)	Leu; Tyr; Ile
Phe (F)	Met; Leu; Tyr
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp; Phe
Val (V)	Ile; Leu

Other substitutions also are permissible and can be determined empirically or in accord with other known conservative or non-conservative substitutions.
As used herein, naturally occurring amino acids refer to the 20 L-amino acids that occur in polypeptides.

As used herein, the term non-natural amino acid refers to an organic compound that has a structure similar to a natural amino acid but has been modified structurally to mimic the structure and reactivity of a natural amino acid. Non-naturally occurring amino acids thus include, for example, amino acids or analogs of amino acids other than the 20 naturally occurring amino acids and include, but are not limited to, the D-stereoisomers of amino acids. Exemplary non-natural amino acids are known to those of skill in the art, and include, but are not limited to, 2-

Aminoadipic acid (Aad), 3-Aminoadipic acid (bAad), β-alanine/β-Amino-propionic acid (Bala), 2-Aminobutyric acid (Abu), 4-Aminobutyric acid/piperidinic acid (4Abu), 6-Aminocaproic acid (Acp), 2-Aminoheptanoic acid (Ahe), 2-Aminoisobutyric acid (Aib), 3-Aminoisobutyric acid (Baib), 2-Aminopimelic acid (Apm), 2,4-Diaminobutyric acid (Dbu), Desmosine (Des), 2,2'-Diaminopimelic acid (Dpm), 2,3-Diaminopropionic acid (Dpr), N-Ethylglycine (EtGly), N-Ethylasparagine (EtAsn), Hydroxylysine (Hyl), allo-Hydroxylysine (Ahyl), 3-Hydroxyproline (3Hyp), 4-Hydroxyproline (4Hyp), Isodesmosine (Ide), allo-Isoleucine (Aile), N-Methylglycine, sarcosine (MeGly), N-Methylisoleucine (MeIle), 6-N-Methyllysine (MeLys), N-Methylvaline (MeVal), Norvaline (Nva), Norleucine (Nle), and Ornithine (Orn).

As used herein, a DNA construct is a single or double stranded, linear, or circular DNA molecule that contains segments of DNA combined and juxtaposed in a manner not found in nature. DNA constructs exist as a result of human manipulation, and include clones and other copies of manipulated molecules.

As used herein, a DNA segment is a portion of a larger DNA molecule having specified attributes. For example, a DNA segment encoding a specified polypeptide is a portion of a longer DNA molecule, such as a plasmid or plasmid fragment, which, when read from the 5' to 3' direction, encodes the sequence of amino acids of the specified polypeptide.

As used herein, the term polynucleotide means a single- or double-stranded polymer of deoxyribonucleotides or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and can be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. The length of a polynucleotide molecule is given herein in terms of nucleotides (abbreviated nt) or base pairs (abbreviated bp). The term nucleotides is used for single- and double-stranded molecules where the context permits. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term base pairs. It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide can differ slightly in length and that the ends thereof can be staggered; thus, all

nucleotides within a double-stranded polynucleotide molecule cannot be paired. Such unpaired ends will, in general, not exceed 20 nucleotides in length.

As used herein, production by recombinant methods refers means the use of the well-known methods of molecular biology for expressing proteins encoded by cloned DNA.

As used herein, heterologous nucleic acid is nucleic acid that encodes products (*i.e.*, RNA and/or proteins) that are not normally produced *in vivo* by the cell in which it is expressed, or nucleic acid that is in a locus in which or at which it does not normally occur, or that mediates or encodes mediators that alter expression of endogenous nucleic acid, such as DNA, by affecting transcription, translation, or other regulatable biochemical processes. Heterologous nucleic acid, such as DNA, also is referred to as foreign nucleic acid. Any nucleic acid, such as DNA, that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which it is expressed, is herein encompassed by heterologous nucleic acid; heterologous nucleic acid includes exogenously added nucleic acid that is also expressed endogenously. Heterologous nucleic acid is generally not endogenous to the cell into which it is introduced, but has been obtained from another cell or prepared synthetically or is introduced into a genomic locus in which it does not occur naturally, or its expression is under the control of regulatory sequences or a sequence that differs from the natural regulatory sequence or sequences.

Examples of heterologous nucleic acid herein include, but are not limited to, a DNA molecule, an RNA molecule, a plasmid, and an antisense oligonucleotide. In the MEV, the heterologous nucleic acid can be encoded on a plasmid. Heterologous nucleic acid, such as DNA, includes nucleic acid that can, in some manner, mediate expression of DNA that encodes a therapeutic product, or it can encode a product, such as a peptide or RNA, that in some manner mediates, directly or indirectly, expression of a therapeutic product.

As used herein, cell therapy involves the delivery of MEVs to a subject to treat a disease or condition. The MEVs are exogenously loaded with cargo, so that they deliver or express products when introduced to a subject. The MEVs also can be endogenously loaded with cargo (see, *e.g.*, copending U.S. provisional application Serial No. 63/349,006, filed on June 03, 2022, which details preparation of

endogenously-loaded MEVs and producer cell lines thereof), and used as described herein. The trafficking of MEVs generally is independent of manner in which they are loaded with cargo. The microalgae can be modified to alter properties of the resulting MEVs. Endogenously-loaded MEVs can be used in the methods and compositions described herein.

As used herein, genetic therapy involves the transfer of heterologous nucleic acid, such as DNA, into certain cells, such as target cells, of a mammal, particularly a human, with a disorder or condition for which such therapy is sought. The nucleic acid, such as DNA, is introduced into the selected target cells in a manner such that the heterologous nucleic acid, such as DNA, is expressed and a therapeutic product(s) encoded thereby is produced. Genetic therapy also can be used to deliver nucleic acid encoding a gene product that replaces a defective gene or supplements a gene product produced by the mammal or the cell in which it is introduced. The introduced nucleic acid can encode a therapeutic compound, such as a growth factor or inhibitor thereof, or a tumor necrosis factor or inhibitor thereof, such as a receptor thereof, that is not normally produced in the mammalian host or that is not produced in therapeutically effective amounts or at a therapeutically useful time. The heterologous nucleic acid, such as DNA, encoding the therapeutic product, can be modified prior to introduction into the cells of the afflicted host in order to enhance or otherwise alter the product or expression thereof. Genetic therapy can also involve delivery of an inhibitor or repressor or other modulator of gene expression.

As used herein, expression refers to the process by which polypeptides are produced by transcription and translation of polynucleotides. The level of expression of a polypeptide can be assessed using any method known in art, including, for example, methods of determining the amount of the polypeptide produced from the host cell. Such methods can include, but are not limited to, quantitation of the polypeptide in the cell lysate by ELISA, Coomassie blue staining following gel electrophoresis, Lowry protein assay and Bradford protein assay.

As used herein, a host cell is a cell that is used to receive, maintain, reproduce and/or amplify a vector. A host cell also can be used to express the polypeptide encoded by the vector. The nucleic acid contained in the vector is replicated when the host cell divides, thereby amplifying the nucleic acids.

As used herein, a vector is a replicable nucleic acid from which one or more heterologous proteins can be expressed when the vector is transformed into an appropriate host cell. Reference to a vector includes those vectors into which a nucleic acid encoding a polypeptide or fragment thereof can be introduced, typically by restriction digest and ligation. Reference to a vector also includes those vectors that contain nucleic acid encoding a polypeptide or RNA. The vector is used to introduce the nucleic acid encoding the polypeptide into the host cell for amplification of the nucleic acid or for expression/display of the polypeptide encoded by the nucleic acid. The vectors typically remain episomal but can be designed to effect integration of a gene or portion thereof into a chromosome of the genome. Also contemplated are vectors that are artificial chromosomes, such as yeast artificial chromosomes and mammalian artificial chromosomes. Selection and use of such vehicles are well-known to those of skill in the art. A vector also includes virus vectors or viral vectors. Viral vectors are engineered viruses that are operatively linked to exogenous genes to transfer (as vehicles or shuttles) the exogenous genes into cells.

As used herein, an expression vector includes vectors capable of expressing DNA that is operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Such additional segments can include promoter and terminator sequences, and optionally can include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or can contain elements of both. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus, or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well-known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

As used herein, primary sequence refers to the sequence of amino acid residues in a polypeptide or the sequence of nucleotides in a nucleic acid molecule.

As used herein, sequence identity refers to the number of identical or similar amino acids or nucleotide bases in a comparison between a test and a reference poly-

peptide or polynucleotide. Sequence identity can be determined by sequence alignment of nucleic acid or protein sequences to identify regions of similarity or identity. For purposes herein, sequence identity is generally determined by alignment to identify identical residues. The alignment can be local or global. Matches, mismatches, and gaps can be identified between compared sequences. Gaps are null amino acids or nucleotides inserted between the residues of aligned sequences so that identical or similar characters are aligned. Generally, there can be internal and terminal gaps. When using gap penalties, sequence identity can be determined with no penalty for end gaps (*e.g.*, terminal gaps are not penalized). Alternatively, sequence identity can be determined without taking into account gaps as the number of identical positions/length of the total aligned sequence x 100.

For purposes herein, sequence identity is determined by aligning the test polypeptide or nucleic acid molecule with the reference molecule and counting the number of differences (or employing software to do so), including gaps and insertions. The number of differences is divided by the length of reference molecule, generally the molecule whose sequence is claimed, and percentage determined. For example, if the polypeptide that is claimed is 100 amino acids in length, and variants include those with 90% sequence identity, then the variants can have 10 amino acid differences, including gaps and insertions.

As used herein, a global alignment is an alignment that aligns two sequences from beginning to end, aligning each letter in each sequence only once. An alignment is produced, regardless of whether there is similarity or identity between the sequences. For example, 50% sequence identity based on global alignment means that in an alignment of the full sequence of two compared sequences each of 100 nucleotides in length, 50% of the residues are the same. It is understood that global alignment also can be used in determining sequence identity even when the length of the aligned sequences is not the same. The differences in the terminal ends of the sequences will be taken into account in determining sequence identity, unless the no penalty for end gaps is selected. Generally, a global alignment is used on sequences that share significant similarity over most of their length. Exemplary algorithms for performing global alignment include the Needleman-Wunsch algorithm (Needleman *et al.* (1970) *J. Mol. Biol.* 48: 443). Exemplary programs for performing global

alignment are publicly available and include the Global Sequence Alignment Tool available at the National Center for Biotechnology Information (NCBI) website (ncbi.nlm.nih.gov/), and the program available at deepc2.psi.iastate.edu/aat/align/align.html.

As used herein, a local alignment is an alignment that aligns two sequences, but only aligns those portions of the sequences that share similarity or identity. Hence, a local alignment determines if sub-segments of one sequence are present in another sequence. If there is no similarity, no alignment will be returned. Local alignment algorithms include BLAST or Smith-Waterman algorithm (*Adv. Appl. Math.* 2: 482 (1981)). For example, 50% sequence identity based on local alignment means that in an alignment of the full sequence of two compared sequences of any length, a region of similarity or identity of 100 nucleotides in length has 50% of the residues that are the same in the region of similarity or identity.

For purposes herein, sequence identity can be determined by standard alignment algorithm programs used with default gap penalties established by each supplier. Default parameters for the GAP program can include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov *et al.* (1986) *Nucl. Acids Res.* 14:6745, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358 (1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Whether any two nucleic acid molecules have nucleotide sequences or any two polypeptides have amino acid sequences that are at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical, or other similar variations reciting a percent identity, can be determined using known computer algorithms based on local or global alignment (*see e.g.*, wikipedia.org/wiki/Sequence_alignment_software, providing links to dozens of known and publicly available alignment databases and programs). Generally, for purposes herein sequence identity is determined using computer algorithms based on global alignment, such as the Needleman-Wunsch Global Sequence Alignment tool available from NCBI/BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&Page_TYPE=BlastHome); LAlign (William Pearson implementing the Huang and Miller algorithm (*Adv. Appl. Math.*

(1991) 12:337-357)); and program from Xiaoqui Huang available at deepc2.psi.iastate.edu/aat/align/align.html. Typically, the full-length sequence of each of the compared polypeptides or nucleotides is aligned across the full-length of each sequence in a global alignment. Local alignment also can be used when the sequences being compared are substantially the same length.

Therefore, as used herein, the term identity represents a comparison or alignment between a test and a reference polypeptide or polynucleotide. In one non-limiting example, at least 90% identical to refers to percent identities from 90 to 100% relative to the reference polypeptide or polynucleotide. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polypeptide or polynucleotide length of 100 amino acids or nucleotides are compared, no more than 10% (*i.e.*, 10 out of 100) of amino acids or nucleotides in the test polypeptide or polynucleotide differ from those of the reference polypeptide. Similar comparisons can be made between a test and reference polynucleotides. Such differences can be represented as point mutations randomly distributed over the entire length of an amino acid sequence or they can be clustered in one or more locations of varying length up to the maximum allowable, *e.g.*, 10/100 amino acid difference (approximately 90% identity). Differences also can be due to deletions, insertions or truncations of amino acid residues. Differences are defined as nucleic acid or amino acid substitutions, insertions, or deletions. Depending on the length of the compared sequences, at the level of homologies or identities above about 85-90%, the result can be independent of the program and gap parameters set; such high levels of identity can be assessed readily, often without relying on software.

In general, determination of sequence identity, such as between two polypeptides, is effected by aligning the polypeptides to maximize the number of matching polypeptides and counting the number of differences in between the two sequences, one sequence is the reference sequence, and the other is the sequence whose identity is determined. If the claim recites a sequence that is 100 amino acids in length and the claim recites that the claimed polypeptide has 95% sequence identity thereto, there can be 5 amino acid differences between the two sequences. The differences include insertions or deletions (missing amino acids).

As used herein, a pharmaceutically effective agent includes any therapeutic agent or bioactive agents, including, but not limited to, for example, anesthetics, vasoconstrictors, dispersing agents, and conventional therapeutic drugs, including small molecule drugs and therapeutic proteins.

As used herein, a therapeutic effect means an effect resulting from treatment of a subject that alters, typically improves, or ameliorates, the symptoms of a disease or condition or that cures a disease or condition.

As used herein, a therapeutically effective amount or a therapeutically effective dose refers to the quantity of an agent, compound, material, or composition containing a compound that is at least sufficient to produce a therapeutic effect following administration to a subject. Hence, it is the quantity necessary for preventing, curing, ameliorating, arresting, or partially arresting a symptom of a disease or disorder.

As used herein, therapeutic efficacy refers to the ability of an agent, compound, material, or composition containing a compound to produce a therapeutic effect in a subject to whom the agent, compound, material, or composition containing a compound has been administered.

As used herein, a prophylactically effective amount or a prophylactically effective dose refers to the quantity of an agent, compound, material, or composition containing a compound that when administered to a subject, will have the intended prophylactic effect, *e.g.*, preventing or delaying the onset, or reoccurrence, of disease or symptoms, reducing the likelihood of the onset, or reoccurrence, of disease or symptoms, or reducing the incidence of viral infection. The full prophylactic effect does not necessarily occur by administration of one dose, and can occur only after administration of a series of doses. Thus, a prophylactically effective amount can be administered in one or more administrations.

As used herein, amelioration of the symptoms of a particular disease or disorder by a treatment, such as by administration of a pharmaceutical composition or other therapeutic, refers to any lessening, whether permanent or temporary, lasting, or transient, of the symptoms that can be attributed to or associated with administration of the composition or therapeutic.

As used herein, an anti-cancer agent refers to any agent that is destructive or toxic to malignant cells and tissues. For example, anti-cancer agents include agents that kill cancer cells or otherwise inhibit or impair the growth of tumors or cancer cells. Exemplary anti-cancer agents are chemotherapeutic agents.

As used herein therapeutic activity refers to the *in vivo* activity of a therapeutic polypeptide. Generally, the therapeutic activity is the activity that is associated with treatment of a disease or condition.

As used herein, a composition refers to any mixture. It can be a solution, suspension, liquid, powder, paste, aqueous, non-aqueous, or any combination thereof.

As used herein, a combination refers to any association between or among two or more items. The combination can be two or more separate items, such as two compositions or two collections, a mixture thereof, such as a single mixture of the two or more items, or any variation thereof. The elements of a combination are generally functionally associated or related.

As used herein, combination therapy refers to administration of two or more different therapeutics. The different therapeutic agents can be provided and administered separately, sequentially, intermittently, or can be provided in a single composition.

As used herein, a kit is a packaged combination that optionally includes other elements, such as additional reagents and instructions for use of the combination or elements thereof, for a purpose including, but not limited to, activation, administration, diagnosis, and assessment of a biological activity or property.
As used herein, a unit dose form refers to physically discrete units suitable for human and animal subjects and packaged individually as is known in the art.

As used herein, a single dosage formulation refers to a formulation for direct administration.

As used herein, a multi-dose formulation refers to a formulation that contains multiple doses of a therapeutic agent and that can be directly administered to provide several single doses of the therapeutic agent. The doses can be administered over the course of minutes, hours, weeks, days, or months. Multi-dose formulations can allow dose adjustment, dose-pooling and/or dose-splitting. Because multi-dose formulations

are used over time, they generally contain one or more preservatives to prevent microbial growth.

As used herein, an emulsion is a system composed of two immiscible liquid phases, intimately mixed and dispersed, one into the other. An emulsion refers to a colloidal dispersion of two immiscible liquids, for example, an oil and water (or other aqueous liquid, *e.g.*, a polar solvent), one of which is part of a continuous phase and the other of which is part of a dispersed phase Emulsions typically are stabilized by one or more surfactants and/or co-surfactants and/or emulsion stabilizers. Surfactants form an interfacial film between the oil and water phase of the emulsion, providing stability. Microemulsion and nanoemulsion are two different types of emulsions. An emulsion is a fine dispersion of minute droplets of one liquid in another in which it is not soluble or miscible. Microemulsions are thermodynamically stable, whereas nanoemulsions are thermodynamically unstable.

As used herein, a microemulsion is a two-phase system composed of a lipid phase and an aqueous phase, finely interspersed. Microemulsions are liquid mixtures, transparent, isotropic and stable, of a lipid phase and an aqueous phase, held together by a surfactant, generally in conjunction with a co-surfactant. Microemulsions are clear, thermodynamically stable, isotropic liquid mixtures of oil, water and surfactant, and optionally a co-surfactant. Microemulsions form spontaneously upon mixing of the aqueous phase and lipid phase. A microemulsion is, therefore, a thermodynamically stable system, with particles dispersed in the continuous phase. The droplet size of the dispersed phase in a microemulsion is less than 100 nm, generally in the range between 5 nm and 50 nm, inclusive. In contrast, a nanoemulsion requires input of energy to form the emulsion; a nanoemulsion is thermodynamically unstable, but is kinetically stable. Eyedrop formulations provided herein include nanoemulsions and microemulsions.

As used herein, "surfactant" refers to synthetic and naturally occurring amphiphilic molecules that have hydrophobic portion(s) and hydrophilic portion(s). Surfactants contain a hydrophilic domain and hydrophobic domain, *i.e.,* amphiphilic molecules. Given their nature, surfactants facilitate the formation of oil-in-water emulsions where the micelles, in order to exist, need to interact with both the water and the oil phases. Due to their amphiphilic (amphipathic) nature, surfactants and co-

surfactants can reduce the surface tension between two immiscible liquids, for example, the oil and water phases in an emulsion, such as a microemulsion, stabilizing the emulsion. Surfactants can be characterized based on their relative hydrophobicity and/or hydrophilicity. For example, relatively lipophilic surfactants are more soluble in fats, oils, and waxes, typically having Hydrophobic-Lipophilic Balance (HLB) values less than 10 or about 10, while relatively hydrophilic surfactants are more soluble in aqueous compositions, for example, water, and typically have HLB values greater than 10 or about 10. Relatively amphiphilic surfactants are soluble in oil and water-based liquids and typically have HLB values close to 10 or about 10. Surfactants for use in the compositions herein are biocompatible.

As used herein, a co-surfactant is a surfactant that acts in addition to another surfactant to further reduce the surface tension of a liquid. For example, recitation that microemulsions contain surfactants refers to the surfactants and the co-surfactants that are included. Co-surfactants are hydrophilic in nature, and reduce the surface tension of water. They generally are used as wetting agents, for example, to increase the spreading abilities of water-based fluid by reducing the surface tension of water. Cosurfactants also are used, and often needed, to increase the solubility of the primary surfactant.

As used herein, “particle size” and “average particle size” refer synonymously to the average diameter of particles in a provided liquid, for example, the droplet diameter or micelle diameter in an emulsion

As used herein, “oil phase” or “lipid phase” refers to the portion (or phase) of a composition hat contains one or more lipophilic ingredients and/or amphiphilic ingredients, such as an oil, and is, in general, the lipid-soluble phase. In an oil-in-water (o/w) microemulsion, the lipid phase typically is the dispersed phase while water is the dispersion phase.

As used herein, oil phase ingredient(s) refers to the components of the provided compositions that are included in the oil Typical oil phase ingredients include non-polar compounds, e.g., non-polar active ingredients; at least one surfactant; oils, such as non-polar solvents; preservatives; and microemulsion

stabilizers. Other lipophilic and/or amphiphilic ingredients can be included in the oil phase.

As used herein, "water phase" or "aqueous phase" refers to the portion (phase) of a composition, such as those provided herein, that contains one or more hydrophilic ingredients and/or amphiphilic ingredients (water phase ingredients) and is, in general, the water-soluble phase. Typically, in the microemulsion compositions provided herein, the water phase is the continuous phase. "Water phase" also is used to refer to the liquid containing the water phase ingredients that is generated while preparing microemulsions.

As used herein, water phase ingredient(s) refers to the components of the compositions that are included in the water phase in the provided methods for making the compositions. Typical water phase ingredients can include, but are not limited to, polar solvents, typically polar protic solvents, such as water and alcohols, typically alcohols having more than one hydroxy group such as dihydroxy and trihydroxy alcohols, such as glycerol and propylene glycol; at least one surfactant; preservatives; and emulsion stabilizers. Other hydrophilic and/or amphiphilic ingredients can be included in the water phase.

As used herein, thermodynamic stability of the microemulsions refers to the stability of the dispersion such that the phases do not separate. The microemulsions provided herein exhibit high thermodynamic stability as shown by their stability at elevated temperatures.

As used herein, room temperature and ambient temperature are used to describe a temperature that is common in one or more enclosed spaces in which human beings typically are or reside. Room temperature can vary, but generally refers to temperatures between 19 °C or about 19 °C and 25 °C or about 25 °C. When a composition is stored at room temperature, it should be understood it is generally kept at a temperature within this range or about within this range.

As used herein, an article of manufacture is a product that is made and sold. As used throughout this application, the term is intended to encompass any of the compositions provided herein contained in articles of packaging.

As used herein, a fluid refers to any composition that can flow. Fluids thus encompass compositions that are in the form of semi-solids, pastes, solutions, aqueous mixtures, gels, lotions, creams, and other such compositions.

As used herein, an isolated or purified polypeptide or protein (*e.g.*, an isolated antibody or antigen-binding fragment thereof) or biologically-active portion thereof (*e.g.*, an isolated antigen-binding fragment) is substantially free of cellular material or other contaminating proteins from the cell or tissue from which the protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. Preparations can be determined to be substantially free if they appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification does not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound, however, can be a mixture of stereoisomers. In such instances, further purification might increase the specific activity of the compound.

As used herein, a cellular extract or lysate refers to a preparation or fraction which is made from a lysed or disrupted cell.

As used herein, a control refers to a sample that is substantially identical to the test sample, except that it is not treated with a test parameter, or, if it is a plasma sample, it can be from a normal volunteer not affected with the condition of interest. A control also can be an internal control.

As used herein, psilocin is the active form of psilocybin. Psilocin is produced by oxidation of psilocybin in the liver. For purposes herein, in the context of MEV-mediated delivery, psilocybin and psilocin should have the same meaning: a mention to 'psilocybin' shall mean to 'psilocin' and vice versa.

As used herein, a tropism of an MEV refers to cells, tissues, and/or organs in where the MEVs, upon administration, accumulate. Unless specified the MEVs provided herein are not modified to provide a specific tropism or targeting property.

As used herein, trafficking of the MEVs upon administration refers to the pathways by which the MEVs travel to their ultimate destination or destinations. For example, as shown and described herein, intranasally administered MEVs traverse nerves and neurons to the locations in the brain connected to or adjacent to the neurons.

As used herein, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to a polypeptide, comprising an immunoglobulin domain includes polypeptides with one or a plurality of immunoglobulin domains.

As used herein, the term "or" is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive.

As used herein, ranges and amounts can be expressed as about a particular value or range. "About" also includes the exact amount. Hence about 5 amino acids means about 5 amino acids and also 5 amino acids. Generally, although depending upon context and the understanding of one of skill in the particular art, "about" includes a range of ±10%.

As used herein, "optional" or "optionally" means that the subsequently described event or circumstance does or does not occur and that the description includes instances where said event or circumstance occurs and instances where it does not. For example, an optionally variant portion means that the portion is variant or non-variant.

As used herein, the abbreviations for any protective groups, amino acids, and other compounds, are, unless indicated otherwise, in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature (see, *Biochem.* (1972) 11(9):1726-1732).

For clarity of disclosure, and not by way of limitation, the detailed description is divided into the subsections that follow.

B. OVERVIEW

Provided are microalgae extracellular vesicles gene therapy vectors (MEV-GTVs), which are MEVs that contain a plasmid encoding a transgene of interest and at least one, generally two ITRs flanking the transgene. Also provided are the circular plasmids that contain ITRs and a transgene or transgenes. For production of the

circular minigene plasmids, also provided are bacterial plasmids that contain the ITRs and the transgene. The bacterial plasmids are propagated in bacterial hosts, and can include, flanking the ITR and transgene constructs, sequences that can be induced to recombine or that recombine to release the ITR-transgene construct, which, when can circularize.

The presence of the ITR in the plasmid confers episomal survival of the plasmid and therefore the long-lasting expression of the payload, including encoded products. The ITRs are the only 'part' of the AAV or other viral vector that is included in the MEV-GTVs; there are no viral proteins (cap, rep), no genetic elements of the virus, such as AAV, with the only exception of the ITRs. There is no need for complex manufacturing procedures so that they are easier and cheaper to manufacture, they have better safety profile (non-viral, non-mammalian), since as shown (copending application International PCT application No: PCT/EP2024/057645 published as International PCT Publication No. WO2024/194423) there is no adverse immune response. The MEV-GTVs provide for delivery and sustained expression encoded products.

The resulting plasmids are introduced in the MEVs, which serve to encapsulate the plasmid constructs, and when administered, the MEVs deliver the encoded products to tissues and organs. As detailed herein and in co-owned publications (see, International PCT publication Nos. WO2023001894, WO 2023/144127, and WO2024088808, and copending application International PCT application No: PCT/EP2024/057645, published as International PCT Publication No. WO2024/194423, and U.S. provisional application Serial No. 63/562,941). MEVs can be produced and exogenously loaded with cargo of interest, and administered to a subject, such as a human, by various routes, including orally, intramuscularly, intranasally, into the eye, and other routes. The route of administration determines the *in vivo* fate of the administered MEVs, and the route can be selected for targeting a tissue or organ.

The resulting MEV-GTVs thus provide encapsulated nucleic acid delivery vectors for gene therapy. The MEVs serve as the capsid containing the circularized minigene plasmid (see, *e.g.*, Figure 4, which depicts the MEV-GTVs (figure 4, image

D) compared to AAV vectors, and prior art exosomes that contain the AAV vector including the capsid). Advantages of the MEV-GTVs are detailed herein.

C. ADENO-ASSOCIATED VIRAL (AAV) VECTORS

Adeno-associated viral (AAV) vectors have been developed and are among the gene therapy vectors approved for use. These small viruses, capable of infecting humans and other primate species, belong to the genus *Dependoparvovirus* within the family *Parvoviridae*. Initially identified as a contaminant in an adenovirus preparation in AAV has since become a pivotal focus for gene therapy applications. Several AAV serotypes have been described, exhibiting variations in their capsid structure, influencing their tropism and immunogenicity in host organisms. Among the naturally occurring serotypes, AAV2 has been the most extensively characterized and frequently employed in research and clinical applications. AAV viruses and vectors with modified or chimeric capsids have been developed, generally to enhance or alter a tropism.

AAVs (depicted in Figure 1) are non-enveloped viruses, less than 30 nm in diameter and with a linear single-stranded DNA (ssDNA) genome of approximately 4.7 kilobases in size. AAVs are known for their ability to maintain their genome in the form of an episome within the host cell, allowing for persistent and stable gene expression without integrating into the host genome. Central to AAV functionality are the Inverted Terminal Repeats (ITRs), each approximately 145 bases in length, flanking the AAV genome. These ITRs play a pivotal role in AAV replication, transcription regulation, and genome packaging, The ITRs are essential for the genetic material delivered by the AAV inside the cell to behave as an episome (and thus to replicate independently from the replication of the host cell); this property is important for long-lasting survival of the vector and long-lasting expression of the encoded product(s). This particular feature of AAV makes them extremely attractive for gene therapy applications as they can eventually assure a long-lasting expression of the desired ('cured') phenotype in the absence of integration of the viral material in the host genome; lack of integration is highly desirable for safety reasons.

Figure 1 provides a schematic diagram of the AAV genome flanked by ITRs, and encoding the Cap and Rep AAV genes [adapted from: Asaad *et al.* (2023). AAV genome modification for efficient AAV production. Heliyon, 9(4), e15071].

The simplicity of the genome and non-pathogenic characteristics of AAVs render them as often used gene therapy vectors. AAV vectors have demonstrated efficacy in treating central nervous system (CNS) disorders, retinal degenerative diseases, various muscular dystrophies, and conditions affecting the heart, lungs, and liver. AAV-based gene therapy vectors face several key challenges, such as a limited packaging capacity of AAV, the complexity and cost of manufacturing, the generation of neutralizing antibodies, the related impossibility to repeat administrations, and the needs to be persisting to cure, among other challenges. As detailed herein, the MEV-GTVs provided herein address these challenges.

1. AAV-based vectors and ITR role and function

AAVs have been genetically engineered to create Adeno-Associated Viral Vectors (AAV vectors) with specific properties suitable for delivering therapeutic genes into target cells. Engineered AAVs serve as efficient and safe vectors for targeted DNA delivery into cells and tissues. AAV vectors are particularly favored in gene therapy for several reasons: (1) they are replication-defective, ensuring they do not reproduce within the host cells; (2) they are non-integrating, minimizing the risk of altering the host genome; and (3) they exhibit low immunogenicity (other than the neutralizing reaction), reducing the likelihood of triggering an immune response.

In the AAV-derived vectors, about 96% of the AAV genome is replaced with a gene cassette of interest, leaving behind only the Inverted Terminal Repeats (ITRs), essential elements within the AAV vectors. ITR sequences, primarily derived from AAV serotype 2, are fundamental for multiple processes, including genome rescue, replication, packaging, and vector persistence. Each AAV construct comprises two ITR sequences, each spanning 145 bases and flanking the transgene. These ITRs form a palindromic structure characterized by a high GC content and the ability to form a hairpin structure (see Figure 2) that facilitates self-priming, allowing primase-independent synthesis of the second DNA strand. Figure 2 shows the secondary structure of the ITR for AAV serotype 2. The characteristic 'T' shape of the ITR is formed by two adjacent inverted repeats (BB' and CC') separated by a single unpaired nucleotide. A third inverted repeat is the central AA', forming a three-way junction. The D sequences do not participate in the T secondary structure. The replicase binding element (RBE) is formed by specific tetranucleotide repeats. The

'flip' and 'flop' sequences exist in equal proportion on either end of AAV genomes [adapted from: Wilmott *et al.* (2019). A User's Guide to the Inverted Terminal Repeats of Adeno-Associated Virus. Human Gene Therapy Methods, 30(6), 206-213].

The ITR sequence possesses intrinsic transcriptional activity, capable of driving expression of the intended transgene in therapeutic applications [see, Earley *et al.* (2020). Adeno-Associated Virus serotype-specific Inverted Terminal Repeat sequence role in vector transgene expression. Human gene therapy, 31(3-4), 151–162]. Numerous ITR sequences, including natural ITRs(derived from naturally occurring AAV serotypes) and synthetic ITRs, can be employed in the plasmids. Figure 3 shows examples of the sequence and structures of ITRs originating from AAV serotypes 1, 2, 3, 4, 6, and 7 with bold letters denoting nucleotides that are not conserved among the ITR sequences. ITR exist in other eukaryotic viruses and can be employed in the ITR-containing plasmids and delivery vehicles containing the plasmids, including the MEV-GTVs provided herein.

For gene therapy applications, ITRs, detailed and shown herein, are the only sequences required *in cis* next to the therapeutic gene. While structural (cap) and packaging (rep) proteins can be delivered *in trans*, ITRs play a critical role in efficient vector replication and transgene expression. AAV vectors undergo replication as an extrachromosomal circular (episomal) DNA molecule, allowing for persistence of the AAV genome in the host cell without integrating into the host genome. Upon entering the host cell, the AAV genetic vector forms an episomal structure that is replicated by the host cell machinery. Episomes can persist stably in the host cell over an extended period, which is favorable for gene therapy applications. Long-term expression of therapeutic genes is possible, and avoids host integration-related complications, including the risk associated with random insertion of the vector into the host genome.

Other ITRs can be employed. The following table provides a list of some known ITRs and sources thereof.

Exemplary Viral and Artificial Inverted Terminal Repeat Sequences			
Organism	**GenBank Accession No.**	**Nucleotide annotation**	**Reference**
Vaccinia virus (Copenhagen)	N/A	Source: 1..175860 ITR: 167703 and 175860	WO2024038175A1 (SEQ ID NO: 4)
Vaccinia virus (Wyeth)	N/A	Source: 1..182664 ITR: 166359 and	WO2024038175A1 (SEQ ID NO: 5)

Exemplary Viral and Artificial Inverted Terminal Repeat Sequences			
Organism	**GenBank Accession No.**	**Nucleotide annotation**	**Reference**
		182664	
Vaccinia virus (Western Reserve)	N/A	Source: 1..181419 ITR: 174482 and 181419	WO2024038175A1 (SEQ ID NO: 6)
Vaccinia virus (MVA)	N/A	Source: 1..163444 ITR: 159457 and 163444	WO2024038175A1 (SEQ ID NO: 7)
Rabbitpox virus (Utrecht)	N/A	Source: 1..186491 ITR: 183030 and 186491	WO2024038175A1 (SEQ ID NO: 2)
Cowpox (Brighton)	N/A	Source: 1..212521 ITR: 206015 and 212521	WO2024038175A1 (SEQ ID NO: 3)
Sheeppox virus	JN118986.1	Source: 1..290 ITR: 1..290	
Sheeppox virus V104	MW020571.1	Source: 1..150211 ITRs: 88..2460; 147752..150124	Viruses 12 (10), E1098 (2020)
Goatpox virus V103	MW020570.1	Source: 1..150578 ITR: 90..2480; 148080..150470	Viruses 12 (10), E1098 (2020)
Variola virus	U23793.1	Source: 1..515 ITRs: 72..140; 141..194	Virology 201 (2), 215-240 (1994)
Variola virus	U22363.1	Source: 1..724 ITR: 72..400	Virology 211 (1), 350-355 (1995)
Deer adenovirus A	OP405339.1	Source: 1..30666 ITRs: 1..29; 30638..30666	
Skunk adenovirus 1	KP238322.1	Source: 1..31848 ITR: 1..181; 31668..31848	Virology 485, 16-24 (2015)
Guinea pig adenovirus strain AUS96	NC_075973.1	Source: 1..37031 ITRs: 1..166; 36866..37031	
Bovine adenovirus 1	M17112.1	Source: 1..159 ITR: 1..159	Gene 55 (1), 85-93 (1987)
bovine adenovirus 5	AF238881.1	Source: 1..67 ITR: 1..67	Virus Genes 22 (2), 175-179 (2001)
bovine adenovirus 4	AF238883.1	Source: 1..59 ITR: 1..59	Virus Genes 22 (2), 175-179 (2001)
Bovine adenovirus 10	AF238882.1	Source: 1..368 ITR: 1..368	Virus Genes 22 (2), 175-179 (2001)
Equine adenovirus 1	AH002313.2	Source: 1..320 5' ITR 1..110; 3' ITR 211..320	Virology 125 (2), 491-495 (1983)
Fowl adenovirus 1	AH003428.2	Source: 1..236 5' ITR 1..68; 3' ITR 169..236	Virology 125 (2), 491-495 (1983)
Simian adenovirus (SA7P)	X01027.1	Source: 1..2338 ITR: 1..183	J. Virol. 5:399-409(1985)
Simian adenovirus 8	AH002309.2	Source: 1..506 5' ITR 1..185; 3' ITR 322..506	Gene 80 (2), 381-384 (1989)
Duck adenovirus 1	M17114.1	Source: 1..52 ITR: 1..52	Gene 55 (1), 85-93 (1987)

Exemplary Viral and Artificial Inverted Terminal Repeat Sequences			
Organism	**GenBank Accession No.**	**Nucleotide annotation**	**Reference**
Canine adenovirus 2	M30882.1	Source: 1..80 ITR: 1..80	Nature 322 (6080), 656-659 (1986)
Canine adenovirus 2	M17111.1	Source: 1..196 ITR: 1..196	Gene 55 (1), 85-93 (1987)
Human adenovirus 19	M17110.1	Source: 1..154 ITR: 1..154	Gene 55 (1), 85-93 (1987)
Human adenovirus 19a	AF271991.1	Source: 1..134 ITR: 1..134	Virology 296 (1), 94-106 (2002)
Human adenovirus 19p	AF271990.1	Source: 1..125 ITR: 1..125	Virology 296 (1), 94-106 (2002)
Human adenovirus 40	M16619.1	Source: 1..163 ITR: 1..163	Virology 156 (2), 414-416 (1987)
Human adenovirus D37	AF271992.1	Source: 1..135 ITR: 1..135	Virology 296 (1), 94-106 (2002)
Human adenovirus B3	M17113.1	Source: 1..195 ITR: 1..195	Gene 55 (1), 85-93 (1987)
Human adenovirus sp.	KY062623.1	Source: 1..208 ITR: 1..208	Viruses 11 (2), 129 (2019)
Canine mastadenovirus A	AH002312.2	Source: 1..434 5' ITR: 1..434	Virology 125 (2), 491-495 (1983)
Canine mastadenovirus A	M16831.1	Source: 1..273 ITRs: 17..56; 57..96; 97..136; 137..176; 177..216; 217..256; 257..273	Virology 159 (1), 76-83 (1987)
Human parvovirus B19	M29711.1	Source: 1..479 3' ITR: 97..479	Virology 175 (1), 247-254 (1990)
Human parvovirus B19	M29710.1	Source: 1..479 5' ITR: 1..383	Virology 175 (1), 247-254 (1990)
Goose parvovirus	NC_001701.1	Source: 1..5106 ITRs: 1..444; 4663..5106	Virology 212 (2), 562-573 (1995)
Muscovy duck parvovirus	KY069274.1	Source: 1..5061 ITRs: 1..381; 4681..5061	
Duck parvovirus strain M8	KR029614.1	Source: 1..5073 ITRs: 1..387; 4687..5073	
Streptococcus phage CP-7	AH001311.2	Source: 1..3422 ITRs: 1..347; 2170..2516	Gene 36 (3), 341-348 (1985)
Enterobacteria phage PRD1	AH003497.2	Source: 1..338 5' ITR 1..110; 3'ITR 230..329	Gene 49 (2), 199-205 (1986)
Enterobacteria phage PR722	AH003496.2	Source: 1..340 5' ITR 1..111; 3' ITR 230..340	Gene 49 (2), 199-205 (1986)
Enterobacteria phage PR4	AH003495	Source: 1..340 5' ITR 1..111; 3' ITR 230..340	Gene 49 (2), 199-205 (1986)
Enterobacteria phage PR5	AH003526.2	Source: 1..338 5' ITR 1..110; 3' ITR 229..338	Gene 49 (2), 199-205 (1986)
Enterobacteria phage L17	AH003524.2	Source: 1..340 5' ITR 1..111; 3' ITR 230..340	Gene 49 (2), 199-205 (1986)

Exemplary Viral and Artificial Inverted Terminal Repeat Sequences			
Organism	**GenBank Accession No.**	**Nucleotide annotation**	**Reference**
Equine herpesvirus 4 (strain NS80567)	AF030027.1	Source: 1..145597 ITRs: 1..27; 112426..112452; 112453..122630; 135420..145597	
Rhodococcus sp. strain: MR2253	L14443.1	Source: 1..2235 5' ITR: 1..583	J. Gen. Microbiol. 139 (Pt 9), 2055-2065 (1993)
Rhodococcus sp. strain: MR2253	L14442.1	Source: 1..2349 3' ITR 1..560	J. Gen. Microbiol. 139 (Pt 9), 2055-2065 (1993)
African swine fever virus	L28109.1	Source: 1..4027 ITRs: 41..54; 45..58; 69..82; 143..156; 157..174; 173..187; 207..220; 221..238; 244..258; 302..1633; 302..335; 1894..2027; 1894..1920	Virology 203 (1), 193-196 (1994)
Kluyveromyces lactis	AH001394.2	Source: 1..580 ITRs: 1..240; 341..580	Basic Life Sci. 30, 433-451 (1985)
Streptomyces rochei	AH000936.2	Source: 1..1295 ITRs: 1..614; 939..1295	Basic Life Sci. 30, 433-451 (1985)
Gallid alphaherpesvirus 1	U27244.1	Source: 1..800 ITR: 1..800	Virus Res. 35 (2), 193-204 (1995)
Dictyostelium discoideum (slime mold)	K03072.1	Source: 1..390 ITR: 1..347	Mol. Cell. Biol. 4 (10), 2207-2213 (1984)
Sterkiella nova (Oxytricha nova)	M17509.1	Source: 1..79 ITR: 1..79	Proc. Natl. Acad. Sci. U.S.A. 79 (10), 3255-3259 (1982)
Rabbit fibroma virus	M14899.1	Source: 1..2886 ITR: 1..2886	Virology 152 (2), 308-321 (1986)
Bythograea thermydron	AJ511262.1	Source: 1..1006 ITR: 971..1006	J. Mol. Evol. 59 (6), 747-760 (2004)
Lachancea kluyveri	M35319.1	Source: 1..598 ITR: 1..598	Mol. Gen. Genet. 206, 377-381 (1987)
Yersinia enterocolitica	Y13309.1	Source: 1..3702 ITR: 252..289	Syst. Appl. Microbiol. 21 (2), 201-211 (1998)
Halobacterium salinarum	X59158.1	Source: 1..932 ITRs: 1..16; 917..932	Nucleic Acids Res. 19 (20), 5788 (1991)
Claviceps purpurea (ergot fungus)	X68490.1	Source: 1..7113 ITRs: 1..574; 6540..7113	Curr. Genet. 23 (4), 315-322 (1993)
Bombyx mori transposon-like element	M26070.1	Source: 1..1219 ITRs: 1..25; 1194..1219	Proc. Natl. Acad. Sci. U.S.A. 86 (18), 6843-6847 (1989)
Artificial sequence (Saccharomyces cerevisiae DNA, plasmid pTLU1 left ITR)	AB036802.1	Source: 1..108 left ITR: 1..15	Plasmid 43 (2), 137-143 (2000)

Exemplary Viral and Artificial Inverted Terminal Repeat Sequences			
Organism	**GenBank Accession No.**	**Nucleotide annotation**	**Reference**
Artificial sequence (Saccharomyces cerevisiae DNA, plasmid pTLU1 right ITR)	AB036803.1	Source: 1..115 right ITR: 1..15	Plasmid 43 (2), 137-143 (2000)
Artificial Sequence (Transposon Tn3)	M13165.1	Source: 1..58 ITR: 1..58	Gene 41 (1), 23-31 (1986)
Artificial Sequence (Plasmid RSF1010)	AH000926.2	Source: 1..485 5' ITR 11..56; 3' ITR 430..475	Mol. Gen. Genet. 184 (2), 241-248 (1981)
Artificial sequence (Erwinia amylovora plasmid pEa34)	M96392.1	Source: 1..6705 ITRs: 1..81; 3660..3686; 4865..4891; 6625..6705	J. Bacteriol. 175 (3), 732-740 (1993)
Artificial sequence (Mus musculus 5' and 3' LTR repeat regions)	AH002036.2	Source: 1..2017 ITRs: 29..32; 578..581; 1135..1138; 1684..1687	J. Virol. 58 (2), 296-306 (1986)
Artificial sequence (Bos taurus 5' LTR repeat region)	AH001143.2	Source: 1..1320 ITRs: 46..51; 571..576; 747..752; 1272..1277	Proc. Natl. Acad. Sci. U.S.A. 81 (15), 4741-4745 (1984)
Artificial sequence (Transposon Tn10 clone pNK)	AH003348.2	Source: 1..2195 ITRs: 1..23; 731..753; 867..889; 2173..2195	Proc. Natl. Acad. Sci. U.S.A. 79 (8), 2608-2612 (1982)
Artificial sequence	AX328971.1	Source: 1..58 ITR: 1..58	EP1164195 (SEQ ID NO: 1)
Artificial sequence	AX328972.1	Source: 1..62 ITR: 1..62	EP1164195 (SEQ ID NO: 2)
Artificial sequence	AX328973.1	Source: 1..54 ITR: 1..54	EP1164195 (SEQ ID NO: 3)
Artificial sequence	AX328974.1	Source: 1..54 ITR: 1..54	EP1164195 (SEQ ID NO: 4)
Artificial sequence	AX328975.1	Source: 1..27 ITR: 1..27	EP1164195 (SEQ ID NO: 5)
Artificial sequence	AX328976.1	Source: 1..27 ITR: 1..27	EP1164195 (SEQ ID NO: 6)
Artificial sequence	AX418199.1	Source: 1..27 ITR: 1..27	WO0192551 (SEQ ID NO: 1)
Artificial sequence	CS260167.1	Source: 1..198 ITR: 1..198	EP1626091 (SEQ ID NO: 1)
Artificial sequence	LQ585715.1	Source: 1..7671 ITR: 17..163; 4674..4819	WO2016106303 (SEQ ID NO: 3)
Artificial sequence	LQ585716.1	Source: 1..7671 ITR: 17..163; 4674..4819	WO2016106303 (SEQ ID NO: 4)
Artificial sequence	LQ585717.1	Source: 1..7671 ITR: 17..163; 4674..4819	WO2016106303 (SEQ ID NO: 5)
Artificial sequence	LV542024.1	Source: 1..7671 ITR: 17..163; 4674..4819	JP 2016538885-A
Artificial sequence	LV542025.1	Source: 1..7505 ITR: 17..163; 4508..4653	JP 2016538885-A
Artificial sequence	LV542026.1	Source: 1..7671 ITR: 17..163;	JP 2016538885-A

Exemplary Viral and Artificial Inverted Terminal Repeat Sequences			
Organism	**GenBank Accession No.**	**Nucleotide annotation**	**Reference**
		4674..4819	
Artificial sequence	LY417217.1	Source: 1..7782 ITR: 1845..4974	KR 1020170002445-A
Artificial sequence	MA132305.1	Source: 1..7671 ITR: 17..163; 4674..4819	JP 2018501791-A
Artificial sequence	LQ841017.1	Source: 1..7862 ITR: 17..163; 4868..5003	WO2018140946 (SEQ ID NO: 1)
Artificial sequence	LQ841018.1	Source: 1..5157 ITR: 17..163; 2171..2316	WO2018140946 (SEQ ID NO: 2)

2. Uses of AAV-based vectors in gene therapy

Adeno-Associated Virus (AAV) vectors have versatility in gene therapy applications, offering a platform for treating a wide array of genetic disorders. These vectors have been employed in targeting diseases affecting various organ systems, including the central nervous system, musculoskeletal system, cardiovascular system, and more. Additionally, AAV vectors have been utilized in both *ex vivo* and *in vivo* gene therapy approaches, further expanding their utility. With advancements in vector engineering and delivery strategies, AAV vectors continue to evolve, catering to the diverse needs of gene therapy applications. To date, several gene therapies for various diseases based on AAVs have been developed and approved for clinical use. Indications include only monogenic hereditary ones, *i.e.*, diseases caused by mutations in one gene.

Glybera was approved in the European Union in 2012 as the first AAV-mediated gene therapy to reach this milestone. Glybera corrected hereditary lipoprotein lipase deficiency (LPLD), which manifests as pancreatitis, recurrent abdominal pain, and eruptive fat-filled spots that result from very high triglyceride levels. The rarity of the disease (1 per million), the cost to the patient, and the expense to maintain therapeutic readiness by the company and regulatory challenges in the US led to market withdrawal. This form of gene therapy was no longer made available after 2018, with only 31 patients treated globally [Keeler *et al.* (2019) Recombinant Adeno-Associated Virus gene therapy in light of Luxturna® (and Zolgensma® and Glybera): where are we, and how did we get here? *Annu. Rev. Virol.* 6:601–621].

Another gene therapy product is Zolgensma®, which was approved by the FDA in May 2019 for the treatment of type 1 spinal muscular atrophy (SMA). Zolgensma® is an AAV-serotype-9 (AAV9)-based vector encoding complementary DNA (cDNA) of the survival motor neuron 1 gene (survival motor neuron, SMN1). Following intravenous administration, AAV9 crosses the blood–brain barrier (BBB) and provides targeted delivery of the SMN gene to neurons [(2019) Zolgensma® - One-time gene therapy for spinal muscular atrophy. *Med. Lett. Drugs Ther.* 61:113–114].

Several other regulatory approvals of gene therapies that use AAV vectors have followed. Luxturna®, intended for the treatment of retinal dystrophy caused by biallelic mutations in the *RPE65* gene (an enzyme of retinal cells involved in light-sensitive pigment regeneration). Drug delivery to the retinal cells leads to synthesis restoration of normal RPE65 protein, which is necessary for the regeneration of the photosensitive pigment [Prado *et al.* (2020) Gene therapy beyond Luxturna®: A new horizon of the treatment for inherited retinal disease. *Curr. Opin. Ophthalmol.* 31:147–154]. In 2022 FDA approved Hemgenix®, a drug for the treatment of hemophilia type B, which is caused by congenital deficiency of factor IX. The drug delivers a functional copy of the *F9* gene using a liver directed AAV5 vector [Von Drygalski *et al.* (2019). Etranacogene dezaparvovec (AMT-061 phase 2b): normal/near normal FIX activity and bleed cessation in hemophilia B. Blood advances, 3(21), 3241–3247]. In 2023, two further AAV-based gene therapies were approved. Elevidys® (delandistrogene moxeparvovec-rokl) was developed by Sarepta Therapeutics for Duchenne gene therapy. The treatment is designed to deliver a gene encoding a micro-dystrophin protein, *i.e.*, a shortened version of the dystrophin protein expressed in normal muscle cells, to all muscles involved in the pathology of DMD [Zaidman *et al.* (2023). Delandistrogene moxeparvovec gene therapy in ambulatory patients (aged ≥4 to <8 years) with Duchenne Muscular Dystrophy: 1-year interim results from study SRP-9001-103 (ENDEAVOR). *Annals of Neurology*, 94(5), 955–968]. BioMarin's Roctavian® (valoctocogene roxaparvovec), approved for treating hemophilia A (factor VIII deficiency), is an AAV5-based gene therapy vector carrying a functional copy of the *F8* gene, driven by a liver-selective promoter [Ozelo

et al. (2022) Valoctocogene roxaparvovec gene therapy for hemophilia A. *N. Engl. J. Med.* 386:1013–1025].

Dozens of other treatments by *in vivo* gene transfer are in development and in clinical trials. The indications include:

- neuromuscular disorders: spinal muscular atrophy (SMA), Duchenne muscular dystrophy (DMD), X-linked myotubular myopathy (XLMTM), and diseases of the central nervous system, including Alzheimer's disease, Parkinson's disease, Canavan disease, aromatic l-amino acid decarboxylase (AADC) deficiency and giant axonal neuropathy (GAN).
- ocular disorders: Leber congenital amaurosis (LCA), age-related macular degeneration (AMD), choroideremia, achromatopsia (ACHM), retinitis pigmentosa, and X-linked retinoschisis (XLRS).
- the bleeding disorder hemophilia.
- and lysosomal storage disorders (LSDs).

Despite the successful development of AAV-based gene therapy medications, many preclinical and clinical studies are suspended or terminated, without resulting in a drug registration; therefore, many diseases, especially the rare ones, remain untreated. Some of the failures in clinical translation result from AAV vector drawbacks related to efficacy limitations and safety concerns.

3. Advantages and disadvantages of AAV-based vectors

Adeno-associated viruses (AAV) have served as the backbone of many gene therapy vectors, exhibiting key properties that contribute to both their advantages and limitations. These properties are key considerations for efficient use of those viral vectors in specific applications while addressing safety concerns.

a. Advantages of AAV-based vectors

1) Ability for long-term expression: A notable strength of AAV vectors lies in their capacity for long-term gene expression. Once introduced into the host cells, AAV genomes can persist in an extrachromosomal state without integrating into the host cell genome. This non-integrative nature is advantageous for many clinical applications where avoiding genomic integration is preferred.

2) Low immunogenicity: AAV vectors demonstrate low immunogenicity, reducing the likelihood of triggering an immune response. This is

advantageous in gene therapy applications, as a robust immune response could hinder the vector's efficacy and lead to adverse effects, as has been well documented for other viral vectors.

3) Safety profile: AAV vectors are engineered with the focus on safety. Although derived from naturally occurring viruses, AAV-based vectors undergo modifications to minimize the risk associated with handling. Crucially, most of the viral genome essential for replication is usually deleted, rendering the virus virtually replication defective. This ensures (or increases the chances) that, post-infection, the AAV requires a helper virus to supply the missing proteins to produce new virions. Such helper viruses might simply be the contamination of the patient with a natural Adenovirus contaminated with wild-type AAV (wild-type AAV naturally carry the genes for the Rep and the Cap protein) which can rescue the AAV vector.

b. Limitations of AAV-based vectors

1) Generation of neutralizing antibodies: Host immune responses against AAV vectors have hampered their widespread application. This includes the generation of neutralizing antibodies in the host. Albeit wild-type AAV is an apparently non-pathogenic virus, infection with wild-type AAV is very common and 30-60% of population is believed to harbor AAV-neutralizing antibodies. Furthermore, there is substantial cross-reactivity among different AAV serotypes [Weber T. (2021). Anti-AAV antibodies in AAV gene therapy: current challenges and possible solutions. Frontiers in Immunology, 12, 658399]. Pre-existing antibodies against AAV can as well impede the vector's ability to effectively deliver genes to target cells, diminishing its therapeutic impact. Additionally, capsid-specific cell-mediated cytotoxic T-cell responses affect transgene expression and result in clearance of transduced cells hindering therapeutic efficacy (Arjomandnejad *et al.* (2023). Immunogenicity of recombinant Adeno-Associated Virus (AAV) vectors for gene transfer. *BioDrugs*, 37(3), 311–329).

2) Size limits on transgene: AAV vectors have constraints regarding the size of the transgene they can carry. Their limited packaging capacity can restrict the delivery of larger therapeutic genes or complex payloads, impacting the vector's versatility. Consequently, many proteins associated with inherited diseases cannot be

expressed with a single AAV vector because the expression cassette size exceeds AAV packaging capacity.

3) Manufacturing complexity and cost: The production of AAV vectors poses manufacturing challenges. Since AAV vectors are replication-defective, they require helper functions for replication and packaging. This is achieved either by co-infecting the producer cells with a helper virus or by providing the necessary helper functions on separate packaging plasmids. Packaging itself is inefficient, resulting in as much as 90% of the capsids empty. Consequently, high-quality, consistent vector production at scale is challenging (Hebben M. (2018) Downstream bioprocessing of AAV vectors: industrial challenges & regulatory requirements. *Cell Gene Ther Insights* 4, 131-146). The intricate modifications required for optimal vector design and production also contribute to the complexity of manufacturing. All this translates into the cost-intensity of manufacturing for AAV vectors. The complex processes involved, coupled with the need for stringent quality control, contribute to the overall cost of employing AAV vectors in gene therapy.

4) Lack of targeting, administration challenges: AAV vectors are generally designed to infect a broad range of cell types, lacking specificity. Different serotypes have distinct transduction specificities or tropisms that allows for transgene delivery to a specific subset of cells. Various AAV serotypes also differ in their efficacy and kinetics of transgene expression. While this is advantageous for certain applications, the inability to target specific cells directly limits the precision in gene delivery. Targeting some cells or tissues can require the use of invasive administration routes due to AAV susceptibility to biological barriers. For example, crossing the blood-brain barrier in AAV-based treatments is mainly performed through intracranial injections, while the back-of-the-eye delivery is performed with subretinal or intravitreal injection.

5) Residual toxicity: for a long time AAV gene therapy safety profile has been considered very favorable; however, this has changed with tragic death of three children in a clinical trial (NCT03199469) aimed at treating X-linked myotubular myopathy with an AAV8 vector promoting the expression of functional MTM1 (Wilson & Flotte (2020). Moving forward after two deaths in a gene therapy trial of myotubular myopathy. *Hum Gene Ther.* 31:695-6). Furthermore, systemic

AAV administration has been associated with neurotoxicity in peripheral nerve ganglia and spinal cord, with neurological signs also seen following direct AAV injection into the brain (Stone *et al.* (2023). Adeno-associated virus vectors and neurotoxicity-lessons from preclinical and human studies. *Gene therapy*, 10.1038/s41434-023-00405-1). AAV vectors have been also shown to activate toll-like receptors, thereby triggering an inflammation response.

D. MEV-BASED GENE THERAPY VECTORS (MEV-GTVS)

The MEV-GTVs provided herein combine the benefits and advantages of AAV vectors as well as other gene therapy vectors, and overcome or avoid the disadvantages and challenges observed with AAV vectors and other gene therapy vectors. It is understood that other delivery vehicles that provide for administration to subject and provide a capsid for the ITR-containing plasmid can be used in place of the MEVs. As described herein, the MEVs confer benefits and advantages that other EVs, such as exosomes, do not confer.

The MEVs comprise a composite body, composed by an envelope made from an extracellular vesicle from microalgae and a payload made of a simple plasmid that bears an ITR (such as an AAV ITR) viral sequence, can be first obtained or produced or synthesized. The MEV-GTVs can be taken up by cells, and the cells properly express message encoded by the plasmid, which migrates to the cell nucleus and can last as an episome. MEV-GTVs mimic the concept of a AAV virus, or an AAV-based vector, but all components of the viral capsid are replaced by a MEV (or other delivery vehicle). Because the MEV acts as an active envelop for the genetic material, the resulting MEV-GTVs share key features with MEVs to confer benefits and advantages of MEVs. The resulting MEV-GTVs behave like MEVs (rather than AAV), with respect to biodistribution, tropism, targeting, delivery, and other such properties.

In contrast, AAV vectors that have been encased in other EVs, such as chimeric nanobodies used by EVOX (evoxtherapeutics.com), contain entire AAV particles loaded into mammalian extracellular vesicles have been prepared. These have not been successfully deployed. Theses chimeric nanobodies require the entire AAV particle (including capsid proteins). In contrast, the plasmids and MEV-GTVs,

that contain a plasmid with the ITRs encapsulated in the MEV, successfully encode and express and deliver gene products.

Thus, among the products provided herein are nanoparticles or nano-sized delivery systems made of two components: (1) an external envelop that is an extracellular vesicles from microalgae (MEV), which is a nano-sized lipid nanoparticle, and (2) an internal payload, that is an expression plasmid that carries at least three genetic elements: (i) AAV-ITR sequences, to confer stability, long-lasting live and episomal behavior to the plasmid, (ii) controlling genetic elements, such as promoters, enhancers, introns, IREs, Kozak sequences, among other possible, and (iii) a transgene, made of a simple or multiple coding sequences that codes for either mRNA, siRNA, miRNA, or any other kind of coding or no coding genetically active molecules.

MEV-GTVs, thus, are most evolved version of a gene therapy vector in which the viral presence is reduced to only the ITRs (the user can employ a viral promoter in the transgene construct), and the enveloped moiety that confers the superior properties of MEVs. Such superior properties, compared to other known delivery systems such as mammalian EVs, LNPs, AAV vectors or other, have been so far reported for MEVs only. They include: the capacity to accommodate large payloads, high exo-loading efficiency, high delivery efficiency, specific biodistribution profiles, non-toxicity, non-immunogenicity, non-generation of neutralizing antibodies, the capacity to overcome biological barriers, the ability to offer non-invasive routes of administration, a safety profile (absence of mammalian viruses), and a series of advantageous features concerning manufacturing, such as convenient/high yield manufacturing, and a clean and sustainable industry.

1. MEV-GTVs

Provided herein are MEV-based gene therapy vectors (or MEV-GTVs), which are MEVs that are loaded with plasmids bearing ITRs, such as viral ITRs, including AAV and other viral ITRs, and other user selected components, including eukaryotic regulatory sequences and nucleic acid encoding genes of interest, including genes with introns. MEVs-GTVs are a non-viral delivery system. The plasmids, which comprise the ITRs circularize and can be produced in bacteria. The plasmids are isolated and then introduced into MEVs.

Described and provided herein are compositions and methods related to the properties, production and use MEV-GTVs, to deliver AAV-ITRs-carrying plasmids for the lasting expression of a variety of therapeutic modalities (such as proteins, polypeptides, peptides, siRNAs, miRNAs, other non-coding regulatory RNAs, as well as editing complex, or a combination of different modalities) to treat diseases or syndromes. The compositions and methods are useful to improve or provide gene therapy treatments, and overcome bottlenecks in gene therapy approaches, and thus to slow, halt, or reverse of genetic disease or syndromes, metabolic diseases, or syndromes and/or multifactorial diseases or syndromes.

MEV-GTVs provide a new type of gene therapy vector that combines the rare and convenient features of MEVs, such as loading capacity and versatility, targeting-tropism, biodistribution, and delivery capacity of MEVs, with the use of plasmids containing ITRs sequences from AAVs (as a payload of the MEVs) that carry the capacity of maintaining long-lasting expression, and the replication of the plasmid as an episomal DNA in human cells.

MEV-GTVs combine the convenient properties of MEVs as a delivery system with those of AAV vectors as a gene therapy vector. Thus, MEV can accommodate and deliver plasmids sized far beyond 5Kb, which is the upper limit imposed by the AAV capsid, to accommodate constructs inside an AAV-based vector. Such a breakthrough in the limitation imposed by AAVs to the size of the payload, allows MEV-GTVs to carry either longer coding regions or multiple coding regions within the same construct.

MEVs can be administered a plurality of times. As shown in previous disclosures (see, International PCT application No: PCT/EP2024/057645) MEVs do not generate a neutralizing immune response; they can thus be administered repeatedly without neutralization of their biological activity by the immune system of the host. AAVs on the contrary are condemned to be applied/administered only once, because the neutralizing response generated by their capsid after the first exposure neutralizes any subsequent administration of such, or related, AAVs. The envelop of MEV-GTVs is made directly by the MEVs, MEV-GTVs are therefore expected to be suitable for repeat treatment of patients.

In other aspect of the present disclosure, MEV-GTVs display biodistribution patterns which are typical for MEVs, according to the particular route of administration

used. For example, while AAV-based gene therapies for brain diseases require the delivery of the vector by invasive approaches such as intracranial of intrathecal administration; MEV-GTVs can be administered by drop instillation intranasally (nose-to-brain delivery by intranasal administration. As described herein MEVs traffic to regions of the brain when administered intranasally in liquid drops or other form for nasal administration. Once in the nose, the MEVs are internalized by sensorial neurons of the olfactory epithelium. From there, they travel through the olfactory bulb to internal structures in both hemispheres of the brain.

AAV-based gene therapies for back-of-the-eye diseases require the delivery of the vector by the invasive and definitively low-patient compliance approach of intraocular injection. The MEV-GTVs, however, can be administered by topical administration of the surface of the eyes.

2. Production of MEV-GTVs

Like any other kind of "loaded MEV" (e.g., an MEV that has been loaded with a particular payload), MEV-GTVs are composite elements. They have two components: the *MEV moiety* (or other delivery vehicle) and the *payload moiety*.

MEV-GTVs are MEVs that have been loaded with a particular expression plasmid. The plasmid carries the following three genetic elements combined in a proper functional array: **(1)** ITRs from AAV origin. The ITRs assure the survival of the plasmid inside the recipient cell as a replicative, and non-integrative episome; **(2)** genetic elements to ensure proper expression, processing, and eventually other activities, of the transgene(s); and **(3)** the transgene. The plasmid can carry either one or two ITRs (see, *e.g.*, Figures 2 and 3). Genetic elements include, but are not limited to, promoters, enhancers, Kozak consensus sequences, IRES, polyA, and other such sequences. The transgene encodes the biologically active principle that is intended to convey the desired modification of the recipient cell. The transgene can encode a variety of genetic elements, such as mRNA, genes, exons, proteins, peptides, and also siRNAs, miRNAs, and other such RNA products, as well as for RNA components of gene editing complexes, among a large diversity of genetic and/or biologically active elements.

MEV-GTVs are produced as follows. The MEV-moiety is produced as described, for example, in any of, International PCT publication Nos.

WO2023001894, WO 2023144127, WO2024088808, WO 2024194423, and priority application, U.S. provisional application Serial No. 63/562,941, and herein. MEVs are produced by growing microalgae in photobioreactors, followed by purifying the produced MEVs from the supernatants of those cultures. The product of such production, clarification, plus purification process consists of purified MEVs ready to be used, characterized, stored and eventually loaded with custom payloads. The plasmid (payload) moiety, the minigene ITR plasmid or ITR plasmid, is produced as described herein, using standard and well-established methods for plasmid production, including the transfection of the plasmid construct (carrying the three genetic elements described above) into suitable bacteria, the cloning or multiplication of the plasmid by growing the transformed bacteria, and the purification of the plasmid from the bacteria cultures following standard, well-known and well established methods for plasmid production and purification.

Once the two moieties (purified MEVs and purified plasmid-payload) have been separately obtained, the plasmid is loaded into the MEVs using the protocols for exo-loading exemplified herein (see, also International PCT publication No: WO2023001894).

Thus, the production, or manufacturing, of MEV-GTVs is simple and straightforward, high-yield, safe, and cheap. As opposed to the production, or manufacturing, of AAV vectors, which is complex, low-yield, not-necessarily safe, and well-known to be expensive, the manufacturing of MEV-GTVs is highly advantageous over the manufacturing of AAV vectors. This advantage and superiority of MEV-GTVs over AAVs, as far as manufacturing is concerned, adds to the multiple advantages of MEV-GTVs over AAVs described throughout the disclosure herein.

3. Structure of the plasmids and methods for loading

The payload carried by the MEV-GTVs is a plasmid. That plasmid is double-stranded. The plasmid contains the ITRs (only viral element carried by the MEV-GTVs), such as AAV ITRs, in addition to the transgene of interest + the necessary expression elements to assure proper expression and processing of the transgene. It is understood herein that the ITR plasmids described herein that contain only the ITRs from viral vector, can be loaded into any suitable delivery vehicle, such as an exosome, lipid nanoparticle, and extracellular vesicle. The delivery vehicle serves as a

capsid for delivering the plasmid nucleic acid. The MEV-GTVs are exemplary, and as detailed herein, provide advantages and benefits that other such delivery vehicles do not provide. The user, however, can select another delivery vehicle to achieve the benefits conferred by the ITR-containing plasmids provided herein.

The plasmids carrying ITRs, such as AAV-ITRs, sequences can be grown in bacteria using standard protocols for plasmid growth, cloning, and preparation. The ITR plasmids or minigene ITR plasmids, are circularized and are double stranded; they contain (1) the necessary sequences for their propagation in bacteria, plus (2) a minigene: the ITR, such as the AAV-ITRs (in a configuration such that the ITRs surround the transgene + expression elements), plus (3) specific sequences that allow and/or induce an internal recombination in the plasmid. The products of such recombination are: (1) a plasmid that carries the transgene expression elements flanked by the ITRs, such as the AAV-ITRs, and (2) a plasmid that carries all other plasmid sequences. These two plasmids can be physically separated, if wanted, to render a purified plasmid containing the ITRs, such as the AAV-ITRs, + the transgene + the expression elements, only.

With respect to the promoter, it is selected based on the particular use or application of the ITR plasmid. The promoter is operatively linked to nucleic acid to be expressed. Promoters include any of suitable strength and specificity, including inducible, tissue specific, heterologous or homologous to the nucleic acid to be expressed. The promoter is user selected. Some ITRs, such as the AAV2 serotype ITRs, can drive transcription activity (see, Earley *et al.* (2020). Adeno-Associated Virus serotype-specific Inverted Terminal Repeat sequence role in vector transgene expression. Human gene therapy, 31(3-4), 151–162); in general, other promoters are included, and are operatively linked (linked for transcription) to the nucleic acid to be transcribed in the ITR plasmid.

The bacterial plasmid that contains the ITR plasmid is propagated in bacteria; the recombination event can be induced in a controlled manner; both plasmid products resulting from the recombination event can be purified from one another.

There are at least two alternative plasmids or payloads for the MEV-GTVs: (1) minicircle plasmid obtained after inducing recombination to eliminate the bacterial plasmid sequences, but not the ITRs and to the transgene, which results in well-

defined and simplified product for use in advanced studies and for human use; or (2) to use the plasmids that still include sequences useful for propagation in bacteria.

The bacterial plasmid vectors contain a standard bacterial plasmid backbone, such as a pUC vector; the minigene is inserted into a convenient cloning site in the plasmid. The plasmids can be isolated and used as is; or the plasmids can include recombination sites, so that the minigene portion can be excised by recombination to produce minicircles that contain the minigene with the ITRs. If further uniformity is needed, the minicircles can be selected by size to choose those with the number of copies of the minigene, such as 1, 2, 3, 4, 5,. . . 10 or more. Large plasmids can be loaded into the MEVs.

As shown and described herein the MEVs readily can accommodate plasmids of up to 10Kb; they are readily loaded into the MEVs. It also is shown that when plasmid of about 10Kb are loaded into the MEVs, the copy number of plasmids in the payload (= number of plasmid molecules/ MEV) is about 2, on average. Thus, the MEVs can accommodate a plasmid of around 20Kb (with a copy number of 1 plasmid molecule/ MEV).

The constructs provided herein are referred as a minigene. The minigene comprises a transgene that contains a user selected coding region and regulatory sequences flanked by a 5' ITR and 3'ITR, such as an AAV ITR. Generally, the minigene constructs contain 2 complementary ITRs to circularize the construct. Thus, the minigene is composed of, at a minimum, a transgene plus 5′ and 3′ ITRs, such as AAV-ITRs. For propagation, the minigene is cloned into a suitable cloning plasmid, such as a pUC backbone or other convenient bacterial plasmid, for propagation in bacteria. Thus, the bacterial plasmid carries the minigene; the minigene carries two ITRs flanking the transgene; the transgene carries the selected coding region of interest plus suitable regulatory sequences (promoter, enhancer, Kozaks, IRES, and other sequences known to those of skill in the art for regulation of gene expression in a eukaryotic host) as detailed herein. These sequences are introduced into a bacterial plasmid for replication/propagation in a bacterial host.

Exemplary genetic structures and sequences of the plasmid constructs used as the genetic material within the MEV-GTVs are described and exemplified herein. Such constructs comprise an internal terminal repeat (ITR) sequence, such as an ITR region

from a viral vectors, such as any ITR sequence set forth in SEQ ID NOs: 1-47 and other such ITRs, such as vaccinia virus ITRs, an enhancer, *e.g.*, SEQ ID NOs: 75-77, a promoter, such as any of those set forth in SEQ ID NOs:78-80; an intron region, see *e.g.*, SEQ ID NOs:81-83; an IRES (internal ribosome entry site) sequence, such as those set forth in SEQ ID NOs:84-86; a Kozak sequence, see *e.g.*, SEQ ID NOs:90-93; a specific transgene as described below, and/or a poly A signal region (*e.g.*, AATAAA). The specific transgene, which can be single or multiple nucleic acid encoding a peptide, a protein, and/or a mRNA, and/or for a non-coding RNA such as a siRNA, a miRNA, or other non-coding RNA, and or gRNA signal to editing, or other. The plasmid can comprise sequences that code for structural or functional proteins, for endogenous or exogenous proteins, and/or sequences that ameliorate the loss-of-function of proteins aimed at improving molecular or cellular processes, such as, as examples, intracellular lipid trafficking or penetrating peptides, or signal peptides to release extracellular proteins. For example, sequences encoding Cas9 protein (e.g., of amino acid sequence described herein, SEQ ID NO: 95) or a Cas9-like protein (e.g. one of amino acid sequences described herein, see, SEQ ID NO: 96-97).

Exemplary ITRs include any selected from among any eukaryotic viral vectors, such as AAV. These include ITRs from any AAV serotype, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, other serotypes or synthetic ones (SEQ ID NOs:1-47). Other ITRs sequences can be obtained through synthetic or other suitable means by reference to published sequences such as are available in the literature or in databases such as, e.g., GenBank, PubMed, and others. For exemplification herein, the ITRs from AAV serotype 2 or ITRs from other suitable serotypes or synthetic sequences can be selected or designed.

MEV-GTVs are made of a plasmid which is loaded into isolated MEVs. We provide methods for loading the minigene-carrying plasmids into the isolated MEVs. Thus, the MEV-moiety provides the means for the delivery of the payload (the minigene-carrying plasmid) to a selected host cell, tissue, or organ.

The transgene is a nucleic acid heterologous to the vector sequences flanking the transgene, which encodes a polypeptide, a protein, or other product of interest (non-coding RNAs (siRNAs, miRNAs, saRNAs, lncRNAs, other) or (editing complexes). The transgene is operationally linked to regulatory components in a manner which

permits proper transgene transcription, translation, and/or expression in a host cell. As used herein, the regulatory elements sequences include both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in trans or at a distance to control the gene of interest.

The composition of the transgene sequence is user selected and is a function of the application. For example, one type of transgene sequence includes a reporter sequence, which upon expression produces a detectable signal. Such reporter sequences include, without limitation, DNA sequences encoding β-lactamase, β-galactosidase (LacZ), alkaline phosphatase, thymidine kinase, fluorescent proteins (EBFP, ECFP, GFP, EGFP, YFP, mOrange, mcherry, DsRed, RFP, others), chloramphenicol acetyltransferase (CAT), luciferases, membrane bound proteins including, for example, CD2, CD4, CD8, the influenza hemagglutinin protein, and others well known in the art, to which high affinity antibodies directed thereto exist or can be produced by conventional means, and fusion proteins comprising a membrane bound protein appropriately fused to an antigen tag domain from, among others, hemagglutinin or Myc, or others.

The transgene also can be a non-marker sequence encoding a product which is useful in biology and medicine, such as proteins, peptides, RNA, enzymes, or catalytic RNAs, regulatory ncRNAs, editing complex or a combination of different sequences useful to treat or and to slow, halt, or reverse of genetic disease or syndromes, metabolic diseases, or syndromes and/or multifactorial diseases or syndromes. Desirable RNA molecules include tRNA, dsRNA, ribosomal RNA, catalytic RNAs, and antisense RNAs, miRNAs, and other. One example of a useful RNA sequence is a sequence which extinguishes expression of a targeted nucleic acid sequence in the treated animal.

The transgene can be used to correct or ameliorate gene deficiencies, which includes, for example, deficiencies in which normal genes are expressed at less than normal levels or deficiencies in which the functional gene product is not expressed. A preferred type of transgene sequence encodes a therapeutic protein or polypeptide which is expressed in a host cell. The vectors can comprise a plurality of transgenes, e.g., to correct or ameliorate a gene defect caused by a multi-subunit protein. In certain situations, a different transgene can be used to encode each subunit of a protein, or to encode different peptides or proteins. This is advantageous when the size of the DNA

encoding the protein subunit is large, e.g., for an immunoglobulin, the platelet-derived growth factor, or a dystrophin protein. The different subunits of a protein can be encoded by the same transgene. In this case, a single transgene includes the DNA encoding each of the subunits, with the DNA for each subunit separated by an internal ribozyme entry site (IRES). Unlike AAV vectors where the size of the DNA encoding each of the subunits is small, e.g., the total size of the DNA encoding the subunits and the IRES is less than 5Kb as for AAV recombinant vectors, the plasmids provided herein do not have such size limitations. For example, polycistronic nucleic acids, such as, as an alternative to an IRES, the DNA can be separated by sequences encoding a 2A peptide, which self-cleaves in a post-translational event. The selected transgene can encode any biologically active product or other product, e.g., a product desirable for study.

Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (*i.e.*, Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. A great number of expression control sequences, including promoters which are native, constitutive, inducible and/or tissue-specific, are known in the art and can be employed. Examples of constitutive promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) (see, e.g., Boshart *et al*, *Cell*, 41:521–530 (1985)), the SV40 promoter, the dihydrofolate reductase promoter, the β-actin promoter, the phosphor-glycerol kinase (PGK) promoter, and the EF1α promoter. Inducible promoters allow regulation of gene expression and can be regulated by exogenously supplied compounds, environmental factors such as temperature, or the presence of a specific physiological state, e.g., acute phase, a particular differentiation state of the cell, or in replicating cells only. Many other systems have been described and can be readily selected by one of skill in the art. Examples of inducible promoters regulated by exogenously supplied compounds include, e.g., the zinc-inducible sheep metallothionine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, and

others. Other types of inducible promoters include, for example, promoters that are regulated by a specific physiological state, such as, but not limited to, temperature, acute phase, a particular differentiation state of the cell, or in replicating cells only.

In another embodiment, the native promoter for the transgene can be used. The native promoter can be used, for example, when it is desired that expression of the transgene mimic the native expression. The native promoter can be used when expression of the transgene must be regulated temporally or developmentally, or in a tissue-specific manner, or in response to specific transcriptional stimuli. In a further embodiment, other native expression control elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences also can be used to mimic the native expression.

Another embodiment of the transgene includes a transgene operably linked to a tissue-specific promoter (e.g., if expression in skeletal muscle is desired, a promoter active in muscle should be used, these include the promoters from genes encoding skeletal β-actin, myosin light chain 2A, dystrophin, muscle creatine kinase, as well as synthetic muscle promoters with activities higher than naturally occurring promoters). Examples of promoters that are tissue-specific are known for liver (albumin, hepatitis B virus core promoter, alpha-fetoprotein (AFP)), for bone expression (bone osteocalcin, bone sialoprotein) for lymphocytes (CD2, immunoglobulin heavy chain; T cell receptor αchain), for neuronal expression such as neuron-specific enolase (NSE) promoter, neurofilament light-chain gene, and the neuron-specific VGF gene, among others for this specific expression and also for other cells types, tissues, or organs.

Optionally, plasmids carrying ITRs, such as AAV-ITRs, and encoding therapeutically useful transgenes or sequences also include, for example, selectable markers and reporter genes, such as, but not limited to, nucleic acid with sequences encoding geneticin, hygromycin or puromycin resistance, among others. Such selectable reporters or marker genes, generally located outside the ITRs, can be used to signal the presence of the plasmids in bacterial cells, such as ampicillin resistance. Other components of the plasmid include, but are not limited to, an origin of replication. Selection of these and other promoters and vector elements are conventional, and many such sequences are available to the construct of the plasmid carrying the ITRS, such as the AAV-ITRs.

The minigene constructs can be isolated from the bacterial plasmids in which they are propagated, or the bacterial plasmids can be isolated. As noted, the minigene constructs can be excised from the bacterial plasmids by including recombination sequences, and inducing recombination to produce the circular minigene constructs. Because the ITRs can concatemerized, the resulting minigene constructs can include one or more copies of the minigene. If desired the resulting constructs can be separated by size to produce a more uniform product. The minigene constructs or the bacterial plasmids can be exogenously introduced into the MEVs to produce MEV-GTVs.

MEV-GTVs are a virus-free, non-viral gene transfer and gene therapy system that provides the capacity to deliver gene editing systems and perform gene editing. MEV-GTVs allow (1) the effective delivery *ex vivo* to a large number of cell types, (2) the effective delivery *in vivo* to specific organs/tissues/cell types determined by the natural tropism of the MEV moiety, (3) the effective delivery of plasmids carrying AAV-ITRs that's allow and support the episomal replication and the non-integration in the host genome, of the genetic material delivered by the MEV-GTV, (4) the effective delivery, expression and biological activity of a large number of modalities such as all modalities of RNAs needed for gene correction, gene replacement or gene editing. MEV-GVTs provide a system for gene editing therapies, for *ex vivo* or *in vivo* approaches, including for hemoglobinopathies, immuno-oncology, autoimmune diseases, among others, involving the brain, the muscles, the lungs, among other. MEV-GTV can be produced by the simple loading of plasmids carrying AAV-ITRs into purified MEVs. More than one modality can be simultaneously loaded into MEVs to generate MEV-GTVs with highly specific functional activity and therapeutic purposes, such as editing complexes (CAS9 coding region (or other such functions, see, *e.g.*, SEQ ID NOs:96-97 for the sequences Cas9, Cas11, and Cas12) plus gRNAs plus RNA modalities needs for the gene editing). Such specific MEV-GTVs are designed to edit specific genes by a precisely cutting (using any kind of molecular scissors), followed by the repair of the DNA, or by other specific processes such as disrupting or deleting sequences, or correcting a sequence, or inserting new sequences.

Because of the structure of the minicircle plasmids that contain only the ITRs and the transgene and regulatory sequences for expression, and their preparation in bacterial plasmid backbones, they can be propagated in bacteria. No host cells or

packaging cells or helper cells or propagation in eukaryotic cells is required. Hence, unlike AAV vectors, there is no need for expression of any AAV capsid protein (AAV CAP region), nor AAV replication proteins (AAV REP region) of the same serotype of the AAV-ITRs in the minigene or a cross-complementing serotype. Nor is there a need for any helper function of another AAV helper virus as needed to package the recombinant adeno-associated virus vectors (rAAV-vectors).

The plasmids are propagated in bacteria and portion flanked by the ITRs are recovered; the ITRs lead to circularization (or in some instances some concatenation to produce larger circular vectors. The minicircles are exogenously loaded into MEVs, produced as described herein and also in copending applications (see, International PCT publication Nos. WO2023001894, WO 2023/144127, and WO2024088808, and copending application International PCT application No: PCT/EP2024/057645 and U.S. provisional application Serial No. 63/562,941, which describe and exemplify preparation of MEVs) to produce MEV-GTVs. MEV-GTVs provide a largely better safety profile than any rAAV-vector. MEV-GTVs can be used as gene therapy vectors for treatments *in vitro*, *ex vivo*, and *in vivo*.

Thus the MEV-based vectors provide the best of the two technologies: the many pros of the MEVs, including the possibility of repeat administration, the capacity to accommodate large payloads, including large transgenes, the ability to overcome stringent biological barriers, the ability to efficiently deliver their payload to recipient cells based upon now-known trafficking pathways and routes of administration, and their non-viral nature; plus the pros of AAVs and other such vectors, with includes the presence of ITRs, which ensure a long-lasting expression of the transgene.

4. MEV-GTVs enhance overall efficacy and improve delivery

AAV vectors have been integral to gene therapy, offering stable, long-term transgene expression. Because of these limitations alternative strategies are needed. MEV-GTVs provide an alternative strategy that leverages the strengths of AAV vectors and solve the challenges of gene therapy. The following are examples of the advantages of the MEV-GTVs, and, for some properties, other delivery vehicles that contain the ITR plasmids. It is understood that that ITR plasmids can be delivered and employed in other delivery vehicles. Some will not provide all of the advantages of

the MEVs, but any that can carry a payload of the size of the ITR plasmid can be loaded therewith. Advantages specific to the plasmid component will be realized with other delivery vehicles.

ITRs assure no replication, no genome integration and genetic stability

AAV vectors are composed in cis only for AAV-ITRs. ITRs confer the capacity to AAV vectors to be replicated by cellular machinery as an episomal genetic material. Therefore, the capacity of AAV vectors to mitigate the risk of uncontrolled viral replication, host genome integration and unintended genetic alterations are related to AAV- ITRs. MEV-GTVs, as they carry AAV-ITRs within a minimal genetic structure, also maintain a non-integrative profile, ensuring genomic stability and minimizing the potential for off-target effects.

Neutralizing immune response

Upon an initial exposure of a host/patient to AAVs vectors, a neutralizing immune response is triggered. Such a neutralizing response is a major obstacle for repeat administration as it renders subsequent administrations useless. MEV-GTVs offer a significant advantage on AAVs. Unlike AAVs, MEV-GTVs do not generate neutralizing antibodies, thus providing a stealthy approach to genetic cargo delivery (see, International PCT publication No. WO 2024/194423).

Limitations in payload size and nature

MEV-GTVs significantly outperform AAV vectors in terms of cargo capacity, accommodating significantly larger genetic payloads, specifically larger plasmids. MEV-GTVs can accommodate, carry and deliver payloads with biological activity such as payloads of DNA, RNA, peptides, proteins... see, Published International PCT application Nos. WO2023001894, WO2023001894, WO2023232976, and WO2023144127 .

Numerous routes for delivery and the ability to cross biological barriers

While AAV vectors face limitations in crossing biological barriers, MEV-GTVs possess a great and rare capacity to navigate complex biological environments, such as passing all the way along the gastrointestinal tract without been destroyed and assuring protection of the payload, targeting the back-of-the-eye following topical administration by drop instillation on the ocular globe, or entering the brain and travelling throughout the entire olfactory tract (to reach relevant regions of the limbic

as well as of the cortical brain, following simple administration by the nose). This characteristic enhances targeted of cargo delivery to specific tissues or cells (see, International PCT publication Nos: WO2023144127 and WO2024088808). Intranasal administration provides a route directly to the brain. Administration as eye drops provides a route to the retina and other tissues at the back of the eye, thereby avoiding intravitreal injection (see, U.S. provisional application Serial No. 63/562,941).

Reduced manufacturing complexity and improved cost-efficiency

Additionally, MEV-GTVs present a significant manufacturing advantage with lower complexity and reduced safety risks compared to AAV vectors. AAV vectors often involve high production and purification costs in addition to the significant burden and complexity associated to the generation of suitable triple-transfected stable producer cell lines. On the other hand, MEV-GTVs offer a cost-effective alternative: MEVs themselves are produced by simply growing unicellular suspension green microalgae with fresh water, light and minerals; the payload is a plasmid grown in bacteria using standard molecular biology techniques, and the loading of the MEVs to generate the MEV-GTVs is based on simple physicochemical protocols. Making MEV-GTVs is a largely economically viable option for large-scale gene therapy applications. See, International PCT publication No WO2023001894.

The ITR plasmids and the MEV-GTVs provide the advantages and strengths of AAV vectors, such as stable transgene expression, while mitigating drawbacks. The increased cargo capacity, reduced immunogenicity, and enhanced ability to navigate biological barriers position MEV-GTVs as a significant advance in the gene therapy landscape, opening avenues for more sophisticated and effective therapeutic interventions. Direct comparison of the features discussed is summarized in the table below.

Feature	**AAV Vector**	**MEV-GTV**
Viral replication capability	No	n/a
Genome Integration	No	No
Risk of genome instability	No	No
Stable, long-term transgene expression	Yes	Yes
Pre-existing neutralizing antibodies	Common	No
Neutralizing immune response	Yes	No

Safety profile	Favorable	Favorable
Capacity for genetic cargo	Up to 5kb	Up to 10kb tested (theory, up to 50kb)
Complex payloads feasible	No	Yes
Ability to cross biological barriers (intestine, brain, retina)	Limited	Favorable
Require invasive administration (intracranial/intrathecal, intraocular injection)	Yes	No
Complexity of manufacturing	High	Low
Manufacturing-related safety concerns	Yes	No
Production scale-up	Challenging	Feasible
Cost of production	High	Low
Cost of purification	High	Low

MEV-GTVs are the ultimate step in the evolution of gene delivery vectors. Figure 4 demonstrates the comparison between the composition of MRV-GTVs and the composition of other (ITR)AAV-based delivery or gene therapy systems: including(A) an ITR-flanked linear ssDNA molecule, (B) a recombinant AAV vector (rAAV) carrying the native or engineered ITR-flanked linear ssDNA molecule inside the capsid, (C) 200-250 nm exosomes loaded with rAAV vectors 20nm in diameter, and (D) an MEV-GTV that comprises AAV-ITR flanked dsDNA expression plasmids (up to 10kb) loaded into an MEV. The MEV-GTVs, provide advantages of ITR-driven gene expression and the excellent *in vivo* properties of MEVs.

E. EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) are biomolecular structures released from plant and animal cells that play a role in cell-to-cell communication. Structurally, EVs are negatively charged lipid bilayer vesicles with a density of 1.13 to 1.19 g/mL. EVs are able to cross barriers such as the plasma (or cytoplasmic) membrane and the blood/brain barrier, and provide for the horizontal transfer of their functional contents (*i.e.*, proteins, lipids, RNA molecules, and circulating DNA) from a donor to a recipient cell (Kuruvinashetti *et al.* (2020) IEEE 20th International Conference on Nanotechnology 354-357). EVs also are naturally stable in various biological fluids, immunologically inert, and can exhibit organ-specific targeting abilities (Picciotto *et al.* (2021) *Biomater. Sci.*, 9(8):2917-2930, doi:10.1039/d0bm01696a). EVs can be

loaded with the ITR-containing plasmids provided herein and exemplified with MEVs. It is understood that other EVs and other nanoparticle delivery vehicles can be loaded with the plasmids. The MEVs, however, confer advantages and benefits other delivery vehicles do not confer. Any delivery vehicle into which the ITR-containing plasmids can be loaded and that serves as a capsid for delivery of the plasmid and for production of encoded products, is contemplated.

EVs contain endogenous lipids, nucleic acids, and proteins. Although results differ due to variations in isolation techniques and methods of analyzing the data, EVs generally contain proteins associated with the plasma membrane, cytosol and those involved in lipid metabolism (see, *e.g.*, Doyle and Wang (2019) *Cells* 8(7):727). Proteins involved in the biogenesis of EVs (*e.g.*, components of the ESCRTs), EV formation and release (*e.g.*, RAB27A, RAB11B, and ARF6), signal transduction, and antigen presentation, as well as tetraspanins, occur in EVs (Abels and Breakefield (2016) *Mol. Neurobiol.* 36(3):301-312, doi: 10.1007/s10571-016-0366-z). EVs are enriched for cholesterol, sphingomyelin, glycosphingolipids, and phosphatidylserine (Kuruvinashetti *et al.* (2020) IEEE 20th International Conference on Nanotechnology 354-357). Although a small number of studies have identified some genomic and mitochondrial DNA in EVs, EVs primarily are enriched with endogenous small RNAs. Studies have identified mRNAs, miRNAs, rRNAs, long and short non-coding RNA, tRNA fragments, piwi-interacting RNA, vault RNA, and Y RNA in EVs. Most of the RNA that naturally occurs in EVs is ~200 nucleotides long (with a small portion up to 4 kb) and thus it is fragmented, although circular RNAs also have been shown to be enriched and stable in EVs. RNA in EVs is protected from RNase digestion in the extracellular environment by the lipid bilayer (Abels and Breakefield (2016) *Mol. Neurobiol.* 36(3):301-312, doi: 10.1007/s10571-016-0366-z). The Exocarta, Vesiclepedia, and EVpedia databases are publicly available and provide data on the protein, nucleic acid, and lipid content of EVs (generally EVs from mammalian origin, such as human origin), as well as the isolation and purification procedures used, from EV studies (Abels and Breakefield (2016) *Mol. Neurobiol.* 36(3):301-312).

EVs are used by cells to mediate several physiological processes or affect various pathological conditions associated with the activation of an immune response

or the spread of disease or infection, and also constitute cross-species communication and are in all kingdoms of life. Sources of EVs include mammalian cells, bacteria, bovine milk, and plants (Adamo *et al.* (2021) *J. Extracell. Vesicles* 10:e12081). Although plants and algae possess a cell wall outside their plasma membrane, which could be a physical barrier for the release of EVs, plants and algae release EVs (Picciotto *et al.* (2021) *Biomater. Sci.*, 9(8):2917-2930, doi:10.1039/d0bm01696a).

As detailed herein, the MEVs confer advantages that are not conferred by other delivery vehicles. The ITR plasmids, as well as the bacterial plasmids containing them, can be delivered in other delivery vehicles to obtain the advantages conferred by the ITR plasmids. Loading them into MEVs to produce MEV-GTVs provides additional benefits and advantages.

MEVs include any microalgae extracellular vesicle, particularly those produced by the family Chlorellaceae, exemplified by *Chlorella* EVs, and also Parachlorella Evs. The MEV is produced in a microalgae, such as a microalgae that is a species of the family Chlorellaceae. Such microalgae include members of the genus *Chlorella* or *Parachlorella*. Exemplary thereof are: *Chlorella ellipsoidea*, *Chlorella pyrenoidosa*, *Chlorella sorokiniana*, *Chlorella vulgaris*, and *Chlorella variabilis*, a species of *Parachlorella*, such as *Parachlorella kessleri*, *Parachlorella beijerinckii*, and *Parachlorella hussii*. MEVs are produced, isolated and purified from microalgae cultures as described herein, and in the commonly owned publications, and exemplified with microalgae from the family of Chlorellaceae, such as *Chlorella vulgaris* and *Parachlorella* species.

1. Types of Extracellular Vesicles (EVs)

a. Exosomes

There are three primary subtypes of EVs; they are classified based on their biogenesis, mode of release, size, content, and function: microvesicles (MVs), exosomes, and apoptotic bodies (Doyle and Wang (2019) *Cells* 8(7):727). Exosomes, or intraluminal vesicles (ILVs) generally are 30-150 nm in diameter and are released through multivesicular bodies (MVBs) in the endosomal pathway. In the endosomal pathway, early endosomes form by inward budding of the plasma membrane and can transform into late endosomes, which accumulate ILVs by inward budding of the endosomal membrane. Late endosomes which contain a number of small vesicles are

called MVBs. MVBs either fuse with the lysosome and are degraded, or the plasma membrane which releases the ILVs as exosomes into the extracellular space. The endosomal sorting complexes required for transport (ESCRT) pathway regulates MVB transportation and exosome formation and is reported to be the primary driver of exosome biogenesis, although other mechanisms of exosome biogenesis exist, including those mediated by the sphingolipid ceramide, which can facilitate membrane invagination, or proteins in the tetraspanin family. The ESCRT accessory proteins Alix, TSG101, HSC70 and HSP90β are often referred to as exosomal marker proteins (Doyle and Wang (2019) *Cells* 8(7):727).

Exosomes are released into the extracellular space by the fusion of the MVB limiting membrane with the plasma membrane. A number of proteins are involved in the release of exosomes, including Rab GTPases, diacylglycerol kinase α, and SNARE proteins (Abels and Breakefield (2016) *Cell Mol. Neurobiol.* 36(3):301-312).

Exosomes have been candidates for drug delivery systems: they have a long circulating half-life; exosomes are tolerated by the human body and can penetrate cell membranes and target specific cell types; and they can be loaded with genetic material, a protein, or a small molecule (Doyle and Wang (2019) *Cells* 8(7):727).

b. Microvesicles

Microvesicles (MVs, or ectosomes) form by outward budding, or pinching, of the cell's plasma membrane, and have a diameter of 100 nm to 1 μm. The formation of MVs involves cytoskeleton components, such as actin and microtubules, molecular motors such as kinesins and myosins, and fusion machinery such as SNAREs and tethering factors. The physiological state and microenvironment of the donor cell effects the number of MVs produced, and the physiological state and microenvironment of the recipient cell effects the number of MVs consumed. MVs also have a number of marker proteins, including cytosolic and plasma membrane associated proteins, as well as cytoskeletal proteins, heat shock proteins, integrins, and proteins containing post-translational modifications, although there are no known specific markers to distinguish MVs from exosomes. Like exosomes, MVs can be loaded with cargo (such as proteins, nucleic acids, and lipids) for delivery to another cell, thereby altering the recipient cell's functions (Doyle and Wang (2019) *Cells* 8(7):727).

c. Apoptotic Bodies

Apoptotic bodies are released by dying cells into the extracellular space, and have a diameter from 50 nm to 5000 nm. Apoptotic bodies are formed when the cell's plasma membrane separates from the cytoskeleton due to increased hydrostatic pressure after the cell contracts. Unlike exosomes and MVs, apoptotic bodies contain intact organelles, chromatin, and small amounts of glycosylated proteins (Doyle and Wang (2019) *Cells* 8(7):727).

d. MEVs

Although other EVs can be loaded with ITR plasmids provided herein, as detailed herein, MEVs, by virtue of their properties, including ease of production, trafficking patterns upon administration, are delivery vehicles of choice for the ITR plasmids provided and described herein. Their properties and production are detailed herein.

2. Uptake of EVs

Cells internalize EVs by fusion with the plasma membrane, or more commonly by endocytosis (Abels and Breakefield (2016) *Cell Mol. Neurobiol.* 36(3):301-312). Uptake via endocytosis can be through several types of endocytotic processes, and different processes have been described in different cell types: clathrin-dependent endocytosis and phagocytosis have been described in neurons, macropinocytosis in microglia, phagocytosis and receptor-mediated endocytosis in dendritic cells, caveolin-mediated endocytosis in epithelial cells, and cholesterol- and lipid raft-dependent endocytosis in tumor cells. Blocking heparin sulfate proteoglycans (HSPGs) on the plasma membrane with heparin reduces the uptake of EVs in cell culture, as does blocking the scavenger receptor type B-1 (SR-B1) with a synthetic nanoparticle mimic of HDL, which suggests a role for HSPGs and SR-B1 in EV uptake (Abels and Breakefield (2016) *Cell Mol. Neurobiol.* 36(3):301-312). Fusion of EVs with the plasma membrane also is a method of uptake and requires low pH conditions; treatment of EVs with the combination of a pH-sensitive fusogenic peptide with cationic lipids resulted in increased cellular uptake of exosomes and the cytosolic release of cargo within the exosomes (Nakase and Futaki (2015) *Sci. Rep.* 5:10112). Low pH conditions occur in tumors (Abels and Breakefield (2016) *Cell*

Mol. Neurobiol. 36(3):301-312), so that EVs for delivering therapeutic payloads to tumor cells can enter cells through fusion with the plasma membrane.

Like cells, EVs have extracellular receptors and ligands on the outside and cytoplasmic proteins and nucleic acid on the inside, and thus communicate with cells in different ways. EVs bind to the cell surface, undergo endocytosis, and/or fuse with the plasma membrane, and release their cargos in the extracellular space. If entering by endocytosis, the EV cargo must escape the degradative pathway; late endosomes can fuse with lysosomes or the plasma membrane, so cargo must exit before it is degraded in a lysosome or re-released through the fusion of MVBs with the plasma membrane. EVs containing cargo, including mRNAs and non-coding RNAs, can be transferred to recipient cells in culture and *in vivo* (Abels and Breakefield (2016) *Cell Mol. Neurobiol.* 36(3):301-312; Maas *et al.* (2017) *Trends Cell Biol.* 27(3):172-188).

3. General Methods for Isolating EVs

a. Ultracentrifugation

Ultracentrifugation methods are used to isolate exosomes; alternative methods also have been developed. Due to the complex nature of the biological fluids from which exosomes are isolated, the overlap in physiochemical and biochemical properties between exosomes and other types of EVs, and the heterogeneity among exosomes, isolation methods can result in complex mixtures of EVs and other components of the extracellular space. Differential ultracentrifugation depends on the initial sedimentation of larger and denser particles from the extracellular matrix, and results in an enrichment of exosomes, but not a complete separation of exosomes from other components in the extracellular space. Density gradient centrifugation is another ultracentrifugation method and is based on separation by size and density in the presence of a density gradient (typically made of sucrose or iodixanol) in the centrifuge tube. Density gradient centrifugation effectively separates EVs from protein aggregates and non-membranous particles but has low exosome recovery, although purity can be improved by coupling differential ultracentrifugation with types of density gradient centrifugation, such as rate-zonal centrifugation or isopycnic centrifugation (Doyle and Wang (2019) *Cells* 8(7):727).

b. Size-Based Techniques

There are a number of size-based techniques for isolating exosomes (Doyle and Wang (2019) *Cells* 8(7):727). Ultrafiltration separates particles based on the size and molecular weight cut-off of the membrane, whereby particles larger than the molecular weight cut-off of the membrane are retained, and particles smaller than the molecular weight cut-off of the membrane are passed through into the filtrate; low isolation efficiency can occur however if the filter becomes clogged and vesicles become trapped. The ExoMir™ Kit (Bioo Scientific; Austin, TX) is a commercially available kit in which two membranes (200 nm and 20 nm) are placed into a syringe and a sample (typically pre-treated with centrifugation and proteinase K) is passed through the syringe; the larger vesicles remain above the first 200 nm filter, the smallest vesicles are passed through the syringe and discarded, and the vesicles between 20 and 200 nm remain between the two filters in the syringe. Sequential filtration also relies on a series of filtration steps to isolate exosomes (Doyle and Wang (2019) *Cells* 8(7):727).

Size Exclusion Chromatography (SEC), often used in parallel with ultracentrifugation methods (in which the exosome pellet obtained from ultracentrifugation is resuspended and further purified using SEC), of exosomes is similar to using SEC to separate proteins. In SEC, a column is packed with a porous stationary phase in which small particles can penetrate and thus elute after larger particles. Typically, SEC methods require several hours of run time; however, the qEV Exosome Isolation Kit (iZON Science, New Zealand) allows for rapid and precise exosome isolation by SEC within 15 minutes (Doyle and Wang (2019) *Cells* 8(7):727).

In Flow Field-Flow Fractionation (FFFF), a sample injected into a chamber is subjected to parabolic flow as it is pushed down the chamber, in addition to a flow perpendicular to the parabolic flow, a crossflow, to separate particles in the sample. Larger particles are more affected by the crossflow and are pushed toward the walls of the chamber, which have a slower parabolic flow, and smaller particles remain in the center. Smaller particles elute earlier, and larger particles later, in FFFF (Doyle and Wang (2019) *Cells* 8(7):727).

In Hydrostatic Filtration Dialysis (HFD), hydrostatic pressure forces a sample through a dialysis tube with a membrane having a molecular weight cut off of 1000

kDa. The result is that small solutes are able to pass through the tube, but larger particles, including exosomes and EVs, remain in the tube and can then be further separated using, for example, ultracentrifugation (Doyle and Wang (2019) *Cells* 8(7):727).

c. Immunoaffinity Capture-Based Techniques

Immunoaffinity capture-based techniques can isolate exosomes based on expression of an antigen on the surface of the exosome, and allow for the isolation of exosomes derived from a particular source. In these methods, an antibody specific for a target antigen can be attached to a plate (*e.g.*, in Enzyme-Linked Immunosorbent Assay, ELISA), magnetic beads (*e.g.*, in magneto-immunoprecipitation), resins and microfluidic devices; these surfaces are then exposed to the exosome sample, resulting in the immobilization of the exosomes expressing the antigen. This assay requires that the protein/antigen for isolating the exosomes be expressed on the surface of the exosomes, and its specificity is limited by the specificity of the antibody that is used, often resulting in a lower yield but higher purity of isolated exosomes. These methods also can be used to separate exosomes within mixed populations of EVs. Immunoaffinity capture-based techniques often are used after ultracentrifugation or ultrafiltration (Doyle and Wang (2019) *Cells* 8(7):727).

d. Exosome Precipitation

Methods for precipitation of exomes include precipitation by polyethylene glycol (PEG) and lectin. In PEG precipitation, the PEG polymer ties-up the water molecules, allowing the other particles, including exosomes, to precipitate out of solution. PEG precipitation is quick and is not limited to the starting volume of solution, but lacks selectivity, as other EVs, extracellular proteins, and protein aggregates are precipitated with EVs. Sample pretreatment using filtration and/or ultracentrifugation can improve exosome yield. Commercially available kits for isolating exosomes using precipitation include, for example, ExoQuick® (System Biosciences, Palo Alto, CA) and the Invitrogen™ Total Exosome Isolation Kit (Thermo Fisher Scientific, Waltham, MA). Alternatively, lectin precipitation can be used, typically after ultracentrifugation, whereby lectins bind to carbohydrates on the surface of exosomes, altering their solubility and leading to their precipitation out of solution (Doyle and Wang (2019) *Cells* 8(7):727).

e. Microfluidic Based Isolation Techniques

Microfluidic based techniques isolate exosomes based on their physical and biochemical properties simultaneously, and are rapid, efficient, and require small starting volumes. In acoustic nanofilter, a matrix containing EVs and other cellular components is injected into a chamber and exposed to ultrasound waves. The particles respond differently to the radiation forces exerted by the waves, depending on their size and density; large particles experience stronger forces and migrate faster toward the pressure nodes. The immuno-based microfluidic isolation technique is similar to that of an ELISA, although, unlike ELISAs, it does not require prior ultrafiltration or ultracentrifugation of exosomes (Doyle and Wang (2019) *Cells* 8(7):727). The ExoChip (Kanwar *et al.* (2014) *Lab Chip.* 14(11):1891-1900) and ExoSearch Chip (Zhao *et al.* (2016) *Lab Chip.* 16(3):489-496) have been developed to isolate exosomes using microfluidic technology.

4. Microalgae and Microalgae-Derived Extracellular Vesicles (MEVs)

Taxonomy and classification of microalgae can vary. According to some schemes there are seven (7) divisions of microalgae: Euglenophyta (Euglenoids), Chrysophyta (Golden-brown algae and Diatoms), Pyrrophyta (Fire algae), Chlorophyta (Green algae), Rhodophyta (Red algae), Paeophyta (Brown algae), and Xanthophyta (Yellow-green algae). Of interest herein are photosynthetic microalgae, such as the species *Chlorella* and *Chlamydomonas*. The methods and uses described herein use MEVs generally from green algae. Exemplary of such algae are *Chlamydomonas* and *Chlorella*, which belong to the classes Chlorophyceae and Trebouxiophyceae, respectively.

Microalgae are bioresources for the production of EVs for use in nanomedicine and other fields. The mechanism of secretion of EVs from microalgae is known in relation to primary and motile cilia/flagella (Picciotto *et al.* (2021) *Biomater. Sci.*, 9(8):2917-2930, doi:10.1039/d0bm01696a). *Chlamydomonas* flagella are devoid of MVBs, thus, ciliary EVs shed from *Chlamydomonas* are classified as ectosomes. Studies have shown the shedding of ectosomes from flagellar and ciliary tips of the chlorophyte *Chlamydomonas reinhardtii*. EVs also have been observed along the length of the cilium in *Chlamydomonas.* Membrane budding and ciliary EV

formation are mediated by components of the endosomal sorting complex required for transport (ESCRT), which are found in isolated ciliary transition zones, ciliary membranes, and ciliary EVs in *Chlamydomonas* and can act as sensors of membrane curvature. The formation of ciliary EVs also can occur when ciliary membrane trafficking is disrupted or during ciliary resorption (Wang and Barr (2018) *Essays Biochem.* 62(2):205-213). Ciliary ectosomes from *Chlamydomonas* contain a lytic enzyme that digests the mother cell wall and is required for the release of daughter cells. *Ift88*-null mutants that do not have flagella were unable to be released from the mother cell, and the addition of ciliary ectosomes from wild-type cells rescued the phenotype, suggesting a role for the flagella and intraflagellar transport (IFT) machinery in EV production (Wang and Barr (2016) *Cell Mol. Neurobiol.* 36(3):449-457).

EVs have been extracted from algal cells using ultra-centrifugation (Kuruvinashetti *et al.* (2020) IEEE 20th International Conference on Nanotechnology 354-357). In accord with this method, algal cells are cultured; the cultured algal cells are collected and centrifuged; the supernatant is collected (and further centrifuged); a sucrose solution is added to the supernatant; and the algal supernatant with the sucrose solution is ultra-centrifuged; because of the sucrose solution, the high-density EVs settle at the bottom of the ultra-centrifugation tube and can be collected using a pipette. Extracted algal EVs can be characterized in size and concentration using Nanoparticle Tracking Analysis (NTA). Studies using this method have isolated green algal EVs that range in size from 25-200 nm, with a concentration of 0.89E8 to 0.94E8 particles/mL (Kuruvinashetti *et al.* (2020) IEEE 20th International Conference on Nanotechnology 354-357).

An ultra-centrifugation protocol also can be used to isolate EVs from marine microalgae grown under various conditions; NTA showed that the nano-particles have a size distribution between 100 and 200 nm, and western blotting of proteins confirmed the presence of EV markers (VES4US, Extracellular vesicles from a natural source for tailor-made nanomaterials, 2020). Subsequent studies have identified microalgal small EVs (sEVs) isolated from the marine photosynthetic microalgal chlorophyte *Tetraselmis chuii*, termed nanoalgosomes. The production of nanoalgosomes is an evolutionarily conserved trait within microalgal strains as similar

results were obtained using sEVs isolated from batch cultures of two other microalgae species, the chlorophyte *Dunaliella tertiolecta*, and the dinoflagellate *Amphidinium sp*. The nanoalgosomes were isolated using differential centrifugation (dUC) and tangential flow filtration (TFF), as well as gradient ultracentrifugation, which was used to further purify samples enriched for small EVs by TFF or dUC. The isolated nanoalgosomes were shown to share characteristics of EVs from other sources. The EV yield (measured by sEV protein content and sEV number) from dUC and TFF was consistent with reported numbers of isolated EVs, around 10^9 EV particles/μg EV proteins. Biophysical analysis of particle size using multi-angle dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), fluorescence nanoparticle tracking analysis (F-NTA), and fluorescence correlation spectroscopy (FCS) yielded consistent size distributions, with the size that appeared the most frequently from DLS (DLS mode) around 70 nm. Compared to exosomes derived from mammalian cells, which have a density of 1.15-1.19 g/mol, nanoalgosomes had a slightly lower density of 1.13 g/mol. Electron microscopy revealed that the nanoalgosomes are spherical, heterogeneous in size and shape, and possess a lipid-bilayer structure. Compared to the microvesicles (or large EVs, lEVs) and lysates, the sEVs were enriched for three of the four target protein biomarkers (Alix, enolase, HSP70 and β-actin). DLS measurements indicated that the nanoalgosomes were resistant to changes in pH and stable in human blood plasma. The tumorigenic MDA-MB-231 breast cancer cell line, the non-tumorigenic 1-7 HB2 cell line, and the human hepatocarcinoma Hep G2 cell line did not show cytotoxic or genotoxic effects after nanoalgosome treatment. Furthermore, the nanoalgosome were taken up by the MDA-MB-231 and 1-7 HB2 cell lines (Adamo *et al.* (2021) *J. Extracell. Vesicles* 10:e12081).

EVs have been isolated from at least eighteen microalgae strains from the main microalgal lineages (*Ankistrodesmus sp., Brachiomonas sp., Chlamydomonas reinhardtii, Dunaliella tertiolecta, Tetraselmis chuii, Chloromonas sp., Rhodella violacea, Kirchneriella sp., Pediastrum sp., Nannochloropsis sp., Cyanophora paradoxa, Cryptomonas pyrenoidifera, Phaeodactylum tricornutum, Phaeothamnion sp., Diacronema sp., Isochrysis galbana, Stauroneis sp.,* and *Amphidinium sp.*) and have been studied. Studied strains include strains with a variety of features such as

saltwater and freshwater inhabitants, small and large sized cells, colonial and single cells, and species with sequenced genomes.

MEVs can be isolated using a differential ultracentrifugation protocol and characterized following the International Society for Extracellular Vesicles (ISEV) guidelines. All strains tested showed the presence of MEVs in the culture medium. EV-producing microalgae strains were established based on the EV protein content, the expression of EV protein markers (*e.g.*, Alix, Hsp70, enolase, and β-actin), the total scatting signal (measured by dynamic light scattering, DLS) or total particle number (measured by NTA), and the sEV average size and size range. These EV-producing strains include *Cyanophora paradoxa, Tetraselmis chuii, Amphidinium sp., Rhodella violacea, Diacronema sp., Dunaliella tertiolecta, Phaeodactylum tricornutum, Pediastrum sp.*, and *Phaeothamnion sp*. (Picciotto *et al.* (2021) *Biomater. Sci.*, 9(8):2917-2930, doi:10.1039/d0bm01696a). The data for *Cyanophora paradoxa* showed ~2×10^9 sEV particles per mL of microalgal-conditioned media, with strong positive signals for EV markers, and a size distribution with a mode of 130 ± 5 nm, in agreement with data from plant-derived vesicles. Cytotoxicity and genotoxicity studies showed that sEVs isolated from *Cyanophora paradoxa*, a freshwater Glaucophyte, did not show toxicity on the tumorigenic MDA-MB-231 breast cancer or C2C12 myoblast cell lines, neither over time nor at different concentrations, nor did MDA-MB-231 cells treated with the sEVs show morphological nuclear changes associated with apoptotic events (Picciotto *et al.* (2021) *Biomater. Sci.*, 9(8):2917-2930, doi:10.1039/d0bm01696a).

EVs also have been isolated from *Synechocystis sp*. PCC6803 (a cyanobacterium), *Chlamydomonas reinhardtii* (a green microalgae), *Euglena gracilis* (an euglenophyte), and *Haematococcus pluvialis* (a chlorophyte) in work done by Zhao *et al.*, who also performed RNomic and proteomic analyses in EVs isolated from *C. reinhardtii* at different stages of cell growth and under different types of abiotic stress (Zhao *et al.* (2020) doi:10.21203/rs.3.rs-38027/v1). EVs were isolated using differential ultracentrifugation and filtration, and the resuspension was shown to contain membrane structures with small clumps of particles 110-120 nm in diameter, in line with the reported diameter of exosomes and small MVs, although there were differences in diameters between the species of microalgae. Specifically, EVs from *C.*

reinhardtii had diameters between 37-710 nm, with an average particle diameter of 120.1 nm. *Synechocystis*-derived EVs had diameters between 24-450 nm, with an average particle size of 94.68 nm. Despite the presence of a cell wall, *Chlamydomonas* cells were able to uptake EVs, as shown by the presence of EVs labeled with a fluorescent lipophilic dye inside microalgal cells. Thus, microalgal EVs can be absorbed by recipient cells. Non-coding RNAs were detected in microalgal EVs at different growth stages and treatment (biotic stress, nitrogen depletion, and nitrogen recovery), and proteomic analyses identified many flagellar-associated membrane proteins in microalgal EVs (Zhao *et al.* (2020) doi:10.21203/rs.3.rs-38027/v1).

These studies show that microalgae produce EVs that can be isolated using traditional or standard methods; microalgal-derived EVs are similar in size and concentration, and exhibit similar markers compared to EVs isolated from other species; EVs isolated from microalgae do not show cytotoxic or genotoxic effects *in vitro*; and that microalgal-derived EVs can be taken up by cells.

It has been shown that EVs from mammalian origin can deliver cargo to a target cell and thus have therapeutic use for delivery of a variety of cargos for use in treating a number of diseases or conditions; this has not been shown for in general for MEVs. Mammalian EVs, except for bovine milk EVs, however, cannot be administered orally because they do not survive the harsh conditions of the stomach. For example, small molecules such as hydrophobic and hydrophilic drugs can be injected into exosomes, or macromolecular proteins and nucleic acids can be embedded into the exosomes. The nucleic acids can include those encoding a gene of interest. Specific targeting ligands, imaging probes, and covalent linkage could be attached to the exosome surface and tracked using NTA, fluorescence, or by bioluminescence.

Besides a mention in a publication that microalgae EVs possibly can be used to deliver a drug of interest to a targeted cell, tissue, or organ (Kuruvinashetti *et al.* (2020) IEEE 20th International Conference on Nanotechnology 354-357), there is no published evidence nor technical descriptions for use of MEVs for delivery for treatment of mammalian disease, disorders, or conditions. There are no publications or technical descriptions describing how knowledge for application of EV technology

to microalgae-derived extracellular vesicles, nor whether it is possible to do so, nor how to do so. Prior studies have not considered *Chlorella* species, nor have the prior studies assessed biodistribution and related properties of the MEVs in general. Hence methods, such as methods of oral delivery, exemplified herein with *Chlorella*, can employ MEVs from other microalgae.

As described and shown herein, however microalgal EVs have a number of advantages over the use of existing drug delivery systems, such as, exosomes derived from mesenchymal stem cells, gold nanoparticles, liposomes and other plant- and animal-derived EVs. Mesenchymal stem cells are a commonly used source of exosomes, and exosomes derived from mesenchymal stem cells are used in drug delivery, for example, anti-cancer vaccines, because they have enhanced passive targeting (a method of preparing a drug carrier system so that it remains circulating in the blood stream). Mesenchymal stem cell derived EVs possess the ability to passively target due to their small size, indigenous nature, and their ability to cross biological barriers. Mesenchymal stem cells, however, have limited secretion of exosomes, and scaling up production of exosomes is difficult due to the need to optimize purification, increase the homogeneity of exosomes, and establish efficient transfection strategies. Nanoparticles can lead to toxicity and current techniques for synthesizing nanoparticles limit their ability to scale for manufacturing purposes. Nanoparticle and liposome-based drug delivery methods also can lead to the formation of a teratoma (a tumor comprised of several different types of tissue). Liposome-based drug delivery methods have been further shown to be less efficient for internalization into a specific cell, tissue, or organ, compared to exosomes. Plant-derived EVs, such as those from curcumin, ginger, grapefruit, and lemon, have been used for drug delivery, but their extraction process and use in treatment has not yet been optimized. The production of EVs from agricultural products, such as fruits and milk, is economically impractical and need 3-4 months to grow, compared to algal EVs, which can be grown anywhere and within a few days. Algal EVs avoid phagocytosis or degradation by macrophages and circulate for prolonged times *in vivo*, and have low immunogenicity. Algal EVs also have a lower risk of teratoma formation. Algae, thus, provide a source from which pure, well-characterized EVs of high quality can be obtained (Kuruvinashetti *et al.* (2020) IEEE 20th International

Conference on Nanotechnology 354-357). Kuruvinashetti *et al.* does not describe the use of *Chlorella* species as a source of EVs, nor its advantages as a source. Prior art does not describe the biodistribution of MEVs per se, nor the implications thereof for administration of MEVs with drugs directed to particular organs, tissues, or systems.

5. Green algae – *Chlorella* species

Chlorella species and other members of the Chlorellaceae family, such as Parachlorella, are advantageous sources of EVs. *Chlorella* and the resulting EVs have advantages for growth, manipulation, and administration of drugs that other species and EVs do not provide. Green algae belong to phylum Chlorophyta, and encompass a diverse group of photosynthetic eukaryotes. Green algae include unicellular and multicellular organisms. Algae originally included in the genus *Chlorella* are among the most widely distributed and frequently encountered algae in freshwater. These algae exist in aqueous environments and on land. They are typically small (~2 to 10 μm in diameter), unicellular, spherical in shape, non-motile, and contain a single chloroplast, and some have a rigid cell wall (Blanc *et al.* (2010) *Plant Cell* 22(9):2943-2955).

Molecular analyses have separated *Chlorella* species into two classes of chlorophytes: the Trebouxiophyceae, which contains the true *Chlorella*; and the Chlorophyceae. For use herein, *Chlorella* species include any that can be or that are used as food complement or that can be consumed by humans or other animals, such as livestock. Exemplary species include, but are not limited to, the species: *Chlorella ellipsoidea, Chlorella pyrenoidosa*, *Chlorella sorokiniana, Chlorella vulgaris*, and *Chlorella variabilis*.

True *Chlorella* species are characterized by glucosamine as a major component of their rigid cell walls. Although most *Chlorella* species are naturally free-living, the Trebouxiophyceae include most of the known green algal endosymbionts, living in lichens, unicellular eukaryotes, plants, and animals (for example mussels and hydra). For example, *Chlorella variabilis* NC64A is a hereditary photosynthetic endosymbiont (or photobiont) of *Paramecium bursaria,* a unicellular protozoan, and NC64A also is a host for a family of large double-stranded DNA viruses that are occur in freshwater (Blanc *et al.* (2010) *Plant Cell* 22(9):2943-2955).

a. Life Cycle

In unicellular organisms, such as microalgae, life cycle is the same as the cell cycle. *Chlorella* is a haploid organism that reproduces asexually by autosporulation. The cell cycle and proliferation of *Chlorella vulgaris* has been investigated using flow cytometric analysis of 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE)-stained algal cells by Rioboo *et al.* (2009) *Aquatic Toxicology*, 94(3):229-237, doi:10.1016/j.aquatox.2009.07.009. Their results indicate that, as generally described for microalgae, the growth of *C. vulgaris* mother cells takes place during light periods, whereas cytoplasmic division and liberation of daughter cells takes place during dark periods. *C. vulgaris* also shows a distinct light/dark cycle, marked by an increase in cell size, cell complexity, and autofluorescence during periods of light, measured over a 96-hour period. A monoparametric histogram of CFSE-stained *C. vulgaris* cells showing only one peak of daughter cells indicates that each mother cell undergoes only one division cycle in 96 hours; the cytoplasmic division was further shown to take place during periods of darkness. Thus, the strain of *C. vulgaris* used exhibits three life cycle phases: 1) growth of mother cells, 2) cell division, and 3) liberation of daughter cells. *C. vulgaris* cells grew during 2 light periods and began to divide during following dark period; cell division occurs once the mother cells are double the size of daughter cells. Furthermore, *C. vulgaris* cells exposed to the herbicide terbutryn need a longer growth period in order to reach a large enough cell size to divide. This suggests there is a critical threshold size needed for *C. vulgaris* to complete the growth phase and begin the division phase, and that this critical threshold can control the progression of the G1 phase of the *C. vulgaris* cell cycle. Finally, this study demonstrates that the intensity of the peak of CFSE-fluorescence of mother cells is four times greater than that of the daughter cells, indicating that 4 daughter cells are produced from each mother cell. Thus, *C. vulgaris* cells undergo a first mitosis followed by cytoplasmic division, and then two other simultaneous mitoses, which result in the liberation of 4 daughter cells (see *e.g.,* Rioboo *et al.* (2009) *Aquatic Toxicology*, 94(3):229-237, doi:10.1016/j.aquatox.2009.07.009).

b. Genomic Analyses of *Chlorella* Species

Although species of *Chlorella* are reported to be non-motile and lack a sexual cycle, genomic analyses of *Chlorella variabilis* NC64A (NC64A) and *Chlorella vulgaris* 211/11P (211/11P) reveal the presence of genes involved in sexual

reproduction and motility (Blanc *et al.* (2010) *Plant Cell* 22(9):2943-2955; Cecchin *et al.* (2019) *Plant J.* 100(6):1289-1305). The NC64A nuclear genome (GenBank Accession No. ADIC00000000.1) is 46.2 Mb, and composed of 12 chromosomes. The meiosis-specific proteins dosage suppressor of MCk1 DMC1, homologous-pairing proteins HOP1 and HOP2, meiotic recombination protein MER3, meiotic nuclear division protein MND1, and mutS homolog protein MSH4 are encoded in NC64A; these genes also occur in most of the other sequenced chlorophyte algal species. Nineteen homologs of the *Chlamydomonas* gametolysin proteins, which promote disassembly of the gametic cells walls and allow gamete fusion, also were identified in NC64A. Additionally, an ortholog of the *Chlamydomonas* GCS1 protein, which is essential for cell fusion, occurs in NC64A (Blanc *et al.* (2010) *Plant Cell* 22(9):2943-2955). The primary genes involved in meiosis also occur in the *Chlorella vulgaris* 211/11P 40 Mb genome (GenBank Accession No. SIDB00000000), in addition to the gene encoding gametolysin (g3347), and a gene encoding a protein that contains a domain with a putative GCS1/HAP2 function (Cecchin *et al.* (2019) *Plant J.* 100(6):1289-1305). Thus, although *Chlorella* species have been observed only in the haploid phase, the presence of meiosis genes indicates that the life cycle of *Chlorella* could include a diploid phase.

Similarly, while flagella have not been observed in NC64A, orthologs of the *Chlamydomonas* flagellar proteins were identified in the NC64A genome, including orthologs to the intraflagellar transport (IFT) proteins IFT52, IFT57, and IFT88, kinesin-2 motor protein FLA8, the kinesin-associated protein KAP, and proteins involved in the axonemal outer dynein arm (Blanc *et al.* (2010) *Plant Cell* 22(9):2943-2955).

Sequencing of three *Chlorella sorokiniana* strains, strain 1228, UTEX 1230, and DOE1412, reveals the presence of sex- and flagella-related genes (Hovde *et al.* (2018) *Algal Research* 35:449-461). The genome of several other *Chlorella* species has been sequenced: *Chlorella protothecoides* sp. 0710 (Gao *et al.* (2014) *BMC Genomics* 15(1):582; GenBank Accession No. APJO00000000); *Chlorella sorokiniana* UTEX 1602 (GenBank Accession No. LHPG00000000) and *Chlorella* sp. strain SAG 241.80 (*Micractinium conductrix*; GenBank Accession No. LHPF00000000) (Arriola *et al.* (2018) *Plant J.* 93(3):566-586); and the *Chlorella*

vulgaris strains UTEX 395 (Guarnieri *et al.* (2018) *Front. Bioeng. Biotechnol.* 6:37; GenBank Accession No. LDKB00000000), UMT-M1 (Teh *et al.* (2019) *Data Brief* 27:104680; GenBank Accession No. VJNP00000000), UTEX 259 (GenBank Accession No. VATW00000000) and NJ-7 (Wang *et al.* (2020) *Mol. Biol. Evol.* 37(3):849-863; GenBank Accession No. VATV00000000).

c. Commercial and Biotechnological Uses of *Chlorella*

The commercial cultivation of microalgae for food purposes began with the production of *Chlorella vulgaris* in Japan and Taiwan in the 1960s. Dried biomass products from *Arthrospira* and *Chlorella* are included in dietary supplements due to reports of high protein content, nutritive value, and health benefits. For example, *Chlorella* extracts have been shown to lower cholesterol and have antioxidant, antibacterial, and antitumor activities. Production of high yields of *Chlorella* is routine, and, as detailed herein, MEVs can be isolated from the cell culture medium. For its use as a pharmaceutical, it is known that ingestion of *Chlorella* is non-toxic and non-immunogenic in humans.

Chlorella has been used in a variety of biotechnology applications, including biofuels, sequestering CO_2, producing molecules of high economic value, or removing heavy metals from wastewaters (Blanc *et al.* (2010) *Plant Cell* 22(9):2943-2955). *Chlorella* species show metabolic flexibility in response to environmental perturbations, and are capable of using nutrients, such as organic carbon and minerals, directly from wastewater for growth. Among microalgae, *Chlorella* species have higher photosynthetic efficiency over other photosynthetic organisms. Additionally, *Chlorella vulgaris* is able to grow either in autotrophic, heterotrophic or mixotrophic conditions (Zuñiga *et al.* (2016) *Plant Physiol.* 172(1):589-602).

Chlorella species also can be genetically modified by *Agrobacterium*-mediated transformation. A study by Cha *et al.* developed a method to genetically transform *Chlorella vulgaris* using the *Agrobacterium tumefaciens* strain LBA4404, and the presence of gene fragments in 30% of the transgenic lines, compared to the wild-type non-infected *Chlorella*, indicates the T-DNA was integrated into the *Chlorella* genome (Cha *et al.* (2012) *World J. Microbiol. Biotechnol.* 28:1771-1779).

d. Chlorella MEVs

As described herein, *Chlorella* species, such as *C. vulgaris*, are advantageous species for the production of EVs, referred to herein as MEVs, for use for delivery of the ITR plasmids, and also the ITR bacterial plasmids. Of particular interest herein are MEVs produced by Chlorellaceae family members, including *Chlorella* species. *Chlorella*, and related species, as a source of EVs provides numerous advantages. *Chlorella* is a haploid organism, which means that specific and targeted variants can be produced by genetic engineering; it readily can be genetically modified or loaded to produce or contain biologically active molecules and small molecules, including the ITR plasmids and the bacterial ITR plasmids. Stable cell lines can be produced, including stable producers of encoded products. They are defined products, and, when exogenously loaded, the resulting compositions contain EVs that contain the same cargo.

Detailed genetic maps can be obtained, and correlations between genotype and phenotype can be established. *Chlorella* genomes have been fully sequenced, so the structure and function of various genes can be known. Phylogenetically, *Chlorella* is at the very crossroads between higher plants and microalgae. As such, *Chlorella* shares with higher plants a significant (and useful) number of molecular biological and metabolic features, but still is a unicellular haploid microalga. Exemplary of molecular biological features shared with eukaryotes is the intracellular machinery that involves the dicer enzyme system for processing exogenous RNA into siRNA. *Chlorella* is autotrophic: unlike mammalian and other animal cells, it can therefore be cultured and reproduced without the need for nutrients or factors of animal origin.

With respect to use of its EVs as therapeutics, *Chlorella* and related species are not toxic. For example, tablets made from *Chlorella* vulgaris biomass (*i.e.*, compressed whole *Chlorella* cells) have been consumed regularly for years by the public worldwide as a dietary supplement, without constraints related to toxicity or immunogenicity. Japan is the world leader in the consumption of *Chlorella* biomass. It also is used, for example, in Japan, for medical treatments because it has shown to have immunomodulatory properties and purported anti-cancer activities, for use for anti-aging applications, such as for cardiovascular diseases, hypertension and cataracts; it reduces the risk of atherosclerosis and stimulates the synthesis of collagen for the skin.

Chlorella cells naturally produce extracellular vesicles (EVs) that respond to the 'standard specifications' of better known EVs (such as mammalian EVs). EVs from plant origin bear a number of features that make them more promising/convenient than synthetic nanoparticles or semisynthetic EVs, for use as a drug delivery system in humans. These include, for example, higher stability, lower toxicity, and lower immunogenicity. Being as close as plants as it is, *Chlorella* provides a source of EVs with similar characteristics to plant EVs. At the same time, mass production of *Chlorella* in large scale is easier and cheaper than for higher plants. The glycosylation pattern of membrane proteins in *Chlorella* is similar/identical to the glycosylation pattern present in higher plants.

The size of the *Chlorella* MEVs ranges between about or between 50 nm and 200m, with an average size of about 130 nm. The morphology resembles plant and mammalian exosomes. For use for administration, the size distribution can be rendered more uniform by separating the MEVs by size and selecting those of a size of interest, which can vary depending upon the intended use and route of administration.

F. EXOGENOUSLY LOADED MICROALGAE EXTRACELLULAR VESICLES (MEVS), CARGO ENCODED AS TRANSGENES, AND TARGETS

Targets and cargo (see discussions below) include any known to those of skill in the art as suitable for gene therapy. Details and examples of the biodistribution of MEVs following administration by various routes, and the implications, uses and methods for targeting or treating particular diseases, disorders, and conditions, and for formulating and administering the MEVs, are described herein and exemplified in the Examples.

1. Isolation of MEVs

Methods for isolation are discussed in the sections above and detailed in the Examples.

2. MEV Loading and Cargos

The MEVs can be loaded with any desired cargo (also referred to as a payload). For purposes herein the cargo comprises the plasmids containing the transgenes, which can encode proteins and other products of interest, including, but not limited to, nucleic acid encoding, RNAi, anti-sense nucleic acids, detectable

marker proteins and tags, small molecule drugs, gene editing systems, and others, and combinations thereof and can be used to deliver gene editing systems, such as CRISPR/Cas and to effect gene editing.

Diseases and conditions that can be treated include any known to those of skill in the art that can be treated by gene therapy or by the delivery and expression of DNA. Diseases, disorders, and conditions, include, for example, genetic disorders that require delivery of a gene product and/or a gene editing system, neurological disorders, infections, and other conditions that are treated by administration of a viral vector. Delivered transgenes include those that encoded protein products, such as antibodies and antigen-binding forms thereof, RNA products, such as, but not limited to, siRNA, miRNA (micro-RNA), lncRNA (long non-coding RNA), saRNA (small activating RNA), shRNA, and mRNA, and other such products. The plasmids can encode reporters genes and other detectable products, such as, for example, a fluorescent protein, such as, but not limited to an enhanced green fluorescent protein (EGFP; SEQ ID NO:99), a luciferase gene (SEQ ID NO:100), the LUX operon (SEQ ID NO:98), and related products

Genes involved in diseases, such as oncogenes, and checkpoints, can be modulated by cargo that encodes a product that inhibits or agonizes expression of a gene, or inhibits or agonizes a gene product. Exemplary of such modulators, are RNAi-type modulators, such as for example, siRNAs, miRNAs, shRNAs, anti-sense oligonucleotides (ASOs), peptides and/or tetratricopeptides.

Gene silencing using RNA interference, including siRNAs and microRNAs, can be used to silence developmental genes, such as, for example, adhesion molecules, cyclin kinase inhibitors, Wnt family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, and neurotransmitters and their receptors; oncogenes; tumor suppressor genes; enzymes; genes associated with a pathological condition; genes associated with autoimmune diseases; anti-angiogenic genes; angiogenic genes; immunomodulator genes; genes associated with alcohol metabolism and liver function; genes associated with neurological disease; genes associated with tumorigenesis or cell transformation; and genes associated with metabolic diseases and disorders (see, *e.g.*, WO 2009/082606,

JP 2014-240428A, WO 2011/072292A2, WO 2010/141724, and WO 2020/097540). These types of products can be delivered in or encoded in MEVs to activate genes or pathways or to provide therapeutic effects. Certain cytokines can be used to treat diseases/disorders, such as certain cancers, in which immune suppression plays a role.

The MEV-GTVs can be used to transfer nucleic acid encoding therapeutic such as microRNA, mRNA, tRNA, rRNA, siRNA, regulatory RNA, non-coding and encoding RNA, DNA fragments, and DNA plasmids (see, *e.g.*, CN105821081A and CN110699382A. Non-limiting examples of proteins that can be encoded by the nucleic acid cargo molecule include, but are not limited to: antibodies, intrabodies, single chain variable fragments, affibodies, enzymes, transporters, tumor suppressors, viral or bacterial inhibitors, cell component proteins, DNA and/or RNA binding proteins, DNA repair inhibitors, nucleases, proteinases, integrases, transcription factors, growth factors, apoptosis inhibitors and inducers, toxins, structural proteins, neurotrophic factors, membrane transporters, nucleotide binding proteins, heat shock proteins, CRISPR-associated proteins, cytokines, cytokine receptors, caspases and any combination and/or derivatives thereof (see, *e.g.*, AU2018365299).

For example, as summarized in the table below, a cocktail of three siRNA oligonucleotides targeting human MYCN with two thymidine residues (dTdT) at the 3'-end of the sequence (purchased from B-Bridge International Inc. (Sunnyvale, CA)) can be used. The anti-MYCN siRNA (siMYCN) and negative control siRNA (nontarget control pool) (siNeg) (both ON-TARGETplus siRNA, Dharmacon, Cambridge, UK) were used (see Ref 1). Exemplary target oncogenes and exemplary sequences of siRNA (see, also, SEQ ID NOs: 101-123) are provided in the table below.

Target oncogene	Type of sequence	Sequence(s)
[1]MYCN	siRNA cocktail	siMYCN-1: sense 5'-CGGAGATGCTGCTTGAGAA-3' siMYCN-2: sense 5'-CGGAGTTGGTAAAGAATGA-3' siMYCN-3: sense 5'-CAGCAGTTGCTAAAGAAAA-3'
[2]MYCN	siRNA	sense 5′-CAGCAGUUGCUAAAGAAAAUU-3′ antisense 5′-UUUUCUUUAGCAACUGCUGUU-3′
[3]MYCN	siRNA	5′-UGAUCUGCAAGAACCCAGA-3′

Target oncogene	Type of sequence	Sequence(s)
[4]MYCN	ASO	5'-AAC GTT GAG GGG CAT-3'
[5]RAS	synthetic miRNA	**Syn-miR-143s** **No.** / **Type** / **Sequence** #1 / Wild / S: 3'-GGUCUCUACGUCGUGACGUGGAGU-5' AS: 5'-UGAGAUGAAGCACUGUAGCUCAGG-3' #12 / F/Ome / S: 3'-GGUCUCUACGUCGUGACGUGGAGU-5' AS: 5'-U^G^AGAUGAAGCACUGUAGCUCA^Dt^Dt-3' N:2'-F RNA **N: 2'-Ome** RNA N:RNA ^: PS _: mismatch
[6]RAS	siRNA	siR-KRAS: 5'-AAUGCAUGA CAACACUGGAUGACCG-3' siR-HRAS: 5'-CCAUCCAGCUGAUCCAGAACCAUUU-3'
[7]RAS	siRNA	GGACUCUGAAGAUGUACCUAGGUACAUCUUCAGAGUCC
[8]mutated K-RAS	ASO	5'-CTACGCCACAAGCTCCA-3'
[9]MYC, BCL2,	miRNA	Homo sapiens microRNA 34a (MIR34A), microRNA
[10]BLC2	3p-siRNA	sense GCAUGCGGCCUCUGUUUGA anti-sense CGUACGCCGGAGACAAACU
[11]BLC2	ASO	TCT CCC AGC GTG CGC CAT
[12]PLK1	modified siRNA	sense: 5'-AGA **U**CA CCC **U**CC **UU**A AA**U** AUU-3' antisense: 5'-UAU UUA A**GG** AGG GUG A**U**C UUU-3' *note: 2'-O-methylated modified nucleotides **in bold***
[13]PLK1	siRNA	5'-AAGGGCGGCUUUGCCAAGUGC-3'
[14]PLK1	ASO	P12: ACC AGT CCG GAG GGG AGG GC

1 Nara *et al.* (2007) *Int. J. Oncol.* 30(5):1189-1196; Silencing of MYCN by RNA interference induces growth inhibition, apoptotic activity and cell differentiation in a neuroblastoma cell line with MYCN amplification

2 Maeshima *et al.* (2020) *Nucleic Acid Ther*. 30(4):237-248; MYCN Silencing by RNAi Induces Neurogenesis and Suppresses Proliferation in Models of Neuroblastoma with Resistance to Retinoic Acid

3 Veas-Perez de Tudela *et al.* (2010) *J. Neurochem*. 113(4):819-825; Human neuroblastoma cells with MYCN amplification are selectively resistant to oxidative stress by transcriptionally up-regulating glutamate cysteine ligase

4 Watson *et al.* (1991) *Cancer Res*. 51(15):3996-4000; Inhibition of c-myc expression by phosphorothioate antisense oligonucleotide identifies a critical role for c-myc in the growth of human breast cancer

5 Yoshikawa *et al.* (2019) *Mol. Ther. Methods Clin. Dev*. 13:290-302; Anti-cancer Effects of a Chemically Modified miR-143 on Bladder Cancer by Either Systemic or Intravesical Treatment

6 Tsujino *et al.* (2019) *Cancer Sci*. 110(7):2189-2199; MicroRNA-143/Musashi-2/KRAS cascade contributes positively to carcinogenesis in human bladder cancer

7 Tirella *et al.* (2019) *Int. J. Pharm*. 561:114-123; CD44 targeted delivery of siRNA by using HA-decorated nanotechnologies for KRAS silencing in cancer treatment

8 Nakada *et al.* (2001) *Pancreatology* 1(4):314-319; Antisense oligonucleotides specific to mutated K-ras genes inhibit invasiveness of human pancreatic cancer cell lines

9 Adams *et al.* (2015) *Expert Opin. Ther. Targets* 20(6):737-753; The Tumor-Suppressive and Potential Therapeutic Functions of miR-34a in Epithelial Carcinomas

10 Poeck *et al.* (2008) *Nat. Med.* 14(11):1256-1263; 5'-Triphosphate-siRNA: turning gene silencing and Rig-I activation against melanoma

11 Szegedi *et al.* (2008) *Pathol. Oncol. Res.* 14(3):275-279; Bcl-2 Antisense Oligonucleotide Inhibits the Proliferation of Childhood Leukemia/lymphoma Cells of the B-cell Lineage

12 Ripoll *et al.* (2018) *RSC Adv.* 8:20758-20763; Co-delivery of anti-PLK-1 siRNA and camptothecin by nanometric polydiacetylenic micelles results in a synergistic cell killing

13 Liu *et al.* (2012) *BMC Cancer* 12(1):519-529; MicroRNA-100 is a potential molecular marker of non-small cell lung cancer and functions as a tumor suppressor by targeting polo-like kinase 1

14 Spänkuch *et al.* (2008) *Neoplasia* 10(3):223-234; Downregulation of Plk1 expression by receptor-mediated uptake of antisense oligonucleotide-loaded nanoparticles

3. Reporter genes, reporter proteins, and/or modulators thereof can be encoded in the plasmids, and other products, can be delivered in the MEV-GTVs

Reporter proteins

Target sequences, in the form of siRNAs, miRNAs, anti-sense oligonucleotides (ASOs), peptides and/or tetratricopeptides, to modulate (inhibition or stimulation) of each of the marker genes, such as a GFP protein, a eukaryotic luciferase, or a prokaryotic Luciferase, such as: Lux operon (luxCDABE) and lux operon (luxABCDE), as well as the reporter genes/proteins can be encoded in the plasmids, for example for diagnostics and gene expression assessments (SEQ ID NOs:124-130, respectively):

Target gene	Type of sequence	Sequence(s)
EGFP	siRNA	sense: 5′-GCAAGCUGACCCUGAAGUUCAUUU-3′ antisense: 5′-AUGAACUUCAGGGUCAGCUUGCCG-3′
firefly luciferase	shRNA	5′-CTGACGCGGAATACTTCGA-3′
luxA (Lux operon)	siRNA	sense: 5′-CAAACAGAGGUAAUGAAAUGGUUG-3′ antisense: 3′-CAACCAUUUCAUUACCUCUGUUUG-5′
luxB (Lux operon)	siRNA	sense: 5′-AUGUUAAGUUGAAUAAGUUCUGCA-3′ antisense: 3′-UGCUCUUGAAUAAGUUGAAUUGAU-5′

Other exemplary encoded products

Chemotherapeutic agents, particularly encoded prodrugs can be encoded in the vectors. . Anti-angiogenic agents such as an antibody, Fc fusion, and cytokine, that binds to a growth factor or growth factor receptor involved in promoting angiogenesis. Examples of anti-angiogenic agents include but are not limited to antibodies that bind to Vascular Endothelial Growth Factor (VEGF) or that bind to VEGF-R, RNA-based therapeutics that reduce levels of VEGF or VEGF-R

expression, VEGF-toxin fusions, Regeneron's VEGF-trap, angiostatin (plasminogen fragment), antithrombin III, angiozyme, ABT-627, Bay 12-9566, BeneFin, bevacizumab, bisphosphonates, BMS-275291, cartilage-derived inhibitor (CDI), CAI, CD59 complement fragment, CEP-7055, Col 3, Combretastatin A-4, endostatin (collagen XVIII fragment), farnesyl transferase inhibitors, fibronectin fragment, GRO-beta, halofuginone, heparinases, heparin hexasaccharide fragment, HMV833, human chorionic gonadotropin (hCG), IM-862, interferon alpha, interferon beta, interferon gamma, interferon inducible protein 10 (IP-10), interleukin-12, kringle 5 (plasminogen fragment), marimastat, metalloproteinase inhibitors (*e.g.*, TIMPs), 2-methoxyestradiol, MMI 270 (CGS 27023A), plasminogen activator inhibitor (PAI), platelet factor-4 (PF4), prinomastat, prolactin 16 kDa fragment, proliferin-related protein (PRP), PTK 787/ZK 222594, retinoids, solimastat, squalamine, SS3304, SU5416, SU6668, SU11248, tetrahydrocortisol-S, tetrathiomolybdate, thalidomide, thrombospondin-1 (TSP-1), TNP470, transforming growth factor beta (TGF-β), vasculostatin, vasostatin (calreticulin fragment), ZS6126, and ZD6474.

Other cargo includes tyrosine kinase inhibitors, which include, but are not limited to quinazolines, such as PD 153035, 4-(3-chloroanilino) quinazoline; pyridopyrimidines; pyrimidopyrimidines; pyrrolopyrimidines, such as CGP 59326, CGP 60261 and CGP 62706; pyrazolopyrimidines, 4-(phenylamino)-7H-pyrrolo(2,3-d) pyrimidines; curcumin (diferuloylmethane, 4,5-bis (4-fluoroanilino) phthalimide); tyrphostins containing nitrothiophene moieties; PD-0183805 (Warner-Lambert); antisense molecules (*e.g.*, those that bind to ErbB-encoding nucleic acid); quinoxalines (U.S. Pat. No. 5,804,396); tyrphostins (U.S. Pat. No. 5,804,396); PTK-787 (Novartis/Schering A G); pan-ErbB inhibitors such as C1-1033 (Pfizer); Affinitac (ISIS 3521; Isis/Lilly); Imatinib mesylate (STI571, Gleevec®; Novartis); PKI 166 (Novartis); GW2016 (Glaxo SmithKline); C1-1033 (Pfizer); EKB-569 (Wyeth); Semaxinib (Sugen); ZD6474 (AstraZeneca); INC-1 C11 (ImClone); or as described in any of the following patent publications: U.S. Pat. No. 5,804,396; PCT WO 99/09016 (American Cyanamid); PCT WO 98/43960 (American Cyanamid); PCT WO 97/38983 (Warner-Lambert); PCT WO 99/06378 (Warner-Lambert); PCT WO 99/06396 (Warner-Lambert); PCT WO 96/30347 (Pfizer, Inc.); PCT WO 96/33978 (AstraZeneca); PCT WO 96/33979 (AstraZeneca); PCT WO 96/33980 (AstraZeneca),

gefitinib (Iressa®, ZD1839, AstraZeneca), and OSI-774 (Tarceva®, OSI Pharmaceuticals/Genentech).

Other encoded cargo includes immunomodulatory agents that increase or decrease production of one or more cytokines, up-or down-regulate self-antigen presentation, mask MHC antigens, or promote the proliferation, differentiation, migration, or activation state of one or more types of immune cells

Other encoded cargo includes cytokines which include, but are not limited to lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormones such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-alpha and-beta; Müllerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-beta; platelet-growth factor; transforming growth factors (TGFs) such as TGF-alpha and TGF-beta; insulin-like growth factor-I and-II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-alpha, beta, and-gamma; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1alpha, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; IL-15, a tumor necrosis factor such as TNF-alpha or TNF-beta; and other polypeptide factors including LIF and kit ligand (KL).

Other exemplary encoded cargo includes cytokines and other agents that stimulate cells of the immune system and enhance desired effector function. For example, agents that stimulate NK cells include IL-2; agents that stimulate macrophages include but are not limited to C5a, formyl peptides such as N-formyl-methionyl-leucyl-phenylalanine. Cargo includes agents that stimulate neutrophils, such as, for example, G-CSF and GM-CSF. Additional agents include, but are not limited to, interferon gamma, IL-3, and IL-7. Other cargo includes nucleic acid

encoding polypeptide inhibitors of complement, such complement protein C3 or C5, including modified proteases that inhibit C3.

The nucleic acids can be operably linked to regulatory elements that are recognized in the particular subject, such as a mammal, in which they are to be delivered.

4. Generation of Payload (cargo)-Loaded MEVs

As shown herein, the isolated MEVs, such as the *Chlorella* MEVs, can be loaded with the plasmid cargo for delivery to humans by any suitable route, including but not limited to intravenous, oral, topical, mucosal, inhalation, and any other routes known to those of skill in the art for delivery of vehicles, such as lipid nanoparticles, vectors, therapeutic bacteria, and therapeutic viruses. Upon administration, the MEV-GTVs are taken up by cells. The transgenes can be designed so that transcription and/or can only occurs targeted cells, such as in instances in which the cargo is a plasmid encoding a therapeutic product. Transcription regulatory signals can be selected so that the encoded product is expressed in targeted cells. For example, for expression in the liver, the encoded product can be expressed under control of a liver-specific promoter, or the product can be targeted to a receptor or target expressed in targeted cells, such as in tumors or in the tumor microenvironment. Loading methods, described above, and in the Examples below, include, but are not limited to:

a. Electroporation
b. Sonication
c. Extrusion
d. Surfactants
e. Other Methods known to those of skill in the art for introducing exosomes into cells.

F. PHARMACEUTICAL COMPOSITIONS, FORMULATIONS, KITS, ARTICLES OF MANUFACTURE AND COMBINATIONS

1. Pharmaceutical Compositions and Formulations

The compositions containing the MEVs and loaded MEVs provided herein can be formulated as pharmaceutical compositions provided for administration by a desired route, such as oral and intramuscular (IM) delivery of the cargo to the immune system, or formulated for administration to the eye. Pharmaceutically acceptable

compositions are prepared in view of approvals for a regulatory agency or other agency prepared in accordance with generally recognized pharmacopeia for use in animals and in humans, and also, for agricultural applications, for plants. Typically, compounds are formulated into pharmaceutical compositions using techniques and procedures well-known in the art (see *e.g.*, Ansel *Introduction to Pharmaceutical Dosage Forms*, Fourth Edition, 1985, 126).

The pharmaceutical compositions provided herein are for use for therapeutic and prophylactic applications. The MEVs and cargo-loaded MEVs provided herein can be formulated with a pharmaceutically acceptable carrier or diluent. Generally, such pharmaceutical compositions include components that do not significantly impair the biological properties or other properties of the cargo. Each component is pharmaceutically and physiologically acceptable so that it is compatible with the other ingredients and not injurious to the subject to whom it is to be administered. The formulations can be provided in unit dosage form and can be prepared by methods well-known in the art of pharmacy, including but not limited to, tablets, pills, powders, liquid solutions or suspensions (*e.g.*, including injectable, ingestible and topical formulations, for example, eye drops, gels, pastes, creams, or ointments), aerosols (*e.g.*, nasal sprays and inhalers), liposomes, suppositories, pessaries, injectable and infusible solutions and sustained release forms. See, *e.g.*, Gilman, *et al.* (eds. 1990) *Goodman and Gilman's: The Pharmacological Bases of Therapeutics*, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, 17th ed. (1990), Mack Publishing Co., Easton, Pa.; Avis, *et al.* (eds. 1993) *Pharmaceutical Dosage Forms: Parenteral Medications* Dekker, NY; Lieberman, *et al.* (eds. 1990) *Pharmaceutical Dosage Forms: Tablets* Dekker, NY; and Lieberman, *et al.* (eds. 1990) *Pharmaceutical Dosage Forms: Disperse Systems* Dekker, NY. When administered systemically, the therapeutic composition is sterile, pyrogen-free, generally free of particulate matter, and in a parenterally acceptable solution having due regard for pH, isotonicity, and stability. These conditions are known to those skilled in the art. Methods for preparing parenterally administrable compositions are well-known or will be apparent to those skilled in the art and are described in more detail in, *e.g.*, "*Remington: The Science and Practice of Pharmacy* (Formerly

Remington's Pharmaceutical Sciences)", 19th ed., Mack Publishing Company, Easton, Pa. (1995).

Pharmaceutical compositions provided herein can be in various forms, *e.g.*, in solid, semi-solid, liquid, powder, aqueous, and lyophilized form. Examples of suitable pharmaceutical carriers are known in the art and include but are not limited to water, buffering agents, saline solutions, phosphate buffered saline solutions, various types of wetting agents, sterile solutions, alcohols, gum arabic, vegetable oils, benzyl alcohols, gelatin, glycerin, carbohydrates such as lactose, sucrose, amylose or starch, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, pentaerythritol fatty acid esters, hydroxy methylcellulose, and powders, among others. Pharmaceutical compositions provided herein can contain other additives including, for example, antioxidants, preservatives, antimicrobial agents, analgesic agents, binders, disintegrants, coloring, diluents, excipients, extenders, glidants, solubilizers, stabilizers, tonicity agents, vehicles, viscosity agents, flavoring agents, emulsions, such as oil/water emulsions, emulsifying and suspending agents, such as acacia, agar, alginic acid, sodium alginate, bentonite, carbomer, carrageenan, carboxymethylcellulose, cellulose, cholesterol, gelatin, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, methylcellulose, octoxynol-9, oleyl alcohol, povidone, propylene glycol monostearate, sodium lauryl sulfate, sorbitan esters, stearyl alcohol, tragacanth, xanthan gum, and derivatives thereof, solvents, and miscellaneous ingredients such as crystalline cellulose, microcrystalline cellulose, citric acid, dextrin, dextrose, liquid glucose, lactic acid, lactose, magnesium chloride, potassium metaphosphate, and starch, among others (see, generally, Alfonso R. Gennaro (2000) *Remington: The Science and Practice of Pharmacy*, 20th Edition. Baltimore, MD: Lippincott Williams & Wilkins). Such carriers and/or additives can be formulated by conventional methods and can be administered to the subject at a suitable dose. Stabilizing agents such as lipids, nuclease inhibitors, polymers, and chelating agents can preserve the compositions from degradation within the body.

The route of administration is in accord with known methods, *e.g.*, injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, subcutaneous, intraocular, intraarterial, intrathecal, inhalation or intralesional routes, topical, rectal,

mucosal, and by sustained release systems. The MEVs or cargo-loaded MEVs can be administered continuously by infusion or by bolus injection. One can administer the MEVs or cargo-loaded MEVs in a local or systemic manner.

The MEVs or cargo-loaded MEVs can be prepared in a mixture with a pharmaceutically acceptable carrier. Techniques for formulation and administration of the compounds are known to one of skill in the art (see *e.g.*, "*Remington's Pharmaceutical Sciences*," Mack Publishing Co., Easton, Pa.). This therapeutic composition can be administered intravenously or through the nose or lung, such as a liquid or powder aerosol (lyophilized). The composition also can be administered parenterally or subcutaneously as desired. When administered systematically, the therapeutic composition should be sterile, pyrogen-free and in a parenterally acceptable solution having due regard for pH, isotonicity, and stability. These conditions are known to those skilled in the art.

Pharmaceutical compositions suitable for use include compositions wherein the MEVs or cargo-loaded MEVs are contained in an amount effective to achieve their intended purpose. Determination of a therapeutically effective amount is well within the capability of those skilled in the art. Therapeutically effective dosages can be determined by using *in vitro* and *in vivo* methods, and/or by a skilled person.

Therapeutic formulations can be administered in many conventional dosage formulations. Dosage formulations of MEVs and cargo-loaded MEVs provided herein are prepared for storage or administration by mixing the compound having the desired degree of purity with physiologically acceptable carriers, excipients, or stabilizers. Such materials are non-toxic to the recipients at the dosages and concentrations employed, and can include buffers such as Tris HCl, phosphate, citrate, acetate and other organic acid salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) peptides such as polyarginine, proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium, and/or

nonionic surfactants such as polysorbates (TWEEN), pluronics, polyethylene glycol, and others.

In particular examples herein, provided are pharmaceutical compositions that contain a stabilizing agent. The stabilizing agent can be an amino acid, amino acid derivative, amine, sugar, polyol, salt or surfactant. In some examples, the stable co-formulations contain a single stabilizing agent. In other examples, the stable co-formulations contain 2, 3, 4, 5 or 6 different stabilizing agents. For example, the stabilizing agent can be a sugar or polyol, such as a glycerol, sorbitol, mannitol, inositol, sucrose or trehalose. In particular examples, the stabilizing agent is sucrose. In other examples, the stabilizing agent is trehalose. The concentration of the sugar or polyol is from or from about 100 mM to 500 mM, 100 mM to 400 mM, 100 mM to 300 mM, 100 mM to 200 mM, 200 mM to 500 mM, 200 mM to 400 mM, 200 mM to 300 mM, 250 mM to 500 mM, 250 mM to 400 mM, 250 mM to 300 mM, 300 mM to 500 mM, 300 mM to 400 mM, or 400 mM to 500 mM, each inclusive.

In examples, the stabilizing agent can be a surfactant that is a polypropylene glycol, polyethylene glycol, glycerin, sorbitol, poloxamer and polysorbate. For example, the surfactant can be a polypropylene glycol, polyethylene glycol, glycerin, sorbitol, poloxamer and polysorbate, such as a poloxamer 188, polysorbate 20 and polysorbate 80. In particular examples, the stabilizing agent is polysorbate 80. The concentration of surfactant, as a % of mass concentration (w/v) in the formulation, is between or about between 0.005% to 1.0%, 0.01% to 0.5%, 0.01% to 0.1%, 0.01% to 0.05%, or 0.01% to 0.02%, each inclusive.

When used for *in vivo* administration, the formulation should be sterile and can be formulated according to conventional pharmaceutical practice. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The MEVs or cargo-loaded MEVs can be stored in lyophilized form or in solution; they can be frozen or refrigerated. Other vehicles such as naturally occurring vegetable oil like sesame, peanut, or cottonseed oil or a synthetic fatty vehicle like ethyl oleate can be included. Buffers, preservatives, and antioxidants can be incorporated according to accepted pharmaceutical practice.

The MEVs or plasmid-loaded MEVs provided herein, can be provided at a concentration in the composition of from or from about 0.1 to 10 mg/mL or higher or

lower amounts, depending upon the application and the subject, such as, for example a concentration that is at least or at least about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10 mg/mL or more. The volume of the solution can be at or about 1 to 100 mL, such as, for example, at least or about at least or 0.5, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 mL or more. In some examples, the MEVs or cargo-loaded MEVs are supplied in phosphate buffered saline.

The MEVs or cargo-loaded MEVs provided herein can be provided as a controlled release or sustained release composition. Polymeric materials are known in the art for the formulation of pills and capsules which can achieve controlled or sustained release of the MEVs and cargo-loaded MEVs provided herein (see, *e.g.*, *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Fla. (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984); Langer and Peppas (1983) *J. Macromol. Sci.* 23:61; see also Levy *et al.* (1985) *Science* 228:190; During *et al.* (1989) *Ann. Neurol.* 25:351; Howard *et al.* (1989) *J. Neurosurg.* 71:105; U.S. Pat. Nos. 5,679,377, 5,916,597, 5,912,015, 5,989,463, 5,128,326; and PCT Publication Nos. WO 99/15154 and WO 99/20253). Examples of polymers used in sustained release formulations include, but are not limited to, poly(2-hydroxy ethyl methacrylate), poly(methyl methacrylate), poly(acrylic acid), poly(ethylene-co-vinyl acetate), poly(methacrylic acid), polyglycolides (PLG), polyanhydrides, poly(N-vinyl pyrrolidone), poly(vinyl alcohol), polyacrylamide, poly(ethylene glycol), polylactides (PLA), poly(lactide-co-glycolides) (PLGA), and polyorthoesters. Generally, the polymer used in a sustained release formulation is inert, free of leachable impurities, stable on storage, sterile, and biodegradable. Any technique known in the art for the production of sustained release formulation can be used to produce a sustained release formulation containing the MEVs or cargo-loaded MEVs provided herein.

In some examples, the pharmaceutical composition contains the MEVs or plasmid-loaded MEVs provided herein and one or more additional agents, such as an antibody or other therapeutic, for combination therapy.

2. Articles of Manufacture/Kits and Combinations

Pharmaceutical compositions of the MEVs or cargo-loaded MEVs can be packaged as articles of manufacture containing packaging material, a pharmaceutical composition which is effective for treating a disease or condition that can be treated by administration of the particular MEVs or cargo-loaded MEVs, such as the diseases and conditions described herein or known in the art, and a label that indicates that the cargo, such as an antibody or nucleic acid molecule, is to be used for treating the infection, disease or disorder. The pharmaceutical compositions can be packaged in unit dosage forms containing an amount of the pharmaceutical composition for a single dose or multiple doses. The packaged compositions can contain a lyophilized powder of the pharmaceutical compositions containing the cargo-loaded MEVs which can be reconstituted (*e.g.*, with water or saline) prior to administration.

The articles of manufacture provided herein contain packaging materials. Packaging materials for use in packaging pharmaceutical products are well-known to those of skill in the art (see, *e.g.*, U.S. Patent Nos. 5,323,907, 5,052,558 and 5,033,252). Examples of pharmaceutical packaging materials include, but are not limited to, blister packs, bottles, tubes, inhalers (*e.g.*, pressurized metered dose inhalers (MDI), dry powder inhalers (DPI), nebulizers (*e.g.*, jet or ultrasonic nebulizers) and other single breath liquid systems), pumps, bags, vials, containers, syringes, bottles, and any packaging material suitable for a selected formulation and intended mode of administration and treatment.

The MEVs or cargo-loaded MEVs can be provided as combinations and as kits. Kits optionally can include one or more components such as instructions for use, devices and additional reagents (*e.g.*, sterilized water or saline solutions for dilution of the compositions and/or reconstitution of lyophilized protein), and components, such as tubes, containers and syringes for practice of the methods. Exemplary kits can include the MEVs or cargo-loaded MEVs provided herein, and can optionally include instructions for use, a device for administering the MEVs or cargo-loaded MEVs to a subject, a device for detecting MEVs or cargo-loaded MEVs in samples obtained from a subject, and a device for administering an additional therapeutic agent to a subject.

The kit can, optionally, include instructions. Instructions typically include a tangible expression describing the MEVs or cargo-loaded MEVs, and, optionally, other components included in the kit, and methods for administration, including methods for determining the proper state of the subject, the proper dosage amount, dosing regimens, and the proper administration method for administering the MEVs or cargo-loaded MEVs. Instructions also can include guidance for monitoring the subject over the duration of the treatment time.

Kits also can include a pharmaceutical composition described herein and an item for diagnosis. For example, such kits can include an item for measuring the concentration, amount or activity of the MEVs and cargo-loaded MEVs, in a subject.

In some examples, the MEVs or cargo-loaded MEVs are provided in a diagnostic kit for the detection of the MEVs or cargo-loaded MEVs or cargo in an isolated biological sample (*e.g.*, tumor cells, such as circulating tumor cells obtained from a subject or tumor cells excised from a subject).

Kits provided herein also can include a device for administering the MEVs to a subject. Any of a variety of devices known in the art for administering medications to a subject can be included in the kits provided herein. Exemplary devices include, but are not limited to, a hypodermic needle, an intravenous needle, a catheter, a nebulizer, and an inhaler. Typically, the device for administering the compositions is compatible with the desired method of administration of the composition.

3. Administration of Plasmid-Loaded MEVs and Routes of Administration

The plasmid-loaded MEVs provided herein can be administered to a subject by any method known in the art for the administration of polypeptides, including for example systemic or local administration. In general, the plasmid-loaded MEVs can be administered by routes, such as parenteral (*e.g.*, routes, such as intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, and intracavity), topical, epidural, or mucosal (*e.g.*, routes, such as topical, intranasal, oral, vaginally, vulvovaginal, esophageal, or esophageal, bronchial, rectal, and pulmonary). The cargo-loaded MEVs can be administered externally to a subject, at the site of the disease for exertion of local or transdermal action. Compositions containing the cargo-loaded MEVs can be administered, for example by infusion, inhalation, by

bolus injection, or by absorption through epithelial or mucocutaneous linings (*e.g.*, topical, oral, vaginal, rectal and intestinal mucosa). Compositions containing the cargo-loaded MEVs can be administered together with or sequentially with other biologically active agents. For example, the cargo-loaded MEVs are administered by infusion delivery, such as by infusion pump or syringe pump, and can be administered in combination with another therapeutic agent or as a monotherapy.

The method and/or route of administration can be altered to alleviate adverse side effects associated with administration provided herein. For example, if a patient experiences a mild or moderate (*i.e.*, Grade 1 or 2) infusion reaction, the infusion rate can be reduced (*e.g.*, reduced by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more). If the patient experiences severe (*i.e.*, Grade 3 or 4) infusion reactions, the infusion can be temporarily or permanently discontinued.

In some examples, if the subject experiences an adverse side effect, such as severe skin toxicity, for example severe acneiform rash, treatment adjustments can be made. For example, after the occurrence of an adverse side effect, administration can be delayed, such as for 1 to 2 weeks or until the adverse side effect improves. In some examples, after additional occurrences of an adverse side effect, the dosage can be reduced. A particular regimen and treatment protocol can be established by the skilled physician or other practitioner.

Appropriate methods for delivery, can be selected by one of skill in the art based on the properties of the dosage amount of the cargo-loaded MEVs or the pharmaceutical composition containing the cargo-loaded MEVs. Such properties include, but are not limited to, solubility, hygroscopicity, crystallization properties, melting point, density, viscosity, flow, stability and degradation profile.

4. Combination Therapies

The cargo-loaded MEVs provided herein can be administered before, after, or concomitantly with one or more other therapeutic regimens or agents. The skilled medical practitioner can determine empirically, or by considering the pharmacokinetics and modes of action of the agents, the appropriate dose or doses of each therapeutic regimen or agent, as well as the appropriate timings and methods of administration. The additional therapeutic regimens or agents can improve the efficacy or safety or other properties of the cargo-loaded MEVs. In some examples,

the additional therapeutic regimens or agents can treat the same disease or a comorbidity. In some examples, the additional therapeutic regimens or agents can ameliorate, reduce or eliminate one or more side effects known in the art or described herein that are associated with administration of the cargo-loaded MEVs or the cargo.

For example, the cargo-loaded MEVs described herein can be administered with other immunomodulatory agents or treatments. The cargo-loaded MEVs can be administered with other anti-pathogen therapeutics and treatments. The cargo-loaded MEVs can be administered in combination with one or more other prophylactic or therapeutic agents, including but not limited to antibodies, cytotoxic agents, chemotherapeutic agents, cytokines, growth inhibitory agents, anti-hormonal agents, kinase inhibitors, anti-angiogenic agents, cardio-protectants, immunostimulatory agents, immunosuppressive agents, agents that promote proliferation of hematological cells, angiogenesis inhibitors, protein tyrosine kinase (PTK) inhibitors, FcγRIIb or other Fc receptor inhibitors, or other therapeutic agents.

The one or more additional agents can be administered simultaneously, sequentially or intermittently with the cargo-loaded MEVs. The agents can be co-administered, for example, as part of the same pharmaceutical composition or same method of delivery. In some examples, the agents can be co-administered at the same time as the cargo-loaded MEVs, but by a different means of delivery. The agents also can be administered at a different time than administration of the cargo-loaded MEVs, but close enough in time to have a combined prophylactic or therapeutic effect. In some examples, the one or more additional agents are administered subsequent to or prior to the administration of the cargo-loaded MEVs separated by a selected time period. In some examples, the time period is 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 2 weeks, 3 weeks, 1 month, 2 months, or 3 months. In some examples, the one or more additional agents are administered multiple times and/or the cargo-loaded MEVs provided herein are administered multiple times.

G. BIODISTRIBUTION OF MEVs FOLLOWING ADMINISTRATION VIA VARIOUS ROUTES

1. Biodistribution of mammalian EVs

Pharmacokinetics and biodistribution in organs and tissues of mammalian EVs have been extensively studied for their pharmacokinetics and distribution in organs

and tissues (Vader *et al.* (2016) *Advanced drug delivery reviews 106(Pt A):*148–156, doi.org/10.1016/j.addr.2016.02.006; Morishita *et al.* (2017) *Journal of pharmaceutical sciences 106(9)*:2265–2269, hdoi.org/10.1016/j.xphs.2017.02.030). Treatments with mammalian cell-derived EVs are generally based on intravenous or intraperitoneal routes of administration. Primary target organs upon systemic administration of mammalian EVs are the liver, spleen and lungs. A comprehensive study (see, Wiklander *et al.* (2015) *J. Extracellular Vesicles 4*:26316) of the tissue distribution of fluorescently-labelled mammalian Evs from various cell sources demonstrated that 24 hours after intravenous (i.v.) injection in mice, the highest fluorescence signal was in the liver, followed by spleen, gastrointestinal tract and lungs. Furthermore, cell source, EV dose, and route of administration was shown to affect EV distribution; for example, injection of higher EV doses resulted in relatively lower liver accumulation compared to lower doses, possibly caused by saturation of the mononuclear phagocyte system (MPS). Comparison between intraperitoneal (i.p.), subcutaneous (s.c.) and i.v. administrations showed that intraperitoneal and subcutaneous doses resulted in reduced EV accumulation in liver and spleen and enhanced pancreas and gastrointestinal tract accumulation compared to i.v. injections. Systemically administered Evs are reported to be rapidly taken up by the mononuclear phagocyte system (MPS), particularly in the liver and spleen. The mechanism of clearance resembles that described for synthetic nanoparticles, such as liposomes (Van der Meel *et al.* (2014) *J. Control. Release 195*:72–85). The majority of splenic accumulation is caused by EV storage in the spleen rather than uptake by the spleen (Lai C.P. *et al.* (2014) *ACS Nano 8*:483-494). Biodistribution of mammalian EVs following other routes of administration also has been investigated. For targeting of the central nervous system, intranasal administration of curcumin-loaded mammalian EVs resulted in EV localization in the brain. Drug levels peaked at 1 hour after administration, and a significant amount detected after 12 hours with no toxic effects observed (Zhuang *et al.* (2011) *Mol. Ther. 19*:1769–1779).

In general, mammalian EVs are not employed for oral delivery because of their low stability at various pH and temperatures, rapid degradation of biomolecules in the digestive tract, and the limitations of industrial scale production for oral dosing (Cheng *et al.* (2019) *Protein Cell 10:*295-299). The only exception so far are bovine

milk-derived EVs, which upon oral delivery to mice have shown a pattern of distribution that, analyzed with whole-body in vivo imaging system (IVIS), included rapid accumulation in the intestine, where the EVs were detectable after 2 and 6 hours, followed by fluorescence signal observed in liver, spleen, lungs, kidney, heart, and the gastrointestinal tract at 24 hours. After 48 hours, the fluorescence signal subsided within most of the organs indicating the clearance of nanovesicles from the system (Samuel *et al.* (2021) *Nat Commun 12*:3950, doi.org/10.1038/s41467-021-24273-8). Thus, mammalian EVs (derived from sources other than milk) cannot be absorbed by the intestinal tract and from the intestines to become bioavailable in target organs (Zhong *et al.* (2021) *Biomaterials. 277:*121126. Doi: 10.1016/j.biomaterials.2021.121126).

Treatments with mammalian cell-derived EVs generally employ intravenous or intraperitoneal routes of administration for systemic administration where the target organs are the liver, spleen and lungs. As noted, most mammalian EVs have not been employed for oral delivery due to their low stability at various pH and temperatures, rapid degradation of biomolecules in the digestive tract, and the limitations of industrial scale production for oral dosing (Cheng *et al.* (2019) *Protein Cell 10(4)*:295-299). The only exception are bovine milk-derived EVs, which upon oral delivery to mice have shown a pattern of distribution that, analyzed with whole-body in vivo imaging system (IVIS), include rapid accumulation in the intestine, where the EVs were detectable after 2 and 6 hours, followed by fluorescence signal observed in liver, spleen, lungs, kidney, heart, and the gastrointestinal tract at 24-hour time point. After 48 hours, the fluorescence signal subsided within most of the organs indicating the clearance of nanovesicles from the system.

As described herein, and in copending commonly owned applications (see, PCT/EP2023/051650), MEVs have different properties from mammalian EVs. For example, they are stable in the harsh environment of the gastrointestinal tract compared to mammalian cell-derived EVs. Thus, the microalgae EVs, as described herein, are particularly suitable for oral administration and drug delivery, as well as other routes of delivery as described herein.

2. Microalgae EVs Biodistribution

MEVs, including those provided herein from *Chlorella*, have properties that are distinct from mammalian EVs, including bovine milk EVs (see, commonly owned PCT/EP2023/051650 published as PCT Publication No. WO2023/144127, which details preparation of MEVs, administration, and biodistribution thereof). For example, a striking difference, as described below, is that the MEVs can be administered orally, and that the primary target is the spleen, particularly the white pulp of the spleen (white spleen). This renders the MEVs of use as vaccines and for delivery of immunomodulatory cargo. MEVs can be administered intranasally, by inhalation into the lung, into the eye, including by topical administration as eyedrops or by injection, intramuscularly, and by any other suitable route. The MEVs can be formulated for administration by each route. Thus, provided are compositions containing MEVs that are for treating particular disease and for particular routes of administration. For immune modulation and vaccination, the MEVs are delivered by routes, such as oral and IM, and mucosally, including by inhalation into the lungs, nose, and intestinal mucosa, that result in delivery of the MEV cargo to organs and tissue of the immune system.

The route of administration determines the fate of the MEVs, and that the ultimate location of the MEVs is a function of the route of administration. Targets and endpoints of the MEVs include, but are not limited to, the liver, spleen, lungs, the intestines, and brain. Routes of administration include, but are not limited to, respiratory (nose, lungs), oral (digestive), intravenous, central nervous system (CNS), and topical. The selection of route depends upon the ultimate target and the payload. It is shown herein that intranasal administration goes to the lungs, intratracheal via a spray goes to the lung(s), intravenous accumulates in the spleen and liver, oral (per Os) goes to the digestive tract and spleen. In contrast, mammalian EVs cannot be taken orally. As described herein, routes for delivery of cargo to the immune system include oral and IM.

MEVs are readily internalized by human cells. For example, *in vitro*, when administered to cells in culture, such as A549 cells, at a ratio of MEV/cell of 1000/1, 93% of the cells internalized the MEVs, and this occurred within 24 to 48 hours after contacting the cells with the MEVs.

DIR-labeled MEVs were administered to mice via four routes: intranasal (IN), intratracheal (IT), intravenous (IV), and oral, and, by full-body imaging as a function of time, the fate of the MEVs was visualized for 3 days, followed by sacrificing the mice to harvest organs for study. As shown in the examples, intravenous administration targets the liver at about 4-12 hours following administration, and the spleen, appearing to be in the red pulp of the spleen (red spleen), at 10-30 hours. Oral administration targets the intestine and spleen. It is shown herein that the MEVs are orally available; they resist passage through the stomach, and reach the intestine at 0.5 hour to 4 hours, and then the spleen at 0.5 hour to 10 hours. Of interest is the route to the spleen; there are two possible routes to the spleen, via the blood (to red spleen), and via lymphocytes (to white spleen), which has implications for targeting and delivering cargo to the immune system, accumulating from 4 hours to 28 hours. This can be effected by internalization by lymphocytes that are activated and end up in the spleen where they multiply, and/or by lymphocytes that phagocytose the MEVs, which are not activated, and go to the white pulp of the spleen (white spleen) from where they are disseminated through the immune system.

3. Diseases and conditions treated by MEVs

Based upon the targeted organs, a variety of diseases and disorders can be treated by MEVs. The MEVs can be loaded or produced to contain therapeutic agents for treating these diseases and conditions. The appropriate route of administration for the targeted organ and disease is selected. For example, for targeting the spleen and intestines, oral administration is selected; and for targeting the lungs, inhalation or nasal administration is selected. Based on the biodistribution and pharmacokinetic data the following organs can be targeted to treat diseases exemplified as follows:

eye: diseases of the eye, including the back of the eye, including the retina and choroid;

brain: diseases involving the brain and CNS (see, commonly owned International PCT publication No: WO 2024/088808, which details MEVs for intranasal administration to the brain, the trafficking pathways, and targeted loci in the brain and CNS;

liver: cancer, cancer metastases, metabolic syndrome, genetic disorders (delivery of gene therapy), alpha-anti-trypsin (AAT) deficiency and other inborn errors of

metabolism, hemophilia, hypercholesterolemia, liver inflammation, steatohepatitis, and other diseases and disorders that can be treated by delivery of a therapeutic to the liver;

spleen: diseases treated by immune modulation, including cancers, and immune cell disorders, and cancer, and other diseases that can be treated by administration to the spleen, particularly by immune cells that occur in or traffic to the white spleen;

intestine: diseases and disorders treated or prevented by vaccines, intestinal infections, microbiota modulation, Crohn's disease, cancer, ulcers, diseases treated by orally administered drugs, such as small molecules and proteins, and other such diseases, disorders, and conditions; and

lungs: infectious diseases, particularly respiratory diseases, chronic obstructive pulmonary disease (COPD), pulmonary hypertension, asthma, other inflammatory lung diseases, cystic fibrosis, ATT-deficiency, lung disease, cancer, cancer metastases, and other such diseases and disorders.

H. EXEMPLARY DISEASES, DISORDERS, AND CONDITIONS FOR TREATMENT WITH MEV-GTVs

As described above, the MEV-GTVs can be loaded with any plasmids encoding any desired cargo, including, but not limited to, nucleic acid molecules encoding therapeutic proteins, detectable marker proteins and tags, small molecule drugs, gene editing systems, and others, and combinations thereof for delivering therapeutic molecules, gene editing systems, such as CRISPR/cas to effect gene editing. Therapeutic products include encoded polypeptides, peptides, and mRNA, Any product that is administered as a DNA vector can be administered encoded in the ITR plasmids and the MEV-GTVs, and encoded gene therapy for effecting gene therapy, such as for treatment of metabolic gene therapy diseases by delivering encoded products, nucleic acids, including CRISPR-CAS and other such gene editing products that can be encoded in a plasmid.

MEVs can carry cargo. Herein the cargo includes the ITR plasmids that encoded products. The MEVS that contain the ITR plasmids are MEV-GTVs. The products include those, for example, for treating a disease characterized by a genetic defect that results in a deficiency of a functional protein, or for treating a disease characterized by overexpression or under expression of a polypeptide. Non-limiting

examples of diseases that can be treated by silencing of a target gene, for example using siRNA or microRNA (see, *e.g.*, International Pub. No. WO 2013/048734) include cancer (*e.g.*, lung cancer, leukemia and lymphoma, pancreatic cancer, colon cancer, prostate cancer, glioblastoma, ovarian cancer, breast cancer, head and neck cancer, liver cancer, skin cancer, and uterine cancer), cardiovascular diseases, ocular diseases (*e.g.*, age-related macular degeneration, herpes stromal keratitis, glaucoma, dry eye syndrome, diabetic retinopathy, and conditions associated with ocular angiogenesis and ocular hypertension), neurological diseases (*e.g.*, amyotrophic lateral sclerosis, Alzheimer's disease, myasthenic disorders, Huntington's disease, spinocerebellar ataxia, frontotemporal dementia, Parkinson's disease, prion diseases, and Lafora disease, and those arising from ischemic or hypoxic conditions), kidney disorders, inflammatory or autoimmune diseases (*e.g.*, ischemia or reperfusion injury, restenosis, Rheumatoid arthritis, inflammatory bowel disease, *e.g.*, Crohn's Disease or ulcerative colitis, lupus, multiple sclerosis, diabetes, *e.g.*, type II diabetes, and diabetic conditions, arthritis, *e.g.*, rheumatoid or psoriatic), respiratory diseases (*e.g.*, asthma, Chronic obstructive pulmonary diseases (COPD), cystic fibrosis, acute respiratory distress syndrome (ARDS), emphysema, and acute lung injury), hearing disorders, epilepsy, spinal cord injuries, oral mucositis, male infertility, uterine disorders, endometrial disorders or conditions, as well as conditions relating to metabolism (*e.g.*, obesity), ischemia, stroke, alcohol metabolism and liver function (see, *e.g.*, International Pub. Nos. WO 2006/029161, WO 2007/022470, WO 2007/130604, WO 2008/021157, WO 2009/104051, WO 2009/142822, WO 2019/217459, WO 2020/123083; European Pub. No. EP 2504435; and U.S. Patent Pub. Nos. U.S. 2011/0223665, U.S. 2012/0116360, U.S. 2012/0071540, U.S. 2016/0257956, U.S. 2015/0196648, and U.S. 2017/0304459). The RNAi molecule can target a gene that encodes, for example, an oncogene, a transcription factor, a receptor, an enzyme, a structural protein, a cytokine, a cytokine receptor, a lectin, a selectin, an immunoglobulin, a kinase, and a phosphatase, where the targeted gene or gene product plays are role in a disease, disorder, or condition of the eye.

MEV-GTVs can be used to deliver DNA encoding products to treat, *e.g.*, with gene silencing, or prevent, *e.g.*, through vaccination, infectious diseases of the eye, diseases such as muscular dystrophy, cystic fibrosis and other such diseases. Gene

silencing also can be used to target a pathogen-associated protein, such as a viral protein involved in immunosuppression of the host, replication of the pathogen, transmission of the pathogen, or maintenance of the infection; or a host protein that facilitates entry of the pathogen into the host, drug metabolism by the pathogen or host, replication or integration of the pathogen's genome, establishment or spread of infection in the host, or assembly of the next generation of pathogen. Pathogens can include, for example, RNA and DNA viruses such as arenaviruses, coronaviruses, influenza viruses, paramyxoviruses, flaviviruses (*e.g.*, West Nile virus), picornaviruses (*e.g.*, Coxsackievirus, Poliovirus, and Rhinovirus), rhabdoviruses, filoviruses, retroviruses (*e.g.*, lentiviruses, and Rous sarcoma virus), adenoviruses, poxviruses, herpes viruses, human papilloma viruses, cytomegaloviruses, hepadnaviruses (*e.g.*, Hepatitis B and C), rotaviruses, respiratory syncytial viruses, polyomaviruses, and others; bacteria; fungi; helminths; schistosomes; trypanosomes; parasites including plasmodiums (*e.g.*, *Plasmodium malariae* and others); and mammalian transposable elements (see, *e.g.*, International Pub. Nos. WO 2010/141724, WO 2011/071860, WO 2011/072292, WO 2013/126803, WO 2020/035620, and WO 2020/097540; Australian Pub. Nos. AU 2004257373 A1, AU 2013203219 B2, and AU 2016225873 A1; European Pub. Nos. EP 2395012, and EP 2888240; U.S. Patent Pub. Nos. U.S. 2011/0223665, U.S. 2014/0256785, and U.S. 2019/0032051; Japanese Pub. No. JP 2018-197239A; and Taiwanese Pub No. TW 201204351A).

MEV-GTVs also can be used to deliver DNA or encoded mRNA molecules that encode therapeutic or therapeutically useful polypeptides. For example, in cases where subjects lack a specific gene product, the gene can be encoded in a nucleic acid molecule, such as a DNA or RNA molecule. The nucleic acid molecule encoding the gene product can be loaded into a MEV and delivered to a subject lacking the gene product. For example, diseases, disorders, or conditions that occur due to the absence or deficiency of a gene product include retinitis pigmentosa and other inherited diseases of the eye, particularly those that lead to vision loss. Other diseases, disorders, and conditions include ophthalmoparesis, and autosomal dominant and recessive progressive external ophthalmoplegia with mitochondrial DNA deletions;

(see, *e.g.*, International Pub. Nos. WO 2011/068810, WO 2019/243574, WO 2019/092287, and WO 2020/099682).

As described, the plasmids and, hence, the MEV-GTVs can encode a clustered, regularly interspaced, short palindromic repeat (CRISPR)/Cas system to effect gene editing to target genes with mutations that results in a disease, disorder, or condition involving the eye. The clustered, regularly interspaced, short palindromic repeat (CRISPR) technology allows for the modification of the genome in a living organism, and is based on the bacterial CRISPR/Cas9 antiviral defense system. The system allows for DNA cleavage at a target site. The type II CRISPR system incorporates sequences from invading foreign nucleic acids, such as DNA from viruses or plasmids, between CRISPR repeat sequences encoded within the host genome. Transcripts from the CRISPR repeat sequences are processed into CRISPR RNAs (crRNAs). Each crRNA harbors a variable sequence transcribed from the foreign DNA and a part of the CRISPR repeat. Each crRNA hybridizes with a second transactivating CRISPR RNA (tracrRNA) and these two RNAs complex with and direct the Cas9 nuclease to cleave the target DNA sequence. By delivering a Cas nuclease complexed with a synthetic guide RNA (gRNA), which contains a fusion of a crRNA and a tracrRNA, into a cell, the cell's genome can be cut at a desired location, allowing existing genes to be removed and/or new ones added *in vivo* (Sander and Joung (2014) *Nat. Biotechnol.* 32(4):347-355). The CRISPR technology can be used with the Cas polypeptide or the single RNA guided endonuclease Cpf1 to effect genome modification, and can be delivered in lipid nanoparticles, EVs and other vesicles (see, *e.g.*, International Pat. Pub. Nos. WO 2017/161010, WO 2019/238626, and WO 2020/097540).

Exemplary of gene therapy approaches include gene replacement, such as gene replacement to replace a gene that encodes a defective product. Gene therapy has been employed to treat diseases of the eye. RNA editing is an alternative to DNA editing. Because unused RNA molecules are rapidly degraded, any errors introduced by a therapeutic are not permanent. The RNA can be introduced by RNA trans-splicing. RNA trans-splicing is a technology that hijacks this naturally occurring phenomenon in order to remove the mutated sections encoding and replace it with a healthy version.

H. EXAMPLES

The following Examples are included for illustrative purposes only and are not intended to limit the scope of the invention(s).

EXAMPLE 1

Production of Chlorella cells and isolation of MEVs.

A. Batch production of the inoculum

Chlorella vulgaris of any strain can be used to produce MEVs. Exemplary strains include, but are not limited to, UTEX 265 strain, UTEX 395 strain, UTEX 26 strain, 15 UTEX 30 strain, UTEX 259 strain, UTEX 2219 strain, UTEX 2714 strain UTEX B 1811 (available from the UTEX Culture Collection), the strain designated CCAP 211/19, GEPEA, University of Nantes, France, and any other suitable strain, either transformed or not, can be used to produce the algal cell material. For exemplary purposes the UTEX 265 strain was used.

Chlorella was stored on nutrient agar slopes until flask/photobioreactor (PBR) inoculation. For different experiments, different scales of production, between 400 mL (flasks) to 170 L (several PBRs with different total volume) cultures, were used. This description relates to the highest volume of PBR used (170 L, HECTOR PBR ["Hector" photobioreactor designed by the Laboratory of Process Engineering - Environment - Agri-food (GEPEA) / CNRS for the culture of microalgae. Reference: 20160067_0017. Year of production: 2016. Maximum size: 56.43 x 37.66 cm/ 170 L / 300 dpi]).

A 5-Liter PBR was filled with 4 L of sterile BG11 medium (see, *e.g.*, utex.org/products/bg-11-medium for a description of its preparation and see table below (Table 1) for autotrophic growth) and inoculated directly from the stock algal slope on nutrient agar. Then, the *Chlorella* strain was grown as a batch culture in a bubble column using the following culture parameters: temperature of 23°C; medium pH 7.5-8.0; light intensity: 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$; light cycle: continuous. Biomass concentration, specific growth rate and biomass productivity of *Chlorella* were estimated daily. Typically, after 6 days of continuous growth the cultures reached biomass concentrations of approx. 1.2 g/L. The total crop volume of 20 L was collected for subsequent production scale-up to the HECTOR PBR.

B. Production scale-up in a semi-industrial Photobioreactor (PBR)

The *Chlorella* cells were cultured further in a 170-Liter photobioreactor system (HECTOR). The inoculum was added to sterile BG11 medium (see Table 1) to the total volume of 150 L and cells were grown autotrophically as a semi-batch culture with bubble column mixing. The following culture parameters were used: temperature of 18±4°C; medium pH 8.0±0.05; light intensity: 150-300 $\mu mol \cdot m^{-2} \cdot s^{-1}$; between 150 µmol·m-2·s-1 the three first days of each batch, 250 µmol·m-2·s-1 days four and five and 300 µmol·m-2·s-1 days six and seven before the harvesting as light cycle: continuous, with gradual increase in light intensity.

Biomass concentration, growth rate and biomass productivity of *Chlorella* were estimated daily. On the 6th day of the cultivation, at the biomass concentration of approx. 1.5 g/L *Chlorella* harvesting was performed.

Table 1. Composition of the BG11 medium

	Conc	**Ml**
Salt Mix	100x	10
Iron*	1000x	1
Nitrate*	100x	10
Trace metals	1000x	1
agar		14
H_2O		up to 1 L
pH 7		

Stock solutions for BG11 medium:

Nitrate 100x	**g/L**	**MW**	**µM**
$NaNO_3$	150	85	1764.71
Iron 1000x			
Fe-EDTA	3	344.06	8.72
Salt Mix 100x			
K_2PO_4	4	173.17	23.10
$MgSO_4 \cdot 7H_2O$	7.5	246.48	30.43
$CaCl_2 \cdot 2H_2O$	3.6	147.01	24.49
Na_2CO_3	2	105.99	18.87
add HCl if needed			
Trace Metals 1000x	g/L	MW	µM
H_3BO_3	2.9	61.84	46.25
$MnCl_2 \cdot 4H_2O$	1.8	197.91	9.15
$ZnSO_4 \cdot 7H_2O$	0.2	287.56	0.77
$Na_2MoO_4 \cdot 2H_2O$	0.4	241.95	1.61
$CuSO_4 \cdot 5H_2O$	0.1	249.68	0.32

$CoCl_2 \cdot 6H_2O$	0.3	237.93	0.14

C. Production of 3 consecutive batches of Chlorella

The *Chlorella* production was performed in 3 semi-batches of 130 L, from which about 80% of the culture volume was aseptically removed for downstream treatment and supplemented with sterile BG11 medium. Following the harvest, the light intensity was lowered to 140 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to avoid excessive photon intake. A seeding line was set up to go from 100 mL of culture to 150 L of culture. Three consecutive batches lasting 6-7 days were carried out with the aim of extracting a vesicle concentrate devoid of microalgae.

Culture parameters monitoring

1. Determination of the protein content

The protein content of cultures was determined by elemental analysis, resorting to Vario el III (Vario EL, Elementar Analyser systeme, GmbH, Hanau, Germany), according to the procedure provided by the manufacturer. The final protein content was calculated by multiplying the percentage of nitrogen given by the elemental analysis by 6.25.

2. Estimation of chlorophyll content

Culture samples were centrifuged at 2547 g for 15 min using a Hermle centrifuge (HERMLE Labortechnik GmbH, Wehingen, Germany). Pigments were extracted from the resulting pellet by bead milling in acetone. The full absorbance spectrum of the extract was obtained with a Genesys™ 10S UV-VIS spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) and iteratively decomposed to the standard pigment spectra to obtain the total chlorophyll content.

3. Growth estimation

Dry weight was obtained by filtration of culture samples using pre-weighed 0.7 µm GF/C 698 filters (VWR, Pennsylvania, USA) and dried at 120°C until constant mass was obtained using a DBS 60-30 electronic moisture analyser (KERN & SOHN GmbH, Balingen, Germany). All dry weight samples were washed with demineralized water to remove growth medium salts.

D. Isolation of Microalgae Extracellular Vesicles: Production of concentrated MEV preparation (Down-Stream Processing: clarification and concentration step)

The culture harvested from the PBR was centrifuged at 2,700 g for 5 minutes at room temperature for cell removal. The supernatant was transferred into fresh bottles and centrifuged again at 2,700 g for 5 minutes at room temperature. The clear MEV-containing solution was then subjected to membrane filtering using a 1.2 µm cut-off cartridge filter. The filtrate was concentrated with the use of a 100 kDa MWCO tangential filtration system. At each isolation step the material was analyzed spectrophotometrically for chlorophyll and particulate matter. Dry weight of the final product was <0.01 g/L and the concentration factor relative to the initial volume of the processed culture was approx. 20. The suspension of MEV thus obtained was stored at -50°C in 1-1.2 L pockets for further purification.

E. Detailed Protocol for Purification of Chlorella Microalgae Extracellular Vesicles

1. Thaw in a cold room at 4°C, overnight, the preparation of MEVs previously clarified, concentrated, and stored (1.0 -1.2 L) as described in section D.
2. When the preparation is thawed, harvest the biomass by centrifugation (set the temperature to 4°C): 2 x 10000g for 10 minutes at 4°C.
3. Collect the supernatant (MEVs) and filter by vacuum filter onto 0.65 µm filters to get rid of the remaining cells.
4. The MEVs are concentrated and purified by tangential flow filtration (TFF) using Sartorius VivaFlow® filtration systems.
    a. The membrane is washed by running water at ≈100 ml/minute, as described by the manufacturer. After that, the circuit is washed with cell-free medium (BG-11 medium) at ≈200 ml/minute (pressure reading at 2/2,5 bars).
    b. The MEV preparation (supernatant) is run in the circuit at ≈200 ml/minute (pressure reading at 2/2,5 bars).

    When the residual volume in the circuit plus the reservoir is about 200mL, the TFF is used to diafiltrate and change the medium from BG-11 to PBS using 1L of PBS.
    c. When the residual volume in the circuit plus the reservoir is about 200 ml in PBS, slow the flow to ≈100 ml/minute (20 minutes, 1 bar).

d. From 30-60 ml of residual volume, slow the flow to a speed lower than 50 ml/ minutes and allow the MEVs in PBS to recirculate for 30 minutes to recover the particles trapped on the membrane surface.

e. MEVs are then filtered using 0.45 µm filters and purified by ultracentrifugation.

f. The filtered MEVs are loaded on the ultracentrifuge tubes and centrifuged for 1h at 4°C, at 100000g (27400rpm) (acceleration and deceleration at 10 max), for example in a SorVall™ WX ultra 80 TST 28.38. Pellets containing the MEVs are resuspended in 1-2 ml of PBS buffer and sterilized by filtration using a 0.2 µm filter and analysed the particles by *nanoparticle tracking analysis* (NTA; dilute up to 1:1000 before the NTA analysis).

F. Protocol for Purification of Chlorella Microalgae Extracellular Vesicles by Size Exclusion Chromatography (SEC)

When higher purity of MEVs is needed, a last step of purification is added. The MEVs previously concentrated by TFF and purified by ultracentrifugation and formulated in PBS at concentration of 10exp11 to 10exp13 per mL are seeded in a pre-packed column qEV1 from IZON. The MEVs are eluted with PBS solution. The elution fractions of 0.5 mL are collected. MEVs are recovered in the first fractions as shown in Figure 5.

MEVs concentrations in the initial sample and in the fractions collected throughout the elution are evaluated with the ZetaView® engine (Nanoparticle Tracking Analyzer from Particle Metrix) as the quantity of proteins by Bradford assay. The most concentrated fractions (4-5) are pooled and stored at 4°C before use.

EXAMPLE 2

MEV characterization

A. Nanoparticle Tracking Analysis (NTA)

MEVs were analyzed for size and dispersity (size distribution) using a NanoSight NS500 system (Malvern Panalytical Instruments). The instrument was equipped with a 488 nm laser, a high sensitivity sCMOS camera and a syringe pump. The MEV samples were diluted in particle-free PBS (0.02 µm filtered) to obtain a concentration within the recommended measurement range (1-10×10^8 particles/mL),

corresponding to dilutions of from 1/1000 to 1/10000 depending on the initial sample concentration.

For each sample, 5 experiment videos of 60 seconds duration were analyzed using NTA 3.4 Build 3.4.003 (camera level 15–16) with syringe pump speed 30. A total of 1500 frames were examined per sample, which were captured and analyzed by applying instrument-optimized settings using a suitable detection threshold so that the observed particles are marked with a red cross and that no more than 5 blue crosses are seen. Further settings were set to "automatic" and viscosity to "water".

For the characterization of the MEVs, MEV samples are produced and purified as described in Example 1 as follows: MEV are concentrated by tangential flow filtration (TFF), diafiltration and ultracentrifugation, purified by SEC and sterilized by 0.22 µm filtration. After SEC purification and filtration, MEVs are diluted 10,000 times in PBS (1X) and measured in the ZetaView® analyzer (Particle Metrix GmbH, Ammersee, Germany).

B. Z potential measurement (NTA)

The zeta potential of MEVs was measured three times at 25°C under the following settings: sensitivity of 85, a shutter value of 70, and a frame rate of 30 frames per second, of while ZetaView software was used to collect and analyze the data. Using the protocol (Particle Metrix GmbH, Ammersee, Germany). Table 2, below, shows the results of 3 independent measurements.

Table 2. MEVs: Z Potential determination

Samples (MEVs after SEC)	**Concentration (MEVs/mL)**	**MEVs Median size (nm)**	**MEVs Zeta potential (mV)**
#1	2.9exp12	159.9	-20
#2	2.9exp12	163.6	-22.58
#3	2.9exp12	161.7	-22.3
MEAN	2.9exp12	161.6	-21.63
SD	5.7exp8	2.6	-2.3

C. Transmission Electron Microscopy (TEM)

To verify the presence of intact MEVs, the preparations were analyzed using transmission electron microscopy (TEM). Fixed (4% formaldehyde, 0.2% glutaraldehyde) MEV samples were allowed to attach to Formvar/carbon-coated grids for 15-20 min, washed again with PBS followed by distilled water and finally stained

with 0.4% uranyl acetate/1.8% methyl cellulose and then dried. The preparations were observed using a JEOL-JEM 1230 transmission electron microscope (TEM) (JEOL Ltd., Tokyo, Japan) at 80 kV and images were acquired using a Morada digital camera and iTEM software (Olympus, Münster, Germany).

The TEM imaging, presented in the Figure 6, demonstrates that the MEVs are round shaped vesicles sized ~50-250 nm in diameter. MEVs are enveloped in a single lipid bilayer membrane, their lumen had slightly higher electron density, and the thickness of the membrane was estimated as ~5-10 nm, which matches the thickness of a plasma membrane. Figure 6 shows exemplary images of MEVs obtained using Transmission Electron Microscopy (TEM).

D. DiR fluorescent labelling

For uptake and internalization studies, and well as for further characterization *in vivo*, MEVs were labelled with DiR, a lipophilic carbocyanine derivative (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide; ThermoFisher Scientific) that has low fluorescence in water, but becomes highly fluorescent upon membrane incorporation, and diffuses laterally within the plasma membrane. Fresh samples of MEVs (prepared as above) were re-suspended in 1 ml of BG11 culture medium. 5 µl of 1 mg/ml DiR solution was added to the samples, following incubation at 37°C for 1 hour. Then, the samples were ultra-centrifuged at 100,000 g for 30 min using a Kontron TST 55.5 rotor at 28,100 rpm. The supernatant was removed, while the pellets were washed twice with 1 ml of PBS and centrifugation at 100,000g for 30 min. Finally, the pellet was re-suspended in 1 ml of PBS. The DiR-labelled MEVs were stored at 4°C and used promptly to ensure highest possible fluorescent intensity. DiR fluorescence of the labelled MEVs was measured using a SpectraMax® fluorescence microplate reader (Molecular Devices, USA) with excitation at 750 nm and emission at 780 nm.

E. PKH26 fluorescent labelling

For uptake and internalization studies, and well as for further characterization *in vivo*, MEVs alternatively are labelled with PKH26 (Sigma-Aldrich), a fluorochrome in the red spectrum with peak excitation (551 nm) and emission (567 nm) that can also be excited by a 488 nm laser. Fresh samples of the P40 fraction (prepared as above) are re-suspended in 1 ml of Diluent C from the PKH26 kit. 6 µl

of PKH26 dye is added to the samples, followed by continuous mixing for 30 seconds by gentle pipetting. After 5-minute incubation at room temperature, the samples are quenched by adding 2 ml of 10% BSA in 1× PBS. The volume is brought up to 8.5 ml in media and 1.5 ml of 0.971 M sucrose solution is added by pipetting slowly and carefully into the bottom of the tube, making sure not to create turbulence. The PKH26-labelled MEVs remain on top of a sucrose cushion. Then, the samples are ultra-centrifuged at 190,000 g for 2 hours at 2-8°C using a Kontron TST 55.5 rotor. The supernatant is removed, while the pellets are washed with 1× PBS by gentle pipetting and centrifuged again at 100,000g for 30 min. Alternatively, labelled MEVs were purified from free dye by size exclusion chromatography using a qVE1/70nm column (IC1-70 – IZON Science) and washed with PBS. The MEVs were recovered in the first two fractions. Finally, the pellet is re- suspended in 1 ml of 1× PBS. The PKH26-labelled MEVs are stored at 4°C and filtered with 0.45 µm filter before adding to cells. Figure 7A shows the labelled MEVs with PKH26 by confocal microscopy.

F. DiD fluorescent labelling

For uptake and internalization studies, MEVs alternatively are labelled with DiD (ThermoFisher Scientific), a fluorochrome in the red spectrum with peak excitation (650 nm) and emission (670 nm). MEVs fresh samples (prepared as describe above) are re-suspended in 1 ml and incubated 2 µl of DiD dye added to the samples, followed by continuous mixing for 30 seconds by gentle pipetting. After 5-minute incubation at room temperature, the samples are quenched by adding 2 ml of 10% BSA in 1× PBS. The labelled MEVs were purified from free dye by size exclusion chromatography using a qVE1/70nm column (IC1-70 – IZON Science) and washed with PBS. The MEVs were recovered in the first two fractions. The PKH26-labelled MEVs are stored at 4°C and filtered with 0.45 µm filter before adding to cells. Figure 7B shows the labelled MEVs with DiD by confocal microscopy. Figures 8A-8C show the uptake of labelled MEVs by cells.

G. Flow cytometry experiments

Flow cytometry analyses were conducted using LSRII flow cytometer with CellQuest™ Pro software (BD Biosciences). Latex beads of 0.3 and 1.1 µm diameters were prepared and used according to the manufacturer's recommendation to define

the MEV gate. Since latex beads typically have higher refractive index and thus lower limits of size detection by flow cytometry than MEVs, the thresholds for forward and side scatter were adjusted to avoid background noise during acquisition. The predefined MEV gate was applied to all samples during analysis. Figure 9 shows the cytometry analysis of labelled MEVs or Loaded MEVs with fluorescent payload. Figures 10A-10B show the uptake of MEVs by different types of cells by cytometry.

EXAMPLE 3

Exogenous-loading of MEVs with plasmids carrying AAV-ITRs: obtention of MEV-GTVs

A. Freeze-thaw cycles - Loading of plasmid DNA (pDNA)

Purified MEVs, as described in Example 1, were diluted in PBS to a specific concentration (10exp8, 10exp9, 10exp10, 10exp11 and 10exp12). Next, various concentrations of cargos (0.2, 1, 2 or 40 µg/ml for pDNA; 0,2 and 2 µg/ml for mRNA) were added to the MEV suspension (total volume of 500 µl). The mixture of MEVs and the cargo was frozen at -80°C or immersed into liquid nitrogen (-196°C). Then the sample was thawed at 37°C in a water bath. This freeze-thaw cycle was repeated 2 or 4 times for each sample.

Table 3. Protocol optimization for freeze-thaw cycles loading of DNA (plasmids); parameters and values used as variables for sensitivity analysis.

MEV concentration (per ml)	**DNA concentration (µg/ml)**	**freezing temperature**	**thawing temperature**	**number of cycles**
8.00E+07 1.00E+08 8.00E+08 9.00E+08 1.00E+09 6.00E+09 8.00E+09 9.00E+09 6.00E+10 9.00E+10 9.00E+11	0.2 1.0 2.0 10 20	-80°C -196°C	37°C	2 4 6 8

B. Extrusion - Loading of plasmid DNA (pDNA)

Purified MEVs, as described in Example 1, were diluted in PBS to a specific

concentration (10exp10). Next, different concentrations of cargos (2 or 20 µg/ml for pDNA; 0.1, 1, 2 and 10 µg/ml for mRNA; 2 or 20 µg/ml for GFP) were added to the MEV suspension (total volume of 500-1000 µl). The mixture of MEVs and the cargo was extruded through a syringe-based hand-held mini-extruder (SKU: 610023-1 EA, Avanti® Polar Lipids). The sample was extruded from 10 to 15 times across a membrane filter with 50 nm to 100 nm diameter pores, using two facing syringes. In some experiments, the sample was extruded sequentially through a 200 nm, a 100 nm and 50 nm diameter pore membranes. The extrusion was performed at room temperature or at 65°C.

Table 4. Protocol optimization for extrusion loading of DNA (plasmids); parameters and values used as variables for sensitivity analysis.

MEV concentration (per mL)	**DNA concentration (µg/mL)**	**temperature**	**extrusion cycles**	**membrane pore diameter (nm)**
4.00E+08 9.00E+08 4.00E+09 4.00E+10 6.00E+10	 0.2 1.0 2.0 10 20	 RT 	 10 11 20 	 100 200
8.00E+10 9.00E+10 1. 00E+11				

C. Electroporation – Loading of plasmid DNA (pDNA)

Purified MEVs, as described in Example 1, were diluted in PBS to a specific concentration (10exp8, 10exp9 or 10exp10). Next, different concentrations of cargos (1, 2, 5, 10 or 20 µg/ml for pDNA; 2 and 20 µg/ml for mRNA) were added to the MEV suspension (total volume of 110-150 µl). The mixture of MEVs and the cargo was transferred into a 100 µl electroporation cuvette and placed into a Super Electroporator NEPA 21 (NEPAGENE) device. The following parameters were set for each electroporation: the voltage (50 V, 100 V, or 200 V), the pulse length (5 ms, 10 ms, or 15 ms), the pulse interval (set to 50 ms), the decay rate (set to 10%), the polarity (set to positive), the number of pulse (1, 9 or 15), and the presence or absence of a transfer pulse (5 pulses of 20 V during 50 ms with 50 ms interval and 10 % decay rate). To assess aggregation, 20 mM of EDTA or 10 mM of citrate have been added before electroporation for each condition.

Table 5. Protocol optimization for electroporation loading of DNA (plasmids); parameters and values used as variables for sensitivity analysis.

MEV concentration (per ml)	**DNA concentration (µg/ml)**	**voltage (V)**	**length (ms)**	**number of pulses**	**transfer pulse(s)**
8.00E+08	0.2				
9.00E+08	1.0	50	5		0
1.50E+09	2.0	100	10	1	1
8.00E+09	10	200	15	9	6
9.00E+09	20				
1.50E+10					
6.00E+10					
8.00E+10					
9.00E+10					
8.00E+11					
9.00E+11					

D. DNase Treatment:

After each loading method, a part of the samples undergone a treatment with a Deoxyribonuclease I (DNase) (10104159001, Roche) to eliminate all free nucleic acids (non-encapsulated by the loading). The DNase and a DNase buffer (Tris-HCl 10 mM, $MgCl_2$ 2.5 mM, CaCl2 0.5 mM, pH 7.6) were mixed with the sample and incubated for 40 minutes in an incubator at 37°C. Finally, Ethylenediaminetetraacetic acid (EDTA) (20 mM) was added to inhibit DNase action. Samples were then stored at 4°C.

E. Purification of MEV after Loading

After treatment with benzonase the loaded MEVS were purified to eliminate the non-digested nucleic acids and the benzonase enzyme used to degrade the nucleic acids. MEVs formulated in PBS at concentration of 10exp11 to 10exp12 per mL are seeded in a pre-packed column qEV1 from IZON. The MEVs are eluted with PBS solution. The elution fractions of 0.7 mL are collected. MEVs are recovered in the first 2 fractions.

MEVs concentrations in the initial sample and in the fractions collected throughout the elution are evaluated with the ZetaView® engine (Nanoparticle Tracking Analyzer from Particle Metrix) as the quantity of proteins by Bradford assay.

EXAMPLE 4

MEV characterization after loading the plasmid payload

A. DNA extraction:

QIAprep® Spin Miniprep Kit (acquired from QIAGEN) was used to extract nucleic acid encapsulated by the MEVs according to the manufacturer's recommendations. At the end of the protocol, nucleic acids were eluted in 50 µL of nuclease-free water.

B. qPCR:

To quantify the plasmid extracted from the MEVs, analysis by quantitative Polymerase Chain Reaction (qPCR) have been performed. DNA concentration was measured in the samples using a NanoDrop™ 2000 analyzer (acquired from ThermoScientific) and the samples were diluted if necessary to be correctly amplified during the qPCR (volumes for each condition are listed in annexes). A plasmid range was realized from 0.4 x 10^{-6} ng to 5.1 x 10^{-6} ng of pDNA. A master mix was prepared with 5 µL of PowerUp™ SYBR™ Green per well (Cat. No. A25776, from Fisher Scientific™ Applied Biosystems™), 0.5 µL of forward primer per well (concentration = 10 µM), and 0.5 µL of reverse primer per well (concentration=10 µM). Primers complementary of the resistance gene sequence of the pDNA have been previously designed to amplify only the plasmid of interest. A MicroAmp™ Optical 384-well reaction plate (Cat. No. 10411785, from Fisher Scientific™, Applied Biosystems™) was used, 6 µL of master mix were put in each well, followed by 4 µL of the sample. Two negative controls were made: nuclease-free water with SYBR™ green dye, and nuclease-free water with SYBR™ green dye and primers. All conditions were done in duplicates. The plate was sealed and briefly centrifuged. The thermocycler was a CFX384 Touch Real Time PCR detection system (from Bio-Rad). The three-step cycling, followed by the melt curve protocols are summarized in the tables below.

Table 6. Three-step cycling protocol for qPCR

Step	**Temperature (°C)**	**Time**	**Number of cycles**
Dual lock DNA polymerase	95	10 minutes	1
Denaturation	95	15 seconds	40
Annealing	60	1 minute	

Table 7. Melt curve protocol for qPCR

Step	Ramp rate	Temperature (°C)	Time
1	1.6°C/second	95	15 seconds
2	1.6°C/second	60	1 minute
3	0.15°C/second	95	15 seconds

For plasmid quantification (dilution by 1000), real-time qPCR was performed in technical duplicates in qPCR plates with a rapid thermal cycler system (LightCycler® 480 II, 384 (well)). The protocol was the following: 3 µl DNA (with defined dilution factor of X1000 were mixed with one forward and one reverse primer (added at optimized concentrations usually between 200 nM and 1.0 µM), mix Takyon No ROX SYBR 2X MasterMix blue dTTP (Eurogentec, UF-NSMT-B0701) with dNTPs, $MgCl_2$, Taq DNA polymerase and buffer, constituting a volume of 10 µl in total. Then, this mix was placed in LightCycler® plates.

The amplification protocol started with an initial incubation at 95°C for 10 min (serving for activation of Taq DNA polymerase), followed by 45 amplification cycles composed of two steps: step I) a 95°C denaturation for 10 seconds, step II) 60°C primer annealing and extension for 40 seconds, (the detection of the fluorescent product was performed at the end of this 60°C extension period with a single acquisition mode). The amplification protocol is followed by the step to perform the melting curve with one cycle of 95°C denaturation for 5 seconds, 60°C annealing for 40 seconds, and a 95°C end point (achieved by a temperature raise with a ramp rate of 0.04°C/s from 60°C to 95°C and accompanied by continuous detection of the fluorescent product). The amplification protocol ended by cooling at 37°C for 10 seconds.

The qPCR's primers and their concentrations are listed below:

Plasmid (pDNA) pcDNA3.1 SEQ ID NO 53 + Primers (SEQ ID NOs:54 and 55):

- Left primer PCDNA3.1+1: GACCACCAAGCGAAACATGG
- Right primer PCDNA3.1+1: CCATGGGTCACGACGAGATC
- PCDNA3.1 LR1(Left primer PCDNA3.1+1, Right primer PCDNA3.1+1): 700 nM

Controls for qPCR assays

For the "DNA efficacy and quantification control" sample, a mix of Plasmid

(pDNA) pcDNA3.1+ was prepared. Subsequently, 9 different concentrations were prepared with RNAse/Dnase-free water (1000 ng, 500 ng, 100 ng, 50 ng, 10 ng, 5 ng, 1 ng, 0.5 ng, 0.1ng), extracted and diluted (identically as the samples, dilution factor of X1000) and mixed with the according primers used on a qPCR plate.

C. dPCR to quantify the number of plasmid molecules loaded in the MEVs.

For the Example, plasmid-loaded MEVs were isolated and purified as describe in Example 1, characterized as describe in Example 2 and loaded as described in Example 3. After that, MEVs were purified by SEC as describe in Example 3 and then quantified by Digital PCR (dPCR) (QIAcuity® System, Qiagen). The control plasmid contains the mcherry coding sequence.

The digital PCR (dPCR) is designed to deliver precise and multiplexed quantification results for mutation detection, copy number variation (CNV), gene expression studies, gene-editing analysis, in the present example is used to determine the exact number of plasmids loaded in an determine amount of MEVs. In the Example, QIAcuity® Digital PCR System (Qiagen), QIAcuity Probe PCR Kit (Qiagen, 250102), and QIAcuity® Nanoplate 26k 24-well (Qiagen, 250001) were used.

MEVs were loaded as describe in Example 3 with the following plasmids:

1- Plasmids encoding the eGFP protein:

pAAV-ssGFP from PlasmidFactory SEQ. ID NO: 48

pBCB-23-0041 plasmid with AAV-ITRs, SEQ. ID NO: 48

pEGFP-N1-FLAG from Adgene, plasmid without AAV-ITRs, SEQ. ID NO:52.

2- Plasmids encoding the Aflibercept (SEQ ID NO:94) protein trap:

pBCB-23-0065, plasmid with AAV-ITRs, SEQ. ID NO:49

pBCB-23-0070, plasmid with AAV-ITRs, SEQ. ID NO:50

The plasmid encoding the mcherry protein was used to normalize the reaction and were not loaded into MEVs:

pBCB-24-0005, plasmid without AAV-ITRs, SEQ. ID NO:51

Table 8. Primers and probes for the reactions: (EGFP plasmid determination) SEQ. ID NO: 56, 57, and 58

OLIGO	Start	Len	tm	gc%	any	3' seq
Left primer	232	20	60.06	50.00	4.00	EGFP(1-1)F5 CACATGAAGCAGCACGACTT
Right Primer	332	18	60.41	61.11	4.00	EGFP(1-1)R5 GCGCGGGTCTCTTGTAGTTG
Hyb Oligo	266	20	67.90	65.00	5.00	EGFPFR5FAM TGCCCGAAGGCTACGTCCAG
Product Size: 101, pair any compl:4.00, Pair 3' compl:2.00						

Table 9. Primers and probes for the reactions: (aflibercept plasmid determination) SEQ. ID NO:59, 60, 61, 62, 63, and 64

For pBCB-23-0070

OLIGO	Start	Len	tm	gc%	any	3' seq
Left primer	775	20	60.65	60.00	5.00	AFLI1010_01F4 GGGACCGTCAGTCTTCCTCT
Right Primer	889	20	59.30	55.00	5.00	AFLI1010_01R4 CTTGACCTCAGGGTCTTCGT
Hyb Oligo	841	20	68.19	65.00	4.00	AFLI1010_01FR4FAM GGTCACATGCGTGCGTGGTGGTGG
Product Size: 101, pair any compl:4.00, Pair 3' compl:2.00						

For pBCB-23-0065

OLIGO	Start	Len	tm	gc%	any	3' seq
Left primer	158	18	59.64	66.67	4.00	AFLI1010_02F4 GAGGGCAGAGAGCTGGTG
Right Primer	321	20	60.10	55.00	4.00	AFLI1010_02R4 AGGCCGATCTCCTTGTAGGT
Hyb Oligo	228	20	68.30	70.00	4.00	AFLI1010_02FR4FAM CCCTGGACACCCTGATCCCC
Product Size: 101, pair any compl:4.00, Pair 3' compl:2.00						

Table 10. Primers and probes for the reactions: (mcherry plasmid determination) SEQ. ID NO: 65, 66, and 67

OLIGO	Start	Len	tm	gc%	any	3' seq
Left primer	1206	18	60.03	55.56	4.00	Cherry(1-1)F1 GAGGGCGAGATCAAGCA
Right Primer	1354	18	61.94	61.11	2.00	Cherry(1-1)R1 CCTCGTTGTGGCTGGTGA
Hyb Oligo	1234	20	67.90	65.00	5.00	CherryFR1HEX CTGAAGGACGGCGGCCACTA
Product Size: 101, pair any compl:4.00, Pair 3' compl:2.00						

Different batches of plasmids loaded MEVs were quantified using dPCR in order to quantify the payload in each batch of MEVs. The dPCR reaction was performed using a concentration of 0.8 mg of each primer and 0.4 mg of the probes, final volume of reaction 40 mL. Each well was measured for all fluorescent dyes (FAM, HEX, and TAMRA).

The number of copies for each plasmid by dPCR was >95% in relation to Nanodrop quantification of the same preparation of plasmid measured the same days of dPCR testing. The results of quantifications are summarized in tables 11-12.

Table 11: Quantification of payload from MEVs loaded with plasmids carrying or not AAV-ITRs (primers and probes for EGFP).

Plasmid Loaded MEVs (lot#)	Plasmid characteristics	Plasmid size (bases)	Quantity of plasmid (plasmid copies/loading)	MEV concentration (particles/mL)	Quantity payload/MEV (plasmid copies/MEV)
MEV-24-026	AAV-ITRs/eGFP	7957	1.23xE12	1.6x10E11	3.4
MEV-24-027	eGFP	4777	2.04xE12	1.2x10E11	5.9
MEV-24-028	AAV-ITRs/variant2	7189	1.37xE12	1.4x10E11	0
MEV-24-029	Aflibercept	7210	1.36xE12	0.8x10E11	0
MEV-24-047	eGFP	4777	2.05xE12	0.6x10E11	6.9
MEV-24-048	eGFP	4777	2.05xE12	0.75x10E11	13.9
MEV-24-050(C. specif)	n/a	mRNA loaded	n/a	0.8x10E11	0
MEV-24-051 (C. neg)	n/a	non-loaded	n/a	0.65x10E11	0
MEV-24-052	AAV-ITRs/eGFP	7957	1.23xE12	0.7x10E11	6.50
MEV-24-053	AAV-ITRs/eGFP	5957	1.23xE12	0.6x10E11	5.75

Table 12: Quantification of payload from MEVs loaded with plasmids carrying or not AAV-ITRs (primers and probes for Aflibercept)

Plasmid Loaded MEVs (lot#)	Plasmid characteristics	Plasmid size (bases)	Quantity of plasmid (plasmid copies/loading)	MEV concentration (particles/mL)	Quantity payload/MEV (plasmid copies/MEV)
MEV-24-026	AAV-ITRs/eGFP	7957	1.23xE12	1.6x10E11	0
MEV-24-027	eGFP	4777	2.04xE12	1.2x10E11	0
MEV-24-028	AAV-ITRs/variant2	7189	1.37xE12	1.4x10E11	4.5
MEV-24-029	Aflibercept	7210	1.36xE12	0.8x10E11	6.3
MEV-24-047	eGFP	4777	2.05xE12	0.6x10E11	0
MEV-24-048	eGFP	4777	2.05xE12	0.75x10E11	0
MEV-24-050(C. specif)	n/a	mRNA loaded	n/a	0.8x10E11	0
MEV-24-051 (C. neg)	n/a	non-loaded	n/a	0.65x10E11	0
MEV-24-052	AAV-ITRs/eGFP	7957	1.23xE12	0.7x10E11	0
MEV-24-053	AAV-ITRs/eGFP	5957	1.23xE12	0.6x10E11	0

D. Flow Cytometry for fluorescent payloads

Flow cytometry analyses were conducted using LSRII flow cytometer with CellQuest™ Pro software (BD Biosciences). Latex beads of 0.3 and 1.1 µm diameters were prepared and used according to the manufacturer's recommendation to define the MEV gate. Since latex beads typically have higher refractive index and thus lower limits of size detection by flow cytometry than MEVs, the thresholds for forward and side scatter were adjusted to avoid background noise during acquisition. The predefined MEV gate was applied to all samples during analysis. Figure 11 shows the percentage of loaded MEVs with a florescent payload.

E. Statistical analysis:

Screening and optimized experimental designs were generated with NemrodW software. The results have been analyzed using R Studio (1.4.1717 version) and Prism (8.3 version). Significance of the variables compared to the controls was evaluated with unpaired parametric t tests. "ns" stands for "not significant" meaning a p-value superior to 0.05, "*" stands for a p-value inferior to 0.05, "**" stands for a p-value inferior to 0.01, and "***" stands for a p-value inferior to 0.001. Loading efficiency were calculated based on the pDNA quantity obtained by qPCR compared to the initial pDNA quantity for each condition. Linear regression modeling was realized to evaluate the effect of tested variables on plasmid encapsulation.

F. Exo-loading Efficiency

The efficiency of exo-loading of MEV obtained, purified, characterized, loaded, and the internalization of payload determined as described in examples 1-3 is shown in the following Table 13. The efficiency of exo-loading using the different loading methods for different payloads is calculated using the parameters: (i) loading efficacy, and/or (ii) loading capacity, or (iii) the percentage of loaded MEV.

Table 13: Summary of conditions and results obtained by different loading methods to load plasmids.

Payload	Method	Tested Conditions	Loading Efficiency	Loading Capacity
DNA (plasmids/ ASOs)	Incubation	9	(0)	(0)
	Saponification	12	(+)	(++)
	Freeze thaw cycle	28	(++)	(+++)
	Electroporation	46	(+++)	(+++)
	Sonication	23	(++)	(++)
	Extrusion	25	(+)	(+)

Payload	Method	Tested Conditions	Loading Efficiency	Loading Capacity
	Serial Extrusion	12	(+)	(+)
	SEAL	(-)	ND	ND

Key: Loading (0) very low; (+) low; (++) acceptable; (+++) best results; ND not determined, where the parameters are as follows:

- Internalized payload is the total amount of payload molecules (DNA) measure into the MEVs after the loading reaction. The internalized payload is determined by a specific quantification method (qPCR), after the elimination of the remaining payload molecules in the loading reaction medium at the end of reaction by specific enzymatic treatment (DNAse).
- Copy number of payloads: Number of copies of payload molecule (DNA) in the either initial amount or the internalized payload amount. The number of copies is obtained using the following calculation:

  Number of copies=(ng×[6.022exp23])/(length ×[1exp9]×650)

  where:

  ng is the amount of nucleic acids (plasmid)

  6.022exp23 = Avogadro's number.

  Length is the length of DNA fragment in base pairs multiply by 1000 (kb)

  Multiply by 1exp9 to convert to nanograms.
- Loading efficiency is the percentage of the initial amount of payload molecule, in the loading reaction medium at the initial timepoint, internalized into extracellular vesicles from microalgae (MEVs).
- Loading capacity is the copy number of payload internalized per MEV. The loading capacity calculated as the ratio between the internalized payload copies and the number of MEVs in the reaction.
- Percentage of loaded MEVs determined by flow cytometry as described in Example 4G and represented by Figure 11.

EXAMPLE 5A

***In vivo* biodistribution of MEVs.**

MEVs can be delivered *in vivo* using several administration routes. Experiments were performed to determine the pathway and fate of MEVs when administered via various routes.

A. MEV labelling using DiR fluorescent

As described in Example 2, the fluorescent, lipophilic carbocyanine DiR (1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide) is weakly fluorescent in water but highly fluorescent and photostable when incorporated into membranes and can be tracked *in vivo* (see, Example 2 above; ThermoFisher Scientific). The DiR labelled (MEVs) are incubated with human cells. Their uptake by human cells is measured by fluorimetry analysis (fluorescence spectroscopy) on microplate readers. DiR has an excitation of 750 nm and an emission of 780 nm.

The methodology for the biodistribution studies is summarized as follows: *Chlorella* cells were grown in the photobioreactor as described above (120L per batch), were isolated and labeled with DiR, and labelling efficiency was assessed as described in Examples 1 and 2.

B. Biodistribution of MEVs in mice

The labeled MEVs were administered to the mouse model animals via one of four routes, via: intranasal, intratracheal, oral, and intravenous administration, to determine the pathways and fate of the MEVs by each route. Treated mice were subjected to full body imaging as a function of time, and at day 3 mice were sacrificed and the organs were imaged *ex vivo*, and by histology in several mice organs after different routes of application.

Animal model

MEVs' pharmacokinetics and biodistribution were studied in Specific Pathogen Free (SPF), 7-week-old male Balb/cByJ mice (Charles River Laboratories). The animals were labelled with unique ear ID tags and acclimatized for at least 2 days. Background (control) mice were housed individually, while MEV-treated mice were housed collectively in disposable standard cages in ventilated racks at 21±3°C (temperature recorded and controlled) with a 12h-12h light/dark cycle. Filtered water and low fluorescence laboratory food for rodents were provided *ad libitum*. All mice were euthanized at the end of the *in vivo* experiments.

***In vivo* administration of DiR-labelled MEVs**

The DiR-labelled MEVs, were used to determine the biodistribution of the MEVs in the whole animal body or in several organs after different routes of application. Prior to the imaging, the fur of each animal was clipped using an electric

clipper in the following areas: abdomen, thorax, head, and whole back. Care was taken in order to clip the fur as homogeneously as possible. Next, DiR-labelled MEV preparations were re-suspended by vortexing before filling the syringes for injection.

Routes of administration

The following routes were used to administer the MEVs to the animals:

Intravenous (*IV*): 50 µL of MEV suspension containing 0.6x10exp12 MEV/mL were injected in a tail vein by disposable plastic syringe and appropriate needle. Dosage of MEV administered: 3x10exp10 MEV/mouse.

Intranasal (*IN*): Animals were induced and maintained under anesthesia during IN administration by a mixture of isoflurane and oxygen as a carrier gas. A volume of 100 µL of MEV suspension containing 0.3×10exp12 MEVs/mL was administered into the nostrils of the mouse using a thin pipette cone. Dosage of MEV administered: 3x10exp10 MEV/mouse.

***Per os* (*PO*)**: 100 µL of MEV suspension containing 0.3×10exp12 MEVs/mL was administered orally using a disposable plastic syringe and an appropriate feeding probe. Dosage of MEV administered: 3x10exp10 MEV/mouse.

Intratracheal (*IT*): Animals were induced and maintained under anesthesia during IT administration by a mixture of isoflurane and oxygen as a carrier gas. Once adequate anesthesia was observed, animals were placed on a mouse intubation platform, suspended from the front incisors in the supine position, to maximize view of the trachea. Then, a cold light was placed on the skin near the trachea localization in order to backlight the trachea. If needed, a laryngoscope was inserted to guide the syringe of Microsprayer® sprayer into the trachea. An injection of 50 µL of MEV suspension containing 0.6×10exp12 MEVs/mL was instilled into the trachea. Anesthesia was maintained throughout the procedure. After injection, the mice were maintained in the same position on the intubating platform for at least 30 seconds, before being replaced in their cage. Dosage of MEVs administered: 3x10exp10 MEV/mouse.

***In vivo* imaging for biodistribution studies**

Fluorescence acquisitions were performed with the IVIS® Spectrum optical imaging system (acquired from PerkinElmer). Bidimensional (2D) fluorescence imaging was performed by sensitive detection of light emitted by DiR-labelled MEVs.

Mice were anesthetized and imaged 1 hour before MEV administration. Next, fluorescence of the material to be administered was confirmed with an *in vitro* acquisition performed on at least 1 drop of a minimum of 20 µL of MEV suspensions, deposited in 2 different Petri dishes. The fluorescence emission was measured and detected with the selected parameters (see below). Finally, *in vivo* fluorescence acquisitions were performed on mice administered MEVs. The animals were induced and maintained under anesthesia with a mixture of isoflurane and oxygen. Mice were positioned in dorsal recumbency to obtain ventral images and in ventral recumbency to obtain dorsal images. At least 2 acquisitions/mouse/timepoint were taken and mice were imaged in groups of maximum 3 mice/acquisition. Images were analyzed and fluorescence quantified on 6 organs (liver, spleen, lung, kidney, intestine, and brain).

The following timepoints were used (T0=MEV administration):

Day 0 - 6 time points : T0 -1h ± 10 min; T0 +30 min ± 5 min; T0 +2 h ± 15min; T0 +4 h ± 30 min; T0 +8h ± 30 min; T0 +10 h ± 30 min

Day 1 - 1 timepoint: T0 + 24 h ± 3 h

Day 2 - 1 timepoint: T0 + 48 h ± 3 h

Day 3 - 1 timepoint: T0 + 72 h ± 3 h

The data were analyzed with the IVIS software (Living Image Software for IVIS).

The following parameters were used for the *in vivo* and ex-vivo fluorescence acquisitions:

Field of View (FOV) 14 x 14 cm (FOV C)

Image number: *in vivo*: For each mouse at each applicable timepoint, at least 2 acquisitions (ventral and dorsal) were performed; 3 mice (at maximum) were imaged in one acquisition.

Ex-vivo: the organs of at least one mouse were imaged in one acquisition

Image format: TIFF format

Fluorescent probe: DiR. Excitation wavelength: 745 nm. Emission wavelength: 800 nm.

Exposition time: Automatic, depending on the fluorescence signal detected. Minimum counts 20000.

Binning : Between 16 and 1 (depending on the fluorescence signal

detected)

F/STOP : 2 (if fluorescence signal reached saturation, it was automatically increased to 4, or if signal was too weak, it was decreased to 1)

Subject height: *In vivo*: 1.5 cm

Ex-vivo: 0.5 cm

***Ex vivo* imaging for biodistribution studies**

Production, purification, characterization, labelling and administration (by different routes) of MEVs was as described above. On Day 3 after administration, mice were euthanized and the organs of interest of each mouse were sampled and positioned in a Petri dish in order to perform *ex vivo* acquisitions. The following organs were sampled: liver, spleen, lungs, kidneys, brain, and intestine. The data were analyzed with the IVIS software (Living Image Software for IVIS).

Pharmacokinetics Results by each route of administration

Figure 12 shows representative patterns of biodistribution according to the route of administration, for the Intravenous (IV), Intratracheal (IT) and Per os (PO) routes.

The pharmacokinetic curves demonstrating the accumulation as a percent of baseline over time (hours) after intravenous, Per os, intranasal, and intratracheal administration are set forth in Figures 13-16 respectively. The results in biodistribution of a representative animal is depicted in Figures 17-20.

Biodistribution in isolated organs (*ex vivo* imaging of organs)

The *ex vivo* fluorescence analysis (total radiant efficiency) in liver; spleen; lungs; and brain after intravenous (IV); Per os (PO); intranasal (IN); and intratracheal (IT) administration in set forth is imaged in Figures 21A-21D.

Summary of results by each route of administration

a. Intravenous administration

- Organs targeted by unmodified MEVs – **liver and spleen**
- PK parameters:
- Peak/plateau time: 2-24 h (liver), 4-24 h (spleen)
- % at t=day 3 compared to peak/plateau approximately 62% (liver), approx. 61% (spleen)
- Longer presence or duration compared to mammalian EVs

(see, *e.g.*, Kang *et al.,* Biodistribution of extracellular vesicles following administration into animals: A systematic review. *J Extracell Vesicles.* 2021;10:e12085. (doi.org/10.1002/jev2.12085)).

- No visible signs of toxicity after 72 hours of treatment.

b. Per os (oral) administration

- Organs targeted by following oral administration: **intestine and spleen**
- Oral availability:
- MEVs are orally available; they resist the passage through the stomach, reach the intestine and subsequently appear in the spleen.
- Mammalian EVs are not orally available, with the exception of bovine milk EVs.
- PK parameters:
- Peak/plateau time: 1-6 h (intestine), 1-10 h (spleen)
- % at t=10h compared to peak/plateau: approx. 33% (intestine), approx. 44% (spleen)

% at t=48h compared to peak/plateau: 0% (intestine), 0% (spleen)

- No visible signs of toxicity after 72 hours of treatment.

c. Distribution in organs following intravenous and oral administration:

Liver: Following intravenous administration, the liver is the primary target organ. Following oral administration, the liver is marginally targeted, if at all.

Spleen: The pharmacokinetics profile depends on whether administration is intravenous or oral (per os). Intravenous administration is longer lasting. At day 3, 50-70% of the peak is still remaining. Oral administration is shorter lasting; at 48 hours, 0% remains.

d. Intranasal administration

- Organs targeted by the unmodified MEVs are the **lungs**
- Preliminary PK parameters:
- Peak/plateau time: 4-28 h
- % at t=day 3 compared to peak/plateau: 70-80%

- No visible signs of toxicity after 72 hours of treatment.

e. Intratracheal administration

- Organs targeted by the MEVs are the **lungs**
- PK parameters:
- Peak/plateau time: 2-72 h
- at t=day 3 compared to peak/plateau: approx. 80%
- No visible signs of toxicity after 72 hours of treatment.

EXAMPLE 5B

Biodistribution of MEVs labelled with PKH26 after per os (PO) administration in BALB/cByJ mouse. Internalization of fluorescence-labelled MEVs in the mouse intestinal epithelium (enterocytes) and GALT cells after per os administration

PKH26 is a lipophilic fluorescent dye used for general membrane labeling. It is used for *in vitro* studies, as well as long term *in vivo* experiments due to enhanced stability (*in vivo* half-life is over 100 days). MEVs were labeled with PKH26 as described above and administered orally to BALB/c mice at a dose of 4x10exp10 MEV particles per animal. Between 0.5 to 24 hours post-administration, the animals were sacrificed, and the intestine was harvested.

A. Organ sampling and processing: Intestine

The jejunum and ileum were gently rinsed with a cold PBS solution. Then, a piece of approximately 0.5 to 1 cm of jejunum and ileum were snap-frozen and lately, each piece was longitudinally placed on bottom of the cryomold for Optimal cutting temperature inclusion (as described below).

B. Snap-frozen:

A small stainless-steel bowl was placed in the bottom of a container containing dry ice in pellet form and liquid nitrogen. Then, isopentane/2-methylbutane was slowly added in the container. When, the dry ice pellets stop bubbling vigorously or/and the isopentane start to become opaque, the isopentane/2-methylbutane is at optimal temperature. Organs were gently immerged in isopentane/2-methylbutane and then placed in cassette (one cassette with each section of liver, the spleen and the piece of jejunum and one cassette with the piece of ileum only). Then, cassettes were stored at ≤-75°C until OCT inclusion.

C. Optical cutting temperature inclusion protocol

A small stainless-steel bowl was placed in the bottom of a container containing dry ice in pellet form and liquid nitrogen. Some pellets of dry ice were also placed directly in the bowl. Then, isopentane/2-methylbutane was slowly added in the container. When, the dry ice pellets stop bubbling vigorously and/or the isopentane starts to become opaque, the isopentane/2-methylbutane is at optimal temperature. The frozen tissue samples were then placed and oriented as mentioned above for each organ in the cryomold. The tissues were gently pushed with forceps to ensure that the bottom of surface of the tissue is placed properly (touching the face of the bottom, center in the mold and properly oriented). The cryomold with frozen tissue samples were placed on the surface of the cold isopentane/2-methylbutane. Optimal cutting temperature compound (OCT compound) was carefully deposited onto the specimen until it is completely covered. After hardening of the OCT compound (between 30 seconds and 1 minute), the OCT embedded block was placed in a bag (such as zip freezer bag). The frozen blocks were temporarily stored in dry ice. Then, slices of 5 µm were performed with a cryostat then glued on untreated slides for the histology and on treated slides for the immunochemistry.

D. HES Staining

The HES staining allowed the observation of the morphology and the structure of tissues. After fixation in acetone, sections were immersed successively in solutions of Harris hematoxylin, eosin and saffron. After dehydration, sections were mounted between slide and cover slip using Entellan® mounting medium. Cytoplasm appeared in pink and nuclei in violet blue. Extracellular matrix was stained in yellow to pink.

E. PKH26 dye

Accumulation of fluorescent labeled MEVs was visualized by a LSM700 laser scanning confocal microscope (from Zeiss) and images were processed with LSM Image Browser. DAPI (4',6-diamidino-2-phenylindole) [Invitrogen, Ref. S36938] was used as nuclear counterstain prior to the imaging. Sections were mounted with aqueous medium with DAPI for fluorescent slide scanner observation (maximum 10 days after collection).

When MEVs are orally administrated to the mice, they follow through the digestive tract. MEVs first reach the stomach, where they resist the stringent

conditions of the gastric juice. In about 30 minutes after administration, they reach the intestine, where they stay for several hours. Results are shown in Figures 22-24.

Once in the lumen of the intestine, as predictable from the Example 5, MEVs are internalized by the cells of the intestinal epithelium, or enterocytes (Figure 22). MEVs also pass through the epithelial layer into the GALT (Figures 23A-23B). The GALT is a lymphoid structure associated to the digestive tract (*gut-associated lymphoid tissues)* located beneath the intestinal epithelium. It is located at specific spots along the intestine. GALT is a dense tissue composed of germinal centers with B and T lymphocytes, plasmacytes and innate immune cells including dendritic cells and macrophages. In the intestine, fluorescence is observed mostly in the GALT. Fluorescence appears (Figure 23A) concentrated in discrete spots around nucleus meaning that MEVs are localized in the cytoplasm of cells occupying all the space in the cytoplasm. This allows revealing of the shape of cells. Cells with MEVs inside correspond to histocytes (resident macrophages) or dendritic cells respectively and are located mainly at the periphery of GALT and in the center of the GALT. No visible MEVs appear in the liver at all times evaluated (30 min to 24h). A few hours after administration, dendritic/macrophage cells with MEVs inside move from the GALT to the spleen and stay in the spleen for several hours. MEVs primarily reach the red pulp (blood cells and some Th and B cells) are and in a lesser extent the white pulp of the spleen (lymphoid cells) (see Figure 24).

In the spleen, fluorescence is visible is several areas corresponding to the white and to the red pulps. The intensity of the fluorescence decreases in a gradient from the red pulps to the white pulps. Fluorescence is detected in spots inside the cell's cytoplasm. Cells with MEVs inside are round or reticular and have similar size; they can be, respectively, for example, histocytes and dendritic cells or macrophages.

Cells can move from the GALT into the bloodstream directly or they can join it after they have first passed through the lymph stream. GALT cells carrying MEVs inside eventually arrived at the liver by the portal vein and/or by mesenteric lymphoid nodes and then to the systemic circulation. In both cases, however, MEVs, are 'invisible' to the liver as they have been internalized by GALT cells that naturally transport them to the spleen (see Figure 25). These MEVS, therefore, are not captured by the liver, as are the MEVs that have been administered intravenously.

This is advantageous for MEVs that carry cargo, such as nucleic acid encoding antigens and/or immune modulators, intended for the immune system.

EXAMPLE 5C

MEV-mediated *in vivo* delivery, expression, and biologic activity of mRNA encoding luciferase enzyme

A. Intratracheal administration of MEVs loaded with mRNA encoding luciferase

In vivo bioluminescence was used to study the biodistribution and MEV-mediated delivery and expression of pDNA encoding luciferase. Eight (8) female BALB/cByJ mice were divided into two groups (4 animals per group) for intra-tracheal (IT) administration. Mice in each group were treated with MEVs loaded with pDNA encoding luciferase. MEVs were loaded and characterized as described above. For the IT administration, the animals were maintained under isoflurane-induced anesthesia and placed on a mouse intubation platform. Then, the trachea was backlit with a cold light and 50 µl/mouse of MEV formulations were administered intra-tracheally using a Microsprayer® aerosolizer (Penn-Century). After administration, mice were maintained in the same position on the intubating platform for at least 30 s before being replaced in their cage. A background mouse also was included in the study; this mouse did not receive any MEV administration and served as a control to measure the background level of the bioluminescence acquisition.

B. Bioluminescence acquisition

Prior to imaging, the fur of each animal was shaved on the abdomen and thoracic area using an electric clipper. Each mouse was intraperitoneally (IP) injected with 200 µL of luciferin solution at a concentration of 16.5 mg/mL. Bioluminescence acquisitions were performed 10 minutes after the luciferin injection, with mice anesthetized with a mix of isoflurane/oxygen. Animals were positioned in dorsal recumbency (ventral images) to visualize the mice abdomen and thorax and particularly the lungs, liver, intestine, and spleen. Bioluminescence acquisition *in vivo* was performed with the IVIS® LUMINA X5 optical imaging system (PerkinElmer).

The bioluminescence was measured at 6 timepoints: 1 h before MEV administration and 6, 30, 48, 54 and 72 h post-administration. The bioluminescence signal was visualized and quantified in the lungs, liver, intestine, and spleen. Animals

were euthanized after the last bioluminescence acquisition. At each timepoint, bioluminescence acquisitions were first performed on the background mouse to measure the background flux level corresponding to the auto-bioluminescence of mice and the noise emitted by the camera of the optical imaging system. Bioluminescence acquisitions then were performed on the experimental mice.

C. Results: Luciferase mRNA delivery, expression and activity *in vivo*

Figures 26A-26B depict whole-body bioluminescence imaging of a representative animal treated with MEVs loaded with pDNA encoding luciferase enzyme. MEV-mediated expression of the protein resulted in enzymatic activity of luciferase in mouse tissues, as evidenced by the luminescence signal detected after the administration of luciferin. The target organs after intratracheal administration include the respiratory system (lungs and naso-buccal epithelium), as well as the gastrointestinal tract (intestinal epithelium) resulting from partial regurgitation of the MEV formulation. Luciferase activity after a single administration of MEVs was detectable starting from 6 hours post-administration and lasted for 3-4 days. The control is a background mouse with no MEV administration.

D. Quantification by digital dPCR of the number of plasmid molecules loaded in the MEVs.

Plasmid-loaded MEVs were isolated and purified as described in Example 1, characterized as described in Example 2, and loaded as described in Example 3. After that, MEVs were purified by SEC as describe in Example 3 and then quantified by Digital PCR (dPCR) (QIAcuity System, Qiagen). The control plasmid contains the *mcherry* coding sequence.

Digital PCR (dPCR) delivers precise and multiplexed quantification results for mutation detection, copy number variation (CNV), gene expression studies, gene-editing analysis. In the present example it is used to determine the exact number of plasmid molecules loaded in a given amount of MEVs.

In the Example, QIAcuity Digital PCR System (Qiagen), QIAcuity Probe PCR Kit (Qiagen, 250102), and QIAcuity Nanoplate 26k 24-well (Qiagen, 250001) were used.

MEVs were loaded as described in Example 3 with the following plasmids:

1- Plasmids encoding the eGFP protein:

pAAV-ssGFP from PlasmidFactory SEQ. ID NO: 48

pBCB-23-0041 plasmid with AAV-ITRs, SEQ. ID NO: 48

pEGFP-N1-FLAG from Addgene, plasmid without AAV-ITRs, SEQ. ID NO:52.

2- Plasmids encoding the Aflibercept (SEQ ID NO:73) protein trap:

pBCB-23-0065, plasmid with AAV-ITRs, SEQ. ID NO:49

pBCB-23-0070, plasmid with AAV-ITRs, SEQ. ID NO:50

The plasmid encoding the *mcherry* protein was used to normalize the reaction and was not loaded into MEVs:

pBCB-24-0005, plasmid without AAV-ITRs, SEQ. ID NO:51

Table 14. Primers and probes for the reactions: (EGFP plasmid determination) SEQ. ID NO: 56, 57, and 58

Oligo	Start	Length	tm	GC%	Any	3' SEQ
Left primer	232	20	60.06	50.00	4.00	EGFP(1-1)F5 (SEQ ID NO:56) CACATGAAGCAGCAGCACGACTT
Right primer	332	18	60.41	61.11	4.00	EGFP(1-1)R5 (SEQ ID NO:57) GCGCGGGTCTTGTAGTTG
Hybrid Oligo	266	20	67.90	65.00	5.00	EGFPFR5FAM (SEQ ID NO:58) TGCCCGAAGGCTACGTCCAG
Product Size: 101, Pair any comple:4.00, Pair 3' COMP 2.00						

Table 15. Primers and probes for the reactions: (aflibercept plasmid determination) SEQ. ID NO:59, 60, 61, 62, 63, and 64.

For pBCB-23-0070

Oligo	Start	Length	tm	GC%	Any	3' SEQ
Left primer	775	20	60.65	60.00	5.00	AFLI1010_01F4 (SEQ ID NO:59) GGGACCGTCAGTCTTCCTCT
Right primer	889	20	59.30	55.00	5.00	AFLI1010_01R4 (SEQ ID NO:60) CTTGACCTCAGGGTCTTCGT
Hybrid Oligo	841	20	68.19	65.00	4.00	AFLI1010_01FR4FAM (SEQ ID NO:61) GGTCACATGCGTGGTGGTGG
Product Size: 115, PAIR ANY COMP:5.00, PAIR 3' COMP 0.00						

For pBCB-23-0065

Oligo	Start	Length	tm	GC%	Any	3' SEQ
Left primer	158	18	59.64	66.67	4.00	AFLI1010_02F4 (SEQ ID NO:62) GAGGGCAGAGAGCTGGTG
Right primer	321	20	60.10	55.00	4.00	AFLI1010_02R4 (SEQ ID NO: 63) AGGCCGATCTCCTTGTAGGT
Hybrid Oligo	228	20	68.30	70.00	4.00	AFLI1010_02FR4FAM (SEQ ID NO:64) CCCTGGACACCCTGATCCCC

Product Size: 164, PAIR ANY COMP:4.00, PAIR 3' COMP 0.00

Table 16. Primers and probes for the reactions: (mcherry plasmid determination) SEQ. ID NO: 65, 66, and 67.

Oligo	Start	Length	tm	GC%	Any	3' SEQ
Left primer	1206	18	60.03	55.56	4.00	Cherry(1-1)F1 (SEQ ID NO:65) GAGGGCGAGATCAAGCA
Right primer	1354	18	61.94	61.11	2.00	Cherry(1-1)R1 (SEQ ID NO:66) CCTCGTTGTGGCTGGTGA
Hybrid Oligo	1234	20	67.90	65.00	5.00	CherryFR1HEX (SEQ ID NO:67) CTGAAGGACGGCGGCCACTA
Product Size: 149, PAIR ANY COMP:4.00, PAIR 3' COMP 2.00						

Different batches of plasmid-loaded MEVs were quantified using dPCR to quantify the payload in each batch of MEVs. The dPCR reaction was performed using a concentration of 0.8 mg of each primer and 0.4 mg of the probes, final volume of reaction 40 mL. Each well was measured for all fluorescent dyes (FAM, HEX, and TAMRA). The number of copies for each plasmid by dPCR was >95% in relation to Nanodrop quantification of the same preparation of plasmid measured the same days of dPCR testing. The results of quantifications are summarized in Tables 17-18.

Table 17: Quantification of payload from MEVs loaded with plasmids carrying or not AAV-ITRs (primers and probes for EGFP).

Plasmid Loaded MEVs (Lot#)	Plasmid Characteristics	Plasmid size (bases)	Plasmid Qty. (PCN/loading)	MEV conc. (particles/ mL)	Payload Qty./MEV (PCN/MEV)
MEV-24-026	AAV-ITRs/eGFP	7957	1.23xE12	1.6x10E11	3.4
MEV-24-027	eGFP	4777	2.05xE12	1.2x10E11	5.9
MEV-24-028 (C.specif)	AAV-ITRs/variant 2 Aflibercept	7189	1.37xE12	1.4x10E11	0
MEV-24-029 (C. specif)	AAV-ITRs/variant 1 Aflibercept	7210	1.36xE12	0.8x10E11	0
MEV-24-047	eGFP	4777	2.05xE12	0.6x10E11	6.9
MEV-24-048	eGFP	4777	2.05xE12	0.75x10E11	13.9
MEV-24-050 (C. specif)	NA	mRNA loaded	NA	0.8x10E11	0
MEV-24-051 (C. neg)	NA	Non loaded	NA	0.65x10E11	0
MEV-24-052	AAV-ITRs/eGFP	7957	1.23xE12	0.7x10E11	6.50
MEV-24-053	AAV-ITRs/eGFP	7957	1.23xE12	0.6X10E11	5.75

*Plasmid copies is abbreviated as PCN

Table 18: Quantification of payload from MEVs loaded with plasmids carrying or not AAV-ITRs (primers and probes for Aflibercept).

Plasmid Loaded MEVs (Lot#)	**Plasmid Characteristics**	**Plasmid size (bases)**	**Plasmid Qty. (PCN/loading)**	**MEV conc. (particles/ mL)**	**Payload Qty./MEV (PCN/MEV)**
MEV-24-026	AAV-ITRs/eGFP	7957	1.23xE12	1.6x10E11	0
MEV-24-027	eGFP	4777	2.05xE12	1.2x10E11	0
MEV-24-028 (C.specif)	AAV-ITRs/variant 1	7189	1.37xE12	1.4x10E11	4.5
MEV-24-029 (C. specif)	AAV-ITRs/variant 2	7210	1.36xE12	0.8x10E11	6.3
MEV-24-047	eGFP	4777	2.05xE12	0.6x10E11	0
MEV-24-048	eGFP	4777	2.05xE12	0.75x10E11	0
MEV-24-050 (C. specif)	NA	mRNA loaded	NA	0.8x10E11	0
MEV-24-051 (C. neg)	NA	Non loaded	NA	0.65x10E11	0
MEV-24-052	AAV-ITRs/eGFP	7957	1.23xE12	0.7x10E11	0
MEV-24-053	AAV-ITRs/eGFP	7957	1.23xE12	0.6X10E11	0

*Plasmid copies is abbreviated as PCN

EXAMPLE 6

Evaluation of toxicity of MEVs in mice.

Experiments were performed to assess the signs of MEV toxicity *in vivo* using Balb/C mouse model after oral and intratracheal administration.

A. Analysis of MEV toxicity *in vivo*:

Toxicity was evaluated in several ways: clinical signs, body weights, hematological analysis, biochemical analysis, histological analysis on main organs (liver, spleen, kidney, lung and brain). The MEV samples were stored at -80°C and thawed just prior to *in vivo* administrations. After thawing, each sample was mixed by vigorous vortexing for 1-2 minutes. Male Balb/C mice at 5 weeks of age and with a mass about 20 g each, were used. Animals were acclimatized. Animals were housed in polyethylene cages (<5 animals/cage), in a controlled environment with 12:12 light-dark at the temperature of 24±1°C (mean ± SD) and fed once daily with an adapted pelleted feed. Water was offered *ad libitum*. The animals were randomly assigned to

experimental groups and acclimatized for at least 7 days before the initiation of the designed study. The experimental groups are described in Table 19, below.

The experiment was designed to determine the eventual toxicity of the test compound after its administration in mice through exemplary routes including oral, and respiratory tract (intratracheal) routes. As described above, male Balb/C mice, with a mass about 20 g each, were used. Animals were acclimatized. After a test item is administered, all mice are closely monitored for 10 days.

Table 19. Experimental groups in MEV toxicity study.

Group no.	Group type	Route of administration	MEV dosage
1	Vehicle control (PBS)	PO (oral)	-
2	Experimental	PO (oral)	4x10exp11 per animal
3	Experimental	PO (oral)	1x10exp12 per animal
4	Vehicle control (PBS)	IT (intratracheal)	-
5	Experimental	IT (intratracheal)	4x10exp11 per animal

Clinical examination

Daily clinical examination of all animals included observation of behavior and signs of suffering (cachexia, weakening, difficulty for moving or feeding, etc.); test item toxicity (hunching, convulsions), and other such parameters. Determination of body weight once a week for each animal, a body weight curve was designed (Mean + SD). Observation of acute reactions was done after administration of the tested compounds.

Clinical Pathology Investigations

After the end of the in-life phase, all animals were euthanized. Clinical pathology investigations were performed at experiment termination.

Blood collection

The blood samples were collected from mice by intracardiac puncture into different vials. Aliquots of blood were collected for various clinical pathology investigations into tubes containing anticoagulants: for hematology analysis with K2 EDTA and for biochemistry analysis with lithium heparin.

Clinical chemistry

Plasma was separated after centrifugation of whole blood samples, 45000 rpm for 15 minutes and analyzed for the following parameters at the end of treatment for all animals. Clinical chemistry analysis parameters are indicated as: Alanine

Aminotransferase (ALT), Aspartate Aminotransferase (AST), and Gamma-glutamyl transferase (GGT) in units per liter (U/L); Urea in g/L and Creatinine in mg/L.

Hematology

The following hematological parameters were determined at the end of treatment of all animals: Hemoglobin (HGB) in g/L; Hematocrit (HCT) in L/L; Mean Corpuscular Volume (MCV) in fL; Eosinophils (EO) and MHCH (BASO) as 10exp9/L.

When animals are euthanatized, an autopsy is performed, and a careful macroscopic evaluation is performed. If any organs look abnormal, a detailed description and analysis is performed.

Histological analysis

Histological analysis is performed for the 5 following organs: Lung, Spleen, Liver, Kidney, and Brain. The organs are collected, weighed, macroscopically observed, fixed in 4% Paraformaldehyde (PFA) and paraffin-embedded, cut into 5-7 µm sections and then observed under fluorescence microscope.

Histopathological scoring

Each H&E (hematoxylin- and eosin-stained) section was thoroughly examined histologically, and lesions observed were recorded in an Excel spreadsheet, their severity graded (minimal, mild, moderate, or severe). Their distribution also was characterized (*i.e.,* as focal, multifocal, focally extensive or diffuse), as well as their localization.

B. Toxicity study results: Clinical examinations

The results of assessment of toxicity of MEVs in a mouse model after oral (PO) or intratracheal (IT) administration at different doses in 4 groups of mice for each parameter are shown in Figures 27E-27I, which show the clinical chemistry and hematology in mice after administration (PO, IT) of MEV.

Clinical chemistry

As shown below, two parameters Urea and Creatinine did not change in all treated groups compared to control groups. The enzymes Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) shown random non-significant deviations. The parameter Gamma-glutamyl transferase value is detected at low level (<6) in all groups indicating a protocol error and are not presented.

The results of the clinical chemistry parameters do not indicate any toxicity at all tested doses in the mice either by oral or intratracheal administration.

Hematology

The measured parameters were not significantly changed compared to controls, indicating that the MEVs do not result in toxicity. MEV toxicity was evaluated by (1) chemistry parameters: ALAT, ASAT, urea and creatine; and (2) by hematology parameters in four groups of mice: Group 1 mice were administered 100 µl of PBS by PO delivery; Group 2 mice were administered 100 µl of $4*10^{11}$ MEV/ mouse by PO delivery (bar with black and white tiles); Group 3 mice were administered 100 µl of $4*10^{12}$ MEV/ mouse by PO delivery (bars with vertical lines); Group 4 mice were administered 100 µl of $4*10^{11}$ MEV/ mouse by IT delivery. The results present blood parameters that can be altered when there is one or more of blood-, kidney-, and liver-related toxicity. Hematocrit, hemoglobin, eosinophil levels, RBC count, and volume (MCV) are hematologic parameters; urea and creatine are biochemical markers of kidney injury; ALAT (or ALT) and ASAT (or AST) are biochemical markers of liver injury. Alanine transaminase (ALT), also called alanine aminotransferase (ALAT), is a transaminase enzyme that occurs in plasma and in various body tissues, but most commonly in the liver. Elevated levels of ALT can be related to liver-related problems, such as hepatitis and/or liver damage. Aspartate transaminase (AST), also known as aspartate aminotransferase (ASAT), is another transaminase enzyme important in amino acid metabolism. Beyond liver toxicity, AST can be elevated also in diseases affecting other organs. Serum ALT level, serum AST level, and their ratio (AST/ALT ratio) are used clinically as biomarkers for liver health. In the instant study, no statistically significant differences between experimental groups in ALT and AST levels were observed (see, Figures 27A-27I).

Eosinophils (eosinophiles) are a variety of white blood cells and one of the immune system components responsible for combating multicellular parasites and certain infections in vertebrates. Eosinophils usually account for less than 7% of the circulating leukocytes. Beyond causes related to infection or parasitic infestation, elevated eosinophil levels (also known as eosinophilia) can be also a sign of allergic or atopic reactions. In the instant study no statistically significant differences between experimental groups in eosinophil levels were observed.

Histology

The main organs were collected, fixed in 4% PFA, paraffin-embedded, cut into sections and stained with H&E.

Lung

Histopathological changes observed in lung sections are primarily limited to minimal to mild focal to multifocal alveolar hemorrhages in 8/10 lung sections examined in animals receiving intratracheal negative control material and test item. Changes were relatively subtle and limited in some of the alveolar spaces. This observation was observed at comparable incidence and severity in negative control and treated animals.

Kidney

Presence of a few basophilic tubules were observed in 1 animal treated PO with $4x10^{11}$ MEV dose. This finding was minimal in severity and often is observed spontaneously at low incidence and severity in laboratory rodent animals. It is therefore considered as incidental in origin in the present study.

Spleen, Liver, and Brain

No histopathological changes were observed in spleen, brain, or liver in all sections examined.

C. Summary

The pulmonary changes observed in most animals receiving the experimental material intra-tracheally were considered not treatment related. There was no treatment-related observed in all organs examined, indicating that MEVs are not toxic to animals under the experimental conditions (see, Table 20).

Table 20. Histopathology results after administration orally or intratracheally of MEVs

Group	**1**	**2**	**3**	**4**	**5**
Organ	**Brain**				
Histopathological Findings					
Number Examined	3	6	5	4	6
Unremarkable	3	6	5	4	6

Organ	**Spleen**				
Histopathological Findings					
Number examined	3	6	6	4	6
Unremarkable	3	6	6	4	6
Organ	**Kidney**				
Histopathological Findings					
Number examined	3	6	6	4	6
Unremarkable	3	5	6	4	6
Basophilic tubules					
Minimal	0	1	0	0	0
Organ	**Liver**				
Histopathological Findings					
Number examined	3	6	6	4	6
Unremarkable	3	6	6	4	6
Organ	**Lung**				
Histopathological Findings					
Number examined	3	6	6	4	6
Unremarkable	3	6	6	1	1
Hemorrhage, alveolar					
Minimal	0	0	0	2	4
Mild	0	0	0	1	1

D. Conclusion

Regarding the clinical aspects, all the animals, at each time of the study, exhibited normal behaviors, body weight, water and food consumption. The clinical findings indicate general tolerance in the mouse model of the test MEV products.

Under the experimental conditions, both negative controls (PBS) and MEV at concentration of 4x10exp11 MEV/ mouse or 4x10exp12 MEV/ mouse by oral administration and at concentration of 4x10exp11 MEV/ mouse by IT administration did not induce any abnormal, toxic effect on animals.

EXAMPLE 7

MEVs as a vaccine delivery system (VDS) by intramuscular or oral administration. MEVs are not immunogenic after repeated dosing *in vivo*.

A. *In vivo* treatment in healthy C57BL/6J mice.

Animals were divided in groups (6 mice per group) and treated follows:

- Group 1: IM administered with OVA (Ovalbumin) in adjuvant (Complete Freund Adjuvant (CFA) on Day 0 or Incomplete Freund Adjuvant (IFA) on Day 21) on Days 0 and 21,
- Group 2: IM administered with OVA-loaded MEVs (MEV-OVA) on Days 0 and 21,
- Group 3: IM administered with adjuvant only (CFA on Day 0 and IFA on Day 21) on Days 0 and 21,
- Group 4: IM administered with MEV only on Days 0 and 21,
- Group 5: PO administered with MEV-OVA on Days 0, 7, 14, and 21,
- Group 6: PO administered with MEV only on Days 0, 7, 14, and 21,
- Group 7: Control group; mock administration
- Group 8: PO administered with MEV only on Days 0, 7, 14, and 21, and with MEV-OVA on Days 40, 47, 54, and 61.

Mice of groups 1- 7 were euthanized on Day 43 after the first vaccinal administration. Mice of groups 8 were euthanized on Day 82 after the first vaccinal administration. Whole blood was sampled just before euthanasia to collect serum for quantification of anti-OVA antibody by Enzyme-Linked Immunosorbent Assay (ELISA), and to perform flow cytometry as defined below.

Just after euthanasia, spleen, inguinal and iliac lymph nodes (LNs, and first fragment of gut to isolated GALT samples were collected and processed to perform flow cytometry analysis to quantify the T cell activation after *in vitro* stimulation with OVA peptides.

B. Blood sampling and serum collection.

According to the experimental schedule, administration, whole blood (WB) was collected by retro-orbital sinus or intracardiac puncture under anesthesia.

1- Whole Blood sampling for flow cytometry acquisitions:

About 300 µl of WB were collected and deposited in microtube previously heparinized (60 µl of heparin) to avoid coagulation.

2- WB sampling for serum collection:

Minimum of 300 µl of WB were sampled and deposited in a Serum Separation Tube (SST) in order to obtain a minimum of 150 µl of serum. The SST were kept at room temperature until clotting and centrifuged at 3000 g for 10 minutes at room temperature. Serum aliquots were stored at ≤-75°C until processing for quantification of anti-OVA antibodies by ELISA.

C. Animal euthanasia and organs sampling and processing.

According to the experimental schedule, at 82 days after first Per os administration, animals were euthanized under anesthesia by cervical dislocation and the selected organs were collected.

- Spleen (free of fat) was cut into small pieces (about 1-2 mm3 per piece) and deposited together in 1mL of Complete Culture Medium (CCM composed of RPMI1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine and 1% Penicillin-Streptomycin).

- Right inguinal and iliac lymph nodes (side of the IM injection site) were also cut into small pieces (about 1-2 mm3 per piece) and deposited together in 0.5mL of CCM.

The spleen pieces with 1 mL of CCM were transferred in a BD™ Medimachine Medicon of 50 µm (pre-rinsed with Cell Wash buffer (BD Biosciences)). The Medicon was inserted in the BD™ Medimachine System, and one cycle of 30 seconds was performed to disaggregate the spleen tissues. The cell suspension obtained was aspirated with a syringe through the syringe port of Medicon. The Medicon was rinsed 2 times with 1 mL of Cell Wash buffer and the cell suspension obtained was also aspirated. Then, the obtained cell suspension was filtered using a BD™ Medimachine Filcon of 100 µm and deposited in a 5 mL tube. The cells were counted using an automatic cell counter. Finally, the cell suspension was split in 2 tubes: 2x10exp6 cells for T cell activation protocol and 1x10exp6 cells

for flow cytometry staining. The tubes were centrifuged at 300 g for 7 minutes at RT. The supernatant was completely removed. Cell pellet of tube 1 (for T cell activation protocol) was resuspended in 100 µL of RPMI complete medium (RPMI, 1% P/S, 10% FBS, 1% Hepes 1M, 1% Sodium pyruvate 100 mM, 1% NEAA 100x) and cell pellet of tube 2 (for flow cytometry staining) was resuspended in 100µL of Cell Wash. Cell suspensions were stored at +5°C ± 3°C until staining for flow cytometry or T cell activation protocol.

The LNs pieces with 0.5 mL of CCM were transferred in a BD™ Medimachine Medicon of 50 µm (pre-rinsed with Cell Wash buffer). The Medicon was inserted in the BD™ Medimachine System, and one cycle of 30 seconds was performed to disaggregate the LNs tissues. The Medicon was rinsed once with 0.5 mL of Cell Wash buffer and the cell suspension obtained was aspirated with a syringe through the syringe port of Medicon. Then, the obtained cell suspension was filtered using a BD™ Medimachine Filcon of 100 µm and deposited in a 5 mL tube. The cells were counted using an automatic cell counter. Finally, the cell suspension was split in 2 tubes: 2x10exp6 cells for T cell activation protocol and 1x10exp6 cells for flow cytometry staining. The tubes were centrifuged at 300 g for 7 minutes at RT. The supernatant was completely removed. Cell pellet of tube 1 (for T cell activation protocol) was resuspended in 100 µL of RPMI complete medium (RPMI, 1% P/S, 10% FBS, 1% Hepes 1M, 1% Sodium pyruvate 100 mM, 1% NEAA 100x) and cell pellet of tube 2 (for flow cytometry staining) was resuspended in 100 µL of Cell Wash. Cell suspensions were stored at +5°C ± 3°C until staining for flow cytometry or T cell activation protocol.

D. Quantification of antibody anti-OVA by ELISA

Immunoglobulins (Ig) quantification was performed by an indirect ELISA according to the dedicated kit from Chondrex: Kit #3011 for Total IgG, Kit #3013 for IgG1, Kit #3018 for IgA. The same protocol was used for the three different kits. Briefly, 100 µL of diluted OVA solution were added in each well and then the plate was incubated at +5 ± 3°C overnight. The plate was washed with 1X wash buffer at least three times and 100 µL of blocking Buffer were added to each well and incubated at RT for 1 hour. The plate was washed a second time and 100 µL of diluted standard [range from to 12.5 ng/mL to 0.2 ng/mL] and serum sample (1/50

dilution) were added in duplicate. The plate was incubated at RT for 2 hours and washed as described before. Then, the secondary antibody was added in each well (100 µL) and the incubation was performed at RT for 1 hour. After incubation, the plate was washed and 100 µL of TMB solution (previously diluted) were added to each well. Reaction with this chromogen solution was done during 25 min at RT and stopped by the addition of 25 µL of 2N sulfuric acid. Finally, the plate was gently mixed, and absorbance was read at 450 nm with a GloMax® plate reader (Promega, Madison, WI). The average of duplicate readings for each standard control were used to plot a standard curve, which was used to quantify the samples in ng/mL of blood.

E. Results:

The administration (at days 0, 7, 14, and 21) in mice, by the PO route, of MEVs loaded with OVA elicits a humoral immune response against OVA. MVEs loaded with OVA not only induce IgG but also IgA antibodies against OVA, showing a strong isotype class switching IgG to IgA antibodies, thus indicating a cellular immune response, as shown in Figures 28A-28C. The control mice were immunized with OVA/adjuvant or only with adjuvant; results shown in Figure 28A.

The immunization with MEVs loaded with OVA by PO administration induce a cellular immune response, involving the CD44/CD62L cell population, and showing an increase in the T virtual central memory activated on TCD4+ and TCD8+ cells.

The immunization with MEVs loaded with OVA by PO administration induce a cellular immune response, involving the CD44(hi)/CD49d cell populations, and showing an increase in the T central memory on TCD4+ and TCD8+ cells.

The immunization with MEVs loaded with OVA by PO administration induces a cellular immune response, involving the CD44(hi)/CD11a(hi) cell populations, and showing an increase on the T central memory on TCD4+ and TCD8+ cells.

F. Conclusions

The repeat treatment with non-loaded MEVs by PO administration does not preclude the appearance of the immune response against OVA when the same animals are subsequently administered with OVA-loaded MEVs (Figure 28C). This observation demonstrates the absence of a neutralizing reaction against MEVs and the absence of a tolerogenic response to MEVs (see below). As shown in the Figure 28C,

orally administered MEVs elicit humoral immune response against an antigenic payload. It is surprising that the oral delivery of protein antigen loaded into MEVs results in generation of antigen-specific antibodies. Oral delivery is the most desirable and patient-accepted route of administration, with over 60% of commercialized small molecule drug products using the oral route. What is more, oral route of vaccine administration provides the advantage of stimulating mucosal immunity. The vaccine-generated antibodies specific to MEV cargo include both IgG and IgA immunoglobulins. Hence, the immune response following per os MEV administration is characterized by significant isotype class switching to IgA antibodies, which indicates MEV potential for triggering mucosal immunity. MEV-based formulations for oral delivery provides offer added value for vaccine development.

The administration of MEVs loaded with an immunogenic payload by the PO route elicits a humoral immune response, and a cellular immune response. The observation of the isotype class switching (from IgG to IgA antibodies) by antigen-loaded MEVs is in full agreement with the observation that antigen-loaded MEVs elicit an antigen-specific cellular response. The observations above support the notion that by PO administration, MEVs elicit a mucosal immune response against their payload.

The use of MEVs by PO administration as a VDS overcomes the general observation that oral/intestinal delivery of antigens is rather tolerogenic (creates tolerance, rather than immune response, against the antigen).

Results presented here show the MEVs are a vaccination delivery system that is non- tolerogenic, as they elicit strong humoral and cellular immune responses against the antigenic payload.

The capacity to use MEVs by repetitive administration without the generation of neutralizing antibodies allows to perform treatments to diseases that needs subsequent administrations using a delivery system largest that oral administration such as the different routes of administration (IV, IM, SC, topical, respiratory, drop instillation in the eyes, IN to brain among others).

EXAMPLE 8

Delivery and expression of transgene (EGFP) in different cell types *in vitro*.

MEVs have the capacity to deliver plasmids with AAV-ITRs into different cell types and, once inside the recipient cell, the plasmid its expressed (transcribed to generate mRNAs encoding functional proteins) and it replicates such that upon cell division, such replication leads to the generation of a stable genetic modification of the cell.

A. Cell Cultures

BEAS-2B human bronchial epithelial cells were obtained from the American Type Culture Collection (ATCC) (CRL-9609). The cells were cultured in BEBM Basal Medium (CC-3171, Lonza) supplemented with BEGM SingleQuots™ Supplement Pack (CC-4175, Lonza) and 100 U/ml of penicillin, and 100 µg/ml of streptomycin (15140122, Gibco) at 37 °C under 5% CO2.

Non-transformed human small intestinal epithelial FHs-74 Int cells were also obtained from ATCC (CCL-241). The cells were maintained in Hybri-Care medium (ATCC-46-X) supplemented with 10 % heat-inactivated fetal bovine, and 100 µg/ml of streptomycin (15140122, Gibco), and 30 ng/ml EGF (SRP3027-500µG, SIGMA) at 37 °C under 5% CO2.

Non-transformed human large intestinal epithelial FHC cells were also obtained from ATCC (CRL-1831). The cells were maintained in DMEM/F-12 media (31330038, Gibco) supplemented with 10 % heat-inactivated fetal bovine, 100 U/ml of penicillin - 100 µg/ml of streptomycin (15140122, Gibco), 20 ng/ml EGF (SRP3027-500µG, SIGMA), 10 ng/ml Cholera Toxin (C8052-1MG, SIGMA), 1 mM HEPES (H0887, SIGMA), 5µg/ml Insulin (I9278), 5µg/ml Transferrin (T8158, SIGMA), 100 ng/ml Hydrocortisone (352450050, Thermo Scientific) at 37 °C under 5% CO2.

ARPE-19 is a spontaneously arising retinal pigment epithelia (RPE) cell line derived from the normal eyes of a 19-year-old male (ATCC, CRL-2302). ARPE-19 cells are cultured in a 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F12 medium with HEPES buffer containing 20% heat inactivated fetal bovine serum, 56 mM final concentration sodium bicarbonate and 2 mM L-glutamine and incubated at 37C in 10% CO2.

A549 cells from ATCC, (CCL-185) are human lung carcinoma cells (epithelial cells morphology).

A549 cells were cultured with F-12K medium (31330038, Gibco), 10% of heat inactivated FBS at 37°C and 5% CO_2.

B. Loading of MEVs

MEVs were loaded with different plasmid constructs SEQ. ID NO: 48 and 52, as described in Example 3. Plasmid sequences of plasmid are described in Example 4, and the quantification of payload was determined by dPCR as describe in Example 4.

C. MEVs internalization and delivery of payload

The human cells were treated at different time points with MEVs loaded with plasmids carrying or not AAV-ITRs at different timepoints between 24h and 144h. After 24h after seeding, cells were counted and a mixture of cell culture media with different conditions of treatments: non-loaded MEVs, MEVs loaded with plasmid carrying AAV-ITRs and codifying for EGFP protein, MEVs loaded with plasmid without AAV-ITRs and codifying for EGFP protein or only PBS as negative control condition. The final concentration of MEVs were between 200 and 1,000 loaded-MEV/cell in the media culture and conditions defined above for each cell type. After incubation, the cells are fixed with PFA 4%, permeabilized with 0.1% Triton™ X-100, and the nuclei are stained with Hoechst, dilution 1:1000). The samples were analyzed by confocal microscopy in order to determine the presence of EGFP protein.

Figure 29 shows the results in BEAS-2B cells after the treatment with MEVs loaded with plasmids. When cells were treated with loaded MEVs with plasmid carrying AAV-ITRs a higher expression level of EGFP and longer persistence of the EGFP expression was detected.

EXAMPLE 9

MEV-GTVs: Delivery, expression, and biological activity of payload *in vitro*

A. Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from ATCC and maintained in endothelial cell medium (ECM) supplemented with 5% heat inactivated fetal bovine serum (HIFBS) and 1% penicillin/streptomycin at 37°C in 5% CO2. The culture medium was changed every 2 days.

B. MEVs Loading

MEVs were loaded with different plasmid constructs, as described in Example 3. Plasmid sequences are described in Example 4, and the quantification of payload was determined by dPCR as describe in Example 4.

C. Angiogenesis assays - Matrigel® tube formation assay with HUVEC cells

The tube formation assay is a recognized *in vitro* assay to model the reorganization stage of angiogenesis. With appropriate Matrigel® support, the endothelial cells form capillary-like structures (also known as tubes). HUVEC cells were plated in 24 wells plates and treated or not with MEVs loaded or not-loaded (as described above) during 36h in fresh medium supplemented with 1.3% FBS at 37°C in humidified air with 5% CO2. After the 36h culture, Matrigel® was thawed and laid into 24-well culture plates to a total volume of 100 µl in each well. Plates were stored at 37°C for 30 min to form a gel layer. After gel polymerization, 1x10exp5 viable (determined as described above) HUVEC cells (from the 36h pre-culture) were seeded on each well and incubated with fresh RPMI 1640 medium supplemented with 2% FBS for 24h at 37°C in humidified air with 5% CO2 for 3h. After 3h, the media was removed, and wells washed twice with PBS. The HUVEC cells were stained overnight with 0.1% crystal violet, 4% paraformaldehyde in PBS (passed through a 0.45µM filter) and 6µM Calcein AM solution in basal medium. The networks of tubes in each well were observed with an EVOS M7000 microscope.

Figure 30A shows the anti-angiogenesis activity of MEVs loaded with plasmid carrying AAV-ITRs and encoding Aflibercept (SEQ ID NOs: 49 and 50) in the tube formation assay.

D. Analysis of tube formation assay images

Images of tube formation were analyzed using the Angiogenesis software plugin in ImageJ. Only complete networks were analyzed. Incomplete networks were excluded.

The experiments were performed in triplicate, and five fields from each chamber were counted and averaged. Figure 30B shows the images of nodes and networks and mesh obtained from the tube formation assay. Figure 31 shows the results of the analysis effected with the Angiogenesis plugin ImageJ software.

EXAMPLE 10

MEV-GTVs: Gene delivery and gene expression *in vivo* (in muscle).

MEVs deliver intact genetic payloads *in vivo*.

The performance of MEV-GTVs as gene delivery/gene expression vehicles is evaluated in murine models of muscle gene transfer and compared to MEVs loaded with plasmids carrying or not AAV-ITRs. The tissular and cellular distribution of delivery and level of expression within the target organ is evaluated using EGFP protein expression vectors by IHC using GFP primary antibody coupling with a florescent secondary antibody.

A. MEV Loading

MEVs were loaded and characterized as described in Example 3 and 4, respectively with different plasmids carrying or not AAV-ITRs, SEQ. ID NOs: 48 and 50. In these plasmid constructs, minigene cassettes are flanked with AAV- ITRs or not. The EGFP gene is used as reporter gene and CMV early promoter and CMV enhancer are used.

B. *In vivo* administration of loaded MEV-GTVs

Loaded MEV-GTVs preparations were re-suspended by vortexing before filling the syringes for injection. For muscle-directed gene transfer, MEVs loaded with plasmids carrying or not AAV-ITRs and eGFP as gene marker are injected into the right tibialis anterior of 4–6-week-old C57BL/6 mice. At different time points (T=0, 1 day, 3 days, 1 week, 2 weeks, 1 month and 3 months) animals were euthanized, and muscles were recovered.

C. Organ sampling and processing: muscle

The right tibialis was carefully separated from the bones. The muscle is excised and gently rinsed with a cold PBS solution. Each muscle was longitudinally placed on bottom of the cryomold for OCT inclusion (as described below).

D. Snap-frozen

A small stainless-steel bowl was placed in the bottom of a container containing dry ice in pellet form and liquid nitrogen. Then, isopentane/2-methylbutane was slowly added in the container. When, the dry ice pellets stop bubbling vigorously or/and the isopentane start to become opaque, the isopentane/2-methylbutane is at optimal temperature. Muscles were gently immerged in isopentane/2-methylbutane and then placed in cassette. Then, cassettes were stored at $\leq$-75°C until OCT inclusion.

E. OCT inclusion protocol

A small stainless-steel bowl was placed in the bottom of a container containing dry ice in pellet form and liquid nitrogen. Some pellets of dry ice were also placed directly in the bowl. Then, isopentane/2-methylbutane was slowly added in the container. When, the dry ice pellets stop bubbling vigorously and/or the isopentane starts to become opaque, the isopentane/2-methylbutane is at optimal temperature. The frozen tissue samples were then placed and oriented as mentioned above for each organ in the cryomold. The tissues were gently pushed with forceps to ensure that the bottom of surface of the tissue is placed properly (touching the face of the bottom, center in the mold and properly oriented). The cryomold with frozen tissue samples were placed on the surface of the cold isopentane/2-methylbutane. Optimal cutting temperature compound (OCT compound) was carefully deposited onto the specimen until it is completely covered. After hardening of the OCT compound (between 30 seconds and 1 minute), the OCT embedded block was placed in a bag (such as zip freezer bag). The frozen blocks were temporarily stored in dry ice. Then, slices of 5 µm were performed with a cryostat then glued on untreated slides for the histology and on treated slides for the immunochemistry.

F. HES Staining

The HES staining allowed the observation of the morphology and the structure of tissues. After fixation in acetone, sections were immersed successively in solutions of Harris hematoxylin, eosin and saffron. After dehydration, sections were mounted between slide and cover slip using Entellan® mounting medium. Cytoplasm appeared in pink and nuclei in violet blue. Extracellular matrix was stained in yellow to pink.

G. Immunohistochemistry (IHC) Staining

The IHC staining allowed the observation of eGFP protein marker in muscles tissues. The slices are washed 2 x 5 minutes in PBS plus 0.025% Triton X-100 with gentle agitation, then blocked in 10% normal serum with 1% BSA in PBS for 2 hours at room temperature, then the slices are drained for a few seconds and wiped around the sections with tissue paper. The primary antibody against eGFP (Roche, ref. 11814460001) diluted in PBS with 1% BSA. The slices are incubated overnight at 4°C. Then the slices are rinsed 2 x 5min PBS 0.025% Triton with gentle agitation, then the secondary antibody is incubated following suppliers' conditions in 1% BSA and incubated 1h at room temperature.

The slices are visualized by a LSM800 laser scanning confocal microscope (from Zeiss) and images were processed with LSM Image Browser. DAPI (4',6-diamidino-2-phenylindole) [Invitrogen, Ref. S36938] was used as nuclear counterstain prior to the imaging. Sections were mounted with aqueous medium with DAPI for fluorescent slide scanner observation (maximum 10 days after collection).

EXAMPLE 11

MEV-GTVs: Gene delivery and gene expression *in vivo* (brain)

The performance of MEV-GTVs as gene delivery/ gene expression vectors is evaluated in murine models of brain gene transfer and compared to MEVs loaded with plasmids carrying or not AAV-ITRs. The tissular and cellular distribution of delivery and level of expression within the target organ is evaluated using EGFP protein expression vectors by IHC using GFP primary antibody coupling with a florescent secondary antibody.

A. MEVs Loading

MEVs were loaded and characterized as described in Example 3 and 4 respectively with different plasmids carrying or not AAV-ITRs (SEQ ID NOs:48 and 52). In these plasmid constructs, minigene cassettes are flanked with AAV- ITRs or not. EGFP gene are used as reporter gene and CMV early promoter and CMV enhancer are used.

B. *In vivo* administration of loaded MEV-GTVs

The experience is performed in 6–8-week-old Swiss mice. During the experiment, mice were housed in cages with filter hoods, in a room where the air is continuously filtered, thereby avoiding contamination. Paired animals were caged at a constant temperature with a day/night cycle of 12/12h.

Animals received water (control tap water) and nutrition ad libitum. Animal health was examined every day to ensure that only animals in good health enter to the testing procedures and follow up the study.

Loaded MEV preparations were re-suspended by vortexing before filling the syringes for injection. For brain-directed gene transfer, MEVs loaded with plasmids carrying or not AAV-ITRs and eGFP as gene marker are administered intranasally (IN).

Intranasal compound administration was made using a dominant hand, the

micropipette was loaded with 20 µl of MEV (for the first nostril). The tip of the filled pipette was placed near the mouse's left nostril, usually at a 45-degree angle. The droplet was placed close enough to the mouse's nostril so that the mouse can inhale the droplet. This procedure was reproduced for the second nostril. A total of 40 µl of MEV per animal was administrated. After full administration, the mouse was held in this position for 15 sec.

At different time points after the IN administration (T=0, 1 day, 3 days, 1 week, 1 month) the mice were euthanized, and the brains were recovered.

C. Organ sampling and processing: Brain

At each time point mice were anesthetized and perfused with 4% (weight/vol.) paraformaldehyde antigen fix solution (PFA). The temporal bone was opened, and the brain was sampled. The brains were taken out from the skull with a special care not to damage the olfactory bulbs, post-fixed in 4% PFA and soaked in 30% sucrose. Each sample was rapidly segmented in five parts as stated, placed in single cryo-blocks, and embedded with OCT using isopentane and stored at -80 °C. Samples were cryo-sectioned, and were stained as describe bellow.

D. Snap-frozen

A small stainless-steel bowl was placed in the bottom of a container containing dry ice in pellet form and liquid nitrogen. Then, isopentane/2-methylbutane was slowly added in the container. When, the dry ice pellets stop bubbling vigorously or/and the isopentane start to become opaque, the isopentane/2-methylbutane is at optimal temperature. Brains were gently immerged in isopentane/2-methylbutane and then placed in cassette. Then, cassettes were stored at ≤-75°C until OCT inclusion.

E. OCT inclusion protocol

A small stainless-steel bowl was placed in the bottom of a container containing dry ice in pellet form and liquid nitrogen. Some pellets of dry ice were also placed directly in the bowl. Then, isopentane/2-methylbutane was slowly added in the container. When, the dry ice pellets stop bubbling vigorously and/or the isopentane starts to become opaque, the isopentane/2-methylbutane is at optimal temperature. The frozen tissue samples were then placed and oriented as mentioned above for each organ in the cryomold. The tissues were gently pushed with forceps to ensure that the bottom of surface of the tissue is placed properly (touching the face of the bottom,

center in the mold and properly oriented). The cryomold with frozen tissue samples were placed on the surface of the cold isopentane/2-methylbutane. Optimal cutting temperature compound (OCT compound) was carefully deposited onto the specimen until it is completely covered. After hardening of the OCT compound (between 30 seconds and 1 minute), the OCT embedded block was placed in a bag (such as zip freezer bag). The frozen blocks were temporarily stored in dry ice. Then, slices of 30 µm coronal section (Figure 32) were performed with a cryostat then glued on untreated slides for the histology and on treated slides for the immunochemistry.

F. HES Staining

The HES staining allowed the observation of the morphology and the structure of tissues. After fixation in acetone, sections were immersed successively in solutions of Harris hematoxylin, eosin and saffron. After dehydration, sections were mounted between slide and cover slip using Entellan® mounting medium. Cytoplasm appeared in pink and nuclei in violet blue. Extracellular matrix was stained in yellow to pink.

G. Immunohistochemistry (IHC) Staining

The IHC staining allowed the observation of eGFP protein marker in brain tissues. The slices are washed 2 x 5 minutes in PBS plus 0.025% Triton X-100 with gentle agitation, then blocked in 10% normal serum with 1% BSA in PBS for 2 hours at room temperature, then the slices are drained for a few seconds and wiped around the sections with tissue paper. The antibody against eGFP (red antibody against eGFP: Roche, ref. 11814460001) is diluted in PBS with 1% BSA. The slices are incubated overnight at 4°C. Then the slices are rinsed 2 x 5min PBS 0.025% Triton with gentle agitation.

The slices are visualized by a LSM800 laser scanning confocal microscope (from Zeiss) and images were processed with LSM Image Browser. DAPI (4',6-diamidino-2-phenylindole) [Invitrogen, Ref. S36938] was used as nuclear counterstain prior to the imaging. Sections were mounted with aqueous medium with DAPI for fluorescent slide scanner observation (maximum 10 days after collection).

EXAMPLE 12

MEV-GTVs: Gene delivery and gene expression *in vivo;* ocular delivery in rabbits (eyes)

Ocular distribution following a single ocular topical administration in albino

rabbits.

A. Loading of MEVs

Isolated and purified MEVs were loaded with plasmids encoding EGFP protein, these plasmids either carrying or not AAV-ITRs (SEQ ID NOs: 48 and 52), as described in Example 3 and characterized as described in Example 4.

B. Animals

Rabbit New Zealand White (albino) is one of the species the most used for ocular biodistribution. Eight 3-month males of weight between 2-2.5 kg were used in the ocular topical administration study. All animals were ear-tagged at their arrival and the identification numbers were also marked in ears using indelible ink following the inclusion examination.

C. Clinical examination and health status

Animals were daily observed for signs of illness and particular attention was paid to their eyes. Only healthy animals without visible ocular defect were used in the study.

1. Housing

Animals were housed individually in standard cages. The temperature was held at 18+/-3°C and the relative humidity at 45-80%. Rooms were continuously ventilated (15-20 air volumes per hour). Animals were routinely exposed (in-cage) to 10-200 lx light in a 12-hour light (from 7:00 a.m. to 7:00 p.m.) and darkness-controlled cycle. Animals had free access to food (90 g/day) and water available ad libitum from plastic bottles.

2. Administration

A 50-µL single instillation on both eyes was applicated to all animals, using an appropriate micropipette, at a dose of 4×10exp10 MEV-GTVs carrying an ITR-plasmid encoding EGFP.

3. Histology and immunohistochemistry

After washing in PBS, both eyeballs were fixed with PFA 4%, rinsed, and embedded in Tissue-Tek® and snap frozen in liquid nitrogen cooling by isopentane until complete freezing of the block. Both eyeballs (2 sagittal and central sections per eye including optic nerve in a 100 µm zone were sectioned on a cryostat into two 10 to 15 µm-thick sections. The sections were dried for at least 1 h at 37°C and

rehydrated with PBS. Mounting medium with red fluorescence GFP immunostaining and DAPI (Vectashield® with DAPI, Vector laboratories, Burlingam, CA, USA) were added to the sections, and they were kept at 4°C until imaging with fluorescent microscopy (Axio Imager Z2 with ApoTome function, Zeiss, Oberkochen, Germany).

EXAMPLE 13

MEV-GTVs: Gene delivery, gene expression, and biological activity *in vivo;* ocular delivery in mice (eyes)

***In vivo* study with mouse laser-induced choroidal neovascularization model.**

Laser-induced choroidal neovascularization (CNV) is a well-established model to mimic the wet form of age-related macular degeneration (AMD).

A. Loading of MEVs

Isolated and purified MEVs were loaded with plasmids encoding EGFP protein. These plasmids either carrying or not AAV-ITRs. (SEQ ID NO: 49 and 50), as described in Example 3 and characterized as described in Example 4.

B. Animals and Anesthesia

Male C57BL/6J mice aged 6-8 weeks old and weighing 18-20 g are used to minimize variability in the study. Animals are housed with food and water ad libitum in a 12-h/12-h light/dark cycle. The humidity and temperature are maintained at 60% and 24-26°C, respectively. During all procedures, the mice are anesthetized with an intraperitoneal injection of 2% pentobarbital sodium (45 mg/kg body weight), and pupils are dilated with topical 5% tropicamide. At different time points after laser-induced photocoagulation, the mice are euthanized by cervical dislocation.

C. Animal model

After dilating pupils, mice are placed on a platform under a slit lamp, and the corneas are anesthetized with oxybuprocaine hydrochloride eye drops. 532 nm argon laser-induced photocoagulation is used to disrupt Bruch's membrane bilaterally in each mouse. Four spots of laser photocoagulation in the posterior pole of the retina are created with a power of 150–200 mW, duration of 100 m s, and 50 μm spot size using a slit lamp delivery system with a handheld coverslip as a contact lens. The laser spots are located approximately two to three optic disc diameters away from the optic nerve head, avoiding the main vessels. The appearance of a white bubble, which indicates a break of Bruch's membrane, is an important factor to obtain CNV, and

only burns causing bubble formation are included in subsequent experiments. Spots with hemorrhage or failing to develop a bubble are excluded from the analysis.

D. Topical administration via drop instillation

Four days after laser photocoagulation, a single instillation of loaded MEVs is applied to experimental animals, using an appropriate micropipette.

E. Intravitreal administration

Four days after laser photocoagulation, mice are anesthetized, and pupils are dilated with topical 5% tropicamide. A shelving puncture of the sclera is made 1 mm behind the limbus with a 32-gauge needle, then a 33-gauge needle (Hamilton Bonaduz, AG, Bonaduz, Switzerland) is inserted with a 45° injection angle into the vitreous. The direction and location of the needle is monitored through the microscope. The right eye is injected with 40 µL of positive control (25 mg/mL aflibercept (Regeneron)). The contralateral eye receives an equal volume of balanced salt solution (BSS). All injections are delivered into the mid vitreous cavity.

F. Fundus Fluorescein Angiography (FFA)

FFA is performed on anesthetized mice using a Spectralis HRA+OCT ophthalmic diagnostic apparatus (Heidelberg, Germany) at the time points. Early-phase and late-phase FFA images are recorded at 1 to 2 min and after 5 min, respectively, after intraperitoneal injection of 0.2 ml 2% fluorescein sodium solution. Images are evaluated by two masked retinal specialists who are not involved in laser photocoagulation or angiography. Then, lesions are graded according to a previously published grading system: 0, “no leakage,” faint hyper-fluorescence or mottled fluorescence; 1, “questionable leakage,” hyper-fluorescence lesion without a progressive increase in size or intensity; 2, “leaky,” hyper-fluorescence increasing in intensity but not in size; and 3, “pathologically significant leakage,” hyper-fluorescence increasing both in size and intensity. Four days after laser photocoagulation, FFA analysis is performed to choose lesions with pathologically significant leakage for further observation. Late-phase images are chosen for analysis of fluorescence leakage and leakage areas, which corresponded with the clearance time of fluorescence from the vasculature and to avoid interference with the observation of hyper-fluorescence from CNV.

G. Spectral Domain-Optic Coherence Tomography (SD-OCT)

SD-OCT is performed using a Heidelberg Spectralis HRA + OCT (Heidelberg, Germany). The cornea is covered with hyaluronan gel before the examination. For each CNV lesion, SD-OCT high-resolution multilinear horizontal scanning images are taken to calculate the thickness of the CNV lesion, which is defined as a spindle-shaped hyperreflective area at the subretinal space. Only the scan that passes through the center of the lesion is chosen to evaluate the CNV central thickness (CCT).

H. Flat mount staining and CNV size measurement

Mice are euthanized, and eyes are enucleated and fixed in 4% paraformaldehyde solution for approximately 30 min. After washing in PBS, the anterior of the eye is cut off, and the whole retinas are removed carefully from the eyecups. To flatten the eyecups, four radial incisions in the remaining RPE-choroid-sclera complexes are made from the edge to the optic nerve head with the RPE facing up. Then, the complexes are washed in Tris-buffered saline (0.5% BSA, 0.2% Tween-20, and 0.1% Triton X-100), incubated with isolectin B4 (1:200, Sigma–Aldrich, St Louis, MO, United States) overnight at 4°C, and mounted onto glass slides. The images are captured with a fluorescence microscope (Carl Zeiss, Germany), and the CNV lesion area is measured using ImageJ software after normalizing for different background fluorescence (NIH, Bethesda, MD, United States).

I. Immunohistochemistry

The eyecups are embedded in an optimal cutting temperature compound (Tissue Tek; Miles Laboratories, Elkhart, IN, United States) and snap frozen. 10-μm thick cryostat sections are cut at-20°C, air dried, and incubated in blocking solution (5% normal donkey serum, 1% BSA, and 0.03% Triton X-100 in 1× PBS) for 1 hour at room temperature. The sections are stained with monoclonal antibodies against NF-κB p65 (CST, 8,242, Beverly, MA, United States) or with polyclonal antibodies against VEGFR2 (CST, 2,478, Beverly, MA, United States) at 4°C overnight and then incubated with corresponding secondary antibodies conjugated to Alexa Fluor 488 (Invitrogen, United States) for 1h at room temperature in the dark, while the nuclei are stained with 4′6-diamidino-2-phenylindole (DAPI; Sigma Aldrich). A fluorescence microscope (Carl Zeiss, Germany) is used to take pictures.

J. Statistical Analysis

The data are expressed as the mean ± SEM where applicable. Statistical

evaluation is conducted by one-way ANOVA followed by Tukey's multiple comparisons test depending on the normality of the data. A value of $p<0.05$ is considered statistically significant. Analysis is performed using the statistical software SPSS 26.0 (SPSS, Inc. Chicago, IL, United States).

EXAMPLE 14

MEV-GTVs: Gene delivery, gene expression, and gene editing *in vivo;* ocular delivery in mice (eyes)

Frequent injections of anti-vascular endothelial growth factor (anti-VEGF) agents are a clinical burden for patients with neovascular age-related macular degeneration (AMD). Genomic disruption of VEGF-A using adeno-associated viral (AAV) delivery of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 can be used effect permanent suppression of aberrant angiogenesis. In the example Cas9 endonuclease, to evaluate the capacity of MEV-GTV to deliver the genome-editing components *in vivo* to drive a successful CRISPR-based suppression of VEGF-A, in a mouse model of laser-induced choroidal neovascularization (CNV).

Cas9 (see, SEQ ID NO:96) endonucleases are derived from bacteria-adaptive immune systems and can be programmed using single guide RNAs (gRNAs) to introduce double-strand breaks (DSBs) at specific genomic loci, based on the presence of protospacer adjacent motif (PAM) sequences in the target gene. The DSBs trigger error-prone nonhomologous end joining (NHEJ) repair, which causes insertion or deletion mutations (indels) that result in nonsense or frameshift mutations that permanently disrupt the target gene (see, Cai B. *et al.* (2018) *Hum. Genet*: 137: 679-688).

A. gRNA Design and Plasmid Constructs

gRNAs targeting exon 1 of the mouse VEGF-A gene were selected using gRNA design software (Benchling, San Francisco, CA) based on the predicted gene cleavage efficiency (on-target score) and probability for off-target binding (off-target score) as described previously. Off-target loci were predicted using the same software (see, Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F; Nat Protoc. 2013; 8(11): 2281-308. doi: 10.1038/ nprot. 2013.143. Epub 2013 Oct 24). The selected gRNAs with the highest on-target scores for Cas9 then were subcloned into the gRNA

scaffold of the plasmid carrying AAV-ITRs. For Cas9, a CMV promoter to drive Cas9 and enhanced GFP were encoded in the plasmid carrying AAV-ITRs, while the VEGF-A targeting sequences were subcloned upstream of the U6-driven gRNA scaffold. All original plasmids used are Addgene plasmids, which were donated by Feng Zhang lab (Addgene, 60957, 60958, 61591). SEQ. ID N0:68 and 69)

B. Animals, MEV-GTV administration, and tissue collection

Six- to eight-week-old male C57BL/6J mice (Jackson Laboratory, USA) were kept in a 12-h light/dark cycle room.

For subretinal injections, animals were anesthetized with isoflurane inhalation, and eyes were dilated with 1% tropicamide and 2.5% phenylephrine. The eyes were prepared with 5% povidone iodine, followed by creation of a small conjunctival peritomy and a paralimbic sclerotomy using a 30G needle and insertion of a 31G Hamilton syringe to subretinal injection of 2-5 μL (4 × 1011 MEV-GVT/eye).

For topical instillation, 20 μL of MEV-GTV formulation was applied (4 × 1011 MEV-GVT/eye). At 1, 3, 6 and 12 weeks after treatments, eyes were enucleated, and RPE-choroid tissues were carefully dissected and homogenized to collect lysates for protein or genomic DNA extraction.

C. Laser-Induced Choroidal Neovascular (CNV) lesion and Fluorescein Angiography (FA)

Animals were anesthetized with isoflurane inhalation, and the pupils were dilated with 1% tropicamide and 5% phenylephrine. Mice were imaged using the MicronIV system (Phoenix, Pleasanton, CA, USA), with topical artificial tear gel (Genteal) applied to prevent corneal damage. Image-guided laser photocoagulation was performed with a 532-nm wavelength laser at 250 mW for 70 ms. CNV was evaluated with FA, OCT, and OCT-A one week after laser photocoagulation, and eyes with severe hemorrhage or where CNV lesion size could not be accurately assessed were excluded from analyses.

D. Scanning Laser Ophthalmoscopy (SLO) and Optical Coherence Tomography (OCT) Imaging

SLO and Optical Coherence Tomography (OCT imaging) were performed using a custom-built multimodal OCT and SLO mouse retinal imaging system developed at the small animal ocular imaging facility (EyePod). One week after

inducing CNV, animals were anesthetized with isoflurane inhalation, and the pupils were dilated with 1% tropicamide and 2.5% phenylephrine prior to imaging. SLO and OCT images were acquired simultaneously with a SLO excitation laser (ORBIS 488LX, Coherent, Santa Clara, CA, USA) running at 488 nm with 100 μW at the mouse pupil and OCT with superluminescent diodes (SLD) light source (Broadlighter 860; Superlum Diodes, Cork, Ireland) operating at 860 nm with full width at half maximum (FWHM) of 132 nm and 600 μW at the mouse pupil, respectively. For SLO, reflectance and fluorescence images were averaged from 50 consecutive images. For OCT and OCT-A, 1080 horizontal B-scans acquired with a series of three multiple B- scans (BM-scans) per one location (spanning 540 × 360 pixels) corresponding to the SLO images were captured for three-dimensional cross-sectional visualization and co-registration. En-face depth average projections of the phase variance signal were used to generate OCT-A images. CNV size was measured by Fiji image-processing software (NIH).

E. Fluorescence Immunohistochemistry

Frozen sections were collected for immunofluorescence labeling as described previously. In brief, eyes cups were fixed with 4% paraformaldehyde (PFA) for 1 h and incubated in 30% sucrose in PBS overnight. Tissues were embedded the next day and sectioned at 12 μm thickness. After blocking with normal donkey serum, tissues were incubated with anti-GFP (Novusbio; NB100-1770) or anti-HA-tag (Cell Signaling; 3724) primary antibodies for either 2h at room temperature or overnight at 4°C, followed by staining with Alexa Fluor 488 or 568-conjugated secondary antibodies and 4′,6-diamidino-2-phenylindole (DAPI). For measuring CNV size on flat-mount immunohistochemistry, eye cups were fixed with 4% PFA for 1 h and washed with PBS. Retinae were carefully removed, and the remaining RPE-choroid and scleral tissues were radially cut and labeled with Alexa Fluor 488-conjugated isolectin B4 (Invitrogen, I21411) overnight at 4°C. Fluorescence immunohistochemical images were captured with confocal microscopy (Olympus FV1000). CNV size was measured using Fiji image processing software (NIH).

F. Enzyme-Linked Immunosorbent Assay (ELISA).

Mouse VEGF-A Quantikine ELISA kits (Millipore, MMV00) were used to quantify VEGF-a protein according to the manufacturer's guidelines. In brief, proteins

were incubated on a plate coated with polyclonal antibodies against mouse VEGF-A protein for 2h followed by horseradish peroxidase conjugate incubation. After washing the plate, substrate was applied and the plate was read at 450 nm with wavelength correction to 570 nm. Quantification of VEGF-A was calculated against a standard curve and normalized with total protein.

G. RPE isolation, genomic DNA extraction, and DNA sequencing

At each time point, RPEs were isolated using an established protocol. In brief, after enucleation of the eye, anterior chamber, lens, and retina were removed, and the posterior eye cup was incubated with 0.05% trypsin-EDTA for 45 min at 37°C. Followed by gentle shaking of the eye cup to isolate the RPEs from the eye cup, cells pallets were collected for genomic DNA extraction.

Genomic DNA was extracted using either a DNeasy blood and tissue kit (QIAGEN, 69504) or a QIAamp DNA FFPE tissue kit (QIAGEN, 56404) according to manufacturer's instructions, and the quantity and quality of gDNA were measured using a NanoDrop (Thermo Fisher). PCR products were amplified from 100 ng of gDNA with forward and reverse primers using Phusion high-fidelity polymerase (NEB, M0530S) for 35 cycles (98°C for 10 s, 60°C for 30 s, and 72°C for 15 s) then purified with a MiniElute PCR purification kit (QIAGEN, 28004). The primer pairs used SEQ. ID N0: 70 and 71. Deep sequencing of 200-bp PCR products of VEGF-A target sites amplified from genomic DNA was conducted at Eurofins.

H. Quantitative Polymerase Chain Reaction (qPCR)

Each 10-μL reaction consists of 10 ng genomic DNA extracted from RPE-choroidal tissues, Universal PCR master mix (Life Technologies, 4318157), 0.25μM of probe for AAV ITR (5′-CACTCCCTCTCTGCGCGCTCG-3′), and 0.9μM forward and reverse primers. (SEQ. ID N0: 72,73, and 74).

EXAMPLE 15

MEVs: penetration, delivery, and biological activity of different payloads

This examples demonstrates the ability of MEVs to penetrate and deliver payloads, and expression of their biological activity in various cells types from the eye and the neural system. In this example, two (2) human primary cells and one human cell line were treated with MEVs either labelled with pKH26 as described in Example 2 or loaded with specific payloads as described in Example 3. Payloads

include the plasmids provided herein encoding biologically active products, which, upon delivery, are expressed in the cells.

Loading of MEVs

Isolated and purified MEVs were loaded with payloads as described in Example 3 using specific protocols, optimized per type of molecule. The MEVs were loaded either with mRNA encoding eGFP protein (e.g. SEQ ID NO:131) or with the ITR plasmid designated pBCB-23-0041-CDS-eGFP protein (SEQ ID NO:48).

Cell lines and culture conditions and MEVs treatment

ARPE-19 (ATCC (CRL-2302) a retinal pigment epithelia (RPE) cell line derived from the normal eyes were cultured and maintained in a 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F12 medium with HEPES buffer (31330038, Gibco) containing 10% fetal bovine serum, 56 mM final concentration sodium bicarbonate, and 2 mM L-glutamine and incubated at 37C in 5% CO2. The culture medium is changed every 2 days. In this example, 1x10Exp5 cells were seeded on a coverslip in 24 well plates at different concentrations using their corresponding medium. After 24 hours, one well from each cell line was used for counting the number of cells, and a mixture of cell culture medium with MEVs (loaded or non-loaded) was prepared with the final MEVs concentration defined for each experiment.

For the long-lasting expression of the GFP protein, after treatment with MEVs loaded with a the ITR plasmid encoding the GFP protein, 5x10Exp4 cells were seeded on a coverslip; after 24 hours the cells were treated with the MEVs. 72 hours after treatment, cells were washed twice with PBS and cultured with fresh medium. The cells were treated as describe bellow (Immunofluorescence) at 72h, 96h, 120h and 144h.

Immunofluorescence and confocal microcopy

For immunostaining of actin: cells were grown on coverslips to reach 70–80% confluency then directly with 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature. Cells were permeabilized by with 0.2% Triton X100-PBS for 10 min then transferred into 0.1% Tween-PBS for 5 min. Coverslips were then incubated with primary antibodies in 0.1% Tween-5% BSA-PBS for 1–2 h, washed with 0.1% Tween-PBS, then incubated with secondary antibodies in Tween 0.1%-BSA 5%-PBS

for 1 h. All the incubations were performed in darkness in a humidified chamber at room temperature. Finally, coverslips were washed again with 0.1% Tween-PBS, incubated with Hoechst (94403, SIGMA) to label DNA for 5 min, and then mounted on glass slides with Prolong (Life). Cells were analyzed by fluorescence microscopy.

For labelled payloads: cells were grown on coverslips to reach 70–80% confluency then directly with 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature, coverslips were washed with PBS, incubated with Hoechst (94403, SIGMA) to label DNA for 5 min, and then mounted on glass slides with Prolong (Life Technologies). Cells were analyzed by and visualized using a LEICA SP5 8 Confocal Microscope with a 63X objective. Figures 33 to 34 show the confocal images of the treatment with the different cells.

For immunostaining of actin: cells were grown on coverslips to reach 70–80% confluency then directly with 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature. Cells were permeabilized by with 0.2% Triton X100-PBS for 10 min then transferred into 0.1% Tween-PBS for 5 min. Coverslips were then incubated with primary antibodies in 0.1% Tween-5% BSA-PBS for 1–2 h, washed with 0.1% Tween-PBS, then incubated with secondary antibodies in Tween 0.1%-BSA 5%-PBS for 1 h. All the incubations were carried out in darkness in a humidified chamber at room temperature. Finally, coverslips are washed again with 0.1% Tween-PBS, incubated with Hoechst (94403, SIGMA) to label DNA for 5 min, and then mounted on glass slides with Prolong (Life). Cells were analyzed by fluorescence microscopy.

Results

Figure 33 shows the penetration of MEVs in ARPE-19 cells. A time response study (data not shown) demonstrated that penetration is maximal at 48 hours of treatment. Panel 1 — T=0h, ARPE-19 cells treated with MEVs labelled with PKH26 (merge DAPI/ Actin (Alexa 488) / MEVs labelled with PKH26 (Alexa 546)); Panel 2— T=24h, ARPE-19 cells treated with MEVs labelled with PKH26 (merge DAPI/ Actin (Alexa 488) / MEVs labelled with PKH26 (Alexa 546)). The expression of GFP from the mRNA delivered by the MEVs into ARPE-19 cells is maximal at 72h (data non shown). The results obtained demonstrate the ability of MEVs to deliver their payloads to retinal pigmented epithelial (RPE) cells and the escape of the encoded payload (mRNA) from the endosomal vesicles, which is translated into protein.

Figures 34 show the long-lasting expression of the GFP protein in ARPE-19 cells treated with MEVs loaded with a plasmid carrying ITR plasmids and encoding GFP protein, at one week after the treatment: a — T=0h, ARPE-19 cells treated with MEVs loaded with a plasmid carrying AAV's ITRs and encoding GFP (merge DAPI/ GFP (Alexa 488)) ; b —T=72h, ARPE-19 cells treated with MEVs loaded with a plasmid carrying AAV's ITRs and encoding GFP (merge DAPI / GFP (Alexa 488)) ; c — T= 96h, ARPE-19 cells treated with MEVs loaded with a plasmid carrying AAV's ITRs and encoding GFP (merge DAPI / GFP (Alexa 488)); d — T=144h , ARPE-19 cells treated with MEVs loaded with a plasmid carrying AAV's ITRs and encoding GFP (merge DAPI / GFP (Alexa 488)). The Figure shows the increasing expression of GFP protein from 72h to 144h, the longest time point studied. The results demonstrate the ability of MEVs to deliver their payloads into RPE cells and the endosomal escape of the payload (plasmid) to reach the nucleus, where it is transcribed into transcribed to mRNA, which then is translated into protein.

EXAMPLE 16

Anti-angiogenesis activity of MEVs loaded with different payloads (Aflibercept protein, mRNA encoding Aflibercept, and ITR plasmid encoding Aflibercept, in *in vitro* HUVEC primary cells

Loading of MEVs

Isolated and purified MEVs were loaded with various payloads as described in Example 3 using specific protocols, optimized per type of molecule. The MEVs were loaded either with Aflibercept (a.k.a. VEGF Trap-Eye) protein (SEQ ID NO: 94), with its coding mRNA (e.g. SEQ ID NO: 135 or 136), or with plasmids encoding Aflibercept designated as pBCB-23-0070-CDS-Aflibercept-1 (SEQ ID NO: 50), or pBCB-23-0065-CDS-Aflibercept-2 (SEQ ID NO: 49).

Cell lines and culture conditions

Human umbilical vein endothelial cells (HUVEC) were purchased from ATCC and maintained in endothelial cell medium (ECM) supplemented with 5% heat inactivated fetal bovine serum (HIFBS) and 1% penicillin/streptomycin at 37°C in 5% CO2. The culture medium is changed every 2 days.

Angiogenesis assays: Tube formation assay

Matrigel® tube formation assay with HUVEC cells: the tube formation assay is a recognized in vitro assay to model the reorganization stage of angiogenesis. With

appropriate Matrigel® support, the endothelial cells form capillary-like structures (also known as tubes). HUVEC cells were plated in 24 wells plates and treated or not with MEVs loaded or not-loaded (as described above) during 36h in fresh medium supplemented with 1.3% FBS at 37°C in humidified air with 5% CO2. After the 36h culture, Matrigel® was thawed and laid into 24-well culture plates to a total volume of 100 µl in each well. Plates were stored at 37°C for 30 min to form a gel layer. After gel polymerization, 1×105 viable (determined as described above) HUVEC cells (from the 36h pre-culture) were seeded on each well and incubated with fresh medium supplemented with 2% FBS at 37°C in humidified air with 5% CO2 for 4h. After 4h, the media was removed, and wells washed twice with PBS. The HUVEC cells were stained overnight with 0.1% crystal violet, 4% paraformaldehyde in PBS (pass through 0.45µM filter) and 6µM Calcein AM solution in basal medium. The networks of tubes in each well were observed with an EVOS M7000 microscope. Figure 35 shows the anti-angiogenesis activity of MEVs loaded with different payloads using the tube formation assay.

Analysis of tube formation assay images

Images of tube formation were analyzed using the software Angiogenesis plugin ImageJ. Only complete networks were analyzed. Incomplete networks were excluded.

The experiments were performed in triplicate, and five fields from each chamber were counted and averaged.

Results

The results demonstrate the anti-angiogenic activity of MEVs loaded either the Aflibercept protein (a.k.a. VEGF Trap-Eye) (SEQ ID NO:134) or with its encoding mRNA (see, SEQ ID NO:135 or 136). These results are shown in Figure 35.

Figure 35 show images of nodes and networks obtained from the tube formation assay, and shows the anti-angiogenic effect of MEVs loaded the anti-angiogenesis product Aflibercept protein, or mRNAs encoding Aflibercept protein. The figure illustrates anti-angiogenic activity following the exposure of HUVEC cells to MEVs loaded either with Aflibercept protein or with mRNAs encoding Aflibercept. It presents images from the tube formation assay: **A-** Cells (Negative control (w/o VEGFa)); **B-** Cells (Positive control (1ng/mL VEGFa)); **C-** Cells (Positive control

(2ng/mL VEGFa)); **D-** Cells treated with Aflibercept protein 0.5 ng/mL + VEGFa 1ng/mL; **E-** Cells treated with MEVs loaded with mRNA (encoding Aflibercept) +VEGFa 1ng/mL**; F-** Cells treated with MEVs loaded with Aflibercept protein + VEGFa 1ng/mL.

Table 21 shows the quantified results of the analysis, which was effected with the Angiogenesis plugin ImageJ software with respect to Figures 35 and previous results using MEVs loaded with aflibercept protein and MEVs loaded with mRNAs encoding aflibercept protein.

Table 21: Anti-angiogenic activity following the treatment of HUVEC cells with MEVs loaded with either aflibercept protein or mRNAs coding for Aflibercept, or with plasmids carrying AAV's ITRs and encoding Aflibercept.

Treatment	**Analyzed Area**	**Nodes (number)**	**Junctions (number)**	**Meshes (number)**
w/o VEGFα	3,145,728	74	87	372
with VEGFα (1ng/mL)	3,145,728	987	282	704,205
with VEGFα (2ng/mL)	3,145,728	1,245	348	832,656
with VEGFα (1ng/mL)+ Aflibercept (0.5ng/mL)	3,145,728	417	187	3584
with VEGFα (1ng/mL)+ MEVs-loaded with Aflibercept mRNA	3,145,728	86	40	440
with VEGFα (1ng/mL)+MEVs loaded with Aflibercept protein	3,145,728	167	131	1583
w/o VEGFα	3,145,728	43	69	315
with VEGFα (1ng/mL)	3,145,728	692	178	170,522
with VEGFα (2ng/mL)	3,145,728	779	227	218,501
with VEGFα (1ng/mL)+ MEVs-loaded with Aflibercept pITRs	3,145,728	417	187	238
with VEGFα (1ng/mL)+MEVs loaded with FKBP-L peptide	3,145,728	86	40	28

EXAMPLE 17

Anti-angiogenesis activity of MEVs loaded with various payloads (Aflibercept protein, mRNA for Aflibercept, or plasmids encoding Aflibercept protein) on in vitro HUVEC cells using the wound healing assay.

Loading of MEVs

Isolated and purified MEVs were loaded with payloads as described in Example 3 using specific protocols, optimized per type of molecule. The MEVs were loaded either with Aflibercept (a.k.a. VEGF Trap-Eye) protein (SEQ ID NO: 94), or

with mRNA encoding Aflibercept (e.g. SEQ ID NO: 135 or 136), or with plasmids encoding Aflibercept designated as pBCB-23-0070-CDS-Aflibercept-1 (SEQ ID NO: 50), or pBCB-23-0065-CDS-Aflibercept-2 (SEQ ID NO: 49).

Cell lines and culture conditions

Human umbilical vein endothelial cells (HUVEC) were purchased from ATCC and maintained in endothelial cell medium (ECM) supplemented with 5% heat inactivated fetal bovine serum (HIFBS) and 1% penicillin/streptomycin at 37°C in 5% CO2. The culture medium is changed every 2 days.

Wound healing assay (WHA) on HUVEC cells

This assay mimics cell migration during wound healing in vivo, as well as the cell proliferation and migration associated with the growing and migration of endothelial cells during angiogenesis. The basic steps involve (i) setting a cell monolayer in vitro, (ii) creating a "scratch" (the wound) in the cell monolayer, (iii) capturing the images at the beginning and at regular intervals during cell migration to close the wound, and (iv) comparing the images to quantify the migration rate of the cells. HUVEC cells were plated in 24 wells plates and treated or not with MEVs loaded or not-loaded (as described) during 36h in fresh medium supplemented with 1.3% FBS at 37°C in 20 humidified airs with 5% CO2. After the 36h culture the medium is washed two times with PBS. In the middle of each well a scratch is done with a pipette tip; fresh medium is added supplemented with 1.3% FBS at cells culture 37°C in 20 humidified airs with 5% CO2. At 2, 4, 6 and 8 hours after doing the scratch cell cultures are imaged using EVOS M7000 microscope. The images of Figure 36A (and the data in Table 22) correspond to the 4 hours register.

Analysis of WHA images

Images of wound healing assays were analyzed using the software Angiogenesis plugin ImageJ. The experiments were performed in triplicate.

Results

The results demonstrate the ability of loaded MEVs to inhibit the neo-angiogenesis process. Figures 36A-36B shows the results obtained 4 hours after the scratch and illustrates the anti-angiogenic activity (cell migration inhibition) following the exposure of HUVEC cells to MEVs loaded either with the Aflibercept protein, with mRNA encoding Aflibercept, or with ITR plasmids c encoding

Aflibercept (a.k.a. VEGF Trap-Eye). Table 22 shows the quantification of the anti-angiogenesis activity of MEVs loaded with anti-angiogenic molecules (Aflibercept, mRNA for Aflibercept, or plasmids encoding Aflibercept) to inhibit the migration of HUVEC cells in the wound healing assay.

Table 22: Quantification of the efficacy of MEVs loaded with various payloads to inhibit neo-angiogenesis, using HUVEC primary cells and the wound healing assay.

Conditions	**Area pixel/Area pixel of Original Wound**	**Wound Healing (1-(Area pixel/ Area pixel of Original Wound))**	**Wound Healing (1-(Area pixel/ Area pixel of Original Wound))*100**	**WH Inhibition Percentage (1-(Area pixel/ Area pixel of Original Wound))*100**
Original scratch	1.00	0.00	0	100
Neg. Control (w/o VEGFα)	0.83	0.17	17	83
Positive Control (VEGFα 1ng/mL)	0.43	0.57	57	43
Positive Control (VEGFα 2ng/mL)	0.44	0.56	56	44
Aflibercept protein (0.5ng/mL) (VEGFα 1ng/mL)	0.66	0.34	34	66
MEVs-Aflibercept protein (VEGFα 1 ng/mL)	0.64	0.36	36	64
MEVs (Aflibercept-mRNA) (VEGFα 1ng/mL)	0.62	0.38	38	62
MEVs (Aflibercept-mRNA2) (VEGFα 1ng/mL)	0.54	0.46	46	54
MEVs (Aflibercept-pITR)+(VEGFα 1ng/mL)	0.55	0.45	45	55
MEVs (Aflibercept-pITR')+(VEGFα 1ng/mL)	0.54	0.46	46	54

EXAMPLE 18

MEVs penetration, delivery, and biological activity of different payloads

Demonstration of the ability of MEVs to penetrate, deliver and allow payloads to express their biological activity in the neural system, which can be accessed by intranasal administration. In this example, two (2) human primary cells and one (1)

human cell line were treated with MEVs either labelled with pKH26 as described in Example 2 or loaded with specific payloads as described in Example 3.

Loading of MEVs

Isolated and purified MEVs were loaded with payloads as described in Example 3 using specific protocols, optimized per type of molecule. The MEVs were loaded either with Ovalbumin (OVA) labelled with Alexa-488, with eGFP protein (SEQ ID NO:132), with mRNA encoding eGFP protein (e.g. SEQ ID NO:131), or with the ITR plasmid designated pBCB-23-0041-CDS-eGFP protein (SEQ ID NO:48).

Cell lines and culture conditions and MEVs treatment

Human umbilical vein endothelial cells (HUVEC) were purchased from ATCC and maintained in endothelial cell medium (ECM) (ATCC) supplemented with 5% heat inactivated fetal bovine serum (HIFBS) and 1% penicillin/streptomycin at 37°C in 5% CO2. The culture medium is changed every 2 days. For the experiment, 1x10Exp5 cells were seeded on a coverslip in 24 well plates at different concentrations using their corresponding media. After 24 hours, one well from each cell line was used for counting the number of cells and a mixture of cell culture media with MEVs (loaded or non-loaded) was prepared with the final MEVs concentration defined for each experiment.

ARPE-19 (ATCC (CRL-2302) retinal pigment epithelia (RPE) cell line derived from the normal eyes were cultured and maintained in a 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F12 medium with HEPES buffer (31330038, Gibco) containing 10% fetal bovine serum, 56 mM final concentration sodium bicarbonate and 2 mM L-glutamine and incubated at 37C in 5% CO2. The culture medium is changed every 2 days. For the experiment, 1x10Exp5 cells were seeded on a coverslip in 24 well plates at different concentrations using their corresponding media. After 24 hours, one well from each cell line was used for counting the number of cells and a mixture of cell culture media with MEVs (loaded or non-loaded) was prepared with the final MEVs concentration defined for each experiment.

For the long-lasting expression of the GFP protein, after treatment with MEVs loaded with the ITR plasmid, 5x10Exp4 cells were seeded on a coverslip, 24 hours

late the cells were treated with the MEVs.72 hours after treatment cells were washed twice with PBS and cultured with fresh medium. Immunofluorescence) was assessed at 72h, 96h, 120h and 144h.

Human neural stem cells (NSCs), hiPSC-derived neural cells, were cultured in a 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F12 medium with HEPES buffer (31330038, Gibco) containing 10% fetal bovine serum, and 2 mM L-glutamine and incubated at 37C in 5% CO2. The culture medium was changed every 3 days. For the experiment and after 30 days of differentiation, neurons were placed on glass coverslips at a density of 2x10Exp5 cells per well and cultured for 15 days to ensure maturation. Prior to the 48-hour incubation with the MEVs, cell density per well was estimated to be 3 x10Exp5 cells.

Immunofluorescence and confocal microcopy

For labelled payloads: cells were grown on coverslips to reach 70–80% confluency then directly with 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature, coverslips were washed with PBS, incubated with Hoechst (94403, SIGMA) to label DNA for 5 min, and then mounted on glass slides with Prolong (Life Technologies). Cells were analyzed by and visualized using a LEICA SP5 8 Confocal Microscope with a 63X objective.

For immunostaining of actin: cells were grown on coverslips to reach 70–80% confluency then directly with 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature. Cells were permeabilized by with 0.2% Triton X100-PBS for 10 min then transferred into 0.1% Tween-PBS for 5 min. Coverslips were then incubated with primary antibodies in 0.1% Tween-5% BSA-PBS for 1–2 h, washed with 0.1% Tween-PBS, then incubated with secondary antibodies in Tween 0.1%-BSA 5%-PBS for 1 h. All the incubations were carried out in darkness in a humidified chamber at room temperature. Finally, coverslips are washed again with 0.1% Tween-PBS, incubated with Hoechst (94403, SIGMA) to label DNA for 5 min, and then mounted on glass slides with Prolong (Life). Cells were analyzed by fluorescence microscopy.

The results, in accord with results described in Examples above, showed the that the MEVS deliver their payloads into RPE cells and that the plasmid payloads reach the nucleus and are transcribed to produce mRNA and then are translated into proteins, which can have a biological effect.

With respect to NSCs, the results show the presence and distribution of the MEVs in the neuronal body as well as along the axons. The alignments of MEVs in the axons is fully compatible the *axonal transport* of MEVs along the olfactory tract, observed in vivo upon intranasal administration of the MEVs; it shows penetration of MEVs (labelled with PKH26) into NSCs cells.

Since modifications will be apparent to those of skill in the art, it is intended that the invention(s) only is/are limited only by the scope of the appended claims.

CLAIMS:

1. An ITR plasmid, comprising an ITR and a transgene, wherein:

the ITR is a eukaryotic viral ITR;

except for promoter and regulatory sequences that are part of the transgene or for expression of the transgene, the ITR is the only element of the plasmid from a eukaryotic virus;

the transgene comprises a nucleic acid encoding a gene product operably linked to a promoter recognized in a eukaryotic cell; and

optional additional eukaryotic transcriptional and/or translational regulatory sequences.

2. The ITR plasmid of claim 1 that consists of the ITR, one or more transgene(s), and optional regulatory sequences.

3. The plasmid of claim 1 or claim 2, wherein the size of the plasmid is up to about 20 kB in size.

4. A bacterial plasmid, comprising an ITR and a transgene, wherein:

the ITR is a eukaryotic viral ITR; and

the bacterial plasmid comprises a bacterial cloning vector for propagation in a bacterium.

5. A bacterial plasmid, comprising the ITR plasmid of any of claims 1-3.

6. The bacterial plasmid of claim 4 or claim 5, wherein the cloning vector is selected from plasmids, bacteriophages, cosmids, and bacterial artificial chromosomes (BACs).

7. The bacterial plasmid of claim 3 or claim 4 that comprises a pUC or pBR322 backbone.

8. The plasmid of any of claims 1-7, wherein the ITR is a eukaryotic virus ITR, such as from a poxvirus, adenovirus, or adeno-associated virus, or is a synthetic ITR.

9. The plasmid of claim 8, wherein the ITR is selected from among the ITRs from an AAV.

10. The plasmid of claim 8, wherein the ITR is selected from among the ITRs whose sequences are set forth in any of SEQ ID NOs: 1-47.

11. The plasmid of claim 8, wherein the ITR is selected from among the ITRs:

Organism	GenBank Accession No.	Nucleotide annotation
Vaccinia virus (Copenhagen)	N/A	Source: 1..175860 ITR: 167703 and 175860
Vaccinia virus (Wyeth)	N/A	Source: 1..182664 ITR: 166359 and 182664
Vaccinia virus (Western Reserve)	N/A	Source: 1..181419 ITR: 174482 and 181419
Vaccinia virus (MVA)	N/A	Source: 1..163444 ITR: 159457 and 163444
Rabbitpox virus (Utrecht)	N/A	Source: 1..186491 ITR: 183030 and 186491
Cowpox (Brighton)	N/A	Source: 1..212521 ITR: 206015 and 212521
Sheeppox virus	JN118986.1	Source: 1..290 ITR: 1..290
Sheeppox virus V104	MW020571.1	Source: 1..150211 ITRs: 88..2460; 147752..150124
Goatpox virus V103	MW020570.1	Source: 1..150578 ITR: 90..2480; 148080..150470
Variola virus	U23793.1	Source: 1..515 ITRs: 72..140; 141..194
Variola virus	U22363.1	Source: 1..724 ITR: 72..400
Deer adenovirus A	OP405339.1	Source: 1..30666 ITRs: 1..29; 30638..30666
Skunk adenovirus 1	KP238322.1	Source: 1..31848 ITR: 1..181; 31668..31848
Guinea pig adenovirus strain AUS96	NC_075973.1	Source: 1..37031 ITRs: 1..166; 36866..37031
Bovine adenovirus 1	M17112.1	Source: 1..159 ITR: 1..159
bovine adenovirus 5	AF238881.1	Source: 1..67 ITR: 1..67
bovine adenovirus 4	AF238883.1	Source: 1..59 ITR: 1..59
Bovine adenovirus 10	AF238882.1	Source: 1..368 ITR: 1..368
Equine adenovirus 1	AH002313.2	Source: 1..320 5' ITR 1..110; 3' ITR 211..320
Fowl adenovirus 1	AH003428.2	Source: 1..236 5' ITR 1..68; 3' ITR 169..236
Simian adenovirus (SA7P)	X01027.1	Source: 1..2338 ITR: 1..183
Simian adenovirus 8	AH002309.2	Source: 1..506 5' ITR 1..185; 3' ITR 322..506
Duck adenovirus 1	M17114.1	Source: 1..52 ITR: 1..52
Canine adenovirus 2	M30882.1	Source: 1..80 ITR: 1..80
Canine adenovirus 2	M17111.1	Source: 1..196 ITR: 1..196

Organism	GenBank Accession No.	Nucleotide annotation
Human adenovirus 19	M17110.1	Source: 1..154 ITR: 1..154
Human adenovirus 19a	AF271991.1	Source: 1..134 ITR: 1..134
Human adenovirus 19p	AF271990.1	Source: 1..125 ITR: 1..125
Human adenovirus 40	M16619.1	Source: 1..163 ITR: 1..163
Human adenovirus D37	AF271992.1	Source: 1..135 ITR: 1..135
Human adenovirus B3	M17113.1	Source: 1..195 ITR: 1..195
Human adenovirus sp.	KY062623.1	Source: 1..208 ITR: 1..208
Canine mastadenovirus A	AH002312.2	Source: 1..434 5' ITR: 1..434
Canine mastadenovirus A	M16831.1	Source: 1..273 ITRs: 17..56; 57..96; 97..136; 137..176; 177..216; 217..256; 257..273
Human parvovirus B19	M29711.1	Source: 1..479 3' ITR: 97..479
Human parvovirus B19	M29710.1	Source: 1..479 5' ITR: 1..383
Goose parvovirus	NC_001701.1	Source: 1..5106 ITRs: 1..444; 4663..5106
Muscovy duck parvovirus	KY069274.1	Source: 1..5061 ITRs: 1..381; 4681..5061
Duck parvovirus strain M8	KR029614.1	Source: 1..5073 ITRs: 1..387; 4687..5073
Streptococcus phage CP-7	AH001311.2	Source: 1..3422 ITRs: 1..347; 2170..2516
Enterobacteria phage PRD1	AH003497.2	Source: 1..338 5' ITR 1..110; 3'ITR 230..329
Enterobacteria phage PR722	AH003496.2	Source: 1..340 5' ITR 1..111; 3' ITR 230..340
Enterobacteria phage PR4	AH003495	Source: 1..340 5' ITR 1..111; 3' ITR 230..340
Enterobacteria phage PR5	AH003526.2	Source: 1..338 5' ITR 1..110; 3' ITR 229..338
Enterobacteria phage L17	AH003524.2	Source: 1..340 5' ITR 1..111; 3' ITR 230..340
Equine herpesvirus 4 (strain NS80567)	AF030027.1	Source: 1..145597 ITRs: 1..27; 112426..112452; 112453..122630; 135420..145597
Rhodococcus sp. strain: MR2253	L14443.1	Source: 1..2235 5' ITR: 1..583
Rhodococcus sp. strain: MR2253	L14442.1	Source: 1..2349 3' ITR 1..560

Organism	GenBank Accession No.	Nucleotide annotation
African swine fever virus	L28109.1	Source: 1..4027 ITRs: 41..54; 45..58; 69..82; 143..156; 157..174; 173..187; 207..220; 221..238; 244..258; 302..1633; 302..335; 1894..2027; 1894..1920
Kluyveromyces lactis	AH001394.2	Source: 1..580 ITRs: 1..240; 341..580
Streptomyces rochei	AH000936.2	Source: 1..1295 ITRs: 1..614; 939..1295
Gallid alphaherpesvirus 1	U27244.1	Source: 1..800 ITR: 1..800
Dictyostelium discoideum (slime mold)	K03072.1	Source: 1..390 ITR: 1..347
Sterkiella nova (Oxytricha nova)	M17509.1	Source: 1..79 ITR: 1..79
Rabbit fibroma virus	M14899.1	Source: 1..2886 ITR: 1..2886
Bythograea thermydron	AJ511262.1	Source: 1..1006 ITR: 971..1006
Lachancea kluyveri	M35319.1	Source: 1..598 ITR: 1..598
Yersinia enterocolitica	Y13309.1	Source: 1..3702 ITR: 252..289
Halobacterium salinarum	X59158.1	Source: 1..932 ITRs: 1..16; 917..932
Claviceps purpurea (ergot fungus)	X68490.1	Source: 1..7113 ITRs: 1..574; 6540..7113
Bombyx mori transposon-like element	M26070.1	Source: 1..1219 ITRs: 1..25; 1194..1219
Artificial sequence (Saccharomyces cerevisiae DNA, plasmid pTLU1 left ITR)	AB036802.1	Source: 1..108 left ITR: 1..15
Artificial sequence (Saccharomyces cerevisiae DNA, plasmid pTLU1 right ITR)	AB036803.1	Source: 1..115 right ITR: 1..15
Artificial Sequence (Transposon Tn3)	M13165.1	Source: 1..58 ITR: 1..58
Artificial Sequence (Plasmid RSF1010)	AH000926.2	Source: 1..485 5' ITR 11..56; 3' ITR 430..475
Artificial sequence (Erwinia amylovora plasmid pEa34)	M96392.1	Source: 1..6705 ITRs: 1..81; 3660..3686; 4865..4891; 6625..6705
Artificial sequence (Mus musculus 5' and 3' LTR repeat regions)	AH002036.2	Source: 1..2017 ITRs: 29..32; 578..581; 1135..1138; 1684..1687
Artificial sequence (Bos taurus 5' LTR repeat region)	AH001143.2	Source: 1..1320 ITRs: 46..51; 571..576; 747..752; 1272..1277
Artificial sequence (Transposon Tn10 clone pNK)	AH003348.2	Source: 1..2195 ITRs: 1..23; 731..753; 867..889; 2173..2195

Organism	GenBank Accession No.	Nucleotide annotation
Artificial sequence	AX328971.1	Source: 1..58 ITR: 1..58
Artificial sequence	AX328972.1	Source: 1..62 ITR: 1..62
Artificial sequence	AX328973.1	Source: 1..54 ITR: 1..54
Artificial sequence	AX328974.1	Source: 1..54 ITR: 1..54
Artificial sequence	AX328975.1	Source: 1..27 ITR: 1..27
Artificial sequence	AX328976.1	Source: 1..27 ITR: 1..27
Artificial sequence	AX418199.1	Source: 1..27 ITR: 1..27
Artificial sequence	CS260167.1	Source: 1..198 ITR: 1..198
Artificial sequence	LQ585715.1	Source: 1..7671 ITR: 17..163; 4674..4819
Artificial sequence	LQ585716.1	Source: 1..7671 ITR: 17..163; 4674..4819
Artificial sequence	LQ585717.1	Source: 1..7671 ITR: 17..163; 4674..4819
Artificial sequence	LV542024.1	Source: 1..7671 ITR: 17..163; 4674..4819
Artificial sequence	LV542025.1	Source: 1..7505 ITR: 17..163; 4508..4653
Artificial sequence	LV542026.1	Source: 1..7671 ITR: 17..163; 4674..4819
Artificial sequence	LY417217.1	Source: 1..7782 ITR: 1845..4974
Artificial sequence	MA132305.1	Source: 1..7671 ITR: 17..163; 4674..4819
Artificial sequence	LQ841017.1	Source: 1..7862 ITR: 17..163; 4868..5003
Artificial sequence	LQ841018.1	Source: 1..5157 ITR: 17..163; 2171..2316

12. A delivery vehicle comprising the plasmid of any of claims 1-11.

13. The delivery vehicle of claim 12 that is an extracellular vesicle, an exosome, or a nanoparticle.

14. The delivery vehicle of claim 12 or claim 13 that is an extracellular vesicle (EV).

15. The delivery vehicle of claim 13 or claim 14 that is a microalgae extracellular vesicle (MEV).

16. A microalgae extracellular vesicle gene therapy vector (MEV-GTV), comprising:

a) a MEV; and

b) an ITR plasmid containing a transgene operably linked to an ITR, wherein:

the transgene encodes a therapeutic product or products, and regulatory elements for controlling/directing expression of the encoded product(s) in a eukaryotic cell;

the transgene optionally encodes a plurality of therapeutic products; and

the plurality can be separated by nucleic acid encoding a 2A peptide for production of a polycistronic message, or each can comprise a separate promoter, or combinations of such structures.

17. A microalgae extracellular vesicle (MEV), comprising a plasmid of any of claims 1-11, whereby the MEV is an MEV-GTV.

18. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-17, wherein the transgene encodes a therapeutic product or products for treating or preventing or modulating a disease, disorder, or condition.

19. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-18, wherein the transgene encodes a protein, peptide, or nucleic acid product.

20. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-19, wherein encoded product comprises a nucleic acid molecule, a polypeptide, a protein, a plasmid, an aptamer, or an antisense oligonucleotide (ASO).

21. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-20, wherein the encoded product is an RNA product or an oligonucleotide.

22. The plasmid, delivery vehicle, or MEV-GTV of claim 21, wherein the encoded product is RNAi or mRNA.

23. The plasmid, delivery vehicle, or MEV-GTV of claim 21, wherein RNAi is silencing RNA (siRNA), or short-hairpin RNA (shRNA), micro-RNA (miRNA), is short activating RNA (saRNA), or long non-coding RNA (lncRNA).

24. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-22, wherein the encoded product is an ASO, an oligonucleotide, dsRNA, a ribozyme, or RNAi.

25. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-24, wherein the encoded product siRNA, shRNA, miRNA.

26. The plasmid, delivery vehicle, or MEV-GTV of any of claims 18-20, wherein the encoded product is a polypeptide or peptide.

27. The plasmid, delivery vehicle, or MEV-GTV of any of claims 18-20, wherein the encoded product is a protein that is a therapeutic product for treating cancer, or an infectious disease, or a neurodegenerative disease or other central nervous system (CNS) disorder, or for ameliorating effects of aging, or slowing or reversing aging, or treating an aging-associated disease, or an ophthalmic disorder, or an immunological disorder.

28. The plasmid, delivery vehicle, or MEV-GTV of any of claims 18-27, wherein the encoded product comprises a gene editing system.

29. The plasmid, delivery vehicle, or MEV-GTV of claim 28, wherein the gene editing system comprises a CRISPR-CAS system.

30. The plasmid, delivery vehicle, or MEV-GTV of claim 29, wherein the gene editing system comprises a CRISPR-associated or CRISPR-like system(s).

31. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-30, wherein the encoded product comprises an immune modulator.

32. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-31, wherein the encoded product comprises an immunomodulatory agent to increase or decrease production of one or more cytokines; up-or down-regulate self-antigen presentation; mask MHC antigens; or promote the proliferation, differentiation, migration, or activation state of one or more types of immune cells.

33. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-32, wherein the encoded product comprises a hormone or a cytokine or a chemokine.

34. The plasmid, delivery vehicle, or MEV-GTV of claim 33, wherein the encoded hormone or cytokine or growth factor selected from among human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-alpha and -beta; Müllerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors, transforming growth factors (TGFs); insulin-like growth factor-I and-II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-alpha, -

beta, and -gamma; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF), granulocyte-macrophage-CSF (GM-CSF), and granulocyte-CSF (G-CSF); or an interleukin (IL).

35. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-34, wherein the encoded product is an agent for treatment of an infection.

36. The plasmid, delivery vehicle, or MEV-GTV of claim 35, wherein the infection comprises a respiratory infection and/or a sinus infections.

37. The plasmid, delivery vehicle, or MEV-GTV of claim 35 or claim 36, wherein the encoded agent is for treating fungal infections or parasitic infections or viral infections.

38. The plasmid, delivery vehicle, or MEV-GTV of any of claims 35-37, wherein the encoded agent is an anti-fungal agent, an anti-bacterial agent, an anti-viral agent, or an anti-parasitic agent.

39. The plasmid, delivery vehicle, or MEV-GTV of any of claims 18-38, wherein encoded product comprises an antibody or antigen-binding fragment thereof.

40. The plasmid, delivery vehicle, or MEV-GTV of claim 39, wherein the antibody is an scFv, a bi-specific antibody, or an antigen-binding fragment thereof.

41. The plasmid, delivery vehicle, or MEV-GTV of claim 39 or claim 40, wherein the antibody or antigen-binding fragment thereof is a checkpoint inhibitor antibody or antigen-binding fragment thereof, or a tumor antigen-specific antibody or antigen-binding fragment thereof, or an anti-oncogene specific antibody or antigen-binding fragment thereof, or is a tumor-specific receptor, or a signaling molecule antibody or antigen-binding fragment thereof.

42. The plasmid, delivery vehicle, or MEV-GTV of claim 39 or claim 40, wherein the antibody or antigen-binding fragment thereof specifically binds to and inhibits one or more of CTLA-4, PD-1, PD-L1, PD-L2, the PD-1/PDL1 pathway, the PD-1/PDL2 pathway, HER2, EGFR, TIM-3, LAG-3, BTLA-4, HHLA-2, CD28, and other checkpoints or immune suppressors, or tumor antigens.

43. The plasmid, delivery vehicle, or MEV-GTV of any of claims 18-42, wherein the encoded product comprises a vaccine for treating or preventing, or reducing the risk of a disease, disorder, or condition.

44. The plasmid, delivery vehicle, or MEV-GTV of claim 43, wherein the vaccine is a DNA, an RNA, or a protein vaccine.

45. The plasmid, delivery vehicle, or MEV-GTV of any of claims 18-44, wherein the encoded product is for treating cancer, or an infectious disease, or a neurodegenerative disease or other central nervous system (CNS) disorder, or for ameliorating effects of aging, or slowing or reversing aging, or treating an aging-associated disease, or an ophthalmic disorder, or an immunological disorder.

46. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-45, wherein the encoded product is a therapeutic for treatment of a disease, disorder, or condition of the eye.

47. The plasmid, delivery vehicle, or MEV-GTV of claim 46, wherein the disease, disorder, or condition is a disease, disorder, or condition of the back of the eye.

48. The plasmid, delivery vehicle, or MEV-GTV of claim 45 or claim 46, wherein the disease, disorder, or condition involves the choroid-retina region, retinal pigment epithelium (RPE) cells, and/or photoreceptor cells.

49. The plasmid, delivery vehicle, or MEV-GTV of any of claims 18-48, wherein the encoded product is a therapeutic for treating a disease, disorder or condition that involves or affects the posterior eye or back of the eye.

50. The plasmid, delivery vehicle, or MEV-GTV of any of claims 45-49 wherein the disease, disorder, or condition involves one or more of choroidal cells, RPE cells, the retina, and photoreceptor cells.

51. The plasmid, delivery vehicle, or MEV-GTV of any of claims 45-50, wherein the disease, disorder, or condition is one or more of an age-related macular degeneration (AMD), a retinal degenerative disease, disorder, or condition, or a disease, disorder, or condition involving choroidal neovascularization, an inheritable disease, disorder, or condition involving the retina, and an ocular hypertension.

52. The plasmid, delivery vehicle, or MEV-GTV of any of claims 45-51, wherein the disease, disorder, or condition is selected from one or more of an age-related macular degeneration (AMD), diabetic retinopathy, retinal vein occlusion, diabetic macular edema (DME), retinal vein occlusion (RVO), and optical neuropathy glaucoma disease.

53. The plasmid, delivery vehicle, or MEV-GTV of any of claims 45-52, wherein the disease, disorder, or condition is wet AMD, geographic atrophy, diabetic retinopathy, macular edema, retinitis pigmentosa, or glaucoma.

54. The plasmid, delivery vehicle, or MEV-GTV of any of claims 45-53, wherein the transgene encodes a product for treating AMD or glaucoma or ocular hypertension.

55. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-54, wherein the transgene encodes a product that inhibits a VEGFR or inhibits VEGF.

56. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-55, wherein the transgene encodes an immunomodulatory agent to increase or decrease production of one or more cytokines; up- or down-regulate self-antigen presentation; mask MHC antigens; or promote the proliferation, differentiation, migration, or activation state of one or more types of immune cells.

57. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-56, wherein the transgene encodes prodrug or an enzyme that converts a prodrug into a drug for treating, preventing, or modulating a disease, disorder, or condition.

58. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-57, wherein the transgene encodes a product for treating, preventing, or modulating a disease, disorder, or condition of the brain.

59. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-57, wherein the transgene encodes prodrug or an enzyme that converts a prodrug into a drug for treating a disease, disorder, or condition of the brain or involving the brain.

60. The plasmid, delivery vehicle, or MEV-GTV of claim 58 or claim 59, wherein the disease, disorder, or condition comprises an infection in or involving the brain.

61. The plasmid, delivery vehicle, or MEV-GTV of any of claims 58-60, wherein the encoded product is for treatment of cancer or tumor in the brain, or for treatment of an infectious disease in the brain, or for treatment of a neurodegenerative disease or other central nervous system (CNS) disorder, or for treating dementia.

62. The plasmid, delivery vehicle, or MEV-GTV of any of claims 58-61, wherein the transgene encodes a product that has an effect on a cell or organism to which it is delivered, or that is detectable or serves as a detectable marker or a

biomarker, to thereby effect treatment, detection, diagnosis, or monitoring of treatment of a disease, disorder, or condition of the brain or involving the brain.

63. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-62, wherein the bioactive molecule is a diagnostic or a therapeutic or a theragnostic for treating, diagnosing, detecting, and/or monitoring treatment of a disease, disorder, or condition of the brain or involving the brain.

64. The plasmid, delivery vehicle, or MEV-GTV of any of claims 58-63, wherein the disease, disorder, or condition is a neurodegenerative disease (such as Parkinson's, or Alzheimer's, or Huntington's, or Creutzfeldt-Jakob disease, or other neurodegenerative disease; or a cognitive disorder (such as dementia, or amnesia, or delirium, or other cognitive disorder); or a brain disorder (such as encephalitis, or seizures, or tumors, or other brain disorder); or a nervous system disorder (such as pain, or seizures, or infections, or other nervous system disorder); or a genetic disease (such as cystic fibrosis, thalassemia, sickle cell anemia, Huntington's Disease, Duchenne's muscular dystrophy, Tay-Sachs disease, Rett syndrome, or other genetic disease); or brain tumor; or Niemann-Pick disease; or prion disease; or Parkinson's disease; or multiple sclerosis; or amyotrophic lateral sclerosis (ALS); or muscular dystrophy; or other disease of the brain or involving the brain.

65. The plasmid, delivery vehicle, or MEV-GTV of any of claims 58-64, wherein the disease, disorder, or condition of the brain or involving the brain is a cancer or is a disease, disorder, or condition treated or prevented by a vaccine.

66. The plasmid, delivery vehicle, or MEV-GTV of any of claims 18-65, wherein the disease, disorder, or condition is caused by or involves an infectious agent.

67. The plasmid, delivery vehicle, or MEV-GTV of claim 66, wherein the infectious agent is one or more of a bacteria, a virus, an oomycete, and a fungus.

68. The plasmid, delivery vehicle, or MEV-GTV of any of claims 58-66, formulated for intranasal administration to deliver encoded product to one or more of neurons, astrocytes, oligodendrocytes, microglial cells, ependymal cells, and/or neural stem cells of the brain.

69. The plasmid, delivery vehicle, or MEV-GTV of any of claims 18-68, wherein the disease, disorder, or condition is one or more of a cognitive, emotional,

behavioral, psychiatric, neurologic, degenerative, genetic, malignant (cancer), and/or traumatic brain disease, disorder, or condition.

70. The plasmid, delivery vehicle, or MEV-GTV of claim 69, wherein the disease, disorder, or condition of the brain or involving the brain results from injury to the brain or the central nervous system (CNS).

71. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-70, where the transgene encodes a product that is psychoactive or treats a psychiatric disorder, or is an immunomodulatory product, or is a detectable product, or treats brain injury or trauma, or treats cancer, or treats neurological brain disorders, or treats CNS disorders, or treats genetic brain disorders, or treats brain cancer, or has anti-aging activity, or has brain regenerative activity.

72. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-71, wherein the transgene encodes a product(s) that comprises one or more of a hormone, a growth factor, an enzyme, an immunomodulatory compound, a receptor, a receptor agonist, or a receptor antagonist.

73. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-72, wherein transgene encodes a product for treating, preventing, or modulating a disease, disorder, or condition that is caused by or involves a pathogen or is an inflammatory disease or involves a signaling pathway activated by a TLR or intracellular receptor or endosomal receptor, or is an autoimmune disease, or any disease, disorder, or condition treated by immunomodulation.

74. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-72, wherein transgene encodes a vaccine product for modulating, inducing, altering an immune response or for treating or preventing a disease, disorder, or condition.

75. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-74 wherein the transgene encodes a product that elicits or contributes to a protective humoral response that comprises serum IgG and IgA and/or mucosal IgG and IgA.

76. The plasmid, delivery vehicle, or MEV-GTV of claim 75, wherein the humoral response that comprises serum IgA and/or mucosal IgA generates or induces IgA-producing memory B-cells to provide systemic and mucosal responses that protect from reinfection.

77. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-76, wherein the transgene encodes one or more of:

an antigen or immunogenic portion thereof, or an epitope, or nucleic acid encoding the antigen or immunogenic portion thereof, or epitope; and/or

an immunomodulator that reduces or eliminates immune-tolerance to previous immunotherapies or vaccines.

78. The plasmid, delivery vehicle, or MEV-GTV of claim 77, wherein the disease, disorder, or condition is cancer or an autoimmune disease.

79. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-77, wherein the transgene encodes a product that is a TLR antagonist or agonist.

80. The plasmid, delivery vehicle, or MEV-GTV of claim 79, wherein the TLR and agonist thereof is one or more of:

TLR Member	**Ligand(s)/Agonists**
TLR1	Triacyl lipopeptides (Pam3CSK4)
TLR2	Zymosan, Porin, Modulin, Lipoproteins, Lipoteichoic acid, Diacyl lipopeptides, Atypical LPS, Peptidoglycan, Triacyl lipopeptides
TLR3	dsRNA
TLR4	Mannans, Taxol, LPS
TLR5	bacterial flagellin, profilin, HMGB1, Small molecule agonists (CBLB502)
TLR6	Zymosan, Porin, Modulin, Lipoproteins, Lipoteichoic acid, Diacyl lipopeptides (Pam2CSK4), Atypical LPS, Peptidoglycan
TLR7	imidazoquinoline, loxoribine, ssRNA, bropirimine, resiquimod
TLR8	ssRNA, small synthetic compounds
TLR9	CpG DNA
TLR10	Diacyl and Triacyl lipopeptides
TLR11	Profilin-like protein, unpathogenic bacteria.

81. The plasmid, delivery vehicle, or MEV-GTV of claim 79, wherein the TLR and antagonist thereof is one or more of:

TLR Member	**Ligand(s)/Antagonists**
TLR1	Small molecule antagonists (CU-T12-9, MMG-11)
TLR2	Small Molecule Antagonists (AT1-AT8, CU-CPT22, CU-T12-9, MMG-11, NPT1220-312), Phloretin, Sulfoglycolipids
TLR3	Small Molecule Antagonists (CU-CPT4a), Monoclonal antibodies (CNTO4685, CNTO5429)
TLR4	Small Molecule Antagonists (Norbinaltorphimine, T4Ics, T5342126, Simvastatin)
TLR5	Small Molecule Antagonist (TH1020)

TLR Member	Ligand(s)/Antagonists
TLR6	Simvastatin
TLR7	Chloroquine, hydroxychloroquine, quinacrine
TLR8	Small Molecule Antagonist (CU-CPT8m, CU-CPT9a)
TLR9	Small Molecule Antagonists (NPT1220-312), chloroquine, hydroxychloroquine, quinacrine; Suppressive or inhibitory oligonucleotides.

82. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-81, wherein the transgene encodes a product or products for treating a disease, disorder, or condition that is cancer or an inflammatory disease, disorder, or condition.

83. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-82, wherein the disease, disorder, or condition is an autoimmune disease.

84. The plasmid, delivery vehicle, or MEV-GTV of claim 82, wherein the disease, disorder, or condition is an inflammatory disease, disorder or condition, or a disease, disorder, or condition in which inflammation plays a role in the etiology of the disease, disorder, or condition.

85. The plasmid, delivery vehicle, or MEV-GTV of any of claims 82-84, wherein the product comprises an immunomodulator that suppresses the inflammatory response.

86. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-71, wherein the transgene encodes a product or products for treating, preventing, or modulating chronic or acute inflammatory diseases, disorders, and conditions.

87. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-86, wherein the transgene encodes a product or products that comprise an immunomodulator that suppresses an inflammatory response but does not suppress an anti-cancer immune response.

88. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-87, wherein the transgene encodes a product or products for treatment of a disease, disorder, or condition involving excessive or persistent inflammation and severe immune pathologies.

89. The plasmid, delivery vehicle, or MEV-GTV of claim 88, wherein the transgene encodes a product or products for treatment of a disease, disorder, or

condition that comprises septic shock, autoimmunity, atherosclerosis, metabolic syndrome and gastric cancer.

90. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-89, wherein the transgene encodes a product or products for treatment of a disease, disorder, or condition that comprises inflammatory bowel diseases, rheumatoid arthritis, sepsis, allergies, Alzheimer's Disease, or Parkinson's disease.

91. The plasmid, delivery vehicle, or MEV-GTV of claim 90, wherein the disease, disorder, or condition comprises ulcerative colitis and Crohn's disease.

92. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-91, wherein the transgene comprises eukaryotic regulatory sequences to control expression the encoded product or products.

93. The plasmid, delivery vehicle, or MEV-GTV of claim 92, where the regulatory sequences comprise a eukaryotic promoter sequence that is recognized by an RNA polymerase II or an RNA polymerase III.

94. The plasmid of claim 93, wherein the promoter is recognized by RNA polymerase II, and is a eukaryotic virus promoter or is a plant or animal promoter.

95. The plasmid of claim 93 or claim 94, wherein the promoter is selected from among a cytomegalovirus promoter, a simian virus 40 promoter, a herpes simplex promoter, an Epstein Barr virus promoter, an adenovirus promoter, a synthetic promoter, an actin promoter, and synthetic chimeric promoters.

96. The plasmid, delivery vehicle, or MEV-GTV of any of claims 92-95, wherein the plasmid further comprises other eukaryotic transcription sequences and eukaryotic translation sequences.

97. The plasmid, delivery vehicle, or MEV-GTV of claim 96, wherein the additional regulatory sequences or elements are selected from among one or more of enhancers, introns, IRES, 2A peptides, and Kozak sequences.

98. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-97, wherein MEV is from a division of microalgae selected from among Euglenophyta (Euglenoids), Chrysophyta (Golden-brown algae and Diatoms), Pyrrophyta (Fire algae), Chlorophyta (Green algae), Rhodophyta (Red algae), Phaeophyta (Brown algae), and Xanthophyta (Yellow-green algae).

99. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-97, wherein the microalgae is a species of the family Chlorellaceae.

100. The plasmid, delivery vehicle, or MEV-GTV of claim 99, wherein the microalgae is a species of the genus *Chlorella* or *Parachlorella*.

101. The plasmid, delivery vehicle, or MEV of claim 100, wherein the *Chlorella* is selected from among *Chlorella ellipsoidea*, *Chlorella pyrenoidosa*, *Chlorella sorokiniana*, *Chlorella vulgaris*, and *Chlorella variabilis*.

102. The plasmid, delivery vehicle, or MEV-GTV of claim 101, wherein the *Chlorella* is *Chlorella vulgaris.*

103. The plasmid, delivery vehicle, or MEV-GTV of claim 100, wherein the microalgae is a species of *Parachlorella* selected from among *Parachlorella kessleri*, *Parachlorella beijerinckii*, and *Parachlorella hussii*.

104. A composition, comprising the plasmid, delivery vehicle, or MEV-GTV of any of claims 1-103.

105. The composition of claim 104 that is formulated for intramuscular administration.

106. The composition of claim 104 that is formulated in a composition for oral administration or is administered orally.

107. The composition of claim 104 that is formulated for administration to the eye.

108. The composition of claim 107 that is formulated for administration to the eye for drop instillation on the eye surface, or intraocular injection, intravitreal injection, supra-choroidal, or subretinal injection.

109. The composition of claim 108, wherein the composition is formulated for intraocular injection.

110. The composition of claim 109, wherein the composition is formulated as eyedrops.

111. The composition of any of claims 104-110, comprising phosphate buffered saline (PBS).

112. The composition of any of claims 104-111, wherein the composition is an emulsion that is a microemulsion or is a nanoemulsion.

113. The composition of claim 104 that is formulated for oral administration, intramuscular administration, inhalation into the lungs or nose, mucosal administration, or local administration, or subcutaneous administration.

114. The composition of any of claims 104-112, wherein the composition is formulated for administration by a route that comprises the gut-associated lymphoid tissue (GALT).

115. A composition comprising the plasmid, delivery vehicle, or MEV-GTV of any of claims 1-103 that is formulated for oral administration for treatment of a disease, disorder, or condition that involves the gastrointestinal tract or the immune system or the white spleen.

116. The composition of any of claims 104-112 that is formulated for oral administration.

117. The composition of any of claims 104-116, wherein the plasmid, delivery vehicle, or MEV-GTV is an MEV-GTV.

118. The composition of claim 117, wherein the MEV is a *Chlorella* MEV.

119. The composition of any of claims 104-118, wherein the composition is formulated for administration by a route that traffics to the spleen.

120. The composition of any of claims 104-118, wherein the composition is formulated for administration by a route that comprises or targets mucosal tissue.

121. The composition of any of claims 104-118 that is formulated for administration by inhalation into the lungs or nose.

122. The composition of claim 121 that is formulated for intranasal administration for treatment of a disease, disorder, or condition of or involving the brain.

123. The composition of any of claims 104-118 that is formulated for intramuscular administration.

124. The composition of any of claims 1-123 that is formulated as tablets, pills, powders, liquid solutions or suspensions (*e.g.*, including injectable, ingestible and topical formulations, for example, eye drops, gels, pastes, creams, or ointments), aerosols (*e.g.*, nasal sprays and inhalers), suppositories, pessaries, injectable and infusible solutions and sustained release forms.

125. A method or use of a product for treating, preventing, or modulating a disease, disorder, or condition, wherein the product is an MEV-GTV as defined in any of claims 1-103.

126. A method of treating, preventing, or modulating a disease, disorder, or condition, comprising administering to a subject a plasmid, delivery vehicle, or MEV-GTV of any of claims 1-103 or a composition of any of claims 104-124.

127. The plasmid, delivery vehicle, MEV-GTV plasmid or method of any of claims 1-126, wherein the disease, disorder, or condition where the plasmid encodes a produced for treatment of a retinal disease or a neovascularization disease.

128. The plasmid, delivery vehicle, MEV-GTV plasmid or method of claim 126 or claim 127, wherein the disease, disorder, or condition involves retinal pigmented epithelial (RPE) cells, neuronal cells, and endothelial cells.

129. The plasmid, delivery vehicle, MEV-GTV plasmid or method of any of claims 1-128, wherein the therapeutic product encoded on the plasmid is a polypeptide, siRNA, or mRNA.

130. The plasmid, delivery vehicle, MEV-GTV plasmid or method of any of claims 1-129, wherein the encoded product inhibits VEGF or VEGFR1, inhibits CD44, or inhibits the HIF-1a pathway.

131. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-103 or a composition of any of claims 104-124 for use for treating, preventing, or modulating a disease, disorder, or condition.

132. The plasmid, delivery vehicle, MEV-GTV or composition of any of claims 1-104 administered by a route, or formulated to be administered by a route, to target organs and tissues, such as the choroid, the retina, endothelial cells, enterocytes, GALT cells, spleen cells, neurons, brain cells other than neurons, lung cells, skin cells, immune cells, blood cells, and/or mucosal cells.

133. The plasmid, delivery vehicle, or MEV-GTV or composition or method of any of claims 1-132, wherein the transgene encodes a therapeutic product for treating an ophthalmic or brain or CNS disease, disorder, or condition.

134. The plasmid, delivery vehicle, or MEV-GTV of any of claims 1-103, or composition of any of claims 104-124, wherein the disease, disorder, or condition

is selected from among metabolic diseases, genetic diseases, lung diseases, bowel diseases, and ophthalmic diseases.

135. The plasmid, delivery vehicle, MEV-GTV of any of claims 1-102, the composition of any of claims 104-124, or method of any of claims 125-134, wherein the plasmid, delivery vehicle, or MEV-GTV is an MEV-GTV for gene therapy.