

Description

Title of Invention: CYTOKINE IMMUNOTHERAPY

Technical Field

- [0001] The present disclosure relates generally to the field of a cytokine immunotherapy against cancer and/or inflammatory disease, specifically to an alphavirus replicon vector comprising a cytokine which is useful for the treatment of a cancer and/or an inflammatory disease.

Background Art

- [0002] Cytokines are molecular messengers of the innate and adaptive immunity that enable cells of the immune system to communicate over short distances, and play critical roles in regulating all aspects of immune responses, including lymphoid development, homeostasis, differentiation, tolerance and memory. Considering the ability of the immune system to recognize and destroy cancer cells, there has been considerable interest over the past decades in harnessing cytokines for the treatment of cancer.
- [0003] Preclinical experiments with interferon alpha (IFNa), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin (IL)-2, IL-12, IL-15, and IL-21 have shown efficacy in multiple murine cancer models.
- [0004] IFNa was the first cytokine approved for the treatment of human cancer, hairy cell leukemia (HCL) in 1986, and after evaluating many treatment regimens, high-dose IL-2 (HDIL-2) was approved for the treatment of metastatic renal cell carcinoma (mRCC) in 1992, and metastatic melanoma (MM) in 1998. Since the initial approval, IFNa has added indications for follicular lymphoma, adjuvant melanoma, mRCC combined with bevacizumab, and AIDS-related Kaposi's sarcoma. Nevertheless, cytokines as monotherapy have not fulfilled the initial excitement they induced.
- [0005] Strategies to address the issue of local versus systemic effects have included local or cavitary cytokine administration and transduction of stimulating or effector cells with genes encoding the cytokine through plasmid or viral delivery to augment the modest success with cytokine monotherapy. Other new approaches include structure-based cytokine engineering to generate "superkines" with increased binding affinity for select receptors to increase antitumor responses and proportionately decrease stimulation of Tregs. The development of chimeric antibody-cytokine fusion proteins and infusion of anticytokines in association with cytokines improve their tumor localization and pharmacokinetics compared to the native molecule. Additional clinical investigations combine cytokines with anticancer vaccines, checkpoint inhibitor (CPI) antibodies (anti-CTLA-4 or anti-PD-1/PD-L1), and the injection of cytokines with cancer-directed monoclonal antibodies to increase the antibody-dependent cellular cytotoxicity

(ADCC) of these antibodies, thereby augmenting their antitumor efficacy (Non Patent literature 1: Kevin C. Conlon et. al, JOURNAL OF INTERFERON & CYTOKINE RESEARCH Volume 39, Number 1, 2019, the contents of the document is herein incorporated by reference).

- [0006] IL-12 is a 70 kDa heterodimeric cytokine comprised of two disulfide-linked proteins, IL-12A p35 (35kDa) and IL-12B p40 (40kDa), naturally produced by dendritic cells, macrophages, neutrophils, and human B-lymphoblastoid cells (NC-37) and is essential for the initiation of effective immune response.
- [0007] IL-12 has emerged as one of the most potent agents for anti-cancer immunotherapy. Local IL-12 expression consistently appears to be one of the most effective methods to overcome the immune suppression due to its central role in T- and NK-cell-mediated inflammatory responses. However, clinical application of IL-12-based therapies remains problematic due to the potential lethal toxicity associated with systemic administration.
- [0008] Oncolytic viruses encoding IL-12 have demonstrated strong anti-tumor effects in pre-clinical models of cancers (Non-Patent Literature 2: Pan, W. Y. et al., Mol. Ther. 20(5), 927-937 (2012), the contents of this document are herein incorporated by reference), however, systemic accumulation of IL-12 after delivery by oncolytic viruses remains potentially lethal to patients. Inefficient transduction of tumor cells with carrier vectors currently limits the overall anti-tumor effect of this approach.
- [0009] More promising drug-inducible IL-12 systems are expected to manage the IL-12 levels over long periods with reducing level of toxicity for the clinical use.

Citation List

Non Patent Literature

[0010]

NPL1

- [0011] Kevin C. Conlon et. al, JOURNAL OF INTERFERON & CYTOKINE RESEARCH Volume 39, Number 1, 2019

NPL2

- [0012] Pan, W. Y. et al., Mol. Ther. 20(5), 927-937 (2012)

Summary of Invention

- [0013] The present disclosure relates to an improved cytokine immunotherapy against cancer and/or inflammatory disease.
- [0014] Specifically, the present disclosure relates to a novel immunologically-active alphavirus replicon vector comprising, a polynucleotide which encodes alphavirus non-structural proteins nsp1, nsp2, nsp3 and nsp4 and a polypeptide comprising a cytokine. The vector is useful for the treatment of cancer and/or inflammatory disease while

minimizing toxicity.

- [0015] In another aspect, the present disclosure provides a composition comprising the above discussed alphavirus replicon vector and a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier may be a delivery vehicle, wherein the vector is encapsulated in the delivery vehicle. In preferred embodiments, the delivery vehicle may be an alphavirus particle consisting of alphavirus structural proteins that may include capsid and/or envelope proteins. In another embodiment, the delivery vehicle may be lipid nanoparticles (LNP).
- [0016] In another aspect, the present disclosure provides the above discussed alphavirus replicon vector or the composition for use in the treatment or immunization against cancer or inflammatory disease.
- [0017] In another aspect, the present disclosure provides a use of the above discussed alphavirus replicon vector or the composition in the manufacture of a medicament for the treatment or immunization against cancer or inflammatory disease.
- [0018] In another aspect, the present disclosure provides a method of treating and/or immunizing against cancer or inflammatory disease in a subject, which comprises administering an effective amount of the above discussed alphavirus replicon vector or composition to the subject in need thereof.

Brief Description of Drawings

- [0019] [Fig.1]A representative construct of an alphavirus replicon vector.
 [Fig.2]Full length VEEV TC-83 vector of the construct including a polynucleotide encoding the construct comprising IL-12-Linker-TM produced in Example 3.
 [Fig.3]Full length VEEV TC-83 vector of the construct including a polynucleotide encoding the construct comprising IL-12 produced in Example 3.
 [Fig.4]Effect of IL-12 alphavirus replicon constructed to express construct 1 on MC-38 tumor model mice.
 [Fig.5]Effect of IL-12 alphavirus replicon constructed to express construct 1 on CT-26 tumor model mice.
 [Fig.6a]Effect of IL-12 alphavirus replicon constructed to express construct 6 on CT-26 tumor model mice.
 [Fig.6b]Effect of IL-12 alphavirus replicon constructed to express construct 6 on CT-26 tumor model mice.
 [Fig.7a]Effect of IL-12 alphavirus replicon constructed to express construct 1 on macrophage M1 and M2 populations in TIL of MC-38 tumor model mice.
 [Fig.7b]Effect of IL-12 alphavirus replicon constructed to express construct 1 on Treg population in TIL of MC-38 tumor model mice.
 [Fig.8]Effect of IL-12 alphavirus replicon constructed to express construct 1 on

B16F10 tumor model mice.

Description of Embodiments

- [0020] “Cytokine(s)” used herein is a polypeptide(s)/glycoprotein(s) derived from a natural lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities), an interleukin (cytokines made by one leukocyte and acting on other leukocytes), or modification thereof. The modified cytokine may be a fragment of the naturally occurring cytokine. In one embodiment, the modified cytokine has at least 70%, 75%, 80%, 85%, 90%, 95% or 98% amino acid sequence identity to a naturally occurring cytokine. In one embodiment, the modified cytokine is a mutant where at most 10% of the amino acids are deleted, substituted, and/or added based on a naturally occurring cytokine.
- [0021] Examples of cytokines include, but are not limited to, interleukin (IL) including over 30 type such as IL-1 α , IL-1 β , IL-2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -12, -13 to -37; interferon (IFN) such as IFN- α , IFN- β and IFN- γ ; tumor necrosis factor (TNF) such as TNF- α and TNF- β ; transforming growth factor (TGF) such as TGF- α and TGF- β ; colony stimulating factor (CSF) such as granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage-colony stimulating factor (GM-CSF), macrophage-colony Stimulating factor (M-CSF), erythropoietin (EPO), stem cell factor (SCF) and monocyte chemotactic and activating factor (MCAF); growth factor (GF) such as epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin like growth 15 factor (IGF), nerve growth factor (NGF), Brain-derived neurotrophic factor (BDNF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), thrombopoietin (TPO), and bone morphogenic protein 20 (BMP); and other polypeptide factors including LIF, kit ligand (KL), MPO (Myeloperoxidase) and CRP (C-reactive protein); COX (Cyclooxygenase) such as COX-1, COX-2 and COX-3, NOS (Nitric oxide synthase) such as NOS-1, NOS-2 and NOS-3; and modified thereof.
- [0022] Cytokines also includes chemokines which are cytokines that induce chemotaxis. There are two major classes of chemokines, CXC and CC. The CXC chemokines, such as neutrophil-activating protein-2 (NAP-2) and melanoma growth stimulatory activity 5 protein (MGSA) are chemotactic primarily for neutrophils and T lymphocytes, whereas the CC chemokines, such as RANTES, Macrophage inflammatory protein (MIP) including MIP-1 α and MIP-1 β , keratinocyte-derived chemokine (KC), the monocyte
- 10 chemotactic proteins (MCP-1, MCP-2, MCP-3, MCP-4, and MCP-5) and the eotaxins (-1 and -2) are chemotactic for, among other cell types, macrophages, T lymphocytes, eosinophils, neutrophils, dendritic cells, and basophils. There also exist the

chemokines lymphotactin-1, lymphotactin-2 (both C15 chemokines), and fractalkine (a CX3C chemokine) that do not fall into either of the major chemokine subfamilies.

[0023] Preferable example of a cytokine is, but not limited to, IL-12.

[0024] In the alphavirus replicon vector of the present disclosure, the gene encoding a cytokine may be fused to a gene encoding a transmembrane domain. As used herein, "transmembrane domain (TM)" is a protein derived either from a natural or from a synthetic source. Where the source is natural, the domain in some aspects is derived from any membrane-bound or transmembrane protein. In one aspect, the membrane-bound or transmembrane protein is a protein heterologous to the cytokine. Examples of the membrane-bound or transmembrane proteins may include the alpha, beta or zeta chain of a T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CDS, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154; toll-like receptors (TLR) such as TLR1-TLR10 in human and TLR1-TLR9, TLR11-TLR13 in mouse; interleukin (IL) receptors such as IL-1-28 receptor, RANTES receptors (CCR1, CCR3, CCR5), MIP-1 receptor, PF4 receptor, M-CSF receptor and NAP-2 receptor belonging to GPCR chemokine receptor; hemagglutinin (HA).

[0025] Examples of transmembrane proteins may also include the followings:

5-Lipoxygenase-Activating Protein, ABC Transporters, ACBP, Amyloid beta (A4), Bcl-2 Inhibitors, BNIPs, CAAX protease, Cytochromes P450, E-NPPs, EPHA1, EPHA2, EPHA3, EPHA4, Fatty Acid Desaturases, Gamma secretase, Glucose transporter, Glycophorins, GPCR, HER2/ErbB2, HER3/ErbB3, HER4/ErbB4, HSD-11 β , Hypoxia-induced Proteins, Immunoglobulins, Insulin receptor, Integrins, Ion channel, MAPEG, MFS, MinK Family, MPPs, Peptidase AD, Peptidase Family M48, Peptidase MA, Protein Jagged, Receptor-type Kinases, SNARE Complex, Sulfatases, TNF receptor, Transmembrane Proteins 14, Transporter, TROBP, VEGF receptors, Aldehyde Dehydrogenases, Ammonia and Urea transporters, FMN-linked Oxidoreductases, Leucine Rich Repeat (LRR)-Containing Transmembrane Proteins, Leukotriene C4 synthase, Lysosome-associated membrane glycoprotein, Major Intrinsic Protein (MIP)/FNT superfamily, Microsomal prostaglandin E synthase, N-(deoxy)ribosyltransferase-like Membrane Proteins, Neutral/alkaline Ceramidases, Oligosaccharyl Transferase, Pentameric Ligand-gated Ion Channels, Rhodopsin-like receptors and pumps, Single-helix ATPase Regulators, Squalene/ phytoene Synthase, Stearoyl -CoA desaturase 1, Stannin (SNN) Membrane Proteins, T-cell Surface Glycoprotein CD3 Zeta Chain, Tetratricopeptide repeat (TPR) Alpha-Helical Repeat Proteins, Transmembrane Proteins with NAD(P)-binding Rossmann-fold Domains.

[0026] In addition, monotypic/peripheral proteins that attached to the lipid bilayer or other integral proteins and peptide may also be used as transmembrane proteins. Examples may include Alpha/Beta-Hydrolase, Annexins, Bet V1-Like Protein, C1 Domain-

Containing Protein, C2 Domain-containing Protein, CoA-Dependent Acyltransferases, CRAL-TRIO Domain-Containing Protein, DNase I-like protein, Fibrinogen , FYVE/ PHD Zinc Finger Protein, Galactose-Binding Domain-Like Protein, Glycolipid Transfer Protein, Immunoglobulin-Like Superfamily (E Set) Protein, Lipocalin, Lipoxigenase, PGBD superfamily, PH Domain-Like Protein, Phosphatidylinositol 3-/4-Kinase, PLC-like Phosphodiesterase, Phosphotyrosine Protein Phosphatases II, P-Loop Containing Nucleoside Triphosphate Hydrolase, Protein kinase superfamily , PX Domain-Containing Protein, Saposin , Synuclein and Transcriptional factor tubby.

- [0027] The expression "transmembrane domain" used in the present disclosure includes at least transmembrane region(s) of the membrane-bound or transmembrane protein. In addition, the transmembrane domain may also include juxtamembrane domain (JMD) and/or cytoplasmic tail of the membrane-bound or transmembrane protein.
- [0028] Alternatively the transmembrane domain in some embodiments is synthetic. In some aspects, the synthetic transmembrane domain comprises predominantly hydrophobic residues such as leucine and valine. There are also highly charged residues flanking the transmembrane domain (stop transport signals). There are also highly charged residues flanking the transmembrane domain (stop transport signals). In some aspects, a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain.
- [0029] Preferable examples of transmembrane domain may be those derived from TLR4 (Toll-like receptor 4) and influenza virus hemagglutinin (HA). Specific examples may include a protein consisting of the flexible juxtamembrane region or flexible linker, the transmembrane domain and the cytoplasmic tail of Influenza virus hemagglutinin "HA (flexible-TM-Cyt)" and a protein consisting of TLR4 (Toll-like receptor 4).
- [0030] By fusing the gene encoding a cytokine with gene encoding a transmembrane domain, especially with the influenza virus hemagglutinin transmembrane domain, the safety of the resulting alphavirus replicon vector will be improved while keeping the effect to induce immunological activities.
- [0031] A cytokine polypeptide and a transmembrane domain may be directly or indirectly fused. In one embodiment, one or two linkers may intervene between them.
- [0032] Also a cytokine polypeptide and a transmembrane domain can be truncated and replaced by short linkers. In some embodiments, a cytokine structural protein and a heterologous transmembrane domain include one or more peptide linkers.
- [0033] An example of a short linker consists of from 2 to 25 amino acids (e.g. 2, 3, 4, 5 or 6 amino acids). Usually, it is from 2 to 15 amino acids in length, such as SG, GS, SGG, GGS SGSG and TRGGS. In certain circumstances, the linker can be only one, such as glycine(G), serine (S) and cysteine (C).
- [0034] When a cytokine polypeptide is chemically conjugated to a heterologous trans-

membrane domain through a chemical cross-linker which is a chemical compound, the examples of the cross-linkers include, but are not limited to, SMPH, Sulfo-MBS, Sulfo-EMCS, Sulfo-GMBS, Sulfo-SIAB, Sulfo-SMPB, Sulfo-SMCC, SVSB and SIA.

- [0035] IgG-derived substances can also be used as a linker. Examples of IgG-derived substances include IgG1 to IgG4 comprising (i) full (hinge-CH₂CH₃) (ii) half (hinge-CH₃) and (iii) short (12aa hinge only). Preferable example is IgG4-CH₃.
- [0036] The construct may also comprise a signal sequence. As used herein, "signal sequence" (sometimes referred to as signal peptide, targeting signal, localization signal, localization sequence, transit peptide, leader sequence or leader peptide) is a polynucleotide or polypeptide, depending on the context. Signal sequence is from about 9 to 200 nucleotides or 3-70 amino acids in length that, optionally, is incorporated at the 5' or N-terminus of the coding region or the protein. Some signal sequences are cleaved from the protein, for example by a signal peptidase after the proteins are transported to the desired site.
- [0037] The signal sequence may be of the target protein to be expressed by the alphavirus replicon.
- [0038] As used herein "alphavirus" is meant to refer to RNA-containing viruses that belong to the Togaviridae family of viruses. Exemplary Togaviridae viruses include but are not limited to Eastern Equine Encephalitis Virus (EEEV), Venezuelan Equine Encephalitis Virus (VEEV), Everglades Virus, Mucambo Virus, Pixuna Virus, Western Equine Encephalitis Virus (WEEV), Sindbis Virus, Semliki Forest Virus, Middleburg Virus, Chikungunya Virus (CHIKV), O'nyong-nyong Virus, Ross River Virus, Barmah Forest Virus, Getah Virus, Sagiya Virus, Bebaru Virus, Mayaro Virus, Una Virus, Aura Virus, Whataroa Virus, Babanki Virus, Kyzylagach Virus, Highlands J virus, Fort Morgan Virus, Ndumu Virus, Buggy Creek Virus and Ockelbo virus.
- [0039] By "alphavirus structural protein" is meant a polypeptide or fragment thereof having at least about 80% amino acid sequence identity to a naturally occurring viral capsid or envelope protein. In one embodiment, the alphavirus structural protein has at least about 85%, 90%, 95% or greater amino acid sequence identity with Eastern Equine Encephalitis Virus (EEEV), Venezuelan Equine Encephalitis Virus (VEEV), Everglades Virus, Mucambo Virus, Pixuna Virus, Western Equine Encephalitis Virus (WEEV), Sindbis Virus, Semliki Forest Virus, Middleburg Virus, Chikungunya Virus (CHIKV), O'nyong-nyong Virus, Ross River Virus, Barmah Forest Virus, Getah Virus, Sagiya Virus, Bebaru Virus, Mayaro Virus, Una Virus, Aura Virus, Whataroa Virus, Babanki Virus, Kyzylagach Virus, Highlands J virus, Fort Morgan Virus, Ndumu Virus, or Buggy Creek Virus. Wild type amino acid sequences of alphavirus structural proteins can be obtained from GenBank.
- [0040] In specific embodiments, the alphavirus is a CHIKV, for example CHIKV strain

37997 or LR2006 OPY-1. In other embodiments, the alphavirus is a VEEV, for example VEEV strain TC-83.

- [0041] By "alphavirus replicon" is meant an RNA molecule which can direct its own amplification in vivo in a target cell. The replicon encodes the polymerase(s) which catalyze RNA amplification (nspl, nsp2, nsp3, nsp4) and contains cis RNA sequences required for replication which are recognized and utilized by the encoded polymerase(s). An alphavirus replicon typically contains the following ordered elements: 5' UTR, sequences which encode alphavirus nonstructural proteins (nspl, nsp2, nsp3, nsp4), 3' UTR, and a poly A signal. An alphavirus replicon also contains one or more viral sub-genomic promoters directing the expression of the gene of interest. Those sequences may have one or more mutations taught in a prior art.
- [0042] In this disclosure, "alphavirus replicon", "alphavirus replicon vector" and "replicon" are used to refer the same substance.
- [0043] An alphavirus replicon provided by the present disclosure may have the construct shown in Figure 1.
- [0044] By "alphavirus replicon particle" (ARP) is meant an alphavirus replicon packaged with alphavirus structural proteins. ARP does not contain polynucleotide encoding any of the alphavirus structural proteins.
- [0045] In this disclosure, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean "includes", "including," and the like; "consisting essentially of" or "consists essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.
- [0046] By "fragment" is meant a portion of a polypeptide or nucleic acid molecule. This portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or amino acids.
- [0047] By "reference" is meant a standard or control condition.
- [0048] A "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset of or the entirety of a specified sequence; for example, a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence. For polypeptides, the length of the reference polypeptide sequence will generally be at least about 16 amino acids, preferably at least about 20 amino acids, more preferably at least about 25 amino acids, and even more preferably about 35 amino acids, about 50 amino acids, or about 100 amino

acids. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least about 50 nucleotides, preferably at least about 60 nucleotides, more preferably at least about 75 nucleotides, and even more preferably about 100 nucleotides or about 300 nucleotides or any integer thereabout or there between.

- [0049] Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e^{-3} and e^{-100} indicating a closely related sequence.
- [0050] By "effective amount" is meant the amount of an agent required to ameliorate the symptoms of a disease relative to an untreated patient. The effective amount of active compound(s) used to practice the present invention for prevention or treatment of a disease varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.
- [0051] A satisfactory effect may be obtained by systemic administration, e.g. intramuscular administration, intertumoral administration, subcutaneous administration or intravenous administration 1-8 times at the amount of 10^3 - 10^{10} Infectious Unit (IU) or 0.01-500 μ g per time, preferably 10^5 - 10^{10} IU or 0.1-100 μ g per time, for example 10^7 - 10^9 IU or 1-50 μ g per one time. The alphavirus replicon vector may preferably be formulated in a vaccine composition suitable for administration in a conventional manner.
- [0052] By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline.
- [0053] As used herein, the terms "treat," "treating," "treatment," and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.
- [0054] As used herein, the terms "prevent," "preventing," "prevention," "prophylactic treatment" and the like refer to reducing the probability of developing a disorder or condition in a subject, who does not have, but is at risk of or susceptible to developing

a disorder or condition.

[0055] Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive.

[0056] Examples of cancer which may be treated include, but are not limited to, melanoma, renal cancer, prostate cancer, breast cancer, colon cancer and non-small cell lung cancer. Other examples of the cancer include, but are not limited to, include bone cancer, pancreatic cancer, skin cancer (e.g. melanoma), cancer of the head or neck, cutaneous or intraocular malignant melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, liver cancer, testicular cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, non-Hodgkin's lymphoma, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, chronic or acute leukemias including acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, solid tumors of childhood, lymphocytic lymphoma, cancer of bladder, cancer of the kidney or ureter, carcinoma of the renal pelvis, neoplasm of the central nervous system (CNS), primary CNS lymphoma, tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, Kaposi's sarcoma, epidermoid cancer, squamous cell cancer, T-cell lymphoma, environmentally induced cancers including those induced by asbestos, and combinations thereof.

[0057] "Inflammatory disease" includes inflammatory conditions with an aetiology including an autoimmune component such as arthritis (for example rheumatoid arthritis, arthritis chronica progrediente and arthritis deformans) and rheumatic diseases, including inflammatory conditions and rheumatic diseases involving bone loss, inflammatory pain, spondyloarthropathies including ankylosing spondylitis, Reiter syndrome, reactive arthritis, psoriatic arthritis, juvenile idiopathic arthritis and enterophathis arthritis, enthesitis, hypersensitivity (including both airways hypersensitivity and dermal hypersensitivity) and allergies. Specific autoimmune diseases include autoimmune haematological disorders (including e.g. hemolytic anaemia, aplastic anaemia, pure red cell anaemia and idiopathic thrombocytopenia), systemic lupus erythematosus (SLE), lupus nephritis, inflammatory muscle diseases (dermatomyositis), periodontitis, polychondritis, scleroderma, Wegener granulomatosis, dermatomyositis, chronic active hepatitis, myasthenia gravis, psoriasis, Steven-Johnson syndrome, idiopathic sprue, autoimmune inflammatory bowel disease (including e.g. ulcerative colitis, Crohn's disease and irritable bowel syndrome), endocrine ophthalmopathy, Graves' disease, sarcoidosis, multiple sclerosis, systemic

sclerosis, fibrotic diseases, primary biliary cirrhosis, juvenile diabetes (diabetes mellitus type I), uveitis, keratoconjunctivitis sicca and vernal keratoconjunctivitis, interstitial lung fibrosis, periprosthetic osteolysis, glomerulonephritis (with and without nephrotic syndrome, e.g. including idiopathic nephrotic syndrome or minimal change nephropathy), multiple myeloma other types of tumors, inflammatory disease of skin and cornea, myositis, loosening of bone implants, metabolic disorders, (such as obesity, atherosclerosis and other cardiovascular diseases including dilated cardiomyopathy, myocarditis, diabetes mellitus type II, and dyslipidemia), and autoimmune thyroid diseases (including Hashimoto thyroiditis), small and medium vessel primary vasculitis, large vessel vasculitides including giant cell arteritis, hidradenitis suppurativa, neuromyelitis optica, Sjogren's syndrome, Behcet's disease, atopic and contact dermatitis, bronchiolitis, inflammatory muscle diseases, autoimmune peripheral neuropathies, immunological renal, hepatic and thyroid diseases, inflammation and atherothrombosis, autoinflammatory fever syndromes, immunohematological disorders, and bullous diseases of the skin and mucous membranes. Anatomically, uveitis can be anterior, intermediate, posterior, or pan-uveitis. It can be chronic or acute. The etiology of uveitis can be autoimmune or non-infectious, infectious, associated with systemic disease, or a white-dot syndrome.

- [0058] In any of the foregoing aspects, the method further comprises administering a second composition comprising a checkpoint inhibitor polypeptide or polynucleotide encoding the same, and an optional pharmaceutically acceptable carrier. In some aspects, the checkpoint inhibitor polypeptide inhibits PD1, PD-L1, CTLA4, or a combination thereof. In some aspects, the checkpoint inhibitor polypeptide is an antibody. In some aspects, the checkpoint inhibitor polypeptide is an antibody selected from an anti-CTLA4 antibody or antigen-binding fragment thereof that specifically binds CTLA4, an anti-PD1 antibody or antigen-binding fragment thereof that specifically binds PD1, an anti-PD-L1 antibody or antigen-binding fragment thereof that specifically binds PD-L1, and a combination thereof. In some aspects, the checkpoint inhibitor polypeptide is an anti-PD-L1 antibody selected from atezolizumab, avelumab, or durvalumab. In some aspects, the checkpoint inhibitor polypeptide is an anti-CTLA-4 antibody selected from tremelimumab or ipilimumab. In some aspects, the checkpoint inhibitor polypeptide is an anti-PD1 antibody selected from nivolumab or pembrolizumab.
- [0059] Unless specifically stated or obvious from context, as used herein, the terms "a", "an", and "the" are understood to be singular or plural.
- [0060] The art will acknowledge that polynucleotide sequences described in the specification and claims will recite "T"s in a representative DNA sequence but where the sequence represents RNA, the "T"s would be substituted for "U"s.
- [0061] Any compositions or methods provided herein can be combined with one or more of

any of the other compositions and methods provided herein.

- [0062] The term "vector" refers to the means by which a nucleic acid sequence can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include plasmids, viruses, bacteriophages, pro-viruses, phagemids, transposons, artificial chromosomes, and the like, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that is not autonomously replicating. In many, but not all, common embodiments, the vectors of the present invention are plasmids or bacmids.
- [0063] Typically, the nucleic acid molecule to be expressed is "operably linked" to a promoter and/or enhancer, and is subject to transcription regulatory control by the promoter and/or enhancer.
- [0064] In one embodiment, an RNA molecule such as an alphavirus replicon may be generated by conventional procedures known to the art from a template DNA sequence. In vitro transcription (IVT) methods permit template-directed synthesis of RNA molecules. IVT methods permit synthesis of large quantities of RNA transcript. Generally, IVT utilizes a DNA template comprising a promoter sequence upstream of a sequence of interest. The promoter sequence is most commonly of bacteriophage origin such as the T7, T3 or SP6 promoter sequence but many other promoter sequences can be tolerated including those designed de novo. Transcription of the DNA template is typically best achieved by using the RNA polymerase corresponding to the specific bacteriophage promoter sequence. Exemplary RNA polymerases include, but are not limited to T7 RNA polymerase, T3 RNA polymerase, or SP6 RNA polymerase, among others. IVT is generally initiated at a dsDNA but can proceed on a single strand. Kits for in vitro transcription such as T7 transcription kit (RiboMax™ Express Large Scale RNA production System, Promega (WI USA)).
- [0065] The method of transfection and the choice of expression vehicle will depend on the host system selected. Transfection methods are described, e.g., in Ausubel et al. (supra); expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (P. H. Pouwels et al., 1985, Supp. 1987) The references cited in this paragraph are herein incorporated by reference.
- [0066] A variety of expression systems exist for the production of the constructs of the invention. Expression vectors useful for producing the constructs include, without limitation, chromosomal, episomal, and virus-derived vectors, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as al-

phavirus (e.g. Chikungunya Virus (CHIKV) and Venezuelan Equine Encephalitis Virus (VEEV)), baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof.

- [0067] Constructs and/or alphavirus replicon vectors used herein comprise alphavirus polynucleotides that encode structural proteins, including envelope proteins or capsid protein as described herein. Also, constructs and/or vectors used herein comprise alphavirus polynucleotides that encode nonstructural proteins nsp1, nsp2, nsp3 and nsp4 and a gene of interest. Specific example of the construct or vector is that shown in Figure 1.
- [0068] The vector may be, for example, a phage, plasmid, viral, or retroviral vector. The constructs and/or vectors that comprise the nucleotides should be operatively linked to an appropriate promoter, such as the CMV promoter, phage lambda PL promoter, the E. coli lac, phoA and tac promoters, the SV40 early and late promoters, and promoters of retroviral LTRs are non-limiting examples. Other suitable promoters will be known to the skilled artisan depending on the host cell and/or the rate of expression desired. The expression constructs will further contain sites for transcription initiation, termination, and, in the transcribed region, a ribosome-binding site for translation. The coding portion of the transcripts expressed by the constructs will preferably include a translation initiating codon at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.
- [0069] Vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin or ampicillin resistance genes for culturing in E. coli and other bacteria. Among vectors preferred are virus vectors, such as baculovirus, poxvirus (e.g., vaccinia virus, avipox virus, canarypox virus, fowlpox virus, raccoonpox virus, swinepox virus, etc.), adenovirus (e.g., canine adenovirus), herpesvirus, and retrovirus. Other vectors that can be used with the invention comprise vectors for use in bacteria, which comprise pQE70, pQE60 and pQE-9, pBluescript vectors, Phagescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5. Among preferred eukaryotic vectors are pFastBacl pWINEO, pSV2CAT, pOG44, pXTI and pSG, pSVK3, pBPV, pMSG, and pSVL. Other suitable vectors will be readily apparent to the skilled artisan.
- [0070] Recombinant constructs can be prepared and used to transfect, can express viral proteins, including those described herein, into eukaryotic cells and/or prokaryotic cells. Thus, in one embodiment, the present disclosure provides for host cells which comprise a vector (or vectors) that contain nucleic acids which encode alphavirus structural proteins, including capsid, E3, E2, 6K, and E1 or portions thereof, and a

vector that comprises nucleic acids which encode alphavirus nsp1, nsp2, nsp3 and nsp4, and at least one of gene of interest encoding a cytokine under conditions which allow the formation of alphavirus replicon particles. The term "alphavirus replicon particles" refers to particles consisting of alphavirus structural proteins in which the alphavirus replicon vector comprising polynucleotide encoding alphavirus nonstructural proteins nsp1, nsp2, nsp3 and nsp4 and a polypeptide comprising a cytokine is incorporated.

- [0071] In one embodiment, said vector is a recombinant baculovirus. In another embodiment, said recombinant baculovirus is transfected into an insect cell. In a preferred embodiment, said cell is an insect cell. In another embodiment, said insect cell is a Sf9 cell.
- [0072] One particular bacterial expression system for polypeptide production is the E. coli pET expression system (Novagen, Inc., Madison, Wis). According to this expression system, DNA encoding a polypeptide is inserted into a pET vector in an orientation designed to allow expression. Since the gene encoding such a polypeptide is under the control of the T7 regulatory signals, expression of the polypeptide is achieved by inducing the expression of T7 RNA polymerase in the host cell. This is typically achieved by using host strains that express T7 RNA polymerase in response to IPTG induction. Once produced, a recombinant polypeptide is then isolated according to standard methods known in the art, for example, those described herein.
- [0073] Depending on the vectors and host cells selected, the constructs are produced by growing host cells transfected by the vectors under conditions whereby the recombinant proteins are expressed and the alphavirus replicon is generated, and constructs containing alphavirus replicon being packaged with the particle of alphavirus structural proteins are formed. In one embodiment, the invention comprises a method of producing a construct, that involves co-transfecting a vector comprising a polynucleotide encoding alphavirus non-structural protein nsp1, nsp2, nsp3 and nsp4, and at least one gene of interest encoding the polipeptide comprising a cytokine, at least one vector each encoding at least one alphavirus structural protein into suitable host cells and expressing said alphavirus structural protein under conditions that allow construct formation. In another embodiment, the eukaryotic cell is selected from the group consisting of, yeast, insect, amphibian, avian or mammalian cells. The selection of the appropriate growth conditions is within the skill or a person with skill of one of ordinary skill in the art.
- [0074] Methods to grow cells that produce alphavirus replicon particles of the disclosure include, but are not limited to, batch, batch-fed, continuous and perfusion cell culture techniques. In one embodiment, cells co-transfected with a vector encoding an alphavirus replicon and a vector comprising a polypeptide encoding capsid, and a vector

comprising a polynucleotide encoding envelope proteins, such as those derived from a CHIKV or VEEV are grown in a bioreactor or fermentation chamber where cells propagate and express protein (e.g., recombinant proteins) for purification and isolation. Typically, cell culture is performed under sterile, controlled temperature and atmospheric conditions. A bioreactor is a chamber used to culture cells in which environmental conditions such as temperature, atmosphere, agitation and/or pH can be monitored. In one embodiment, the bioreactor is a stainless steel chamber. In another embodiment, said bioreactor is a pre-sterilized plastic bag (e.g., Cellbag.RTM., Wave Biotech, Bridgewater, N.J., the contents of the cited document is herein incorporated by reference). In other embodiment, said pre-sterilized plastic bags are about 50 L to 1000 L bags.

[0075] As used herein, the term “pharmaceutically acceptable carrier” means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration to a human or other vertebrate animal, including any and all aqueous solvents (e.g., water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles, such as sodium chloride, and Ringer's dextrose), non-aqueous solvents (e.g., propylene glycol, polyethylene glycol, vegetable oil, and injectable organic esters, such as ethyloleate), dispersion media, coatings, surfactants, antioxidants, preservatives (e.g., antibacterial or antifungal agents, anti-oxidants, chelating agents, and inert gases), isotonic agents, absorption delaying agents, salts, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, fluid and nutrient replenishers, such like materials and combinations thereof, as would be known to one of ordinary skill in the art. The pH and exact concentration of the various components in a pharmaceutical composition are adjusted according to well-known parameters.

Encapsulating substances refers to a delivery vehicle where the polynucleotide or vector is packaged, such as a replicon particle (e.g. the alphavirus replicon particle described in US patent publication No. 2019-0185822, the contents of this document is herein incorporated by reference) and a lipid delivery system (e.g. liposome).

[0076] In some embodiments, the compositions or formulations of the present disclosure comprise a lipid delivery system, e.g., a liposome, a lipoplexes, a lipid nanoparticle, or any combination thereof. The alphavirus replicon vector described herein can be formulated using one or more liposomes, lipoplexes, or lipid nanoparticles. Liposomes, lipoplexes, or lipid nanoparticles can be used to improve the efficacy of the polynucleotides directed protein production as these formulations can increase cell transfection by the polynucleotide; and/or increase the translation of encoded protein. The liposomes, lipoplexes, or lipid nanoparticles can also be used to increase the stability of the polynucleotides.

- [0077] Liposomes are artificially-prepared vesicles that may primarily be composed of a lipid bilayer and may be used as a delivery vehicle for the administration of pharmaceutical formulations. Liposomes can be of different sizes. A multilamellar vesicle (MLV) may be hundreds of nanometers in diameter, and may contain a series of concentric bilayers separated by narrow aqueous compartments. A small unicellular vesicle (SUV) may be smaller than 50 nm in diameter, and a large unilamellar vesicle (LUV) may be between 50 and 500 nm in diameter. Liposome design may include, but is not limited to, opsonins or ligands to improve the attachment of liposomes to unhealthy tissue or to activate events such as, but not limited to, endocytosis. Liposomes may contain a low or a high pH value in order to improve the delivery of the pharmaceutical formulations.
- [0078] The formation of liposomes may depend on the pharmaceutical formulation entrapped and the liposomal ingredients, the nature of the medium in which the lipid vesicles are dispersed, the effective concentration of the entrapped substance and its potential toxicity, any additional processes involved during the application and/or delivery of the vesicles, the optimal size, polydispersity and the shelf-life of the vesicles for the intended application, and the batch-to-batch reproducibility and scale up production of safe and efficient liposomal products, etc.
- [0079] In some embodiments, the alphavirus replicon vector described herein may be encapsulated by the liposome and/or it may be contained in an aqueous core that may then be encapsulated by the liposome.
- [0080] In some embodiments, the alphavirus replicon vector described herein can be formulated in a cationic oil-in-water emulsion where the emulsion particle comprises an oil core and a cationic lipid that can interact with the polynucleotide anchoring the molecule to the emulsion particle. In some embodiments, the vector described herein can be formulated in a water-in-oil emulsion comprising a continuous hydrophobic phase in which the hydrophilic phase is dispersed.
- [0081] In some embodiments, the alphavirus replicon vector described herein can be formulated in a lipid- polycation complex. As a non-limiting example, the polycation can include a cationic peptide or a polypeptide such as, but not limited to, polylysine, polyornithine and/or polyarginine and the cationic peptides.
- [0082] In some embodiments, the alphavirus replicon vector described herein can be formulated in a lipid nanoparticle (LNP).
- [0083] Lipid nanoparticle formulations typically comprise one or more lipids. In some embodiments, the lipid is a cationic or an ionizable lipid. In some embodiments, lipid nanoparticle formulations further comprise other components, including a phospholipid, a structural lipid, a quaternary amine compound, and a molecule capable of reducing particle aggregation, for example a PEG or PEG-modified lipid. In some em-

bodiments, the amount of the cationic and ionizable lipids in the lipid composition ranges from about 0.01 mol% to about 99 mol%.

- [0084] LNPs contain a pH-sensitive ionizable cationic lipid that attract anionic nucleic acids to form the core of self-assembling nanoparticle to ensure high encapsulation. At physiological pH, LNPs are neutral, eliminating a mechanism of toxicity seen with permanently cationic molecules.
- [0085] These same pH-sensitive lipids are responsible for responding to the acidic environment of the endosome and triggering the disruption of the endosome and release of the nucleic acid into the cell.
- [0086] This replicon based technology is a unique platform technology for the vaccination as a RNA can self-amplify to produce the vaccine antigen and deliver into the cellular organ. Moreover, this replicon based vaccine technology overcomes the challenges commonly associated with DNA based vaccines, such as risk of genome integration or the high doses and devices needed for administration, e.g. electroporation, and expects the higher immunogenicity with minimum dose based on the self-replication system over the mRNA technology.
- [0087] According to the present disclosure, a novel immunologically-active alphavirus replicon vector which comprises a nucleic acid encoding a cytokine protein(s)/polypeptide(s), which is useful for the treatment of a cancer and/or an inflammatory disease while minimizing toxicity.
- [0088] The invention will be described in detail with reference to the following examples, which, however, are not intended to limit the scope of the present application.

Example 1

- [0089] Each gene encoding shown below constructs 1-6 was synthesized by Integrated DNA Technologies, Inc. (<https://www.idtdna.com/pages>).
- [0090] Construct 1:
Gene encoding Mouse IL-12 sequence described below.
- [0091] [Chem.1]

ssmIL-12B	mIL12-B (p40 w/o SS)	linker	mIL12A (p35 w/o SS)
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MACPQKLTISWFAIVLLVSPLMAMWELEKDVYVVEVDWTPDAPGETVNLTC
 CDTPEEDDITWTSDQRHGVIGSGKTLTITVKEFLDAGQYTCHKGGGETLSHSHLL
 LHKKENGWSTEILKNFKNKTFKCEAPNYSGRFTCSWL VQRNMDLKFNIKSS
 SSPPDSRAVTCGMASLSAEKVTL DQRDYEKYSVSCQEDVTCPTAEETLPIELAL
 EARQQNKYENYSTSFFIRDIIKPDPPKNLQMKPLKNSQVEVSWEYPDSWSTPHS
 YFSLKFFVRIQRKKEKMKETEEGCNQKGAFLVEKTSTEVQCKGGNVVCVQAQD

RYYNSSCSKWACVPCRVRSVPGVGVPGVGRVIPVSGPARCLSQSRNLLKTDD
 MVKTAREKLKHYSCTAEDIDHEDITRDQTSTLKTCLPLELHKNESCLATRETSS
 TTRGSCLPPQKTSMMTLCLGSIYEDLKMYQTEFQAINAALQNHNHQQIILDK
 GMLVAIDELMQSLNHNGETLRQKPPVGEADPYRVKMKLCILLHAFSTRVVTIN
 RVMGYLSSA (SEQ ID NO: 1)

The linker is underlined.

[0092] Mouse IL-12B (p40)

MACPQKLTIWFAIVLLVSPLMAMWELEKDVYVVEVDWTPDAPGETVNLT
 CDTPEEDDITWTSQRHGVIGSGKTLTITVKEFLDAGQYTCHKGGETLSHSHLL
 LHKKENGWSTEILKNFKNKTFLKCEAPNYSGRFTCSWLVRNMDLKFNIKSS
 SSPDSRAVTCGMASLSAEKVTLTDQRDYEKYSVSCQEDVTCPTAEETLPIELAL
 EARQQNKYENYSTSFFIRDIKPDPPKNLQMKPLKNSQVEVSWEYPSWSTPHS
 YFSLKFFVRIQRKKEKMKETEEGCNQKGAFLVEKTSTEVQCKGGNVCVQAQD
 RYYNSSCSKWACVPCRVR (SEQ ID NO: 2)

Mouse IL-12A (35p) without signal sequence

RVIPVSGPARCLSQSRNLLKTDDMVKTAREKLKHYSCTAEDIDHEDITRDQ
 TSTLKTCLPLELHKNESCLATRETSSSTRGSCLPPQKTSMMTLCLGSIYEDLK
 MYQTEFQAINAALQNHNHQQIILDKGMLVAIDELMQSLNHNGETLRQKPPVG
 EADPYRVKMKLCILLHAFSTRVVTINRVMGYLSSA (SEQ ID NO: 3)

Linker

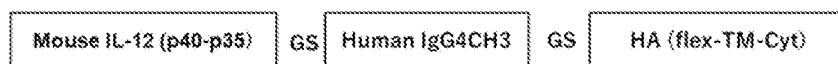
VPGVGVPGVG (SEQ ID NO: 4)

[0093] In the following constructs 2-6, "Mouse IL-12(p40-p35)" corresponds to Construct 1.

[0094] Construct 2:

Gene encoding Mouse IL-12(p40-p35) fused to human IgG4CH3 and HA (flexible domain-Transmembrane(TM)-Cytoplasmic Tail(Cyt)) with linkers.

[0095] [Chem.2]



MACPQKLTIWFAIVLLVSPLMAMWELEKDVYVVEVDWTPDAPGETVNLT
 CDTPEEDDITWTSQRHGVIGSGKTLTITVKEFLDAGQYTCHKGGETLSHSHLL
 LHKKENGWSTEILKNFKNKTFLKCEAPNYSGRFTCSWLVRNMDLKFNIKSS
 SSPDSRAVTCGMASLSAEKVTLTDQRDYEKYSVSCQEDVTCPTAEETLPIELAL
 EARQQNKYENYSTSFFIRDIKPDPPKNLQMKPLKNSQVEVSWEYPSWSTPHS
 YFSLKFFVRIQRKKEKMKETEEGCNQKGAFLVEKTSTEVQCKGGNVCVQAQD
 RYYNSSCSKWACVPCRVRSVPGVGVPGVGRVIPVSGPARCLSQSRNLLKTDD
 MVKTAREKLKHYSCTAEDIDHEDITRDQTSTLKTCLPLELHKNESCLATRETSS

TTRGSCLPPQKTSMMTLCLGSIYEDLKMYQTEFQAINAALQNHNHQQIILDK
 GMLVAIDELMQSLNHNGETLRQKPPVGEADPYRVKMKLCILLHAFSTRVVTIN
 RVMGYLSSAGSGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWE
 SNGQPENNYKTTTPVLDSGDSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHN
 HYTQKSLSLSLGKSGVKLESMGIYQILAIYSTVASSLVLLVSLGAISFWMCSN
 GSLQCRICI (SEQ ID NO: 5)

The underlined "GS" are linkers.

[0096] human IgG4 CH3: GQPREPQVYTLPPSQEEMTKNQVSLTCLVKG-
 FYPSDIAVEWE SNGQPENNYKTTTPVLDSGDSFFLYSRLTVDKSRWQEGNVF-
 SCSVMHEALHNHYTQKSLSLSLGK (SEQ ID NO: 6)

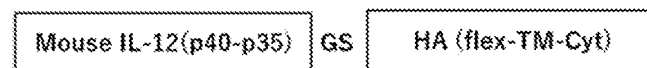
GS: linker (GGATCC)

HA(flexible-TM-Cyt): GVKLESMGIYQILAIYSTVASSLVLLVSLGAISFWMCS-
 NGSLSLQCRICI (SEQ ID NO: 7)

[0097] Construct 3:

Gene encoding Mouse IL-12(p40-p35) fused to HA (flexible-TM-Cyt) with a linker

[0098] [Chem.3]



MACPQKLTIWFIVLLVSPLMAMWELEKDVYVVEVDWTPDAPGETVNLT
 CDTPEEDDITWTSDQRHGVIGSGKTLTITVKEFLDAGQYTCHKGGETLSHSHLL
 LHKKENGWSTEILKNFKNKTKFLKCEAPNYSGRFTCSWLVRNMDLKFNIKSS
 SSPDSRAVTCGMASLSAEKVTLDRDYEKYSVSCQEDVTCPTAEETLPIELAL
 EARQQNKYENYSTSFIRDIIKPDPPKNLQMKPLKNSQVEVSWEYPSWSTPHS
 YFSLKFFVRIQRKKEKMKETEEGCNQKGAFLVEKTSTEVQCKGGNVVCVQAQD
 RYYNSSCSKWACVPCRVRSPGVGVPGVGRVIPVSGPARCLSQSRNLLKTTDD
 MVKTAREKLKHYSCTAEDIDHEDITRDQTSTLKTCLPLELHKNESCLATRETSS
 TTRGSCLPPQKTSMMTLCLGSIYEDLKMYQTEFQAINAALQNHNHQQIILDK
 GMLVAIDELMQSLNHNGETLRQKPPVGEADPYRVKMKLCILLHAFSTRVVTIN
 RVMGYLSSAGSGVKLESMGIYQILAIYSTVASSLVLLVSLGAISFWMCSN
 GSLQCRICI (SEQ ID NO: 8)

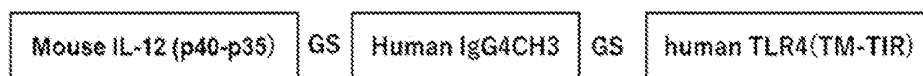
The underlined "GS" is a linker.

[0099] Construct 4:

Gene encoding mouse IL-12(p40-p35) fused to human IgG4CH3 and human
 TLR4(TM-Toll/interleukin-1 receptor domain (TIR)) with linkers

[0100]

[Chem.4]



MACPQKLTIWFAIVLLVSPLMAMWELEKDVYVVEVDWTPDAPGETVNLTC
 DTPEEDDITWTSQQRHGVIGSGKTLTITVKEFLDAGQYTCHKGGETLSHSHLLL
 HKKENGWSTEILKNFKNKTKFLKCEAPNYSGRFTCSWLVRNMDLKFNIKSSS
 SPPDSRAVTCGMASLSAEKVTLQDRDYEKYSVSCQEDVTCPTAEETLPIELALE
 ARQQNKYENYSTSFFIRDIKPDPPKNLQMKPLKNSQVEVSWEYPDSWSTPHS
 YFSLKFFVRIQRKKEKMKETEEGCNQKGAFLVEKTSTEVQCKGGNVVCVQAQD
 RYYNSSCSKWACVPCRVRSPVGVGVGVGRVIPVSGPARCLSQSRNLLKTTDD
 MVKTAREKLKHYSCTAEDIDHEDITRDQTSTLKTCLPLELHKNESCLATRETSS
 TTRGSCLPPQKTSMMTLCLGSIYEDLKMYQTEFQAINAALQNHNHQIILDK
 GMLVAIDELMQSLNHNGETLRQKPPVGEADPYRVKMKLCILLHAFSTRVVTIN
 RVMGYLSSAGSGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWE
 SNGQPENNYKTTTPVLDSGDSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHN
 HYTQKSLSLSLGKSGSKIIGVSVLSVLVSVVAVLVYKFYFHLMLLAGCIKYG
 RGENIYDAFVIYSSQDEDWVRNELVKNLEEGVPPFQLCLHYRDFIPGVAIAANI
 IHEGFHKSARKVIVVVSQHFQSRWCIFEYEIAQTWQFLSSRAGIIFIVLQKVEKTL
 LRQQVELYRLLSRNTYLEWEDSVLGRHIFWRRLRKALLDGKSWNPEGTVGTG
 CNWQEATSI (SEQ ID NO: 9)

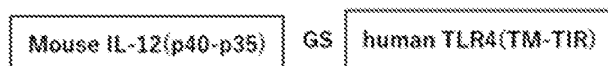
The underlined "GS" are linkers.

[0101] human TLR4(TM-TIR): KTIIGVSVLSVLVSVVAVLVYKFYFHLMLLAGCIK
 YG RGENIYDAFVIYSSQDEDWVRNELVKNLEEGVPPFQLCLHYRDFIPGVA-
 IAANIIHEGFHKSARKVIVVVSQHFQSRWCIFEYEIAQTWQFLSSRAGIIFIVLQK
 VEKTLRQQVELYRLLSRNTYLEWEDSVLGRHIFWRRLRKALLDGKSWNPEG
 TVGTGCNWQEATSI (SEQ ID NO: 10)

[0102] Construct 5:

Gene encoding mouse IL-12(p40-p35) fused to human TLR4 (TM-TIR) with linker

[0103] [Chem.5]



MACPQKLTIWFAIVLLVSPLMAMWELEKDVYVVEVDWTPDAPGETVNLTC
 CDTPEEDDITWTSQQRHGVIGSGKTLTITVKEFLDAGQYTCHKGGETLSHSHLL
 LHKKENGWSTEILKNFKNKTKFLKCEAPNYSGRFTCSWLVRNMDLKFNIKSS
 SSPPDSRAVTCGMASLSAEKVTLQDRDYEKYSVSCQEDVTCPTAEETLPIELAL
 EARQQNKYENYSTSFFIRDIKPDPPKNLQMKPLKNSQVEVSWEYPDSWSTPHS

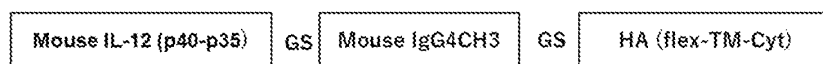
YFSLKFFVRIQRKKEKMKETEEGCNQKGAFLVEKTSTEVQCKGGNVCVQAQD
 RYYNSSCSKWACVPCRVRSPGVGVPGVGRVIPVSGPARCLSQSRNLLKTTDD
 MVKTAREKLKHYSCTAEDIDHEDITRDQTSTLKTCLPLELHKNESCLATRETSS
 TTRGSCLPPQKTSLMMTLCLGSIYEDLKMYQTEFQAINAALQNNHQQIILDK
 GMLVAIDELMQSLNHNGETLRQKPPVGEADPYRVKMKLCILLHAFSTRVVTIN
 RVMGYLSSAGSKTIIGVSVLSVLVVSVAVLVYKFYFHLMLLAGCIKYGRGEN
 IYDAFVIYSSQDEDWVRNELVKNLEEGVPPFQLCLHYRDFIPGVAIAANIIHEGF
 HKSRKVIVVVSQHFIQSRWCIFEYEAQTWQFLSSRAGIIFIVLQKVEKTLLRQQ
 VELYRLLSRNTYLEWEDSVLGRHIFWRRLRKALLDGKSWNPEGTVGTGCNW
 QEATSI (SEQ ID NO: 11)

The underlined "GS" is a linker.

[0104] Construct 6:

Gene encoding Mouse IL-12(p40-p35) fused to Mouse IgG4CH3 and HA (flexible domain-Transmembrane(TM)-Cytoplasmic Tail(Cyt)) with linkers.

[0105] [Chem.6]



MACPQKLTIWFAIVLLVSPLMAMWELEKDVYVVEVDWTPDAPGETVNLT
 CDTPEEDDITWTSDQRHGVIGSGKTLTITVKEFLDAGQYTCHKGGETLSHSHLL
 LHKKENGWSTEILKNFNKTFLKCEAPNYSGRFTCSWLVRNMDLKFNIKSS
 SSPPDSRAVTCGMASLSAEKVTLTDQRDYEKYSVSCQEDVTCPTAEETLPIELAL
 EARQQNKYENYSTSFFIRDIIKPDPPKNLQMKPLKNSQVEVSWEYPDSWSTPHS
 YFSLKFFVRIQRKKEKMKETEEGCNQKGAFLVEKTSTEVQCKGGNVCVQAQD
 RYYNSSCSKWACVPCRVRSPGVGVPGVGRVIPVSGPARCLSQSRNLLKTTDD
 MVKTAREKLKHYSCTAEDIDHEDITRDQTSTLKTCLPLELHKNESCLATRETSS
 TTRGSCLPPQKTSLMMTLCLGSIYEDLKMYQTEFQAINAALQNNHQQIILDK
 GMLVAIDELMQSLNHNGETLRQKPPVGEADPYRVKMKLCILLHAFSTRVVTIN
 RVMGYLSSAGSGRPKAPQVYTIPPPKEQMAKDKVSLTCMITNFFPEDITVEWQ
 WNGQPAENYKNTQPIMDTDGSYFVYSKLVQKSNWEAGNTFTCSVLHEGLH
 NHHTEKSLSHSPGKGSGVKLESMDGIYQILAIYSTVASSLVLLVSLGAISFWMCS
 NGSLQCRICI (SEQ ID NO: 12)

The underlined "GS" are linkers.

[0106] Mouse IgG4CH3

GRPKAPQVYTIPPPKEQMAKDKVSLTCMITNFFPEDITVEWQWNGQPAENY
 KNTQPIMDTDGSYFVYSKLVQKSNWEAGNTFTCSVLHEGLHNHHTEKSLSH
 SPG (SEQ ID NO: 13)

[0107] HA(flexible-TM-Cyt) and IgG4 are also disclosed as follows.

HA(flexible-TM-Cyt)

<https://www.ncbi.nlm.nih.gov/protein/P03452>

UniProtKB/Swiss-Prot: P03452.2

Human IgG4

<https://www.uniprot.org/uniprot/P01861>

UniProtKB: P01861

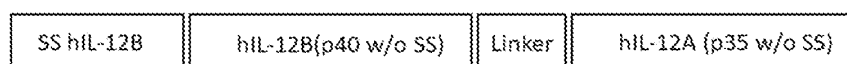
Example 2

[0108] Each gene encoding Construct 7 and 8 was prepared.

Construct 7:

Human IL-12 sequence described below.

[0109] [Chem.7]



MCHQQLVISWFSLVFLASPLVAIWELKKDVYVVVELDWYPDAPGEMVVLTC
DTPEEDGITWTLDQSSEVLGSGKTLTIQVKEFGDAGQYTCHKGGEVLSHSLLL
LHKKEDGIWSTDILKDQKEPKNKTFLRCEAKNYSGRFTCWWLTTISTDLTFSV
KSSRGSSDPQGVTCGAATLSAERVGRDNKEYEYSVECQEDSACPAAEESLPIE
VMVDAVHKLKYENYTSSFFIRDIKPDPPKNLQLKPLKNSRQVEVSWEYPDTW
STPHSYFSLTFCVQVQGKSKREKKDRVFTDKTSATVICRKNASISVRAQDRYY
SSSWSEWASVPCSVPGVGVPGVGRNLPVATPDPGMFCLHHSQNLLRAVSNM
LQKARQTLEFYPCCTSEEIDHEDITKDKTSTVEACLPLELTKNESCLNSRETSFIT
NGSCLASRKTSFMMALCLSSIEDLKMYQVEFKTMNAKLLMDPKRQIFLDQN
MLAVIDELMQALNFNSETVPQKSSLEEPDFYKTKIKLCILLHAFRIRAVTIDRV
MSYLNAS (SEQ ID NO: 14)

The first underline represents the signal sequence (SS) of human IL-12B (p40) and the second underline represents a linker.

[0110] Human IL-12B (p40)

MCHQQLVISWFSLVFLASPLVAIWELKKDVYVVVELDWYPDAPGEMVVLTC
DTPEEDGITWTLDQSSEVLGSGKTLTIQVKEFGDAGQYTCHKGGEVLSHSLLL
LHKKEDGIWSTDILKDQKEPKNKTFLRCEAKNYSGRFTCWWLTTISTDLTFSV
KSSRGSSDPQGVTCGAATLSAERVGRDNKEYEYSVECQEDSACPAAEESLPIE
VMVDAVHKLKYENYTSSFFIRDIKPDPPKNLQLKPLKNSRQVEVSWEYPDTW
STPHSYFSLTFCVQVQGKSKREKKDRVFTDKTSATVICRKNASISVRAQDRYY
SSSWSEWASVPCS (SEQ ID NO: 15)

The underlined is the signal sequence (SS) of human IL-12B(p40).

Linker(underline)

VPGVGVP (SEQ ID NO: 4)

Human IL-12A (p35) w/o signal sequence

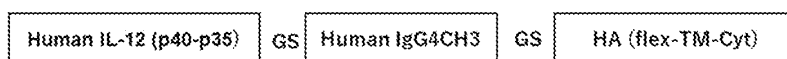
(The signal sequence "MCPARSLLLVATLVLLDHLSLA (SEQ ID NO: 16)" is deleted)

RNLVPVATPDPMFPCLLHHSQNLLRAVSNMLQKARQTLEFYPTCTSEEIDHEDIT
KDKTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIED
LKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNSETVPQKSS
LEEDFYKTKIKLCILLHAFRIRAVTIDRVMSYLNAS (SEQ ID NO: 17)

[0111] Construct 8:

Gene encoding human IL-12(p40-p35) fused to human IgG4CH3 and HA (flexible domain-Transmembrane(TM)-Cytoplasmic Tail(Cyt)) with linkers.

[0112] [Chem.8]



In the above structure, "Human IL-12(p40-p35)" corresponds to Construct 7.

MCHQQLVISWFSLVFLASPLVAIWELKKDVYVVELDWYPDAPGEMVVLTC
DTPEEDGITWTLQDSSEVLGSGKTLTIQVKEFGDAGQYTCHKGGVLSHSLLL
LHKKEDGIWSTDILKDQKEPKNKTFLRCEAKNYSGRFTCWWTITSTDLTFSV
KSSRGSSDPQGVTCGAATLSAERVGRDNKEYEYSVEQCQEDSACPAAEESLPIE
VMVDAVHKLKYENYTSSFFIRDIKPDPPKNLQLKPLKNSRQVEVSWEYPDTW
STPHSYFSLTFCVQVQGKSKREKKDRVFTDKTSATVICRKNASISVRAQDRYY
SSSWSEWASVPCSVPGVGVPGVGRNLPVATPDPMFPCLLHHSQNLLRAVSNM
LQKARQTLEFYPTCTSEEIDHEDITKDKTSTVEACLPLELTKNESCLNSRETSFIT
NGSCLASRKTSFMMALCLSSIEDLKMYQVEFKTMNAKLLMDPKRQIFLDQN
MLAVIDELMQALNFNSETVPQKSSLEEDFYKTKIKLCILLHAFRIRAVTIDRV
MSYLNASGSGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESN
GQPENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMEALHNHY
TQKSLSLGKGSGVKLESMGIYQILAIYSTVASSLVLLVSLGAISFWMCNNGS
LQCRICI (SEQ ID NO: 18)

The underlined "GS" are linkers.

[0113] Human IL-12B (p40)

UniProt: P29460

<https://www.uniprot.org/uniprot/P29460>

Human IL-12A (p35)

UniProt: P29459

<https://www.uniprot.org/uniprot/P29459>

Example 3

[0114] Preparation of vector

Schematic construct of the alphavirus replicon is shown in Figure 1.

[0115] Each of constructs 1-6 prepared in Example 1 and constructs 7 and 8 prepared in Example 2 was used as gene of interest. Nucleotides encoding the construct was cloned into the VEEV replicon vector under the control of SG promoter. The VEEV replicon plasmid encoding each fragment was created by inserting *AscI* and *SbfI* restriction sites to obtain the full-length VEEV TC-83 replicon construct. See Figure 2 for the construct with TM and Figure 3 for the construct without TM.

[0116] Nucleotide sequences of SG promoter, 5'UTR, 3'UTR and Poly A tail are as follows. RNA sequences were obtained by using those DNA sequences as template.

SG promoter: cctgaatggactacgacatagctagtcgcccaag (SEQ ID NO: 19)

5'UTR: ataggcggcgcgatgagagaagcccagaccaattacacccaaa (SEQ ID NO: 20)

3'UTR: gcgatcgcatcacagcagcaattggcaagctgctacatagaactcgcggcgattggcatgccgcct-taaaattttattttttttttttttccgaatcgattttgttttaattttc (SEQ ID NO: 21)

Poly A tail: aa (SEQ ID NO: 22)

[0117] VEEV TC-83 Replicon nsP1-4 amino acid sequence is as follows.

MEKVHVDIEEDSPFLRALQRSFPQFEVEAKQVTDNDHANARAFSHLASKLIE
TEVDPSTILDIGSAPARRMYSKHKYHCICPMRCAEDPDRLYKYATKLKNCK
EITDKELDKKMKELAAVMSDPDLETETMCLHDDDESCRYEGQVAVYQDVYAV
DGPTSLYHQANKGVRVAYWIGFDTTPFMFKNLAGAYPSYSTNWADETVLTA
RNIGLCSSDVMERSRRGMSILRKKYLKPSNNVLFVSGSTIYHEKRDLLRSWHL
SVFHLRGKQNYTCRCETIVSCDGYVVKRIAISPGLYGKPSGYAATMHREGFLC
CKVTDTLNGERVSPVCTYVPATLCDQMTGILATDVSADDAQKLLVGLNQRI
VVNGRTQRNTNTMKNYLLPVVAQAFARWAKEKEYKEDQEDERPLGLRDRQLV
MGCCWAFRRHKITSIYKRPDTQTIKVN SDFHSFVLPRIGSNTLEIGLRTRIRKM
LEEHPSPPLITAEDIQEAKCAADEAKEVREAEELRAALPPLAADFEEPTLEAD
VDLMLQEAGAGSVETPRGLIKVTSYAGEDKIGSYAVLSPQAVLKSEKLSCHPL
AEQVIVITHSGRKGRYAVEPYHGKVVVPEGHAIPVQDFQALSESATIVYNEREF
VNRYLHHIATHGGALNTDEEYYKTVKPSEHDGEYLYDIDRKQCVKKELVTGL
GLTGELVDPPFHEFAYESLRTRPAAPYQVPTIGVYGVPGSGKSGIISAVTKKD
LVVSAKKENCAEIIRDVKMKGLDVNARTVDSVLLNGCKHPVETLYIDEAFA
CHAGTLRALIAIIRPKKAVLCGDPKQCGFFNMMCLKVHFNHEICTQVFHKSISR
RCTKSVTSVVSTLFYDKRMRTTNPKETKIVIDTTGSTKPKQDDLILTCFRGWVK
QLQIDYKGNEIMTAAASQGLTRKGVYAVRYKVNENPLYAPTSEHVNVLTRT
EDRIVWKTLAGDPWIKILTAKYPGNFTATIEEWQAEHDAIMRHILERPDPTDVF
QNKANVCWAKALVPVLKTAGIDMTTEQWNTVDYFETDKAHS AEIVLNQLCV
RFFGLDLDSGLFSAPTVP LSIRNNHWDNSPSPNMYGLNKEVVRQLSRRYPQLP
RAVATGRVYDMNTGTLRNYDPRINLVPVNRRLPHALVLHHNEHPQSDFSSFV

SKLKGRTVLVVGEKLSVPGKKVDWLSDQPEATFRARLDLGIPGDVPKYDIVFI
 NVRTPYKYHHYQQCEDHAIKLSMLTKKACLHLNPGGTCTVSIGYGYADRASESI
 IGAIARQFKFSRVCKPKSSHEETEVLVFIGYDRKARTHPYKLSSTLTNIYTGS
RLHEAGCAPSYHVVRGDIATATEGVIINAANSKGQPGGGVCGALYKKFPESFD
LQPIEVGKARLVKGAAKHIIHAVGPNFNKVSEVEGDKQLAEAYESIAKIVNDN
NYKSVAIPLLSTGIFSGNKDRLTQSLNHLLTALDTTDADVAIYCRDKKWEMTL
KEAVARREAVEEICISDDSSVTEPDAELVRVHPKSSLAGRKGYSTSDGKTFSYL
EGTKFHQAADIAEINAMWPVATEANEQVCMYILGESMSSIRSKCPVEESEAS
TPPSTLPCLCIHAMTPERVQRLKASRPEQITVCSSFPLPKYRITGVQKIQCSQPIL
FSPKVPAYIHPRKYLVEPPVEETPESPAENQSTEGTPEQPALVNVDATRTRMP
EPIIIIIIIEDSISLLSDGPTHQVLQVEADIHGSPSVSSSSWSIPHASDFDVSLSIL
DTLDGASVTSGAVSAETNSYFARSMEFRARPVPAPRTVFRNPPHPAPRTRTPPL
AHSRASSRTSLVSTPPGVNRVITREELEALTPSRAPSRASRTSLVSNPPGVNRV
ITREEFEAFVAQQQXRFDAGAYIFSSDTGQGHLLQKQSVRQTVLSEVVLERTELE
 ISYAPRLDQEKEELLRKQLNPTPANRSRYQSRRVENMKAITARRILQGLGH
 YLKAEGKVECYRTLHPVPLYSSSVNRAFSSPKVAVEACNAMLKENFPTVAS
 CIIPEYDAYLDMVDGASCCLDASFCPAKLRSFPKKHSYLEPTIRSAVPSAIQNT
 LQNVLAATAKRN CNVTQMRELPLVDSAAFNVECFKKYACNNEYWETFKENPI
 RLTEENVVNYITKLKGPKAAALFAKTHNLNMLQDIPMDRFVMDLKRQVQVTP
 GTKHTEERPKVQVIQAADPLATADLCGIHRELVRRLNAVLLPNIHTLFDMSAE
 DFDAAIAEHFQPGDCVLETDIASFDKSEDDAMALTALMILEDLGVD AELLTLIE
 AAFGEISSIHLPTKTKFKFGAMMKSGMFLTLFVNTVINIVIASRVLRRERLTGSPC
 AAFIGDDNIVKGVKSDKLMADRCATWLNMEVKIIDA VVGEKAPYFCGGFILC
 DSVTGTACRVADPLKRLFKLGKPLAVDDEHDDRRRALHEESTRWNRVGILP
 ELCKAVESRYETVGTSHVMAMTTLASSVKSFSYLRGAPITLYG (SEQ ID NO:
 23)

Amino acid sequence corresponding to nsp3 is underlined.

[0118] In this example, amino acid sequence of nsp3 which is corresponding from 1330-1886 in SEQ ID NO: 23 was replaced with the sequence shown below. The underlined sequence was different from SEQ ID NO: 23.

APSYHVVRGDIATATEGVIINAANSKGQPGGGVCGALYKKFPESFDLQPIEV
 GKARLVKGAAKHIIHAVGPNFNKVSEVEGDKQLAEAYESIAKIVNDNNYKS
 VA IPLLSTGIFSGNKDRLTQSLNHLLTALDTTDADVAIYCRDKKWEMTLKEAVA
 RREAVEEICISDDSSVTEPDAELVRVHPKSSLAGRKGYSTSDGKTFSYLEGTKF
 HQAADIAEINAMWPVATEANEQVCMYILGKSMSSIRSKCPVEESEASTPPSTL
 PCLCIHAMTPERVQRLKASRPEQITVCSSFPLPKYRITGVQKIQCSQPILFSPKVP
 AYIHPRKYLVEPPVDETPESPAENQSTEGTPEQPPLITEDETRTRTPEPIIIIIIEE
 EDSISLLSDGPTHQVLQVEADIHGPPSVSSSSWSIPHASDFDVSLSILDTLEGAS

VTSGATSAETNSYFAKSMEFLARPVPAPRTVFRNPPHPAPRTRTPSLAPSRACS
RTSLVSTPPGVNRVITREELEALTPSRTPSRSVSRTSLVSNPPGVNRVITREEFEA
FVAQQQXRFDAGA (SEQ ID NO: 24)

Example 4

[0119] Preparation of alphavirus replicon particles (ARP)

10 µg of the full-length replicon plasmid for each of constructs 1-8 prepared in Example 3, 1 µg of VEEV Env expression plasmid and 1 µg of VEEV Capsid NLS mutant (or 1µg VEEV Capsid expression plasmid) were transfected into HEK293T cells. The supernatant was harvested 48-96 hours after transfection. The replicon particles were purified by using an ion exchange column. The HEK293T or Vero cells was infected with the purified particles to determine the infectious titer. The purified replicon particles were used for the treatment of therapy.

Example 5

[0120] Effects of the alphavirus replicon constructed to express construct 1

IL-12 alphavirus replicon particles prepared in Example 4 constructed to express construct 1 were evaluated. Mouse MC-38 subcutaneous syngeneic model was employed for the evaluation. MC-38 is a cell line derived from C57BL6 murine colon adenocarcinoma cells. C57BL/6 female mice (48 mice) were injected with 10^6 cells, subcutaneously, into the rear flank. All mice were randomized into 6 groups (n=8 per group) when the average tumor volume reached 75-125 mm³. Dosing was initiated within 24 hours of randomization (Day 0). Animals were dosed according to Table 1 below. The replicon Groups 3 and 4 assessed a control vector, constructed to express GFP as the gene of interest, and Groups 5 and 6 were constructed to express mouse IL-12 (Construct 1). The mice were injected with 1×10^9 infectious unit dose (IU) of the indicated replicon intratumorally (i.t.) at one day intervals (total of 8 times) or with 10mg/kg of anti-mouse PD-1 monoclonal antibody (clone RMP1-14, aPD-1 mAb) given by intraperitoneal injection (IP) biweekly for a total of 6 times. Animals were monitored, and tumors measured twice weekly for the duration of the study. The results are shown in Figure 3. Figure 3 shows mean and errors (s.e.m.) of tumor size in each group.

[0121]

[Table 1]

Group	Treatment	N	Dose Route	Dosing Frequency & Duration	Dose Level (mg/kg)	Dose Volume (mL/kg)
1	Vehicle (TNE buffer)	8	i.t.	0,2,4,6,8, 10, 12, 14	NA	50 ul for each dose (Fixed volume)
2	aPD-1 mAb	8	IP	BiWx3 weeks (Total 5 dose)	10	5
3	Control ARP (GFP)	8	i.t.	0,2,4,6,8, 10, 12, 14	10 ⁸ IU/dose	50 ul for each dose (Fixed volume)
4	Control ARP (GFP)	8	i.t.	0,2,4,6,8, 10, 12, 14	10 ⁸ IU/dose	50 ul for each dose (Fixed volume)
	aPD-1 mAb	8	IP	BiWx3 weeks (Total 6 dose)	10	5
5	ARP mIL-12	8	i.t.	0,2,4,6,8, 10, 12, 14	10 ⁸ IU/dose	50 ul for each dose (Fixed volume)
6	ARP mIL-12	8	i.t.	0,2,4,6,8, 10, 12, 14	10 ⁸ IU/dose	50 ul for each dose (Fixed volume)
	aPD-1 mAb	8	IP	BiWx3 weeks (Total 5 dose)	10	5

Results are shown in Figure 4.

[0122] The data indicated that the IL-12 alphavirus replicon and the combination of IL-12 alphavirus replicon and anti-PD-1 antibody demonstrated superior anti-tumor effect over control and anti-PD-1 single immunotherapy.

Example 6

[0123] Effects of the alphavirus replicon constructed to express construct 1

IL-12 alphavirus replicon particles (ARP) prepared in Example 4 constructed to express construct 1 were evaluated by using another cancer cell line. In this example, mouse CT-26 subcutaneous syngeneic mouse model was used. CT-26 is an N-nitroso-N-methylurethane-(NNMU) induced, undifferentiated colon carcinoma cell line. Balb/c female mice (48 mice) were injected with 5×10^5 cells subcutaneously into the rear flank. All mice were randomized into 6 groups (n=8 per group) when the average tumor volume reached 75-125 mm³. Dosing was initiated within 24 hours of randomization (Day 0). Animals were dosed according to Table 2. The alphavirus replicon particles Group 3 and 4 were constructed to express GFP as a control vector and Groups 5 and 6 were constructed to express mouse IL-12 (Construct 1). The mice were injected with 4×10^8 infectious units (IU) of the indicated replicon intratumorally (i.t.) at one day intervals (total 8 injections) or with 10mg/kg of an anti-mouse PD-1 monoclonal antibody (clone RMP1-14, aPD-1 mAb) given by intraperitoneal injection (IP), biweekly, for a total of 6 times. Animals were monitored and tumors were measured twice weekly for the duration of the study. The results are shown in Figure 5. Figure 5 shows mean and standard error of the mean (s.e.m.) of tumor size in each group.

[0124]

[Table 2]

Group	Treatment	N	Dose Route	Dosing Frequency & Duration	Dose Level (mg/kg)	Dose Volume (mL/kg)
1	Vehicle (TNE buffer)	8	i.t.	0,2,4,6,8, 10,12,14	NA	50 ul for each dose (Fixed volume)
2	aPD-1 mAb	8	IP	BIWX3 weeks (Total 6 dose)	10	5
3	Control ARP (GFP)	8	i.t.	0,2,4,6,8, 10,12,14	4×10^8 IU/dose	50 ul for each dose (Fixed volume)
4	Control ARP (GFP)	8	i.t.	0,2,4,6,8, 10,12,14	4×10^8 IU/dose	50 ul for each dose (Fixed volume)
	aPD-1 mAb	8	IP	BIWX3 weeks (Total 6 dose)	10	5
5	ARP IL-12	8	i.t.	0,2,4,6,8, 10,12,14	4×10^8 IU/dose	50 ul for each dose (Fixed volume)
6	ARP IL-12	8	i.t.	0,2,4,6,8,10,12,14	4×10^8 IU/dose	50 ul for each dose (Fixed volume)
	aPD-1 mAb	8	IP	BIWX3 weeks (Total 6 dose)	10	5

[0125] The data indicated that IL-12 alphavirus replicon and the combination of IL-12 alphavirus replicon and anti-PD-1 antibody demonstrated superior anti-tumor effect over control and anti-PD-1 single immunotherapy.

Example 7

[0126] Effects of the alphavirus replicon constructed to express construct 6

IL-12 alphavirus replicon particles prepared in Example 4 constructed to express construct 6 were evaluated. Balb/c female mice were injected with 1.75×10^8 CT-26 cells subcutaneously into the rear flank. All mice were randomized into 2 groups (n=8 per group) when the average tumor volume reached 50-100 mm³. Dosing was initiated within 24 hours of randomization (Day 0). Animals were immunized with alphavirus replicon particles prepared in Example 4 expressing construct 6. The mice were injected with 4×10^8 infectious units (IU) of the indicated replicon intratumorally (i.t.) at one day intervals (total 8 injections). Animals were monitored and tumors were measured twice weekly for the duration of the study. The results are shown in Figure 6a. Figure 6a shows mean and standard error of the mean (s.e.m.) of tumor size in each group. Figure 6b show tumor size of individual mouse (Left, vehicle, right, IL-12_HA). The cross symbols represent when each individual mouse was sacrificed due to large size of tumor.

[0127] The data indicates that IL-12 alphavirus replicon demonstrated potent anti-tumor effect.

Example 8

[0128] Effects of the alphavirus replicon constructed to express construct 1

IL-12 alphavirus replicon particles prepared in Example 4 constructed to express construct 1 were evaluated. C57BL6 female mice were injected with MC-38 cells subcutaneously into the rear flank. All mice were randomized into 4 groups (n=8 per

group) when the average tumor volume reached 50-100 mm³. Dosing was initiated within 24 hours of randomization (Day 0). The alphavirus replicon particles were constructed to express GFP as a control vector or to express mouse IL-12 (Construct 1). The mice were injected with 2.1×10^8 infectious units (IU) of the indicated replicon intratumorally (i.t.) at one day intervals (total 4 injections) on Day 0, 2, 4 and 6 or with 10mg/kg of an anti-mouse PD-1 monoclonal antibody (clone RMP1-14, aPD-1 mAb) given by intraperitoneal injection (IP) on day 0 and 6. Animals were sacrificed on Day 9 and tumors collected and analyzed tumor-infiltrating lymphocytes (TIL) by FACS. The results are shown in Figure 7. Figure 7a shows M1 and M2 macrophage populations in macrophages contained in the TIL. Among the CD45⁺CD11b⁺F4/80⁺ cells in the viable cells in TIL, M1 and M2 populations were identified by the markers of CD206 and IA/EA. Figure 7b shows percentage of T regulatory cell (Treg) population in CD4⁺T cells contained in the TIL. Among the CD45⁺CD3⁺CD4⁺ cells in the TIL, the percentage of FoxP3⁺ cells was determined as Treg population. The mice immunized with replicon expressing mouse IL-12 shows that 1) population of M1 macrophage was much higher compared to population of M2 macrophage and 2) the population of Treg cells in TIL was reduced.

[0129] Although not intended to be constrained by theory, the present inventor considers that the alphavirus replicon constructed to express IL-12 can provide potent anti-tumor effect by increasing the M1 macrophage population over the M2 macrophage and also by reducing the Treg population in the TIL, in addition to the other known effects exerted by IL-12.

Example 9

[0130] Effects of the alphavirus replicon constructed to express construct 1

C57BL6 female mice were injected with B16F10 cells subcutaneously into the rear flank. All mice were randomized into 6 groups (n=8 per group) when the average tumor volume reached 50-100 mm³. B16F10 is a mouse cell line derived from melanoma. Dosing was initiated within 24 hours of randomization (Day 0). The alphavirus replicon particles were constructed to express GFP as a control vector or express mouse IL-12 (construct 1). The mice were injected with 1×10^9 infectious units (IU) of the indicated replicon intratumorally (i.t.) at one day intervals (total 8 injections) or with 10mg/kg of an anti-mouse PD-1 monoclonal antibody (clone RMP1-14, aPD-1 mAb) given by intraperitoneal injection (IP) (total 6 injections). Animals were monitored and tumors were measured twice weekly for the duration of the study. The results are shown in Figure 8. Figure 8 shows mean and standard error of the mean (s.e.m.) of tumor size in each group.

[0131] The data indicated that IL-12 alphavirus replicon and the combination of IL-12 al-

phavirus replicon and anti-PD-1 antibody demonstrated superior anti-tumor effect over control and anti-PD-1 single immunotherapy.