Compositions and vaccines for treating and/or preventing viral infections, including coronavirus infections, and methods of using the same

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefits under 35 USC § 119 to U.S. provisional Application 62/994,057, filed March 24,2020, the entire contents of which are incorporated herein by reference in their entirety.

BACKGROUND

[0002] Outbreaks of severe acute respiratory syndrome (SARS, 2002–2004 [Ksiazek et al., 2003; Drosten et al., 2003]) and Middle East respiratory syndrome (MERS, 2012-current [Zaki et al., 2012]) in the last two decades are a significant threat to global public health.

[0003] Respiratory syndromes caused by coronaviruses (CoVs) that are transmitted from person-to-person via close contact, result in high morbidity and mortality in infected individuals. Although SARS and MERS initially present as mild, influenza-like illnesses with fever, dyspnea, and cough, progression to more severe symptoms is characterized by an atypical interstitial pneumonia and diffuse alveolar damage. Both SARS-CoV and MERS-CoV are capable of causing acute respiratory distress syndrome (ARDS), the most severe form of acute lung injury where alveolar inflammation, pneumonia, and hypoxic lung conditions lead to respiratory failure, multiple organ disease, and death in 50% of ARDS patients [Lew et al., 2003].

[0004] Over the decades, research effort has gone into developing antiviral drugs and these are directed largely at nonstructural proteins involved in viral replication and assembly since many of these proteins are highly conserved and can have broad spectrum antiviral activity. Structural and accessory proteins tend to be less conserved and are susceptible to a high mutation rate allowing escape of mutant viruses from the effect of the antiviral drugs. Examples of successful antiviral drugs include Oseltamivir (Tamiflu) and Zanamivir (Relenza), both neuraminidase inhibitors used to treat and prevent influenza A and influenza B (flu), and Ribavirin which is a guanosine analog with *in vitro* activity against a large number of highly lethal emerging viruses.

[0005] Monoclonal antibodies (mAbs) have potential utility in combating highly pathogenic viral diseases, by prophylactic and therapeutic neutralization of structural proteins on virions. Unfortunately, these mAbs have to be directed at the surface exposed structural proteins and these tend to mutate at a high frequency. Hence, it was found that mAbs that were effective against CoV infection in animal models targeted the highly variable Spike glycoprotein, but these mAbs lack cross-protection against other related CoVs [Agnihothram et al., 2014]. Pre-clinical and clinical mAb formulations may include a cocktail of multiple mAbs that target different epitopes to ensure that viruses cannot escape neutralization.

[0006] Vaccines have long been considered the gold standard for infectious disease prevention and eradication targeted at human populations as well as conferring the benefits of longlived immune protection for the individual. Unfortunately, in human infections of highly pathogenic coronaviruses SARS-CoV and MERS-CoV, the most vulnerable populations are patients over the age of 65 and patients with comorbidities, and design of efficacious vaccines for patients in these groups is difficult. Vaccine formulations that have been developed against SARS-CoV not only fail to protect animal models of aged populations, but also result in immunopathology in younger populations, where SARS disease is enhanced in vaccinated groups that are subsequently challenged with SARS-CoV [Bolles et al., 2011; Sheahan et al., 2011].

[0007] Due to the diversity of Bat-CoVs, it seems unlikely that current therapeutic strategies targeting specific SARS-CoV or MERS-CoV antigens will be efficacious against future coronaviruses that emerge into the human population. Vaccines formulated against the SARS-CoV epidemic antigens do not offer effective protection against SARS-like Bat-CoVs that are currently circulating in bat populations [Menachery et al., 2015].

[0008] Accordingly, new compositions and methods are needed for effective stimulation of antiviral immunity. The present invention satisfies these needs.

SUMMARY OF THE INVENTION

[0009] The present disclosure is directed to compositions comprising: (a) a vector comprising a plasmid that encodes at least one viral antigen; and (b) a vector comprising a CD1d-recognized antigen; and (c) at least one pharmaceutically acceptable carrier, wherein at least one of vector

(a) and vector (b) is an intact, bacterially-derived minicell or killed bacterial cell.

[0010] In another aspect, vector (a) is a first intact, bacterially derived minicell or killed bacterial cell, and vector (b) is a second intact, bacterially derived minicell or killed bacterial cell. In yet another aspect, vector (a) and vector (b) are the same intact, bacterially derived minicell or killed bacterial cell, comprising the CD1d-recognized antigen and the plasmid that encodes at least one viral antigen.

[0011] In one embodiment of the compositions described herein, the one of vector (a) and vector (b) is not an intact, bacterially derivd minicell or killed bacterial cell and the other of vector (a) and vector (b) is an intact, bacterially derived minicell or killed bacterial cell.

[0012] In all of the compositions described herein, the viral antigen can comprise or characterizes a virus selected from the group consisting of an Alphacoronavirus; a Colacovirus such as Bat coronavirus CDPHE15; a Decacovirus such as Bat coronavirus HKU10 or Rhinolophus ferrumequinum alphacoronavirus HuB-2013; a Duvinacovirus such as Human coronavirus 229E; a Luchacovirus such as Lucheng Rn rat coronavirus; a Minacovirus such as a Ferret coronavirus or Mink coronavirus 1; a Minunacovirus such as Miniopterus bat coronavirus 1 or Miniopterus bat coronavirus HKU8; a Myotacovirus such as Myotis ricketti alphacoronavirus Sax-2011; a nyctacovirus such as Nyctalus velutinus alphacoronavirus SC-2013; a Pedacovirus such as Porcine epidemic diarrhea virus or Scotophilus bat coronavirus 512; a Rhinacovirus such as Rhinolophus bat coronavirus HKU2; a Setracovirus such as Human coronavirus NL63 or NL63-related bat coronavirus strain BtKYNL63-9b; a Tegacovirus such as Alphacoronavirus 1; a Betacoronavirus; a Embecovirus such as Betacoronavirus 1, Human coronavirus OC43, China Rattus coronavirus HKU24, Human coronavirus HKU1 or Murine coronavirus; a Hibecovirus such as Bat Hp-betacoronavirus Zhejiang2013; a Merbecovirus such as Hedgehog coronavirus 1, Middle East respiratory syndrome-related coronavirus (MERS-CoV), Pipistrellus bat coronavirus HKU5 or Tylonycteris bat coronavirus HKU4; a Nobecovirus such as Rousettus bat coronavirus GCCDC1 or Rousettus bat coronavirus HKU9, a Sarbecovirus such as a Severe acute respiratory syndrome-related coronavirus, Severe acute respiratory syndrome coronavirus (SARS-CoV) or Severe acute respiratory syndrome coronavirus 2 (SARS-

CoV-2, COVID-19); a Deltacoronavirus; an Andecovirus such as Wigeon coronavirus HKU20; a Buldecovirus such as Bulbul coronavirus HKU11, Porcine coronavirus HKU15, Munia coronavirus HKU13 or White-eye coronavirus HKU16; a Herdecovirus such as Night heron coronavirus HKU19; a Moordecovirus such as Common moorhen coronavirus HKU21; a Gammacoronavirus; a Cegacovirus such as Beluga whale coronavirus SW1; and an Igacovirus such as Avian coronavirus.

[0013] In another aspect, the viral antigen can be encoded by a polynucleotide comprising the sequence of SARS-CoV-2, or a polynucleotide having at least 80% sequence identity to the polynucleotide comprising the sequence of SARS-CoV-2. In yet another aspect, the viral antigen can comprise or is characteristic of human coronavirus 229E, human coronavirus OC43, SARS-CoV, HCoV NL63, HKU1, MERS-CoV, or SARS-CoV-2. Further, the viral antigen can comprise or is characteristic of SARS-CoV-2.

[0014] In another aspect, the plasmid encodes at least one of spike (S) protein, nucleocapsid (N) protein, membrane (M) protein, and envelope (E) protein of of SARS-CoV-2. Further, the plasmid can encode the spike (S) protein, nucleocapsid (N) protein, membrane (M) protein, and the envelope (E) protein.

[0015] In one embodiment, the CD1d-recognized antigen comprises a glycosphingolipid. For example, the CD1d-recognized antigen can be selected from the group consisting of α-galactosylceramide (α-GalCer), C-glycosidific form of α-galactosylceramide (α-C-GalCer), 12 carbon acyl form of galactosylceramide (β-GalCer), β-D-glucopyranosylceramide (β-GlcCer), l,2-Diacyl-3-O-galactosyl-sn-glycerol (BbGL-II), diacylglycerol containing glycolipids (Glc-DAG-s2), ganglioside (GD3), gangliotriaosylceramide (Gg3Cer), glycosylphosphatidylinositol (GPI), α-glucuronosylceramide (GSL-1 or GSL-4), isoglobotrihexosylceramide (iGb3), lipophosphoglycan(LPG), lyosphosphatidylcholine (LPC), α-galactosylceramide analog (OCH), threitolceramide, and a derivative of any thereof.

[0016] In another aspect, the CD1d-recognized antigen comprises α-GalCer. In addition, the CD1d-recognized antigen can comprise a synthetic α-GalCer analog. For example, the CD1d-recognized antigen can comprise a synthetic α-GalCer analog selected from 6′-deoxy-6′-

acetamide α-GalCer (PBS57), napthylurea α-GalCer (NU-α-GC), NC-α-GalCer, 4ClPhC-α-GalCer, PyrC-α-GalCer, α-carba-GalCer, carba-α-D-galactose α-GalCer analog (RCAI-56), 1-deoxy-neo-inositol α-GalCer analog (RCAI-59), 1-O-methylated α-GalCer analog (RCAI-92), and HS44 aminocyclitol ceramide.

[0017] In one aspect, the CD1d-recognized antigen is an IFNγ agonist.

[0018] The compositions described herein can be formulated for any pharmaceutically acceptable use. Examples of pharmaceutically acceptable formulations include but are not limited to oral administration, injection, nasal administration, pulmonary administration, or topical administration.

[0019] The disclosure also encompasses methods of treating and/or vaccinating against a viral infection, comprising administering to a subject in need a composition described herein.

[0020] In one aspect, the subject is suffering from or at risk of developing lymphopenia. In another aspect, the subject is deemed at risk for severe illness and/or serious complications from the viral infection. For example, an "elderly" subject at higher risk for severe illness and/or serious complications from the viral infection is about age 50 or older, about age 55 or older, about age 60 or older, or about age 65 or older.

[0021] In another aspect of the methods described herein, the subject suffers from one or more pre-existing conditions selected from the group consisting of diabetes, asthma, a respiratory disorder, high blood pressure, and heart disease. In yet another aspect, the subject is immunocompromised. For example, the subject can be immunocompromised due to AIDS, cancer, a cancer treatment, hepatitis, an auto-immune disease, steroid receiving, immunosenescence, or any combination thereof.

[0022] In one embodiment, administration of a composition described herein increases the chance of survival following exposure to a coronavirus. For example, the chance of survival can be increased by about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or about 100%, as measured using any clinically recognized technique.

[0023] In yet another aspect, administration of a composition described herein reduces the risk of transmission of coronavirus. For example, the reduction in risk of transmission can be by about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or about 100%, as measured using any clinically recognized technique.

[0024] In all of the methods described herein, the administration step can be via any pharmaceutically acceptable methods.

[0025] In another aspect, the subject can be exposed to or is anticipated to be exposed to an individual who is contagious for a coronavirus. In addition, the individual who is contagious for a coronavirus can have one or more symptoms selected from the group consisting of fever, cough, shortness of breath, diarrhea, sneezing, runny nose, and sore throat.

[0026] In one embodiment, the subject of the methods described herein is a healthcare worker, aged 60 years or older, frequent traveler, military personnel, caregiver, or a subject with a preexisting condition that results in increased risk of mortality with infection.

[0027] In another aspect, the method further comprises administering one or more antiviral drugs. For example, the one or more antiviral drugs can be selected from the group consisting of chloroquine, darunavir, galidesivir, interferon beta, lopinavir, ritonavir, remdesivir, and triazavirin.

[0028] In the methods of the disclosure, the CD1d- recognized antigen induces a Th1 cytokine response in the subject. For example, the cytokine can comprise IFNγ.

[0029] In another aspect, a first minicell comprising the CD1d- recognized antigen and a second minicell comprising the plasmid encoding at least one viral antigen are administered to the subject simultaneously. In yet another aspect, a first minicell comprising the CD1d- recognized antigen and a second minicell comprising the plasmid encoding at least one viral antigen are administered to the subject sequentially. Alternatively, the disclosure encompasses a method wherein first minicells comprising the CD1d- recognized antigen and second minicells comprising the plasmid encoding at least one viral antigen are administered to the subject repeatedly.

[0030] In the methods described herein, first minicells comprising the CD1d- recognized antigen and second minicells comprising the plasmid encoding at least one viral antigen can be administered to the subject at least once a week, twice a week, three times per week, or four times per week.

[0031] Both the foregoing summary and the following description of the drawings and detailed description are exemplary and explanatory. They are intended to provide further details of the invention, but are not to be construed as limiting. Other objects, advantages, and novel features will be readily apparent to those skilled in the art from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0032] FIG. 1 is a graphical depiction of composition comprising a combination of an EnGeneIC Dream Vehicle (EDV™), *i.e.*, an intact, bacterially derived minicell, loaded with the CD1d-restricted iNKT cell antigen α-galactosylceramide (α-GalCer), which stimulates IFNγ, and a bacterial minicell loaded with a plasmid encoding viral antigens.

[0033] FIG. 2 shows peripheral blood mononuclear cells (PBMCs) from patient 1-CB04-1 (72 year-old male) with end-stage hepatocellular carcinoma, showed an elevation in CD8+ cytotoxic T cells (**Fig. 2A**), NK cells (**Fig. 2B**), NKT cells (**Fig. 2C**) and iNKT cells (**Fig. 2D**) by cycle 2 and 3 following treatment with EGFR-targeted, PNU-packaged intact, bacterially derived minicells + α-galactosyl ceramide packaged intact, bacterially derived minicells. It is to be noted that the patient is elderly and severely immune-compromised. PNU is PNU-159682, which is a morpholinyl anthracycline derivative.

[0034] FIG. 3 shows PBMCs from a 45 year-old female with end-stage colorectal cancer, showing activation of key immune cells. The patient's CD8+ effector cytotoxic T cells (CD45RA+ CCR7-) increased significantly by cycles 2 and 3 (**Fig. 3A**). Similarly, the subject's PBMCs showed an increase in NK cells (**Fig. 3B**) by cycles 2 and 3. Interestingly, ELISA analysis of the patient's serum, 3 hrs post each intact, bacterially derived minicell dose, showed a spike in IFNγ (**Fig. 3C**) which would occur if the α-galactosyl ceramide were effectively

presented by antigen presenting cells (APCs) to the iNKT cells which would then trigger off the release of IFNγ, a critical mediator in fighting viral infections.

[0035] FIG. 4 shows the white blood cell counts (average of 9 patients) at pre-dose and 3 hrs post dose. 8 out of the 9 patients were elderly and all were severely immune-compromised with Stage IV pancreatic cancer and all having failed all lines of conventional therapy. Yet, interestingly, 3 hrs post dose there was a significant increase in white blood cells (WBC) and this occured at every dose after dose 2, suggesting that the early doses of intact, bacterially derived minicells recruit fresh monocytes from the bone marrow following activation signals from the macrophages, dendritic cells and NK cells and by dose 3 they are sufficiently activated and matured to result in proliferation.

[0036] FIG. 5 shows PBMCs from a 45 year-old female with end-stage colorectal cancer, showing activation of key immune cells. The patient's CD8+ effector cytotoxic T cells (CD45RA+ CCR7-) increased significantly by cycles 2 and 3 (**Fig. 5A**). Similarly, the patient's PBMCs showed an increase in NK cells (**Fig. 5B**) by cycles 2 and 3. Interestingly, ELISA analysis of the patient's serum, 3 hrs post each intact, bacterially derived minicell (EDV™) dose, showed a spike in IFNγ (**Fig. 5C**). This would occur if the α-galactosyl ceramide were effectively presented by antigen presenting cells to the iNKT cells, which then would trigger the release of IFNγ, a critical mediator in fighting viral infections.

[0037] FIG. 6A shows onstruction of the expression cassette; **FIG 6B** shows that bacterial minicells loaded with Covid- αGC ($EDV_{Covid\text{-}\alpha GC}$) were able to successfully deliver αGC to JAWSII cells. **FIG 6**C shows Western blot analysis demonstrating the successful incorporation of the spike protein into the EDV membrane.

[0038] FIG 7A shows the results of measuring serum IgG titre at 1 week following administration of various bacterial minicell (EDV) formulations to mice, where it was found that intramuscular (IM) injection of bacterial minicells loaded with Covid- αGC ($EDV_{Covid\text{-}\alpha GC}$), produced the highest S-protein specific IgG titre as compared to subcutaneous (SC) injection. **FIG 7B** shows a bar graph of total AUC for IgG at 1 week following administration of various bacterial minicell (EDV) formulations to mice, where AUG analysis showed the highest AUG in

IM injected mice. Figures 7C-G show that mice injected with $EDV_{Covid-\alpha GC}$ through IM had the highest levels of serum IFNα (**FIG 7C**), IFNγ (**FIG 7D**), IL12 (**FIG 7E**), IL6 (**FIG 7F**) and TNFα (**FIG 7G**) 8h post-injection.

[0039] FIGs 8A and 8B show that mice injected with bacterial minicells loaded with Covid-αGC ($EDV_{Covid-\alpha GC}$) had the highest levels of serum IgM (**FIG 8A**) IgG (**FIG 8B**) at 4 weeks (boost on day 21) post-initial injection. **FIG 8C** shows an ELISA analysis demonstrating that bone-marrow derived B cells were able to produce spike protein specific IgG *ex vivo* when incubated with spike protein. **FIG 8D** shows a neutralising antibody analysis at 4 weeks post-initial dose. **FIG 8E** shows an IgG subtype analysis of the EDVCovid and $EDV_{Covid-\alpha GC}$.

[0040] FIG 9A shows a FACS analysis of mouse splenocytes demonstrating that $EDV_{Covid-\alpha GC}$ injected mice had the highest amount of antigen-specific memory CD137+CD69+ cytotoxic T-cell at 4 weeks (1 boost at day 21) post-initial injection, e.g., there were significantly high number of CD137+ CD69+ population within the cytotoxic T-cell population in the $EDV_{Covid-\alpha GC}$ treated mice as compared to all other treatment groups. **FIG 9B** shows an AIMS assay demonstrating that bacterial minicells loaded with Covid-αGC ($EDV_{Covid-\alpha GC}$) treated cytotoxic T-cells from the spleen expressed viral antigen-specific CD69 single positive cytotoxic T-cells following stimulation of the spike protein in a similar fashion to that of stimulated using PHA (e.g., when exposed to the spike protein *ex vivo*). Splenocytes from EDV_{Covid} treated mice exhibited a similar characteristic but to a less degree. This was not found in other treatment groups.

[0041] FIG. 10A shows a pUC57-Kan construct, with 5' KpnI and 3'SalI sites of construct insertion. **FIG 10B** shows *in vitro* synthesis of a synthetic modified-lactamase promoter. Nucleotide sequences of the native P-lactamase promoter (A1) and the synthetic, modified version (B2). The -35 and -10 regions, the +1 transcriptional start site, the ribosome binding site (RBS), and the ATG translation start codon are shown. The newly introduced EcoRI, XhoI, NdeI, and BamHI restriction enzyme sites are also indicated.

[0042] FIGS. 11A and **11B** show that treatment with JAWSII cells with $EDV_{Covid-\alpha GC}$ resulted in the expression of αGC through CD1d ligand onto the surface of the cells. The level of expression

was better than JAWSII cells treated with free αGC alone (**FIG. 11A**). Western blot analysis of $EDV_{Covid\text{-}\alpha GC}$ showed that the spike protein is incorporated into the structure EDVs (**FIG. 11B**).

[**0043**] **FIGs 12A-E** show a detailed ELISA analysis of initial interferon response in mouse serum following I.M. injections of EDV, $EDV_{\alpha GC}$, $EDV_{Control}$, $EDV_{Control\text{-}\alpha GC}$, EDV_{Covid}, and $EDV_{Covid\text{-}\alpha GC}$. The results demonstrated that the early interferon response in mice was predominantly induced by the administration of αGC carried by EDVs with or without an accompanying antigen-specific plasmid (e.g., administration of $EDV_{\alpha GC}$ with or without the combination of an $EDV_{Plasmid}$), as IM injections resulted in a dramatic increase in IFNα, IFNγ, TNFα, IL12, IL6 8h post initial treatment. See **FIG. 12A** (serum IFNα concentration 8h post-IM injection); **FIG. 12B** (serum IFNγ concentration 8h post-IM injection); **FIG. 12C** (IL6 serum concentration 8h post-IM injection); **FIG. 12D** (serum TNFα, concentration 8h post-IM injection); and **FIG. 12E** (IL12p40 serum concentration 8h post-IM injection).

[**0044**] **FIGs 13A** and **13B** show a FACS analysis of extracted mouse spleen showed that there is a high percentage of CD3+ CD8+ cytotoxic T-cells in the $EDV_{Covid\text{-}\alpha GC}$ treated mice (**FIG. 13A**). The stimulation of the splenocytes with Covid-19 spike protein induced the number of CD69+ CD137+ cells within the cytotoxic T-cell population at a greater extend compared to that of stimulated using PHA (+ve control) (**FIG 13B**).

[**0045**] **FIGs 14A, B,** and **C** show that high levels of spike protein specific IgG were found in the serum of the mouse treated with $EDV_{Covid\text{-}\alpha GC}$ 4 weeks post-initial injection (**FIG 14A**); this was also found for spike protein specific IgM (**FIG 14B**). Interestingly, while the serum of mice treated with $EDV_{Covid\text{-}\alpha GC}$ exhibited the highest degree of inhibition of the binding of spike protein to hACE receptor protein, the treatment of containing $EDV_{\alpha GC}$ also demonstrated ability to prevent spike protein binding (**FIG 14C**).

[**0046**] **FIG 15A** and **15B** show the results of an experiment where B-cells extracted from the bone marrow of $EDV_{Covid\text{-}\alpha GC}$ treated mice at a 4 week time point secreted the highest level of spike protein specific IgG (**FIG 15A**) and IgM (**FIG 15B**) as detected by a modified version of ELISA.

DETAILED DESCRIPTION

I. Overview

[0047] The present disclosure is directed to novel compositions useful in treating and/or vaccinating subjects against viral infections, including but not limited to coronavirus infections. The compositions comprise a combination of (a) a vector comprising a plasmid that encodes at least one viral antigen from the virus to be treated/vaccinated against; and (b) a vector comprising a CD1d-recognized antigen, wherein at least one of the two vectors is an intact, bacterially-derived minicell or killed bacterial cell, and wherein the two vectors are present in at least one pharmaceutically acceptable carrier. An exemplary CD1d-recognized antigen is α-galactosylceramide (α-GalCer), which stimulates IFNγ, which is critical to viral immunity. In another aspect, both of the two vectors are intact, bacterially-derived minicells or killed bacterial cells, including either two separate bacterially-derived minicells or killed bacterial cells or together in a single bacterially-derived minicell or killed bacterial cell. In another aspect, the vector or intact, bacterially derived minicell can comprise all 4 major structural proteins of a coronavirus, or antigenic fragments thereof, e.g., the spike (S) protein, nucleocapsid (N) protein, membrane (M) protein, and the envelope (E) protein.

[0048] In another aspect, one or the other (but not both) of the plasmid payload and the CD1d-recognized payload, as described above, can be administered via a vector that is not an intact, bacterially derived minicell or a killed bacterial cell. Exemplary of such non-minicell vectors are liposomes, polymeric vectors, reconstituted virus envelopes (virosomes), and immune stimulating complexes (ISCOMs). For instance, see Bungener et al. (2002), Kersten et al. (2003), Daemen et al. (2005), Chen et al. (2012) and Yue et al. (2013). *See*
https://www.ncbi.nlm.nih.gov/pubmed/12428908 (Bungener);
https://www.meta.org/papers/liposomes-and-iscoms/12547602 (Kersten);
https://www.ncbi.nlm.nih.gov/pubmed/15560951 (Daemen);
https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0039039 (Chen);
https://pubs.rsc.org/en/content/articlelanding/2013/bm/c2bm00030j#!divAbstract (Yue).

[0049] A mature SARS-CoV-2 virus has four structural proteins, namely, envelope, membrane,

nucleocapsid, and spike. It is believed that all these proteins may serve as antigens to stimulate neutralizing antibodies and increase CD4+/ CD8+ T-cell responses.

[0050] The composition can be administered via any pharmaceutically acceptable method, such as but not limited to injection (parenteral, intramuscular, intravenous, intraportal, intrahepatic, peritoneal, subcutaneous, intratumoral, or intradermal administration), oral administration, application of the formulation to a body cavity, inhalation, insufflation, nasal administration, pulmonary administration, or any combination of routes also may be employed.

[0051] The compositions can be administered to subjects at risk of viral infection as a vaccine, or the compositions can be administered as a theraepeutic to a subject who is suffering from a viral infection.

[0052] It has been shown that in the end-stage cancer patients who are highly immuno-compromised, intact bacterial minicell therapy (also referred to as "EnGeneIC Dream Vector™" or EDV™) results in: (1) activation and proliferation of CD8+ T cells, Macrophages, NK cells, Dendritic cells, and iNKT cells. This result is exactly what is desired in the CoV-2 therapeutic/vaccine.

[0053] According to one aspect, the present disclosure provides for use of recombinant, intact bacterial minicells in the preparation of a composition, the minicells comprising a plasmid encoding viral proteins for use in a method of treating and/or preventing a disease by administration of the composition to a virally infected person, or a person at risk of viral infection. The disease treated in this context is a viral infection.

[0054] The present disclosure is directed to compositions useful in treating and/or vaccinating against viral infections. An exemplary viral infection to be treated or vaccinated against includes coronaviruses, including but not limited to the coronavirus SARS-CoV-2, infection which causes Coronavirus Disease 2019 (COVID-19). Thus, by way of example, this description describes the development of an intact, bacterially derived minicell -based therapeutic and/or vaccine against SARS-CoV-2 coronavirus infections in humans.

[0055] In yet another aspect, encompassed is a composition comprising a combination of (a) an

intact, bacterially-derived minicell comprising at least one viral antigen from SARS-CoV-2 and (b) an intact, bacterially-derived minicell comprising the CD1d-recognized antigen α-GalCer. Further, the intact, bacterially-derived minicell comprising at least one viral antigen from SARS-CoV-2 can comprise all four of the constituent proteins of SARS-CoV-2.

[0056] The major areas being currently explored for the treatment/vaccines against SARS-CoV2 include: (1) antiviral drugs (e.g. Gilead Sciences; nucleotide analog Remdesivir); (2) Cocktail monoclonal antibodies (e.g. Regeneron); and (3) Attenuated viruses as vaccines to stimulate a potent antibody response to the viral proteins. Each of these strategies face difficulties but most importantly, none of these approaches is able to solve the problem of lymphopenia in the elderly and immune-compromised patients to be able to overcome the viral infection. In the absence of a robust immune system, this population of patients will still be most vulnerable and likely to succumb to the disease.

[0057] In prior EnGeneIC disclosures, the use of plasmid-packaged minicells in the treatment of neoplastic diseases has been demonstrated, where the primary function of the plasmid was to encode siRNAs or miRNAs to silence genes in cancer cells that were responsible for cell proliferation or drug resistance.

[0058] In the present disclosure, the function of the plasmid-packaged minicell component of the full composition (which includes a CD1d-recognized antigen such as an α-GC-packaged minicell) has a novel function not shown or described before. Specifically, the plasmid is used to encode viral proteins in the parent bacterial cell and the proteins segregate into the minicell at the time of asymmetric cell division. These viral proteins are delivered into the lysosomes of antigen processing and presenting cells (APCs) such as macrophages and dendritic cells. Post-antigen processing, the viral protein epitopes are displayed on the APC surface via MHC Class I and Class II molecules, which is predicted to result in a potent antibody response to the viral proteins. Additionally, the plasmid itself being a double stranded nucleic acid is recognized by nucleic acid sensing proteins in the APC and this then triggers the secretion of Type I interferons (IFNα and IFNβ).

[0059] This unique dual trigger of antibody response to viral proteins and Type I interferon

response results in not only mopping up viral particles released from infected cells but also results in cells of the immune system being able to recognize virally infected cells and kill them. This dual trigger has not been described before, particularly the ability of Type I interferon to trigger a heretofore uncharacterized mechanism by which virally infected cells can be recognized and killed.

[0060] In the present disclosure, post-presentation of α-GC/CD1d to the iNKT cell receptor, the trigger of IFNγ is the key to augmenting anti-viral immunity. The exact mechanism of action is unknown, but IFNγ is critical in identifying and destroying virally infected cells.

[0061] In the United States, several clinical trials have been conducted where anticancer-agent loaded intact, bacterially derived minicells, and microRNA mimic loaded intact, bacterially derived minicells, have been administered to humans in methods of treating cancer. *See, e.g.*, ClinicalTrials.gov Identifier Nos. NCT02766699, NCT02687386, and NCT02369198. In addition, in Australia a bacterial minicell loaded with α-GC is being administered to patients in a Phase IIa clinical trial in end-stage cancer patients. The results have shown that intact, bacterially derived minicells loaded with alpha-GC are a potent stimulator of IFN-γ. *See* Trial ID No. ACTRN12619000385145. Thus, *in vivo* efficacy in humans of intact, bacterially derived minicells loaded with a CD1d-recognized antigen has been shown, and additionally efficacy in humans of intact, bacterially derived minicells loaded with a target compound (e.g., an anticancer compound instead of a viral antigen) has been shown.

[0062] Additionally, the disclosed composition has another critical function that allows elderly and immune-compromised patients to recover from lymphopenia (rapid depletion of lymphocytes including macrophages, dendritic cells, NK cells and CD8+ T cells), which is the main reason viruses like SARS-CoV-2 takes over in these patients and they end up with with Respiratory distress syndrome and eventual death. Specifically, the minicells of the composition themselves activate the macrophages via recognition of pathogen associated molecular patterns (PAMPs) like LPS. This provides the activation, maturation and proliferation signals to the resting monocytes in the bone marrow resulting in a significant increase in activated macrophages and dendritic cells. Additionally, the minicell-associated PAMPs also activate NK

cells and these are also provoked into proliferation. Further still, the activated macrophages and dendritic cells home into the infected area and engulf the apoptotic virally infected cells. They then migrate into the draining lymph nodes and activate the naïve CD8+ T cells which then get activated and proliferate.

[0063] Therefore, the minicell component of the composition, by virtue of the PAMP signals is able to overcome the lymphopenia in these elderly and immune-compromised patients and the activation of these lymphocytes helps to overcome the viral infection and prevent the patient from tipping over into respiratory distress and death.

A. Background regarding Coronavirus Infections

[0064] Coronaviruses are a family of hundreds of viruses that can cause fever, respiratory problems, and sometimes gastrointestinal symptoms. SARS-CoV-2 is one of seven members of this family known to infect humans, and the third in the past three decades to jump from animals to humans. Since emerging in China in December 2019, this new coronavirus has caused a global health emergency, sickening 350,000+ people worldwide, and as of March 22, 2020, ~15,0000 deaths have been attributed to COVID-19 worldwide. So far, it appears the coronavirus is more deadly than the seasonal flu. However, there is still a lot of uncertainty around the mortality rate of COVID-19. The annual flu typically has a mortality rate of around 0.1% in the U.S., and to date in the 2019-20 flu season there is a 0.05% mortality rate in the U.S, according to the Centers for Disease Control and Prevention (CDC). In comparison, recent data suggests that COVID-19 has a mortality rate more than 20 times higher, of around 2.3%, according to a study published Feb. 18 by the China CDC Weekly. The death rate varied by different factors such as location and an individual's age.

[0065] Patients infected with SARS-CoV or MERS-CoV initially present with mild, influenza-like illnesses with fever, dyspnea, and cough. Most patients recover from this illness. However, the the most vulnerable populations are patients over the age of 65 and patients with comorbidities that result in immune-suppression such as cancer, HIV, etc., where the disease progresses to more severe symptoms and is characterized by an atypical interstitial pneumonia and diffuse alveolar damage. Both SARS-CoV and MERS-CoV are capable of

causing acute respiratory distress syndrome (ARDS), the most severe form of acute lung injury where alveolar inflammation, pneumonia, and hypoxic lung conditions lead to respiratory failure, multiple organ disease, and death in 50% of ARDS patients. As the disease progresses, lymphopenia is commonly observed. Most of the deaths that occur from CoV-2 infection are a result of the severe lymphopenia in immune-compromised patients and the disease takes over resulting in ARDS.

[0066] Coronavirus (SARS-CoV-2; COVID-19) causes atypical pneumonia in infected people and the symptoms include fever, dry cough, and fatigue. Most patients have lymphopenia (drop in white blood cell counts particularly T cells, B cells and NK cells). Current observations indicate that the patients most likely to die from this disease are those that are immune-compromised (elderly and those with immunosuppressive disease, such as cancer) and patients with diabetes and other underlying healh conditions, such as high blood pressure, heart disease, and respiratory disorders. The former group of patients most likely succumb due to the lymphopenia and hence the viral replication and infection of both lungs becomes uncontrolled resulting in Acute Respiratory Distress Syndrome (ARDS).

[0067] The viral proliferation takes over once the major cells of the immune system e.g. T cells, B cells, macrophages and NK cells are depleted. In elderly patients, immune function is not as robust as it is in younger people. Studies have shown that in most people, their immune function is fine in their 60s, or even in their 70s. The immune functions go down rather quickly after age 75 or 80.

[0068] COVID-19 spreads rapidly by human-to-human transmission with a median incubation period of 3.0 days (range, 0 to 24.0) and the time from symptom onset to developing pneumonia is 4.0 days (range, 2.0 to 7.0) (Guan et al., 2020). Fever, dry cough, and fatigue are common symptoms at onset of COVID- 19 (Huang et al., 2020). Most patients have lymphopenia and bilateral ground-glass opacity changes on chest CT scans (Huang et al., 2020; Duan and Qin, 2020). No specific antiviral treatments or vaccines are available. Development of SARS-CoV-2-based vaccines is urgently required.

[0069] The entire virus particle-based preparation of vaccines, including inactivated and

attenuated virus vaccines is thought to be advisable, because it is based on previous studies about the prevention and control of seasonal influenza vaccines (Grohskopf et al., 2018). The genomic sequence of the first SARS-CoV-2 (Wuhan-Hu-1) has been completed (Genbank Accession no. MN908947.3; Wu et al., 2020). Large-scale culture of SARS-CoV-2 has been carried out and an inactivated virus vaccine has been prepared through the employment of established physical and chemical methods such as UV light, formaldehyde, and β-propiolactone (Jiang et al., 2005). The development of attenuated-virus vaccines is also possible by screening the serially propagated SARS-CoV-2 with reduced pathogenesis such as induced minimal lung injury, diminished limited neutrophil influx, and increased antiinflammatory cytokine expressions compared with the wild-type virus (Regla-Nava et al., 2015). Both inactivated and attenuated virus vaccines have their own disadvantages and side effects (Table 1; reproduced from Shang et al., 2020).

Table 1. Advantages and disadvantages of different vaccine strategies.

Vaccine strategy	Advantages	Disadvantages	References
Inactivated virus vaccines	Easy to prepare; safety; high-titer neutralizing antibodies	Potential inappropriate for highly immunosuppressed individuals	[35]
Attenuated virus vaccines	Rapid development; induce high immune responses	Phenotypic or genotypic reversion possible; can still cause some disease	[36]
Subunit vaccines	High safety; consistent production; can induce cellular and humoral immune responses; high-titer neutralizing antibodies	High cost; lower immunogenicity; require repeated doses and adjuvants	[13,14]
Viral vector vaccines	Safety; induces high cellular and humoral immune responses	Possibly present pre-existing immunity	[12]
DNA vaccines	Easier to design; high safety; high-titer neutralizing antibodies	Lower immune responses in humans, repeated doses may cause toxicity	[37]
mRNA vaccines	Easier to design; high degree of adaptability; induce strong immune responses	Highly unstable under physiological conditions	[38]

[0070] All new therapies under development are (i) anti-viral drugs to stem the proliferation of the virus systemically or (ii) attenuated viruses as vaccines to stimulate a potent antibody response to the viral proteins.

[0071] None of these therapies are likely to stall the death of immune-compromised patients who get infected just as is currently seen in the case of influenza virus infected patients. Each year the largest number of deaths from flu infections occurs in immune-compromised patients and the elderly.

[0072] Effective immunotherapy strategies for the treatment of diseases such as cancer depend on the activation of both innate and adaptive immune responses. Cells of the innate immune system interact with pathogens via conserved pattern-recognition receptors, whereas cells of the

adaptive immune system recognize pathogens through diverse, antigen-specific receptors that are generated by somatic DNA rearrangement. Invariant natural killer T (iNKT) cells are a subset of lymphocytes (Type I NKT) that bridge the innate and adaptive immune systems. iNKT cells express an invariant α chain T cell receptor (Vα24-Jα18 in humans and Vα14-Jα18 in mice) that is specifically activated by certain glycolipids presented in the context of the non-polymorphic MHC class I-like protein, CD1d. CD1d binds to a variety of dialkyl lipids and glycolipids, such as the glycosphingolipid α-galactosylceramide (α-GalCer). iNKT cell TCR recognition of the CD1d-lipid complex results in the release of pro-inflammatory and regulatory cytokines, including the Th1 cytokine interferon gamma (IFNγ). The release of cytokines in turn activates adaptive cells, such as T and B cells, and innate cells, such as dendritic cells and NK cells.

[0073] α-GalCer, also known as KRN7000, chemical formula $C_{50}H_{99}NO_9$, is a synthetic glycolipid derived from structure-activity relationship studies of galactosylceramides isolated from the marine sponge *Agelas mauritianus*. α-GalCer is a strong immunostimulant and shows potent anti-tumor activity in many *in vivo* models. A major challenge to using α-GalCer for immunotherapy is that it induces anergy in iNKT cells because it can be presented by other CD1d expressing cells, such as B cells, in the peripheral blood. Delivery of α-GalCer also has been shown to induce liver toxicity.

B. Background regarding Coronaviruses and SARS-CoV-2

[0074] The outbreak of severe acute respiratory syndrome (SARS) in 2003 and, more recently, Middle-East respiratory syndrome (MERS) has demonstrated the lethality of CoVs when they cross the species barrier and infect humans.

[0075] Coronaviruses (are enveloped viruses with a positive sense, single-stranded RNA genome. With genome sizes ranging from 26 to 32 kilobases (kb) in length, CoVs have the largest genomes for RNA viruses. Based on genetic and antigenic criteria, CoVs have been organised into three groups: α-CoVs, β-CoVs, and γ-CoVs. Coronaviruses (CoVs) primarily cause enzootic infections in birds and mammals but, in the last few decades, have shown to be capable of infecting humans, causing disease to varying degrees, from upper respiratory tract infections (URTIs) resembling the common cold, to lower respiratory tract infections (LRTIs)

such as bronchitis, pneumonia, and even severe acute respiratory syndrome (SARS).

[0076] The lack of effective, licensed treatments for CoV infections underpin the need for a more detailed and comprehensive understanding of coronaviral molecular biology, with a specific focus on both their structural proteins as well as their accessory proteins.

[0077] The coronaviral genome encodes four major structural proteins: the spike (S) protein, nucleocapsid (N) protein, membrane (M) protein, and the envelope (E) protein, all of which are required to produce a structurally complete viral particle. Some CoVs do not require the full ensemble of structural proteins to form a complete, infectious virion, suggesting that some structural proteins might be dispensable or that these CoVs might encode additional proteins with overlapping compensatory functions. Individually, each protein primarily plays a role in the structure of the virus particle, but they are also involved in other aspects of the replication cycle. The S protein mediates attachment of the virus to the host cell surface receptors and subsequent fusion between the viral and host cell membranes to facilitate viral entry into the host cell. In some CoVs, the expression of S at the cell membrane can also mediate cell-cell fusion between infected and adjacent, uninfected cells. This formation of giant, multinucleated cells, or syncytia, has been proposed as a strategy to allow direct spreading of the virus between cells, subverting virus-neutralising antibodies.

[0078] It has been shown that the SARS-CoV-2 spike (S) glycoprotein binds to the cell membrane protein angiotensin-converting enzyme 2 (ACE2) to enter human cells. COVID-19 has been shown to bind to ACE2 via the S protein on its surface. During infection, the S protein is cleaved into subunits, S1 and S2. S1 contains the receptor binding domain (RBD) which allows coronaviruses to directly bind to the peptidase domain (PD) of ACE2. S2 then likely plays a role in membrane fusion.

[0079] Unlike the other major structural proteins, N is the only protein that functions primarily to bind to the CoV RNA genome, making up the nucleocapsid. Although N is largely involved in processes relating to the viral genome, it is also involved in other aspects of the CoV replication cycle and the host cellular response to viral infection. Transient expression of N was shown to

substantially increase the production of virus-like particles (VLPs) in some CoVs, suggesting that it might not be required for envelope formation, but for complete virion formation instead.

[0080] The M protein is the most abundant structural protein and defines the shape of the viral envelope. It is also regarded as the central organiser of CoV assembly, interacting with all other major coronaviral structural proteins. Homotypic interactions between the M proteins are the major driving force behind virion envelope formation but, alone, is not sufficient for virion formation. Binding of M to N stabilises the nucleocapsid (N protein-RNA complex), as well as the internal core of virions, and, ultimately, promotes completion of viral assembly. Together, M and E make up the viral envelope and their interaction is sufficient for the production and release of VLPs.

[0081] The CoV envelope (E) protein is the smallest of the major structural proteins. It is an integral membrane protein involved in several aspects of the virus' life cycle, such as assembly, budding, envelope formation, and pathogenesis. During the replication cycle, E is abundantly expressed inside the infected cell, but only a small portion is incorporated into the virion envelope. The majority of the protein is localised at the site of intracellular trafficking, where it participates in CoV assembly and budding. Recombinant CoVs lacking E exhibit significantly reduced viral titres, crippled viral maturation, or yield propagation incompetent progeny, demonstrating the importance of E in virus production and maturation.

[0082] Coronaviruses are viruses whose genome is a single-stranded mRNA, complete with a 3'-UTR and poly-A tail. In a subset ofcoronaviruses that include 2019-nCoV, SARS and MERS, the3'-UTR contains a highly-conserved sequence (in an otherwise rather variable message) that folds into a unique structure, calledthe s2m (stem two motif). Although the s2m appears to be extremely conserved in sequence, and is required for virus viability, its exact function is not known. The 2019 Wuhan Novel Coronavirus (COVID-19, formerly 2019-nCoV) posesses almost exactly the same s2m sequence (and therefore structure) as SARS.

[0083] SARS-CoV-2 genome sequences are being released and have been published on https://www.ncbi.nlm.nih.gov/genbank/sars-cov-2-seqs/ (downloaded on March 24, 2020), including the multiple complete nucleotide sequences from viruses around the world, as well as

sequences of particular viral genes, such as the S gene, N gene, M gene, etc. Examples include GenBank accession numbers MN908947.3, MN975262.1, NC_045512.2, MN997409.1, MN985325.1, MN988669.1, MN988668.1, MN994468.1, MN994467.1, MN988713.1, and MN938384.1. SARS-CoV-2, is an enveloped, single- and positive-stranded RNA virus with a genome comprising 29,891 nucleotides, which encode the 12 putative open reading frames responsible for the synthesis of viral structural and nonstructural proteins (Wu et al., 2020; Chen et al., 2020). A mature SARS-CoV-2 has four structural proteins, namely, envelope, membrane, nucleocapsid, and spike (Chen et al., 2020). All of these proteins may serve as antigens to stimulate neutralizing antibodies and increase CD4+/ CD8+ T-cell responses (Jiang et al., 2015). However, subunit vaccines require multiple booster shots and suitable adjuvants to work, and certain subunit vaccines such as hepatitis B surface antigen, PreS1, and PreS2 may fail to yield protective response when tested clinically. The DNA and mRNA vaccines that are easier to design and proceed into clinical trials very quickly remain experimental. The viral vector-based vaccines could also be quickly constructed and used without an adjuvant. However, development of such vaccines might not start until antigens containing the neutralizing epitopes are identified. The E and M proteins have important functions in the viral assembly of a coronavirus, and the N protein is necessary for viral RNA synthesis. Deletion of E protein abrogated the virulence of CoVs, and several studies have explored the potential of recombinant SARS-CoV or MERS-CoV with a mutated E protein as live attenuated vaccines. The M protein can augment the immune response induced by N protein DNA vaccine against SARS-CoV; however, the conserved N protein across CoV families implies that it is not a suitable candidate for vaccine development, and the antibodies against the N protein of SARS-CoV-2 do not provide immunity to the infection. The critical glycoprotein S of SARS-CoV-2 is responsible for virus binding and entry. The S precursor protein of SARS-CoV-2 can be proteolytically cleaved into S1 (685 aa) and S2 (588 aa) subunits. The S2 protein is well conserved among SARS-CoV-2 viruses and shares 99% identity with that of bat SARS-CoVs. The vaccine design based on the S2 protein may boost the broad-spectrum antiviral effect and is worth testing in animal models. Antibodies against the conserved stem region of influenza hemagglutinin have been found to exhibit broadly cross-reactive immunity,

but are less potent in neutralizing influenza A virus. In contrast, the S1 subunit consists of the receptor-binding domain (RBD), which mediates virus entry into sensitive cells through the host angiotensin-converting enzyme 2 (ACE2) receptor. The S1 protein of 2019-nCoV shares about 70% identity with that of human SARS-CoVs. The highest number of variations of amino acids in the RBD is located in the external subdomain, which is responsible for the direct interaction between virus and host receptor.

C. Overview of how the disclosed compositions function to treat and/or vaccinate against viral infections

[0084] The present invention aims to intervene pre-infection, or at an early stage post-infection, with a virus, such as a coronavirus such as SARS-CoV or MERS-CoV. The compositions and methods address issues including **(i)** overcoming lymphopenia to prevent the viral infection/disease from overtaking a patient's own immune defences, **(ii)** stimulating a high titer of systemic antibodies to proteins exposed on the surface of the virus to rapidly mop up viral particles released from infected cells and thereby limit the infection of other healthy cells, and **(iii)** stimulating a potent Type I and Type II interferon response, which is well known to rapidly combat a range of different viral infections through a plethora of effects such as specific stimulation of antiviral immunity and virally infected cell elimination.

[0085] To address these and other needs, the present invention provides, in accordance with one aspect, a composition comprising a combination of **(i)** a vector, which can be intact bacterial-derived minicells which are optionally recombinant, packaged with a plasmid encoding viral proteins which function to stimulate an antibody response to the viral proteins and stimulate Type I interferons; **(ii)** a vector, which can be intact bacterially-derived minicells which are optionally recombinant, packaged with a CD1d-recognized antigen, and (iii) at least one pharmaceutically acceptable carrier. The vector packaged with a CD1d-recognized antigen, such as α-GalCer, functions to stimulate Type II interferon. The minicell vector itself functions to stimulate the activation, maturation and proliferation of cells of the immune system. In another aspect, the intact bacterially-derived minicells can also be replaced with killed bacterial cells.

[0086] Thus, described herein, in certain embodiments, are compositions comprising an

immunogenically effective amount of a combination of (a) a vector or intact, bacterially derived minicells or killed bacterial cells that encapsulate one or more viral antigens and a plasmid and (b) a vector or intact, bacterially derived minicells or killed bacterial cells that encapsulate a CD1d-recognized antigen, such as α-galactosylceramide (α-GalCer). In some embodiments, the encapsulated CD1d-recognized antigen is capable of uptake by a phagocytic cell, such as a dendritic cell or a macrophage. Following uptake, the CD1d-recognized cell antigen form complexes with CD1d within the lysosomes of the phagocytic cells and is subsequently transported to the surface of the phagocytic cells where the CD1d-recognized antigen bound to CD1d is presented for recognition by an iNKT cell. In some embodiments, the CD1d-recognized cell antigen induces a Th1 cytokine response particularly IFNγ by an iNKT cell that recognizes the CD1d-recognized cell antigen bound to CD1d on the surface of the phagocytic cell. IFNγ is also known to trigger a potent antiviral immune response. The ability of CD1d-restricted NKT cells to activate innate and adaptive immune responses has led to the idea that these cells can modulate immunity to infectious agents. In addition, CD1d-restricted iNKT cells may directly contribute to host resistance as they express a variety of effector molecules that could mediate an antimicrobial effect. The CD1 proteins are antigen-presenting molecules that present lipid antigens to T cells.

[0087] In one aspect, the intent of administering a composition described herein to a subject in need would be to rapidly lift the subject out of lymphopenia and simultaneously activate the key cells of the immune system to fight against the virus infection, particularly in elderly and immune-compromised patients. This would prevent exacerbation of the viral infection and resultant death of these patients. Consequently, infected subjects would suffer milder flu-like symptoms and recover more rapidly as the body's own immune system tips the balance over to recovery.

[0088] In one aspect of the disclosure, all four SARS-CoV-2 structural protein (Envelope, Membrane, Nucleocapsid and Spike) encoding genes are cloned in a plasmid that carries a bacterial origin of replication and the genes are transcribed using a bacterial gene expression promoter so that the proteins are only expressed in the EDV™-producing bacterial cell and

segregated into the EDV™ cytoplasm. Thus, all four of the SARS-CoV-2 proteins can be expressed from a single bacterial expression promoter. Alternatively, the genes can be transcribed under a mammalian gene expression promoter so the proteins are expressed only by mammalian cells. The recombinant plasmid can be transformed into a minicell producing strain of *Salmonella typhimurium*. Such a recombinant intact, bacterially derived minicell therapeutic is expected to elicit a potent antibody response to all four CoV-2 proteins.

[0089] Additionally, when the recombinant intact, bacterially derived minicells are administered systemically in a CoV-2 virus infected patient, the intact, bacterially derived minicells are rapidly taken up by professional phagocytic cells such as macrophages and dendritic cells and the intact, bacterially derived minicells are broken down in the lysosomes releasing the plasmid DNA. This DNA is then recognized by intracellular DNA sensors like cGAS, AIM2, IFI16 and others and this will trigger a Type I interferon (IFNα and IFNβ) response. These interferons are known to be potent inducers of antiviral defence.

[0090] It is well recognized that early in infection, IFN stimulation results in altered cellular transcriptional programs, leading to an antiviral state characterized by the activation of a large set of host genes with partially defined antiviral functions [Schoggins et al., 2011].

[0091] In some embodiments, the CD1d-recognized antigen is a glycosphingolipid. In some embodiments, the glycosphingolipid is selected from among α-galactosylceramide (α-GalCer), C-glycosidific form of α-galactosylceramide (α-C-GalCer), 12 carbon acyl form of galactosylceramide (β-GalCer), β-D-glucopyranosylceramide (β-GlcCer), 1,2-Diacyl-3-O-galactosyl-sn-glycerol (BbGL-II), diacylglycerol containing glycolipids (Glc-DAG-s2), ganglioside (GD3), gangliotriaosylceramide (Gg3Cer), glycosylphosphatidylinositol (GPI), α-glucuronosylceramide (GSL-1 or GSL-4), isoglobotrihexosylceramide (iGb3), lipophosphoglycan(LPG), lyosphosphatidylcholine (LPC), α-galactosylceramide analog (OCH), threitolceramide, and a derivative of any thereof. In some embodiments, the glycosphingolipid is α-GalCer. In some embodiments, the glycosphingolipid is a synthetic α-GalCer analog. In some embodiments, the synthetic α-GalCer analog is selected from among 6′-deoxy-6′-acetamide α-GalCer (PBS57), napthylurea α-GalCer (NU-α-GC), NC-α-GalCer, 4ClPhC-α-GalCer, PyrC-α-

GalCer, α-carba-GalCer, carba-α-D-galactose α-GalCer analog (RCAI-56), 1-deoxy-neo-inositol α-GalCer analog (RCAI-59), 1-O-methylated α-GalCer analog (RCAI-92), and HS44 aminocyclitol ceramide. In some embodiments, the CD1d-recognized antigen is derived from a bacterial antigen, a fungal antigen, or a protozoan antigen.

[0092] In some embodiments, the immune response produced in the target cells comprises the production of Type I interferon, including interferon-α and/or interferon-β.

[0093] This bacterial minicell treatment should reduce the severity of the disease in almost all patients and reduce the duration of the disease making it more like just a common cold.

[0094] Alternatively, the treatment may be administered in a healthy person as a vaccine to protect against the viral infection where the virus carries the proteins encoded by the recombinant plasmid carried in the minicell.

[0095] In one embodiment, the adjuvant composition comprises (a) an immunogenically effective amount of an encapsulated CD1d-recognized antigen and (b) a minicell carrying a recombinant plasmid encoding one or more viral antigens.

[0096] In one embodiment, the CD1d-recognized antigen and the recombinant plasmid are packaged within two intact bacterially derived minicells or killed bacterial cells.

[0097] The CD1d-recognized antigen is comprised within a first intact bacterially-derived minicell or killed bacterial cell, and the recombinant plasmid encoding viral antigens is comprised within a second intact bacterially-derived minicell or killed bacterial cell.

[0098] In some embodiments, the encapsulated CD1d-recognized antigen (e.g., α-GalCer) and the minicell carrying the recombinant plasmid encoding at least one viral antigen are administered simultaneously. In some embodiments, the encapsulated CD1d- recognized antigen (e.g., α-GalCer) and the minicell carrying the recombinant plasmid encoding viral antigens are administered sequentially. In some embodiments, the encapsulated CD1d- recognized antigen (e.g., α-GalCer) and the minicell carrying the recombinant plasmid encoding viral antigens are administered multiple times. In some embodiments, the encapsulated CD1d- recognized antigen (e.g., α-GalCer) and the minicell carrying the recombinant plasmid encoding viral antigens are

administered at least once a week or twice a week or three times per week or four times per week until the disease is resolved.

[0099] Following infection with SARS-CoV-2, the aim of this therapy would be to achieve the following: (1) stimulate innate and adaptive immunity via recruitment of fresh monocytes and dendritic cells from the bone marrow and activation of NK cells. This would keep the immune status high in the patients as the disease progresses and prevent the development of lymphopenia. (2) Physiologically well tolerated secretion of Type I (IFNα and IFNβ) and Type II (IFNγ) interferons. It is well recognized that early in viral infection, IFN stimulation results in altered cellular transcriptional programs, leading to an antiviral state characterized by the activation of a large set of host genes with partially defined antiviral functions. This activation would enable rapid elimination of virally infected cells along with a reduction in viral replication. (3) Secrete antibodies to the four structural proteins of the virus (Envelope, Membrane, Spike and Nucleocapsid) and this would aim to mop up a significant number of viral particles that are released from infected cells. All of the above would be expected with minimal to no toxicity.

II. Intact bacterially-derived minicells

[0100] The term "minicell" is used herein to denote a derivative of a bacterial cell that lacks chromosomes ("chromosome-free") and is engendered by a disturbance in the coordination, during binary fission, of cell division with DNA segregation. Minicells are distinct from other small vesicles, such as so-called "membrane blebs" (about 0.2 μm or less in size), which are generated and released spontaneously in certain situations but which are not due to specific genetic rearrangements or episomal gene expression. Bacterially derived minicells employed in this disclosure are fully intact and are distinguished from other chromosome-free forms of bacterial cellular derivatives characterized by an outer or defining membrane that is disrupted or degraded, even removed. The intact membrane that characterizes the minicells of the present disclosure allows retention of the therapeutic payload within the minicell until the payload is released.

[0101] Intact, Bacterially-derived minicells or EDVs™ are anucleate, non-living nanoparticles produced as a result of inactivating the genes that control normal bacterial cell division, thereby

de-repressing polar sites of cell. Moreover, in contrast to current stealth liposomal drug carriers like DOXIL (liposomal doxorubicin), for example, that can package only ~14,000 molecules per particle, or "armed antibodies," which can carry fewer than 5 drug molecules, bacterial minicells can readily accommodate payloads of up to 1 million drug molecules.

[0102] The minicells employed in the present disclosure can be prepared from bacterial cells, such as *E. coli* and *S. typhymurium*. Prokaryotic chromosomal replication is linked to normal binary fission, which involves mid-cell septum formation. In *E. coli*, for example, mutation of min genes, such as minCD, can remove the inhibition of septum formation at the cell poles during cell division, resulting in production of a normal daughter cell and an chromosome-less minicell.

[0103] In addition to min operon mutations, chromosome-less minicells also are generated following a range of other genetic rearrangements or mutations that affect septum formation, for example, in the divIVB1 in *B. subtilis*. Minicells also can be formed following a perturbation in the levels of gene expression of proteins involved in cell division/chromosome segregation. For instance, over-expression of minE leads to polar division and production of minicells. Similarly, chromosome-less minicells can result from defects in chromosome segregation, e.g., the smc mutation in *Bacillus subtilis*, the spoOJ deletion in *B. subtilis*, the mukB mutation in *E. coli*, and the parC mutation in *E. coli*. Further, CafA can enhance the rate of cell division and/or inhibit chromosome partitioning after replication, resulting in formation of chained cells and chromosome-less minicells.

[0104] Accordingly, minicells can be prepared for the present disclosure from any bacterial cell, be it of Gram-positive or Gram-negative origin due to the conserved nature of bacterial cell division in these bacteria. Furthermore, the minicells used in the disclosure should possess intact cell walls (i.e., are "intact minicells"), as noted above, and should be distinguished over and separated from other small vesicles, such as membrane blebs, which are not attributable to specific genetic rearrangements or episomal gene expression.

[0105] In a given embodiment, the parental (source) bacteria for the minicells can be Gram positive, or they can be Gram negative. In one aspect, the parental bacteria are one or more

selected from Terra-/Glidobacteria (BV1), Proteobacteria (BV2), BV4 including Spirochaetes, Sphingobacteria, and Planctobacteria. Pursuant to another aspect, the bacteria are one or more selected from Firmicutes (BV3) such as Bacilli, Clostridia or Tenericutes/Mollicutes, or Actinobacteria (BV5) such as Actinomycetales or Bifidobacteriales.

[0106] Pursuant to the disclosure, killed bacterial cells are non-living prokaryotic cells of bacteria, cyanobateria, eubacteria and archaebacteria, as defined in the 2nd edition of *Bergey's Manual Of Systematic Biology*. Such cells are deemed to be "intact" if they possess an intact cell wall and/or cell membrane and contain genetic material (nucleic acid) that is endogenous to the bacterial species. Methods of preparing killed bacterial cells are described, for instance, in U.S. 2008/0038296.

[0107] In yet a further aspect, the bacteria are one or more selected from Eubacteria (Chloroflexi, Deinococcus-Thermus), Cyanobacteria, Thermodesulfobacteria, thermophiles (Aquificae, Thermotogae), Alpha, Beta, Gamma (Enterobacteriaceae), Delta or Epsilon Proteobacteria, Spirochaetes, Fibrobacteres, Chlorobi/Bacteroidetes, Chlamydiae/Verrucomicrobia, Planctomycetes, Acidobacteria, Chrysiogenetes, Deferribacteres, Fusobacteria, Gemmatimonadetes, Nitrospirae, Synergistetes, Dictyoglomi, Lentisphaerae Bacillales, Bacillaceae, Listeriaceae, Staphylococcaceae, Lactobacillales, Enterococcaceae, Lactobacillaceae, Leuconostocaceae, Streptococcaceae, Clostridiales, Halanaerobiales, Thermoanaerobacterales, Mycoplasmatales, Entomoplasmatales, Anaeroplasmatales, Acholeplasmatales, Haloplasmatales, Actinomycineae, Actinomycetaceae, Corynebacterineae, Nocardiaceae, Corynebacteriaceae, Frankineae, Frankiaceae, Micrococcineae, Brevibacteriaceae, and Bifidobacteriaceae.

[0108] For pharmaceutical use, a composition of the disclosure should comprise minicells or killed bacterial cells that are isolated as thoroughly as possible from immunogenic components and other toxic contaminants. Methodology for purifying bacterially derived minicells to remove free endotoxin and parent bacterial cells are described, for example, in WO 2004/113507. Briefly, the purification process achieves removal of (a) smaller vesicles, such as membrane blebs, which are generally smaller than 0.2 μm in size, (b) free endotoxins released

from cell membranes, and (c) parental bacteria, whether live or dead, and their debris, which also are sources of free endotoxins. Such removal can be implemented with, *inter alia*, a 0.2 μm filter to remove smaller vesicles and cell debris, a 0.45 μm filter to remove parental cells following induction of the parental cells to form filaments, antibiotics to kill live bacterial cells, and antibodies against free endotoxins.

[0109] Underlying the purification procedure is a discovery by the present inventors that, despite the difference of their bacterial sources, all intact minicells are approximately 400 nm in size, i.e., larger than membrane blebs and other smaller vesicles and yet smaller than parental bacteria. Size determination for minicells can be accomplished by using solid-state, such as electron microscopy, or by liquid-based techniques, e.g., dynamic light scattering. The size value yielded by each such technique can have an error range, and the values can differ somewhat between techniques. Thus, the size of minicells in a dried state can be measured via electron microscopy as approximately 400 nm ± 50 nm. Dynamic light scattering can measure the same minicells to be approximately 500 nm ± 50 nm in size. Also, drug-packaged, ligand-targeted minicells can be measured, again using dynamic light scattering, to be approximately 400 nm to 600 nm ± 50 nm.

[0110] Another structural element of a killed bacterial cells or a minicell derived from Gram-negative bacteria is the O-polysaccharide component of lipopolysaccharide (LPS), which is embedded in the outer membrane via the lipid A anchor. The component is a chain of repeat carbohydrate-residue units, with as many as 70 to 100 repeat units of four to five sugars per repeat unit of the chain. Because these chains are not rigid, in a liquid environment, as *in vivo*, they can adopt a waving, flexible structure that gives the general appearance of seaweed in a coral sea environment; i.e., the chains move with the liquid while remaining anchored to the minicell membrane.

[0111] Influenced by the O-polysaccharide component, dynamic light scattering can provide a value for minicell size of about 500 nm to about 600 nm, as noted above. Nevertheless, minicells from Gram-negative and Gram-positive bacteria alike readily pass through a 0.45 μm filter, which substantiates an effective minicell size of 400 nm ± 50 nm. The above-mentioned

scatter in sizes is encompassed by the present invention and, in particular, is denoted by the qualifier "approximately" in the phrase "approximately 400 nm in size" and the like.

[0112] In relation to toxic contaminants, a composition of the disclosure preferably comprises less than about 350 EU free endotoxin. Illustrative in this regard are levels of free endotoxin of about 250 EU or less, about 200 EU or less, about 150 EU or less, about 100 EU or less, about 90 EU or less, about 80 EU or less, about 70 EU or less, about 60 EU or less, about 50 EU or less, about 40 EU or less, about 30 EU or less, about 20 EU or less, about 15 EU or less, about 10 EU or less, about 9 EU or less, about 8 EU or less, about 7 EU or less, about 6 EU or less, about 5 EU or less, about 4 EU or less, about 3 EU or less, about 2 EU or less, about 1 EU or less, about 0.9 EU or less, about 0.8 EU or less, about 0.7 EU or less, about 0.6 EU or less, about 0.5 EU or less, about 0.4 EU or less, about 0.3 EU or less, about 0.2 EU or less, about 0.1 EU or less, about 0.05 EU or less, or about 0.01 EU or less.

[0113] A composition of the disclosure also can comprise at least about 10^9 minicells or killed bacterial cells, e.g., at least about 1 x10^9, at least about 2 x 10^9, at least about 5 x 10^9, or at least 8 x 10^9. In some embodiments, the composition comprises no more than about 10^{11} minicells or killed bacterial cells, e.g., no more than about 1 x 10^{11} or no more than about 9 x 10^{10}, or no more than about 8 x 10^{10}.

III. CD1d-recognized antigens

[0114] The present compositions and methods comprise a vector, which can be an intact bacterially derived minicell, that comprises a CD1d-recognized antigen. Such antigens result in an increases the level (*e.g.*, the activity or expression level) of type II interferons, e.g., IFN-γ (gamma). IFN-γ is involved in the regulation of the immune and inflammatory responses; in humans, there is only one type of interferon-gamma. It is produced in activated T cells and natural killer cells. IFN-γ potentiates the effects of type I IFNs. IFN-γ released by Th1 cells recruits leukocytes to a site of infection, resulting in increased inflammation. It also stimulates macrophages to kill bacteria that have been engulfed. IFN-γ released by Th1 cells also is important in regulating the Th2 response.

[0115] IFN gamma cytokines are released by innate Natural Killer (NK) cells upon binding of

natural antigen, but glycosphingolipid compounds can function as potent activators of both innate and acquired immune responses. Exposure to a glycosphingolipid induces a potent cytokine response by innate natural killer T (iNKT) cells, including the type II interferon, IFN-γ, and a number of Interleukins (Th1-, Th2-, and/or Th17-type cytokines). iNKT cells then induce DC maturation and display T cell helper–like functions that result in the development of cytotoxic T cell responses.

[0116] Examples of glycosphingolips useful to induce a IFN type II response are described herein and include C-glycosidific form of α-galactosylceramide (α-C-GalCer), α-galactosylceramide (α-GalCer), 12 carbon acyl form of galactosylceramide (β-GalCer), β-D-glucopyranosylceramide (β-GlcCer), l,2-Diacyl-3-0-galactosyl-sn-glycerol (BbGL-II), diacylglycerol containing glycolipids (Glc-DAG-s2), ganglioside (GD3), gangliotriaosylceramide (Gg3Cer), glycosylphosphatidylinositol (GPI), α-glucuronosylceramide (GSL-1 or GSL-4), isoglobotrihexosylceramide (iGb3), lipophosphoglycan(LPG), lyosphosphatidylcholine (LPC), α-galactosylceramide analog (OCH), and threitolceramide. In a particular embodiment the minicell disclosed herein comprises α-galactosylceramide (α-GalCer) as a type II IFN agonist.

[0117] α-GC, an INF type II agonist is known to stimulate the immune system through activation of a type of white blood cell known as natural killer T cell (NKT cell).

[0118] The minicell can deliver type II IFN agonists directly to cells of the immune system, with a view to enhancing iNKT cell activation and type II interferon IFN-γ production *in vivo*. Non-targeted intact, bacterially derived minicells are taken up by phagocytic cells of the immune system, where they are broken down in endosomes, and αGC is presented to iNKT cells for immune activation. Accordingly, in some embodiments the minicell provides targeted delivery of type II interferon agonists. In other embodiments, the composition disclosed herein comprises a non-targeted minicell comprising a type II interferon agonist.

[0119] IFN-γ production is controlled by cytokines secreted by antigen presenting cells (APCs), most notably interleukin (IL)-12 and IL-18. These cytokines serve as a bridge to link infection with IFN-γ production in the innate immune response. Macrophage recognition of many

pathogens induces secretion of IL-12 and chemokines. These chemokines attract NK cells to the site of inflammation, and IL-12 promotes IFN-γ synthesis in these cells. In macrophages, natural killer cells and T cells, the combination of IL-12 and IL-18 stimulation further increases IFN-γ production. Accordingly, any of these proteins or their combinations are suitable agents for the purpose of this disclosure.

[0120] Negative regulators of IFN-gamma production include IL-4, IL-10, transforming growth factor β and glucocorticoids. Proteins or nucleic acids that inhibit these factors will be able to stimulate the production of IFN-γ.

[0121] Also suitable for use in this context are polynucleotides that encode IFN-γ or genes that activate the production and/or the secretion of IFN-γ.

[0122] The agent that increases the level of IFN-γ may also be a viral vaccine. A number of viral vaccines are available that can induce IFN-γ production without causing infection or other types of adverse effects. Illustrative of this class of viral-vaccine agent is a flu (influenza) vaccine.

[0123] Serum concentration of IFN-γ required for effectively activating host immune response to is low when the patient also receives administration of drug-loaded, bispecific antibody-targeted minicells or killed bacterial cells. Thus, in one aspect the inventive methodology results in increase of serum IFN-γ concentration that is not higher than about 30,000 pg/mL. In another aspect, the serum IFN-γ concentration is increased to not higher than about 5000 pg/mL, 1000 pg/mL, 900 pg/mL, 800 pg/mL, 700 pg/mL, 600 pg/mL, 500 pg/mL, 400 pg/mL, 300 pg/mL, 200 pg/mL, or 100 pg/mL. In a further aspect, the resulting serum IFN-gamma concentration is at least about 10 pg/mL, or at least about 20 pg/mL, 30 pg/mL, 40 pg/mL, 50 pg/mL, 60 pg/mL, 70 pg/mL, 80 pg/mL, 90 pg/mL, 100 pg/mL, 150 pg/mL, 200 pg/mL, 300 pg/mL, 400 pg/mL or 500 pg/mL.

[0124] Pursuant to some aspects, the agent is an IFN-γ protein or an engineered protein or analog. In some aspects, the administration achieves from about 0.02 ng to 1 microgram of IFN-γ per ml of host blood. In one aspect, the achieved IFN-gamma concentration in the host blood is from about 0.1 ng to about 500 ng per ml, from about 0.2 ng to about 200 ng per ml, from

about 0.5 ng to about 100 ng per ml, from about 1 ng to about 50 ng per ml, or from about 2 ng to about 20 ng per ml.

IV. Loading Viral Antigens and CD1d-recognized antigens into Minicells or Killed Bacterial Cells

[0125] Viral antigens as well as CD1d-recognized antigens can be packaged into minicells or killed bacterial cells directly, by co-incubating a plurality of intact minicells or killed bacterial cells with the antigens in a buffer. The buffer composition can be varied, as a function of conditions well known in this field, to optimize the loading of the antigens in the intact minicells. The buffer also may be varied in dependence on the antigen (e.g., dependent upon the nucleotide sequence or the length of the nucleic acid to be loaded in the minicells in the case of a nucleic acid payload). An exemplary buffer suitable for loading includes, but is not limited to, phosphate buffered saline (PBS). Once packaged, the antigen remains inside the minicell and is protected from degradation. Prolonged incubation studies with siRNA-packaged minicells incubated in sterile saline have shown, for example, no leakage of siRNAs.

[0126] Antigens such as proteins that can be encoded for by a nucleic acid, can be introduced into minicells by transforming into the parental bacterial cell a vector, such as a plasmid, that encodes the antigen. When a minicell is formed from the parental bacterial cell, the minicell retains certain copies of the plasmid and/or the expression product, e.g., the antigen. More details of packaging and expression product into a minicell is provided in WO 03/033519.

[0127] Data presented in WO 03/033519 demonstrated, for example, that recombinant minicells carrying mammalian gene expression plasmids can be delivered to phagocytic cells and to non-phagocytic cells. WO 03/033519 also described the genetic transformation of minicell-producing parent bacterial strains with heterologous nucleic acids carried on episomally-replicating plasmid DNAs. Upon separation of parent bacteria and minicells, some of the episomal DNA segregated into the minicells. The resulting recombinant minicells were readily engulfed by mammalian phagocytic cells and became degraded within intracellular phagolysosomes. Moreover, some of the recombinant DNA escaped the phagolysosomal membrane and was transported to the mammalian cell nucleus, where the recombinant genes

were expressed. In other embodiments, multiple antigens can be packaged in the same minicell.

[0128] Antigens can be packaged in minicells by creating a concentration gradient of the antigen between an extracellular medium comprising minicells and the minicell cytoplasm. When the extracellular medium comprises a higher antigen concentration than the minicell cytoplasm, the antigen naturally moves down this concentration gradient, into the minicell cytoplasm. When the concentration gradient is reversed, however, the antigen does not move out of the minicells. More details of the active agent loading process and its surprising nature are found, for instance, in U.S. Patent Application Publication No. 2008/0051469.

V. Formulations

[0129] The disclosure includes within its scope compositions comprising a combination of (a) a vector, intact bacterial minicell, or killed bacterial cell comprising as a payload at least one viral antigen; and (b) a vector, intact bacterial minicell, or killed bacterial cell comprising as a payload at least one CD1d-recognized antigen, both of which are present in at least one pharmaceutically acceptable carrier. The at least one viral antigen and at least one CD1d-recognized antigen can be in the same or different vector, intact bacterial minicell, or killed bacterial cell. At least one of the viral antigen and CD1d-recognized antigen is present in an intact bacterial minicell.

[0130] In another aspect, one of the viral antigen and at least one CD1d-recognized antigen are present in a non-bacterial cell carrier, such as a liposomal carrier.

[0131] In some aspects, the CD1d-recognized antigen is the interferon type II agonist α-galactosyl ceramide.

[0132] Compositions of the disclosure can be presented in unit dosage form, *e.g.*, in ampules or vials, or in multi-dose containers, with or without an added preservative. The composition can be a solution, a suspension, or an emulsion in oily or aqueous vehicles, and can comprise formulatory agents, such as suspending, stabilizing and/or dispersing agents. A suitable solution is isotonic with the blood of the recipient and is illustrated by saline, Ringer's solution, and dextrose solution. Alternatively, formulations can be in lyophilized powder form, for reconstitution with a suitable vehicle, *e.g.*, sterile, pyrogen-free water or physiological saline.

The formulations also can be in the form of a depot preparation. Such long-acting formulations can be administered by implantation (for instance, subcutaneously or intramuscularly) or by intramuscular injection. In some embodiments, administering comprises enteral or parenteral administration. In some embodiments administering comprises administration selected from oral, buccal, sublingual, intranasal, rectal, vaginal, intravenous, intramuscular, and subcutaneous injection.

[0133] In some aspects, a minicell-comprising composition that includes a therapeutically effective amount of a viral antigen, as well as a a therapeutically effective amount of a CD1d-recognized antigen, is provided. A "therapeutically effective" amount of an antigen is an amount that invokes a pharmacological response when administered to a subject, in accordance with the present disclosure.

[0134] In the context of the present disclosure, therefore, a therapeutically effective amount can be gauged by reference to the prevention or amelioration of the viral infection, either in an animal model or in a human subject, when minicells carrying a therapeutic payload are administered, as further described below. An amount that proves "therapeutically effective amount" in a given instance, for a particular subject, may not be effective for 100% of subjects similarly treated for the viral infection, even though such dosage is deemed a "therapeutically effective amount" by skilled practitioners. The appropriate dosage in this regard also will vary as a function, for example, of the stage and severity of the viral infection, as well as whether the subject has any underlying adverse medical conditions, is aged 60+, or is immunocomprised.

a. Administration Routes

[0135] Formulations of the disclsoure can be administered via various routes and to various sites in a mammalian body, to achieve the therapeutic effect(s) desired, either locally or systemically. Delivery may be accomplished via any pharmaceutically acceptable route, for example, oral administration, application of the formulation to a body cavity, inhalation, nasal administration, pulmonary administration, insufflation, or by injection (e.g., parenteral, intramuscular, intravenous, intraportal, intrahepatic, peritoneal, subcutaneous, intratumoral, or intradermal administration). A combination of routes also may be employed.

b. Purity

[0136] Bacterial minicells are substantially free from contaminating parent bacterial cells. Thus, minicell-comprising formulations preferably comprise fewer than about 1 contaminating parent bacterial cell per 10^7 minicells, fewer than about 1 contaminating parent bacterial cell per 10^8 minicells, fewer than about 1 contaminating parent bacterial cell per 10^9 minicells, fewer than about 1 contaminating parent bacterial cell per 10^{10} minicells, or fewer than about 1 contaminating parent bacterial cell per 10^{11} minicells.

[0137] Methods of purifying minicells are known in the art and described in PCT/IB02/04632. One such method combines cross-flow filtration (feed flow is parallel to a membrane surface; Forbes, 1987) and dead-end filtration (feed flow is perpendicular to the membrane surface). Optionally, the filtration combination can be preceded by a differential centrifugation, at low centrifugal force, to remove some portion of the bacterial cells and thereby enrich the supernatant for minicells.

[0138] Particularly effective purification methods exploit bacterial filamentation to increase minicell purity. Thus, a minicell purification method can include the steps of (a) subjecting a sample containing minicells to a condition that induces parent bacterial cells to adopt a filamentous form, followed by (b) filtering the sample to obtain a purified minicell preparation.

[0139] Known minicell purification methods also can be combined. One highly effective combination of methods is as follows:

Step A: Differential centrifugation of a minicell producing bacterial cell culture. This step, which may be performed at 2,000 g for about 20 minutes, removes most parent bacterial cells, while leaving minicells in the supernatant;

Step B: Density gradient centrifugation using an isotonic and non-toxic density gradient medium. This step separates minicells from many contaminants, including parent bacterial cells, with minimal loss of minicells. Preferably, this step is repeated within a purification method;

Step C: Cross-flow filtration through a 0.45 μm filter to further reduce parent bacterial cell contamination.

Step D: Stress-induced filamentation of residual parent bacterial cells. This may be

accomplished by subjecting the minicell suspension to any of several stress-inducing environmental conditions;

Step E: Antibiotic treatment to kill parent bacterial cells;

Step F: Cross-flow filtration to remove small contaminants, such as membrane blebs, membrane fragments, bacterial debris, nucleic acids, media components and so forth, and to concentrate the minicells. A 0.2 μm filter may be employed to separate minicells from small contaminants, and a 0.1 μm filter may be employed to concentrate minicells;

Step G: Dead-end filtration to eliminate filamentous dead bacterial cells. A 0.45 um filter may be employed for this step; and

Step H: Removal of endotoxin from the minicell preparation. Anti-Lipid A coated magnetic beads may be employed for this step.

c. Administration Schedules

[0140] In general, the formulations disclosed herein may be used at appropriate dosages defined by routine testing, to obtain optimal physiological effect, while minimizing any potential toxicity. The dosage regimen may be selected in accordance with a variety of factors including age, weight, sex, medical condition of the patient; the severity of the condition to be treated, the route of administration, and the renal and hepatic function of the patient.

[0141] Optimal precision in achieving concentrations of minicell and drug within the range that yields maximum efficacy with minimal side effects may require a regimen based on the kinetics of the minicell and antigen availability to target sites and target cells. Distribution, equilibrium, and elimination of a minicell or antigen may be considered when determining the optimal concentration for a treatment regimen. The dosages of the minicells and antigens may be adjusted when used in combination, to achieve desired effects.

[0142] Moreover, the dosage administration of the formulations may be optimized using a pharmacokinetic/pharmacodynamic modeling system. For example, one or more dosage regimens may be chosen and a pharmacokinetic/pharmacodynamic model may be used to determine the pharmacokinetic/pharmacodynamic profile of one or more dosage regimens. Next, one of the dosage regimens for administration may be selected which achieves the desired

pharmacokinetic/pharmacodynamic response based on the particular pharmacokinetic/pharmacodynamic profile. See, e.g., WO 00/67776.

[0143] Specifically, the formulations may be administered at least once every day for a few days (three to four) or until the symptoms of viral infection subside. In one embodiment, the formulations are administered at least once a day until viral disease subsides.

[0144] More specifically, the formulations may be administered at least once a day for about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, or about 31 days. Alternatively, the formulations may be administered about once every day, about once every about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30 or about 31 days or more.

[0145] The compositions may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three, or four times daily.

VI. Definitions

[0146] Unless defined otherwise, all technical and scientific terms used in this description have the same meaning as commonly understood by those skilled in the relevant art.

[0147] For convenience, the meaning of certain terms and phrases employed in the specification, examples, and appended claims are provided below. Other terms and phrases are defined throughout the specification.

[0148] The singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

[0149] As used herein, the term "about" will be understood by persons of ordinary skill in the art and will vary to some extent depending upon the context in which it is used. If there are uses of the term which are not clear to persons of ordinary skill in the art given the context in which it is

used, "about" will mean up to plus or minus 10% of the particular term.

[0150] As used herein, except where the context requires otherwise, the term "comprise" and variations of the term, such as "comprising," "comprises" and "comprised" are not intended to exclude other additives, components, integers or steps.

[0151] The phrases "biologically active" and "biological activity" are used to qualify or to denote, as the case may be, the effect(s) of a compound or composition on living matter. Thus, a material is biologically active or has biological activity if it has interaction with or effect on any cell tissue in a human or animal body, *e.g.*, by reacting with protein, nucleic acid, or other molecules in a cell.

[0152] "Individual," "subject," "host," and "patient," terms used interchangeably in this description, refer to any mammalian subject for whom diagnosis, treatment, or therapy is desired. The individual, subject, host, or patient can be a human or a non-human animal. Thus, suitable subjects can include but are not limited to non-human primates, cattle, horses, dogs, cats, guinea pigs, rabbits, rats, and mice.

[0153] The terms "treatment," "treating," "treat," and the like refer to obtaining a desired pharmacological and/or physiologic effect in a patient. The effect can be prophylactic in terms of completely or partially preventing viral infection or a symptom thereof and/or the effect can be therapeutic in terms the viral infection. Alternatively or additionally, a desired treatment effect can be an increase of overall patient survival, progress-free survival, or a reduction of adverse effect.

[0154] The phrase "pharmaceutical grade" denotes a lacking of parental cell contamination, cell debris, free endotoxin and other pyrogens that is sufficient to meet regulatory requirements for human intravenous administration. See, *e.g.*, "Guidance for Industry – Pyrogen and Endotoxins Testing," U.S. Food and Drug Administration (June 2012).

[0155] "Payload" in this description identifies or qualifies biologically active material that is to be loaded or that has been loaded into a minicell for delivery to a targeted host cell.

[0156] The term "substantially" generally refers to at least 90% similarity. In some

embodiments, in the context of a first X-ray powder diffraction pattern being substantially as shown in a second X-ray powder diffraction pattern, "substantially" refers to ± 0.2°. In some embodiments, in the context of a first differential scanning calorimetry thermogram being substantially as shown in a second differential scanning calorimetry thermogram, "substantially" refers to ±0.4 °C. In some embodiments, in the context of a first thermogravimetric analysis being substantially as shown in a second thermogravimetric analysis, "substantially" refers to ±0.4% weight. In some embodiments, "substantially purified" refers to at least 95% purity. This includes at least 96, 97, 98, or 99% purity. In further embodiments, "substantially purified" refers to about 95, 96, 97, 98, 99, 99.5, or 99.9% purity, including increments therein.

[0157] As used herein, "therapeutic activity" or "activity" may refer to an activity whose effect is consistent with a desirable therapeutic outcome in humans, or to desired effects in non-human mammals or in other species or organisms. Therapeutic activity may be measured *in vivo* or *in vitro*. For example, a desirable effect may be assayed in cell culture.

[0158] As used herein, the phrase "therapeutically effective amount" shall mean the drug dosage that provides the specific pharmacological response for which the drug is administered in a significant number of subjects in need of such treatment. It is emphasized that a therapeutically effective amount of an antigen that is administered to a particular subject in a particular instance will not always be effective in treating the viral infection described herein, even though such dosage is deemed to be a therapeutically effective amount by those of skill in the art.

[0159] The present technology, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present technology.

EXAMPLES

Example 1

[0160] Figure 1 depicts and exemplary composition, comprising a first intact, bacterial minicell comprising a plasmid encoding viral protein, which function to stimulate antibody responses to the viral proteins. Plasmid double-stranded DNA is recognized by intracellular nucleic acid

sensors and triggers IFNalpha and IFNbeta response. Also shown is a second intact, bacterially-derived minicell comprising an IFNgamma stimulating compound, alpha-galactosyl ceramide.

[0161] Since the genomic sequence of the SARS-CoV-2 virus is known, a plasmid expressing all four of the SARS-CoV2 proteins expressed from a single bacterial expression promoter can be made. The plasmic then can be encapsulated in an intact bacterially-derived minicell (i.e., an EnGeneIC Nanocell Dream Vector (EDV™)). A second component would be an intact bacterially-derived minicell packaged with a glycolipid (α-galactosyl ceramide; EDVα-GC).

[0162] The product can be lyophilized. The intact bacterially-derived minicell based products are very stable and lyophilized vials with anti-cancer compounds and intact bacterially-derived minicell loaded with α-GC have already shown stability for more than 3 years when the vials are simply stored at 4°C in a normal fridge at the hospital pharmacy. They can be shipped anywhere in the world via a courier, which has previously been demonstrated for US cancer trials using EDVs.

[0163] Patient dosing: When a patient is to be dosed, the vial can be reconstituted in 1 ml of sterile physiological saline and injected i.v. as a bolus injection.

[0164] The plasmid can be transformed into the intact bacterially-derived minicell producing strain and it would express the viral proteins in the bacterial cytoplasm. When the intact bacterially-derived minicell is produced during asymmetric bacterial division a lot of the protein is segregated into the intact bacterially-derived minicell cytoplasm. This has been demonstrated in in several studies where heterologous foreign proteins have been expressed in intact bacterially-derived minicell producing bacterial cells and the proteins segregate into the intact bacterially-derived minicell cytoplasm.

[0165] The expected results from plasmid-packaged intact bacterially-derived minicells is an antibody response to all 4 virus proteins, plus a Type I interferon response.

[0166] The injected intact bacterially-derived minicells would be rapidly engulfed by the cells of the immune system (macrophages, NK cells and dendritic cells) in the lymph nodes, liver and spleen. The intact bacterially-derived minicells normally enter the endosomes and are broken

down in the lysosomes and the plasmid is released which escapes into the cytoplasm.

[0167] Cytosolic DNA sensors, which would recognize the plasmid DNA, are a class of pattern recognition receptors (PRRs), which induce the production of type I interferons (IFNα and IFNβ) and trigger the induction of a rapid and efficient innate immune response. It is well known that Type I interferons have a potent antiviral effect.

[0168] The viral proteins are released from the broken down intact bacterially-derived minicells in the lysosomes and undergo antigen processing and presentation via MHC Class II on to the cell surface. This triggers a potent antibody response to the viral antigenic epitopes. This further provokes a CD4+/CD8+ T cell response against virally infected cells and this should augment the anti-viral response.

[0169] The activation maturation and proliferation of fresh bone marrow derived monocytes along with the activation and proliferation of macrophages, dendritic cells, NK cells, B cells and T cells would be expected to overcome the observed lymphopenia in the elderly and immune-compromised SARS-CoV2 patients.

[0170] Expected results from α-galactosyl ceramide packaged intact bacterially-derived minicells – induction of IFN-γ response: EDV™α-GC are also engulfed by cells of the immune system (macrophages, NK cells and dendritic cells) in the lymph nodes, liver and spleen. The intact bacterially-derived minicells are broken down in the intracellular lysosomes and the α-GC is released which is picked up by lysosomally associated CD1d (MHC Class I like molecule which is involved in the presentation of foreign glycolipids) and transported to the cell surface. This α-GC/CD1d complex is recognized by the invariant T cell receptor on invariant NKT cells and this results in the rapid release of IFN-γ. IFN-γ is known to be a potent stimulator of a specific anti-viral immune response which would then be expected to augment the rejection of the viral infection.

[0171] The intact bacterially-derived minicell therapeutics have already been shown to be safe in human cancer patients where over 1,500 doses have been administered in over 140 patients with minimal to no side effects despite repeat dosing.

Example 2

[0172] FIG. 2 shows peripheral blood mononuclear cells (PBMCs) from patient 1-CB04-1 (72 year old male) with end-stage hepatocellular carcinoma, showing an elevation in CD8+ cytotoxic T cells (**Fig. 2A**), NK cells (**Fig. 2B**), NKT cells (**Fig. 2C**) and iNKT cells (**Fig. 2D**) by cycle 2 and 3 following treatment with epidermal growth factor receptor (EGFR)-antibody targeted, PNU-packaged intact bacterially-derived minicells (i.e., EDV™) + α-galactosyl ceramide packaged intact bacterially-derived minicells (i.e., EDV™).

[0173] It is to be noted that the patient is elderly and severely immune-compromised. PNU is PNU-159682, which is a morpholinyl anthracycline derivative.

Table 2

Patient #	Age	Gender	Stage IV cancer
1-CB04-1	72	M	Hepatocellular Ca.

X-axis = Cycle and dose number e.g. C2D7 = Cycle 2, dose 7

[0174] Preparation of epidermal growth factor receptor (EGFR)-antibody targeted, PNU-packaged intact bacterially-derived minicells is described, for example, in WO 2020/021437.

[0175] The results detailed in Figs. 2A-D demonstrate the positive effects on the immune system following administration of a combination bacterial minicell composition comprising an intact bacterially-derived minicells-packaged anticancer compound (PNU-159682) combined with an intact bacterially-derived minicell-packaged + CD1d-recognized antigen (α-galactosyl ceramide). In particular, Fig. 2A shows a graph of percent CD8+ T cells (y axis) vs T cell subsets for naïve (first 4 columns) and effector (last four columns). T cell subsets shown are C1D1, C1D9, C2D7, and C3D7.

[0176] Groups of specific, differentiated T cells have an important role in controlling and shaping the immune response by providing a variety of immune-related functions. One of these functions is immune-mediated cell death, and it is carried out by T cells in several ways: CD8+ T cells, also known as "killer cells", are cytotoxic - this means that they are able to directly kill virus-infected cells as well as cancer cells. CD8+ T cells are also able to utilize small signalling proteins, known as cytokines, to recruit other cells when mounting an immune response.

[0177] Fig. 2B shows the percent of leukocytes vs subsets of NK cells (C1D1, C1D9, C2D7, and C3D7). Fig. 2C shows the percent of T-cells vs subsets of NKT cells (C1D1, C1D9, C2D7, and C3D7). Finally, Fig. 2D shows the percent NKT cells vs subsets of iNKT cells (C1D1, C1D9, C2D7, and C3D7).

Example 3

[0178] **FIG. 3** shows PBMCs from a 45 year-old female with end-stage colorectal cancer, showing activation of key immune cells. The patient's CD8+ effector cytotoxic T cells (CD45RA+ CCR7-) increased significantly by cycles 2 and 3 (**Fig. 3A**). Similarly, the subject's PBMCs showed an increase in NK cells (**Fig. 3B**) by cycles 2 and 3. Interestingly, ELISA analysis of the patient's serum, 3 hrs post each intact bacterially-derived minicell dose, showed a spike in IFNγ (**Fig. 3C**) which would occur if the α-galactosyl ceramide were effectively presented by antigen presenting cells (APCs) to the iNKT cells which would then trigger off the release of IFNγ, a critical mediator in fighting viral infections.

Table 3

Patient #	Age	Gender	Stage IV cancer
1-CB10-1	45	F	Colorectal ca + renal failure/on dialysis

X-axis = Cycle and dose number e.g. C2D7 = Cycle 2, dose 7

[0179] Similar to Example 2, the intact bacterially-derived minicells administerd to the subject included a combination bacterial minicell composition comprising an intact bacterially-derived minicell-packaged anticancer compound (PNU-159682) combined with an intact bacterially-derived minicell-packaged + CD1d-recognized antigen (α-galactosyl ceramide).

[0180] Fig. 3A shows a graph of the percent CD8+ T cells vs CD8+ memory T cell subsets, with the first 4 columns corresponding to the naïve test results, followed by the second four columns corresponding to the effector test results. The patient's CD8+ effector cytotoxic T cells (CD45RA+ CCR7-) increased significantly by cycles 2 and 3.

[0181] Fig. 3B shows a graph of the percent leukocytes vs NK cell subsets (C1D1, C1D9, C2D7, and C3D7). The results show that the subject's PBMCs showed an increase in NK cells by

cycles 2 and 3.

[0182] Finally, Fig. 3C shows IFNgamma (pg/mL) vs IFNgamma measured per dose. The ELISA analysis of the patient's serum, 3 hrs post each intact bacterially-derived minicell dose, showed a spike in IFNγ, which would occur if the α-galactosyl ceramide were effectively presented by antigen presenting cells (APCs) to the iNKT cells, which would then trigger off the release of IFNγ.

Example 4

[0183] FIG. 4 shows the white blood cell counts (average of 9 patients) at pre-dose and 3 hrs post dose. 8 out of the 9 patients were elderly and all were severely immune-compromised with Stage IV pancreatic cancer and all having failed all lines of conventional therapy. Yet, interestingly, 3 hrs post dose there was a significant increase in white blood cells (WBC) and this occured at every dose after dose 2, suggesting that the early doses of intact bacterially-derived minicells recruit fresh monocytes from the bone marrow following activation signals from the macrophages, dendritic cells and NK cells and by dose 3 they are sufficiently activated and matured to result in proliferation.

Table 4

Patient #	Age	Gender	Stage IV cancer
CB01	67	M	Pancreatic Ca.
CB05	62	M	Pancreatic Ca.
CB06	71	M	Pancreatic Ca.
CB07	68	M	Pancreatic Ca.
CB12	47	M	Pancreatobiliary Ca.
CB14	65	F	Pancreatic Ca.
CB15	70	M	Pancreatic Ca.
CB16	70	F	Pancreatic Ca.
CB22	72	F	Pancreatic Ca.

Every data point is an average value from 9 end-stage pancreatic cancer patients

[0184] The results, as detailed in Figure 4, are significant as proliferation of macrophages, dendritic cells and NK cells is critical to a successful immune defense of a viral infection.

Example 5

[0185] **FIG. 5** shows PBMCs from a 45 year-old female with end-stage colorectal cancer, showing activation of key immune cells. The patient's CD8+ effector cytotoxic T cells (CD45RA+ CCR7-) increased significantly by cycles 2 and 3 (**Fig. 5A**). Similarly, the patient's PBMCs showed an increase in NK cells (**Fig. 5B**) by cycles 2 and 3. Interestingly, ELISA analysis of the patient's serum, 3 hrs post each intact bacterially-derived minicell dose, showed a spike in IFNγ (**Fig. 5C**) which would occur if the α-galactosyl ceramide were effectively presented by antigen presenting cells to the iNKT cells, which then would trigger the release of IFNγ, a critical mediator in fighting viral infections.

Table 5

Patient #	Age	Gender	Stage IV cancer
1-CB04-1	72	M	Hepatocellular Ca.
1-CB10-1	45	F	Colorectal ca + renal failure/on dialysis
2-CB12-1	47	M	Pancreatobiliary Ca.

Example 6

[0186] This example is directed to a study evaluating the feasibility of using bacterial minicells loaded with $EDV_{Covid\text{-}\alpha GC}$ ($EDV_{Covid\text{-}\alpha GC}$) as a vaccine against SARS-CoV-2.

[0187] Alpha-GC and the spike protein along with the plasmids encoding the spike protein DNA sequence can be successfully incorporated into one single EDV ($EDV_{Covid\text{-}\alpha GC}$). The EDVs were then administered through subcutaneous (SC), intravenous (IV) and intra-muscular (IM) injections. It was found that administration through intra-muscular injections yielded the strongest initial interferon response 8h post-injection as well as the highest spike protein specific IgG titres 1 week post-injection compared to all other strategies tested.

[0188] $EDV_{Covid\text{-}\alpha GC}$ and corresponding controls were then administered through intra-muscular injections and the incorporation of αGC in the EDVs resulted in a dramatic increase in IFNα, TNFα, IFNγ, IL12 and IL6 production 8h post-treatment. This was accompanied by an increase in the amount of cytotoxic T-cells in the spleens of $EDV_{Covid\text{-}\alpha GC}$ treated mice. These T-cells

responded to the stimulation of the spike protein *ex vivo* and expressed CD69+ CD137+.

[0189] At 4 weeks post-initial treatment, mice injected with $EDV_{Covid\text{-}\alpha GC}$ contained the highest amount of spike protein specific IgG and IgM compared to all the controls tested. B-cells extracted from these mice were able to produce IgG and IgM *ex vivo* in response to spike protein stimulation. In addition, splenocytes from EDVCovid-αGC treated mice contained the highest amount of anti-viral CD69+ CD137+ cytotoxic T-cells and *ex vivo* stimulation of these splenocytes using the spike protein yielded an increase in viral antigen specific CD69+ cytotoxic T cells. Moreover, the serum of $EDV_{Covid\text{-}\alpha GC}$ injected mice exhibited the strongest inhibition of spike protein binding to the hACE receptor *in vitro*, indicating the antibodies produced were neutralizing. Interestingly, the serum from mice that received any form of αGC also exhibited measurable but non-antigen-specific antiviral effect.

[0190] In summary, the incorporation of αGC into EDV_{Covid} is important for achieving maximum anti- SARS-CoV-2 spike protein efficacy. The results of this study indicate that I.M. administration of $EDV_{Covid\text{-}\alpha GC}$ is a viable strategy for combating the current Covid-19 pandemic.

[0191] Materials and methods

[0192] *SARS-CoV-2 Spike protein bacterial expression plasmid design*: The expression cassette was generated by placing the coding nucleotide sequence for SARS-Cov-2 (Covid-19) Spike protein (Genebank MN908947.3) on the 3'-end of a modified β-lactamase promoter, which has been previously tested for expression in *Salmonella typhimurium* strains (Su, Brahmbhatt et. al., *Infection and Immunity*, *60*(8):3345-3359 (1992)). The expression cassette was then inserted between the Kpn 5' and Sal I 3' sites of the M13 multiple cloning site of PUC57-Kan backbone plasmid to create P-Blac-Cov2S. The control plasmid, P-Blac was created by removing the Cov2S sequence from the P-Blac-Cov2S (Figures 10A and 10B).

[0193] *Cloning of P-Blac-Cov2S and P-Blac-Cov2S into Salmonella Typhimurium EDV producing strain and the subsequent incorporation of P-Lac-Cov2S and the spike protein into the EDVs*: P-Blac-Cov2S and P-Blac-Cov2S were electroporated using a Gene Pulser Xcell™ (Bio-Rad, Hercules CA) into a chemically competent *Salmonella typhimurium* intermediate strain (4004), which lacks plasmid restriction mechanism, using settings 200ohm, 25Hz, 2.5 mV.

Transformants were recovered in TSB medium for 1.5 hrs at 37°C before plating on TSB agar plates containing 75 µg/ml kanamycin (#K4000, Sigma-Aldrich, St. Louis, Missouri). Isolates were picked into TSB broth with 75 µg/ml kanamycin and plasmid DNA extracted using the Qiagen miniprep kit as per manufacturer's instructions (#27104, Qiagen, Hilden, Germany). Subsequently, the extracted plasmid DNA from 4004 strain was electroporated as above into EnGeneIC Pty. Ltd. EDV producing *Salmonella typhimurium* strain (ENSm001). The bacteria that contained P-Blac-Cov2S would produce the encoded Covid2 spike protein, which alone with the plasmid DNA, would be incorporated into the EDVs to produce EDV_{COVID}. The EDVs containing P-Blac (EDVCONT) would be used as a control.

[0194] To determine the plasmid content of EDV_{COVID} and EDV_{CONT}, plasmids were extracted from $2x10^9$ EDVs using a Qiaprep Spin miniprep kit (Qiagen) following the manufacturer's instructions. Empty EDV were treated the same was and used as controls. The quantity of DNA plasmids were then measured by absorption at 260nm using a Biophotometer (Eppendorf). The copy number of the plasmids were calculated using:

$$\text{Number of copies} = \frac{\text{amount} * 6.022x10^{23}}{\text{length} * 1x10^9 * 660}$$

[0195] *Western Blot*: Proteins from $2x10^{10}$ EDVCOVID were extracted using 100µL B-PER™ (Thermo Fisher) bacterial protein extraction reagent supplemented with 10% (v/v) lysozyme (Sigma-Aldrich) and 1% (v/v) DNaseI (Qiagen). The extracted samples were then centrifuged at 12,000 g for 10min and the supernatant was collected. The left-over pellet was also collected and resuspended in 100 µl PBS. 23 µl of the supernatant and pellet protein samples were co-incubated with 5 µl of loading buffer and 2 µl DTT (Sigma-Aldrich) at 80oC for 20min before the entire content of each sample was loaded onto a NuPAGE 4-12% Bis-Tris mini gel (Life Technologies) and run at 190 V for ~80min. The sample was then transferred using an iBlot 2 machine and the membrane was blocked using Superblock blocking buffer (Thermo Fisher) and subsequently stained with 1:2000 Rabbit poly-clonal SARS-CoV2 spike antibody (also cross-reacts with the S1 subunit, Sino Biological, Beijin, China) and incubated overnight at 4°C. The

membrane was then washed with PBST and incubated with HRP conjugated anti-rabbit secondary antibody (1:5000) (Abcam) for 1h at room temperature. The blot was developed using Lumi-Light Western Blot substrate (Roche) and visualised using a Chemidoc MP (Biorad).

[0196] *Alpha-galactosylceramide loading into EDVCOVID and cell culture:* Alpha-galactosylceramide glycolipid adjuvant (α-GC) was loaded into EDVCOVID to created EDVCOVID-αGC using a proprietary method developed at EngeneIC.

[0197] JAWSII cells (ATCC) were treated with EDVCOVID-αGC in a 96-well Perfecta3D hanging drop plate (Sigma) at $1x10^4$ EDVCOVID-αGC per cell. JAWSII cells treated with 4μg/mL α-GC was used as a positive control. The cultures were then incubated for 24h at 37°C with 5% CO_2 and cells were collected and stained with a CD1d-αGC antibody (ThermoFisher) and analysed using a Gallios flow cytometer (Beckman). The results were analysed using Kaluza Analysis software (Beckman).

[0198] *Animal studies:* Female Balb/c mice, 6-7 weeks old were obtained from the Animal Resources Company in Western Australia. The mice were acclimatized for one week before the experiments commenced. The mice was injected with appropriate treatments through SC and IM injections and serum was collected 8h, 1 week and 4 weeks post-injection through the tail vein and the spleen and bone marrows were collected.

[0199] *Enzyme-linked immunosorbent assay:* The levels of IL-12p40, IFN-γ, TNFα, IL-6, IL2, IFNα and IFNβ in the mouse serum were measured by standard sandwich enzyme-linked immunosorbent assay (ELISA) from R&D Systems according to manufacturer's instructions. The concentrations of the protein present were determined by calculating absorbance of the samples again standards curves constructed within the same assay using purified proteins.

[0200] For analysis of anti-RBD specific IgG and IgM antibodies, 96-well plates (Immulon 4 HBX; Thermo Fisher Scientific) were coated at 4°C with 50μl per well of a 2μg/ml solution of anti-covid spike RBD protein (Genetex) suspended in PBS (GIBCO). On the following day, the coating protein solution was removed and the samples in each well were blocked using 100μl per well of 3% non-fat milk prepared in PBS with 0.1% Tween 20 (PBST) at room temperature for 1h. During this time, serial dilutions of mouse serum were prepared in 1% non-fat milk prepared

in PBST. The blocking solution was then removed and 100µl of each serial diluted serum sample was added to the plates and incubated for 2h at room temperature. At the end of incubation period, the plates were washed three times with 250µl per well of 0.1% PBST, before adding 100µl of 1:3,000 dilution of goat anti-mouse IgG/IgM–horseradish peroxidase (HRP) conjugated secondary antibody (ThermoFisher) prepared in 0.1% PBST. The samples were incubated at room temperature for 1h and then were again washed three times with 0.1% PBST. Once completely dry, the samples were visualised by incubating with TMD. The reactions were then terminated and the samples were read at 490nm using a KC Junior plate reader (BioTek Instruments).

[0201] Antibody titre was determined using ELISA by generating 1:3 serial dilution of the treated mouse serum samples and is expressed as the inverse of the highest dilution with a positive result.

[0202] *Statistical analysis:* Student's T-tests and One-way ANOVA was conducted using Prism 8 (GraphPad). A P value of <0.05 is considered to be statistically significant.

[0203] Results

[0204] To achieve effective and efficient delivery of the vaccine with one single injection, αGC was co-loaded into EDV_{Covid} to create $EDV_{Covid\text{-}\alpha GC}$. The function of the co-loaded αGC was tested by examining its presentation on JAWSII cells via CD1d ligand following $EDV_{Covid\text{-}\alpha GC}$ treatment. It was found that a high percentage of JAWSII cells expressed CD1d-αGC following the treatment at a comparable or higher level than those that were treated with 3µg/mL of free αGC (Figure 11A). Western blot analysis was conducted to ensure the spike protein incorporated into the EDVCovid-αGC was not affected by the secondary incorporation of αGC (Figure 11B).

[0205] The effect of different delivery methods for the $EDV_{Covid\text{-}\alpha GC}$ was then assessed *in vivo*. Mouse serum samples were collected from treatments administrated through subcutaneous (S.C.), intravenous (I.V.) and Intra-muscular (I.M.) injections and analysed via ELISA levels of IFNα (Fig.7C; serum IFNα concentration 8h post-injection), IFNγ (Fig. 7D; serum IFNγ concentration 8h post-injection), IL12 (Fig. 7E; IL12p40 serum concentration 8h post-injection), IL6 (Fig. 7F; IL6 serum concentration 8h post-injection) and TNFα (Fig. 7G; serum TNFα,

concentration 8h post-injection) . It was found that $EDV_{Covid\text{-}\alpha GC}$ administered through I.M. injection was vastly superior at inducing the production of all the cytokines tested in mice at 8h post-injection.

[0206] The difference between the different methods of administration of $EDV_{Covid\text{-}\alpha GC}$ was further demonstrated when spike protein specific antibodies were analyzed at 1 week post-initial injection. High spike protein specific IgG titre was detected in the serum of EDVCovid-αGC treated mice through I.M. injections compared that of through S.C. injections (Figure 7A). It was concluded that due to the high levels of initial interferon response and subsequent high IgG titres, administration of $EDV_{Covid\text{-}\alpha GC}$ through I.M. injection was the preferred delivery strategy.

[0207] Detailed analysis of initial interferon response following I.M. injections of EDV, $EDV_{\alpha GC}$, $EDV_{Control}$, $EDV_{Control\text{-}\alpha GC}$, EDV_{Covid}, $EDV_{Covid\text{-}\alpha GC}$ showed that the early interferon response in mice was predominantly induced by the administration of αGC carried by EDVs with or without an accompanying antigen-specific plasmid. *See* Fig. 12A (serum IFNα concentration 8h post-IM injection); Fig. 12B (serum IFNγ concentration 8h post-IM injection); Fig. 12C (IL6 serum concentration 8h post-IM injection); Fig. 12D (serum TNFα, concentration 8h post-IM injection); and Fig. 12E (IL12p40 serum concentration 8h post-IM injection)

[0208] FACS analysis of mouse splenocytes at 1 week post-injection showed that there is an increase in CD3+ CD8+ cytotoxic T-cell number in the $EDV_{Covid\text{-}\alpha GC}$ injected mice as compared to the saline group (Figure 13A). AIMS assay was conducted on the *ex vivo* splenocytes and it was found that there is an increase in viral antigen-specific CD69+ CD137+ population within the cytotoxic T-cell population when stimulated with the spike protein, at a higher level as compared to the PHA stimulated positive controls (Figure 13B).

[0209] At 4 weeks post-initial injection, the highest levels of spike protein specific IgG (Figure 14A) and IgM (Figure 14B) were observed in the serum of the mice that were treated with $EDV_{Covid\text{-}\alpha GC}$ administered through I.M. injections. Interestingly, it was also found that the serum of mice treated with $EDV_{Control\text{-}\alpha GC}$ also contained spike protein "specific" antibodies. This finding was confirmed by neutralizing antibody analysis. While the serum of mice treated with $EDV_{Covid\text{-}\alpha GC}$ contained the highest amount of neutralizing antibodies, serums of mice treated

with $EDV_{Control-\alpha GC}$, EDV_{Covid} and $EDV_{\alpha GC}$ also resulted in measurable degree of spike protein to hACE receptor binding inhibition (Figure 14C). It appeared that αGC alone has anti-viral properties in which the administration of the compound could result in the inhibition of viral binding to the cells in the body. On the other hand, injecting EDV_{Covid} by itself without the addition of αGC was capable of producing neutralizing antibodies in the serum, albeit at much lower levels compared to that of treated with $EDV_{Covid-\alpha GC}$. This demonstrated the importance of incorporating αGC as an immuno-adjuvant in this system as a vital part of a functional vaccine.

[0210] To further demonstrate the specificity of the antibody response, B-cells were extracted from the bone marrow of the treated mice at 4 weeks post-initial injection and stimulated with spike protein for 48h *in vitro*. B cells from mice treated with $EDV_{Covid-\alpha GC}$ produced the highest level of spike protein specific IgG (Figure 15A) and IgM (Figure 15B) as compared to all other treatment groups.

[0211] FACS analysis of *ex vivo* splenocytes from treated mice showed that $EDV_{Covid-\alpha GC}$ treatment resulted in an increase in CD69+ CD137+ cytotoxic T-cells as compared to all other treatment conditions (Figure 9A). It was also observed that when the ex vivo spherocytes were stimulated with the spike protein, there was an increase in viral antigen specific CD69+ CD137- cells within the cytotoxic T-cell population at a similar rate compared to the PHA stimulated positive controls from $EDV_{Covid-\alpha GC}$ and EDV_{Covid} treated mice (Figure 9B). This was not observed in all the other treatment groups. It indicates that, unlike the anti-viral response triggered by $EDV_{Covid-\alpha GC}$ treatment, the anti-viral property of αGC may be broad spectrum and not antigen-specific.

[0212] While certain embodiments have been illustrated and described, it should be understood that changes and modifications can be made therein in accordance with ordinary skill in the art without departing from the technology in its broader aspects as defined in the following claims.

[0213] The embodiments, illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising," "including," "containing," etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as

terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the claimed technology. Additionally, the phrase "consisting essentially of" will be understood to include those elements specifically recited and those additional elements that do not materially affect the basic and novel characteristics of the claimed technology. The phrase "consisting of" excludes any element not specified.

[0214] The present disclosure is not to be limited in terms of the particular embodiments described in this application. Many modifications and variations can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. Functionally equivalent methods and compositions within the scope of the disclosure, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing descriptions. Such modifications and variations are intended to fall within the scope of the appended claims. The present disclosure is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled. It is to be understood that this disclosure is not limited to particular methods, reagents, compounds, or compositions, which can of course vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

[0215] In addition, where features or aspects of the disclosure are described in terms of Markush groups, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member or subgroup of members of the Markush group.

[0216] As will be understood by one skilled in the art, for any and all purposes, particularly in terms of providing a written description, all ranges disclosed herein also encompass any and all possible subranges and combinations of subranges thereof, inclusive of the endpoints. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, etc. As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, etc. As will also be understood by one skilled in the art all language such as "up

to," "at least," "greater than," "less than," and the like, include the number recited and refer to ranges which can be subsequently broken down into subranges as discussed above. Finally, as will be understood by one skilled in the art, a range includes each individual member.

[0217] All publications, patent applications, issued patents, and other documents referred to in this specification are herein incorporated by reference as if each individual publication, patent application, issued patent, or other document was specifically and individually indicated to be incorporated by reference in its entirety. Definitions that are contained in text incorporated by reference are excluded to the extent that they contradict definitions in this disclosure.

[0218] Other embodiments are set forth in the following claims.

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