

NUCLEOCAPSID ANTIGEN IMMUNOTHERAPY FOR COVID-19 FUSION PROTEINS AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the priority benefit of U.S. Provisional Patent Application Serial No. 63/488,565, filed March 6, 2023, entitled NUCLEOCAPSID ANTIGEN IMMUNOTHERAPY FOR COVID-19 FUSION PROTEINS AND METHODS OF USE, incorporated by reference in its entirety herein.

SEQUENCE LISTING

[0002] The following application contains a sequence listing filed electronically as a Standard ST.26 compliant XML file with a file name of “ABC-047PCT.xml,” a creation date of February 15, 2024, and a file size of 23,503 bytes, the entire contents of which are incorporated by reference herein.

TECHNICAL FIELD

[0003] The present technology relates to fusion proteins comprising a truncation of the SARS-CoV-2 nucleocapsid N protein or an analog thereof linked to human Fc fragments and their use in relation to the 2019 Novel Coronavirus (COVID-19).

BACKGROUND

[0004] The following description of the background is provided simply as an aid in understanding the present technology and is not admitted to describe or constitute prior art to the present technology.

Fc Fusion Proteins

[0005] Fc fusion proteins are comprised of a species-specific immunoglobulin Fc domain that is linked to another peptide such as a protein or peptide with therapeutic potential. As used herein, the terms “fusion protein” and “Fc fusion protein” refer to a protein comprising more than one part, for example from different sources (e.g., different proteins, polypeptides, cells, etc.), that are covalently linked through peptide bonds. Fc fusion proteins are preferably covalently linked by (i) connecting the genes that encode for each part into a single nucleic acid molecule and (ii) expressing in a host cell (e.g., HEK cell or CHO cell) the protein for which the nucleic acid molecule encodes. The fully recombinant synthesis approach is preferred over methods in which the therapeutic protein and Fc fragments are synthesized separately and then

chemically conjugated. The chemical conjugation step and subsequent purification process increase the manufacturing complexity, reduce product yield, and increase cost.

[0006] The terms “Fc fragment,” “Fc region,” “Fc domain,” or “Fc polypeptide,” are used herein to define a C-terminal region of an immunoglobulin heavy chain. The Fc fragment, region, domain, or polypeptide may be a native sequence Fc region or a variant/mutant Fc region. Although the boundaries of the Fc region of an immunoglobulin heavy chain may vary, they generally comprise some or all of the hinge region of the heavy chain, the CH2 region of the heavy chain, and the CH3 region of the heavy chain. The hinge region of a Fc fragment (e.g., a canine or human Fc fragment) comprises amino acid sequences that connect the CH1 domain of the heavy chain to the CH2 region of the heavy chain and contains one or more cysteines that form one or more interheavy chain disulfide bridges to form a homodimer of an Fc fusion protein from two identical but separate monomers of the Fc fusion protein. The hinge region may comprise all or part of a naturally occurring amino acid sequence or a non-naturally occurring amino acid sequence.

[0007] The presence of the Fc domain increases the plasma half-life due to its interaction with the neonatal Fc-receptor (FcRn) in addition to slower renal clearance of the Fc fusion protein due to the large molecule size, resulting in *in vivo* recycling of the molecule achieving prolonged activity of the linked peptide and improved solubility and stability of the Fc fusion protein molecule. The Fc domain also enables Fc fusion proteins to interact with Fc receptors on immune cells. In some examples, the therapeutic protein or peptide is linked to the immunoglobulin Fc domain via a linker. The therapeutic protein or peptide and linker effectively replace the variable region of an antibody while keeping the Fc region intact.

[0008] An Fc receptor (FcR) refers to a receptor that binds to an Fc fragment or to the Fc region of an antibody. In examples, the FcR is a native sequence of the canine or human FcR, and the FcR is one which binds an Fc fragment or the Fc region of an IgG antibody (a gamma receptor) and includes without limitation, receptors of the Fc(gamma) receptor I, Fc(gamma) receptor IIa, Fc(gamma) receptor IIb, and Fc(gamma) receptor III subclasses, including allelic variants and alternatively spliced forms of these receptors. “FcR” also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgG molecules to the fetus and is also responsible for the prolonged *in vivo* elimination half-lives of antibodies and Fc-fusion proteins *in vivo*. In examples, FcR of human origin are used *in vitro* (e.g., in an assay) to measure the binding of Fc fusion proteins comprising Fc fragments of any mammalian origin so as to assess their FcR binding properties. Those skilled in the art will understand that mammalian FcR from one species (e.g., FcR of human origin) are sometimes capable of *in vitro* binding of Fc fragments from a second species (e.g., FcR of canine origin).

SUMMARY OF THE PRESENT TECHNOLOGY

[0009] Described herein are fusion proteins, each comprising a respective viral nucleocapsid protein fragment and an Fc fragment, wherein the viral nucleocapsid fragment and the Fc fragment are connected by a peptide linker. In one or more embodiments, the nucleocapsid fragment comprises a SARS-CoV-2 nucleocapsid protein fragment comprising a functional fragment, analog, or variant/mutant thereof. In one or more embodiments, the nucleocapsid protein fragment comprises a nucleocapsid fragment of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 12, SEQ ID NO: 13, or a functional fragment, analog, or variant/mutant thereof. In one or more embodiments, the Fc fragment comprises a sequence or functional fragment of SEQ ID NO: 1. In one or more embodiments, the linker comprises SEQ ID NO: 14. In one or more embodiments, the fusion protein comprises, consists essentially or even consists of a sequence of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 19, or SEQ ID NO: 20. In one or more embodiments, the fusion protein is a homodimer. In one or more embodiments, the Fc fragment is glycosylated.

[0010] Also described herein are immunogenic compositions which comprise or consist essentially of a fusion protein(s) according to any embodiments or combinations of embodiments described herein, and a pharmaceutically-acceptable carrier. In one or more embodiments, the fusion protein is dispersed in the carrier. In one or more embodiments, the compositions further comprise an adjuvant. In one or more embodiments, the adjuvant is Montanide™ ISA-720. In one or more embodiments, the fusion protein is emulsified with the adjuvant. In one or more embodiments, the emulsification is prepared onsite before administration. In one or more embodiments, the prepared emulsification is refrigeration (4°C) or room temperature stable for at least 8 hours, preferably up to 24 hours. In one or more embodiments, the composition is an injectable formulation. In one or more embodiments, the composition is adapted for subcutaneous administration. In one or more embodiments, the composition is adapted for prophylactic vaccination. In one or more embodiments, the composition is adapted for therapeutic vaccination.

[0011] Also described herein are various methods for increasing antibody production in a subject against an antigenic agent. The methods generally comprise administering a therapeutically effective amount of a fusion protein(s) or immunogenic composition(s) according to any embodiments or combinations of embodiments described herein to the subject. In one or more embodiments, the subject has a measurable antibody titer against the antigenic agent prior to administration of the fusion protein or immunogenic composition. In one or more embodiments, the subject is antibody naïve prior to administration of the fusion protein or

immunogenic composition. In one or more embodiments, the fusion protein or immunogenic composition is administered via injection. In one or more embodiments, the fusion protein or immunogenic composition is administered subcutaneously or intramuscularly. In one or more embodiments, the fusion protein or immunogenic composition is provided as a unit dosage form. In one or more embodiments, the fusion protein or immunogenic composition is co-administered with an adjuvant. In one or more embodiments, the methods further comprise preparing the fusion protein or immunogenic composition for administration, wherein the preparation comprises pre-mixing the fusion protein or immunogenic composition with an adjuvant before administration. In one or more embodiments, pre-mixing comprises emulsifying the adjuvant and fusion protein to yield an emulsion and administering the emulsion to the subject. In one or more embodiments, the prepared emulsification is refrigeration (4°C) or room temperature stable for at least 8 hours, preferably up to 24 hours after preparation.

[0012] Also described herein are methods of inducing an immune response in a subject against viral infection, preferably SARS-CoV-2 virus, more preferably COVID-19. The methods generally comprise administering a therapeutically effective amount of a fusion protein(s) or immunogenic composition(s) according to any embodiments or combinations of embodiments described herein to the subject. In one or more embodiments, the subject has a measurable antibody titer against the viral infection prior to administration of the fusion protein or immunogenic composition. In one or more embodiments, the subject is antibody naïve prior to administration of the fusion protein or immunogenic composition. In one or more embodiments, the fusion protein or immunogenic composition is administered via injection. In one or more embodiments, the fusion protein or immunogenic composition is administered subcutaneously or intramuscularly. In one or more embodiments, the fusion protein or immunogenic composition is provided as a unit dosage form. In one or more embodiments, the fusion protein or immunogenic composition is co-administered with an adjuvant. In one or more embodiments, the methods further comprise preparing the fusion protein or immunogenic composition for administration, wherein the preparation comprises pre-mixing the fusion protein or immunogenic composition with an adjuvant before administration. In one or more embodiments, pre-mixing comprises emulsifying the adjuvant and fusion protein to yield an emulsion and administering the emulsion to the subject. In one or more embodiments, the prepared emulsification is refrigeration (4°C) or room temperature stable for at least 8 hours, preferably up to 24 hours after preparation.

[0013] Also described herein are methods of producing a fusion protein according to any embodiments or combinations of embodiments described herein. The methods generally comprising transiently transfecting a nucleic acid encoding for the fusion protein into a

HEK293, wherein the transfected HEK293 cell expresses the fusion protein. In one or more embodiments, the fusion protein is secreted by the cells into cell culture media, further comprising purifying or isolating the fusion protein from the media. Advantageously, the yield of the purified or isolated fusion protein is greater than 100 mg/L in any of the foregoing expression systems.

[0014] Also described herein are cells engineered to express a fusion protein according to any embodiments or combinations of embodiments described herein. In one or more embodiments, the cell is a HEK293 cell.

[0015] As described herein, the fusion protein(s) or immunogenic composition(s) according to any embodiments or combinations of embodiments described herein can be used in therapy and/or as a medicament.

[0016] As described herein, the fusion protein(s) or immunogenic composition(s) according to any embodiments or combinations of embodiments described herein can be used in increasing antibody production in a subject.

[0017] As described herein, the fusion protein(s) or immunogenic composition(s) according to any embodiments or combinations of embodiments described herein can be used in treatment and/or prophylaxis of a viral infection, preferably SARS-CoV-2 virus, more preferably COVID-19.

[0018] As described herein, the fusion protein(s) or immunogenic composition(s) according to any embodiments or combinations of embodiments described herein can be used as a prophylactic, therapeutic and/or booster vaccine.

[0019] Particular embodiments concern fusion protein(s) selected from the group consisting of: SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 19, SEQ ID NO: 20, or pharmaceutical compositions thereof for use in treatment and/or prophylaxis of a viral infection, preferably SARS-CoV-2 virus, more preferably COVID-19.

[0020] As described herein, the fusion protein(s) or immunogenic composition(s) according to any embodiments or combinations of embodiments described herein can be used in the manufacture of a medicament for the treatment and/or prophylaxis of a viral infection.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] FIG. 1 shows a schematic representation of an insulin-Fc fusion protein homodimer.

[0022] FIG. 2 shows a schematic representation of an exemplary SARS-CoV-2 N-Fc fusion protein homodimer.

[0023] FIG. 3 shows Fc(gamma) receptor I binding for the insulin-Fc fusion proteins of SEQ ID NO: 5 and SEQ ID NO: 7.

[0024] FIG. 4 shows titers of anti-insulin-antibodies (AIA) against RHI averaged over 200-fold dilutions for 6 beagles with chemically induced diabetes over a series of 8 doses of the insulin-Fc fusion protein of SEQ ID NO: 5.

[0025] FIG. 5 shows percentage change in the titers of anti-insulin antibodies (AIA) against RHI from Day 0 of the trial for 6 beagles with chemically induced diabetes over a series of 8 weekly doses of the insulin-Fc fusion protein of SEQ ID NO: 5.

[0026] FIG. 6 shows the normalized AIA titers for 8 client dogs treated for diabetes with the insulin-Fc fusion protein of SEQ ID NO: 5 according to Protocol 1 as described in Example 18 or Protocol 2 as described in Example 19.

[0027] FIG. 7 shows the normalized AIA titer for a single dog treated for diabetes with the insulin-Fc fusion protein of SEQ ID NO: 5 with an interruption in treatment.

[0028] FIG. 8 shows a graphical representation of the number of dogs that showed AIA after being treated for diabetes with the insulin-Fc fusion protein of SEQ ID NO: 5 manufactured in either an HEK transient cell pool or a CHO stable cell pool.

[0029] FIG. 9 shows the normalized AIA titers for 8 dogs treated for diabetes with the insulin-Fc fusion protein of SEQ ID NO: 7.

[0030] FIG. 10 shows a graphical representation of the number of dogs that showed AIA after being treated for diabetes with the insulin-Fc fusion protein of SEQ ID NO: 5 or SEQ ID NO: 7.

[0031] FIG. 11 illustrates APC processing of Fc-fusion proteins via the Fc(gamma) receptors.

[0032] FIG. 12 illustrates the absence of anti-SP/RBD mouse IgG Titers when BALB/c mice are dosed with the insulin-Fc fusion protein of SEQ ID NO: 15 on Day 0, Day 21 and Day 46.

[0033] FIG. 13 illustrates the presence of anti-nucleocapsid protein mouse IgG titers when BALB/c mice are dosed with the insulin-Fc fusion protein of SEQ ID NO: 15 on Day 0, Day 21 and Day 46.

[0034] FIG. 14 illustrates a side-by-side sequence comparison of the SARS-CoV-2 nucleocapsid fragment of SEQ ID NO: 10 and the shortened nucleocapsid fragment of SEQ ID NO: 11.

[0035] FIG. 15 illustrates a side-by-side sequence comparison of the SARS-CoV-2 nucleocapsid fragment of SEQ ID NO: 10 and the further shortened nucleocapsid fragment of SEQ ID NO: 12.

[0036] FIG. 16 illustrates a side-by-side sequence comparison of the SARS-CoV-2 nucleocapsid fragment of SEQ ID NO: 10, SEQ ID NO: 12 and the nucleocapsid fragment of SEQ ID NO: 13.

[0037] FIG. 17 illustrates a side-by-side sequence comparison of the SARS-CoV-2 nucleocapsid fragment of SEQ ID NO: 10 and the nucleocapsid fragment of SEQ ID NO: 8.

[0038] FIG. 18 illustrates a side-by-side sequence comparison of the SARS-CoV-2 nucleocapsid fragment of SEQ ID NO: 9 and the nucleocapsid fragment of SEQ ID NO: 8.

[0039] FIG. 19 illustrates a side-by-side sequence comparison of the SARS-CoV-2 nucleocapsid fragment of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 12 and the nucleocapsid fragment of SEQ ID NO: 13.

[0040] FIG. 20 illustrates a side-by-side sequence comparison of the SARS-CoV-2 nucleocapsid fragment of SEQ ID NO: 12 and the nucleocapsid fragment of SEQ ID NO: 13.

[0041] FIG. 21 illustrates a 96 well microplate such as that which may be used for a general serology assay for evaluating existing SARS-CoV-2 antibody titer in serum.

DETAILED DESCRIPTION

Novel Coronavirus Disease 2019

[0042] Novel Coronavirus Disease 2019 (COVID-19) is a severe and acute respiratory illness caused by the SARS-CoV-2 virus. SARS-CoV-2 has spread worldwide and by the end of 2021 had caused more than 400 million infections. The consensus among experts is that society cannot return to normal unless and until there is a sufficient level of immunity conferred on the population. Achieving natural herd immunity is estimated to require at least 70% of the population to have been infected which would result in millions of deaths worldwide, an ethically unacceptable outcome.

ACE2 Receptor

[0043] Angiotensin-Converting Enzyme 2 (ACE2) is the host cell receptor responsible for mediating infection by SARS-CoV-2 (i.e., to which the receptor binding domain of the spike protein of SARS-CoV-2 binds in order to infect cells). ACE2 is a type 1 transmembrane metallopeptidase. Polymerase Chain Reaction (PCR) analysis shows that ACE2 is expressed on lung epithelium, blood vessel endothelium, and specific neuronal cells that appears to account for the dominant clinical manifestations of COVID-19, including pulmonary, cardiovascular, and neurological complications, respectively. Based on the sequence similarities of the receptor binding domain between SARS-CoV-2 and SARS-CoV, researchers have shown that SARS-CoV-2 can use ACE2 expressed on the surface of human cells to gain entry into ACE2 expressing HeLa cells.

Convalescent Sera for Treatment of Virus Patients

[0044] Human convalescent serum has been used as an option for the prevention and

treatment of COVID-19, in particular in immunocompromised patients.

[0045] Convalescent serum is a form of passive antibody therapy, through which sera from infected and recovered individuals containing anti-virus antibodies is transfused to a susceptible or infected individual, providing that individual with some level of immunity to either prevent or reduce the severity of the disease. This treatment is different from a vaccine, which works by inducing an immune response in an individual such that the individual produces their own antibodies against the virus. Experience from the SARS-CoV outbreak in 2002 and the 2009-2010 H1N1 influenza outbreak has shown that sera from patients that have contracted and recovered from the virus (human convalescent sera) contains antibodies capable of neutralizing the virus and is useful as an intervention for individuals with severe disease symptoms or as a prophylactic vaccine.

[0046] The use of human convalescent sera has risks and limitations. Firstly, the transfer of blood substances from one person to another comes with it the risk of inadvertent infection of another infectious disease as well as the risk of reactions to other serum constituents. Another challenge in using convalescent sera is that some patients who recover from viral diseases do not have high titers of neutralizing antibody. In one case with respect to another human coronavirus, Middle East respiratory syndrome (MERS-CoV), three patients in South Korea were treated with convalescent serum, but only two of the recipients had neutralizing antibody in their serum. Of those that do have neutralizing antibodies after recovering from viral disease, some may not have sufficiently high titers of neutralizing antibody to be a viable donor. A further survey related to SARS-CoV found that of 99 samples of convalescent sera from patients with SARS, 87 had neutralizing antibody, with a geometric mean titer of 1:61. These and various other studies suggest that few patients made high-titer responses and also that neutralizing antibody titer declines with time. There are a number of companies looking to overcome this challenge by producing recombinant antibodies instead of solely relying on antibodies from recovered patients; however, the scale of production is insufficient, and the medical intervention required to administer effective doses to patients every few weeks to few months, most likely through intravenous injection or infusion, is highly burdensome. A significant limitation of recombinant antibody treatment is that as the SARS-CoV-2 virus mutates, existing monoclonal antibody treatments may become ineffective. In fact, as of November 2022, all six monoclonal antibody treatments authorized by the FDA in the United States between 2020 and 2022 which had been previously used for treating COVID-19 had FDA authorization rescinded due to their lack of ability to neutralize new Omicron subvariants of the SARS-CoV-2 virus. This has led to renewed use of convalescent sera for immunocompromised people who contract COVID-19, as such sera had a large number of different antibodies, giving it a breadth of treatment that

monoclonal antibodies don't have.

[0047] A limitation however is that the use of convalescent sera to treat COVID-19 patients relies on preparations with high titers of SARS-CoV-2 neutralizing antibodies. This requires a significant population of donors who have recovered from the disease or developed antibodies from vaccination or boosters and can donate convalescent serum. Determining who has already had the disease and has developed some immunity presents challenges. COVID-19 presents with a wide variety of severity of symptoms and many individuals with mild cases may not know that they have had the disease.

[0048] However, even with the ability to identify recovered patients with high titers of neutralizing antibodies, it is unlikely that a single individual's plasma can treat more than a few patients. Therefore, while current approaches to convalescent sera treatment may be able to prevent or treat COVID-19 in a small number of patients, this solution does not address the greater need of humanity during and after this pandemic.

Overview and Challenges of Current Vaccines

[0049] Clinicians and researchers around the world have developed various solutions to mitigate the pandemic caused by the SARS-CoV-2 virus. These solutions have included vaccines that can prevent or reduce the severity of COVID-19 and antiviral treatments to reduce the severity and symptoms of the illness when contracted. The expectation of the foreseeable future is that natural and vaccine-induced immunity most likely will not be long-lived, and therefore a cost-effective and safe booster vaccine administered as frequently as every 6 months, if necessary, is required to maintain robust immunity among the population. Thus, the critical design features of an effective prophylactic COVID-19 vaccine are: i) a potent capacity to induce SARS-CoV-2 viral neutralizing IgG titers and a significant T helper type 1 (Th1) cell response, preferably after a single dose, in both antibody naïve subject and subjects with existing antibody titers; ii) an acceptable safety and tolerability profile, especially with respect to inflammation caused by reactogenicity (systemic effect) and injection site (local effect), a favorable cost-of-goods (COGs) with respect to manufacturability and vaccine potency which dictate dose-frequency and dose-level, and a suitable supply-chain path including a sufficient storage shelf-life and robust test article preparation and administration procedures.

[0050] Live-attenuated or inactive whole virus vaccines represent a classic strategy. A major advantage of whole virus vaccines is their inherent immunogenicity and ability to stimulate toll-like receptors (TLRs) including TLR 3, TLR 7/8, and TLR 9. However, live virus vaccines often require extensive additional testing to confirm their safety. This is especially an issue for coronavirus vaccines, given the findings of increased infectivity following immunization with live or killed whole virus SARS coronavirus vaccines. Johnson & Johnson created Janssen's

AdVac® adenoviral vector manufactured in their PER.C6® cell line technology to generate their lead vaccine, JNJ-78436735. This technology was an attempt to produce a viral vector to replace the whole virus with a purportedly benign adenoviral vector that carries a portion of the SARS-CoV-2 virus DNA. However, use of JNJ-78436735 encountered significant serious adverse events (SAEs) that caused clinical trial pauses.

[0051] Two additional hurdles in the early development of SARS coronavirus vaccines have been the finding of 1) undesired immunopotentiality in the form of Th2-mediated eosinophilic infiltration and 2) increased viral infectivity driven by ADE, which is noted to occur following challenge infections after immunizations with whole virus vaccines and complete SP vaccines. The risk of Th2-mediated eosinophilic infiltration and lung pathology is still under investigation in SARS-CoV-2 infection, but it has been found in infants and animals challenged with respiratory syncytial virus (RSV) or with immunization with whole RSV vaccines.

[0052] ADE is an adverse characteristic of other viral vaccines, including those for the original SARS-CoV, dengue virus, and Zika viral infections, in which vaccine-induced Ab concentrations or affinities are too low to neutralize virus infection, but rather form immune complexes with virus that tend to interact with Fcγ receptors on myeloid cell surface through Fc domains of Abs. Such Abs do not neutralize viral infection or induce Fcγ-mediated viral clearance (Li), but aid virus infection by directly increasing virus uptake through Fcγ receptor or boosting virus replication intracellularly via activating downstream pathways to antagonize the innate immunity (reviewed in Sun). In both ADE and Th2-immunopotentiality, there is evidence that feline IgG2a mAbs (possibly of the Th2 isotype) can mediate both adverse conditions while IgG1 mAbs (known to have strong effector function, i.e., Th1 isotype) avoid such effects.

[0053] In addition to their risk of causing ADE and/or Th2-immunopotentiality, another challenge with viral vector vaccines is the relatively low manufacturability throughput and therefore high cost of goods (COGs) due to either chicken egg-based production or cell expression systems (Ewer).

[0054] As an alternative, nucleic acid expression vector vaccine platforms for COVID-19 encode the major coronavirus target antigen (Ag), the Spike Protein (SP), that mediates the virus' infective mechanism via its binding the host receptor, ACE2. mRNA monovalent vaccines encoding the full-length SP have been developed by BioNTech/Pfizer and Moderna. Recently, bivalent COVID-19 vaccines from Pfizer-BioNTech and Moderna have been authorized for use in many countries around the world for use as a booster. These bivalent booster vaccines target the original strain and the Omicron BA.4 and BA.5 variants and have been released in an attempt to create a broader immune response and improve the strength and

duration of protection against circulating variants. The concept of immunizing with RNA or DNA began with promising results in mice in 1993 showing protective immunity against influenza, but for decades these findings have not translated to similar findings in humans. Moreover, while non-replicative, many of these RNA and DNA expression vector vaccines continue to endogenously produce the target viral Ag well after induction of the intended immune response, an aspect that could ultimately create immune tolerance to the virus which is a growing concern and may become a practical risk with such current COVID-19 mRNA vaccines. Other challenges of these nucleic acid vaccines are the low durability of the response that may require too frequent dosing, and an unfavorable COGs due to cumbersome manufacturability via chemical synthesis. Furthermore, due to the inherent instability of RNA, the products must be kept and transported under frozen conditions, making them very difficult for most of the world to access. The monovalent vaccines demonstrated efficacy in protecting from symptomatic SARS-CoV-2 viral infection of greater than 90% of the original SARS-CoV-2 virus and the bivalent vaccines afford protection against some Omicron variants. However continued mutation of the spike protein may lead to decreasing efficacy of the vaccines.

[0055] As an additional alternative, recombinant subunit vaccines rely on eliciting an immune response against the SP to prevent its docking with the host target protein, ACE2. Such vaccines comprise all or a portion of the SP, rather than the DNA or RNA encoding for the protein, which is then mixed with an adjuvant to enhance the immune response. Due to the inherent stability of proteins relative to RNA and DNA, the storage and transportation requirements are less strict for subunit vaccines. Companies developing recombinant subunit vaccines include Novavax who has developed and produced immunogenic virus-like nanoparticles based on recombinant expression of SP, NVX-Cov2373, that are formulated with a saponin-based adjuvant system, Matrix-M™, and Clover Biopharmaceuticals who is developing a subunit vaccine consisting of a trimerized SARS-CoV-2 SP using their patented Trimer-Tag® technology. However, the full-length SP target Ag is known to have low expression yields in cell-expression systems and when used in SARS vaccines is known to induce anti-SP IgG titers against non-neutralizing epitopes of SP that again could mediate increased viral infectivity (i.e., ADE) and inflammation caused by lung eosinophilia (i.e., Th2-mediated immunopotentialization, discussed below).

[0056] A consortium led by Texas Children's Hospital Center for Vaccine Development at Baylor College of Medicine has developed and tested a subunit vaccine comprised of only the receptor-binding domain (RBD) of the SARS SP, and when formulated with alum, this RBD-based vaccine can elicit high levels of protective immunity upon homologous virus challenge, in addition to avoiding ADE and immunopotentialization (Hotez. P. J et al. A novel SARS

immunogenic composition (2014) US20160376321). Initial findings that the SARS and SARS-CoV-2 RBDs exhibit more than 80% amino acid similarity and bind the same ACE2 target offer an opportunity to develop either protein Ag as a subunit vaccine. Indeed, such a subunit vaccine proof-of-concept has been successfully demonstrated with coronavirus SP/RBD Ag's of MERS and SARS infections.

[0057] Each vaccine strategy has unique advantages and challenges with respect to manufacturing, safety, and efficacy that must be simultaneously managed in an optimal manner.

[0058] The SARS-CoV-2 virus has had many spike protein variants and subvariants. Additionally, a significant number of naturally occurring mutations to the SARS-CoV-2 spike protein have been identified in the SP/RBD, which may enhance the ability of such variants to circumvent immunity conferred by natural infection or vaccination. Existing vaccines targeting all or a portion of the spike protein of SARS-CoV-2 may have become less effective with the development of SARS-CoV-2 variants and mutations, which have lowered the efficacy of such vaccines, leading to a requirement for a new generation of vaccines for SARS-CoV-2 capable of preventing infection from SARS-CoV-2 variants.

[0059] The present disclosure is directed to methods for making and using nucleocapsid based novel Fc fusion proteins (SARS-CoV-2 N-Fc fusion proteins) which allow for the cost-effective production of large quantities of a recombinant subunit vaccine that is robust against variants of the SARS-CoV-2 virus spike protein region, and which can be transported and stored at mild temperatures. The present disclosure is specifically directed to methods for making and using nucleocapsid-based Fc fusion proteins for use in a prophylactic, therapeutic or booster vaccine which is efficacious for causing patients to create anti-virus antibodies to the SARS-CoV-2 virus, for example to decrease the SARS-CoV-2 viral load in cells decreasing the ability of the virus to replicate and ameliorating the severity of symptoms. Using a novel SARS-CoV-2 N-Fc fusion protein prophylactic vaccine to cause a patient to create endogenous antibodies targeted to the SARS-CoV-2 virus is expected to be significantly more cost effective than recombinantly generating anti-SARS-CoV-2 therapeutic monoclonal antibodies to later be injected into a patient and which will become ineffective as the spike protein and RBD regions of the SARS-CoV-2 virus mutates.

[0060] The main structural proteins of the SARS-CoV-2 virus include the membrane protein (M protein), the spike protein (S protein), the envelope protein (E protein) and the nucleocapsid protein (N protein). The N protein is more conservative and has a lower mutation rate compared to the spike protein, and the N protein is highly immunogenic and is abundantly expressed during infection. (Marra MA, Jones SJ, Astell CR, et al. The Genome sequence of the SARS-associated coronavirus. Science. 2003;300(5624):1399-1404; Drosten C, Günther S, Preiser W,

et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Holmes KV, Enjuanes L. Virology. The SARS coronavirus: a postgenomic era. Science. 2003;300(5624):1377-1378; Rota PA, Oberste MS, Monroe SS, *et al.* Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science. 2003;300(5624):1394-1399; Zhu Y, Liu M, Zhao W, *et al.* Isolation of virus from a SARS patient and genome-wide analysis of genetic mutations related to pathogenesis and epidemiology from 47 SARS-CoV isolates. Virus Genes. 2005;30(1):93-102. Engl J Med. 2003;348(20):1967-1976.; Cong Y, Ulasli M, Schepers H, *et al.* Nucleocapsid protein recruitment to replication-transcription complexes plays a crucial role in coronaviral life cycle. J Virol. 2020;94(4):e01925-e19.) Previous studies have shown that high levels of anti-N protein antibody were detected in COVID-19 patents, indicating that the N protein could stimulate human immune responses to the SARS-CoV-2 virus. (Algaissi A, Alfaleh MA, Hala S, *et al.* SARS-CoV-2 S1 and N-based serological assays reveal rapid seroconversion and induction of specific antibody response in COVID-10 patents. Sci Rep. 2020;10(1):16561; Jiang HW, Li Y, Zhang HN, *et al.* SARS-CoV-2 [0001] proteome microarray for global profiling of COVID-19 specific IgG and IgM responses. Nat Commun. 2020;11(1):3581.)

[0061] In an example, a pharmaceutical composition of a novel SARS-CoV-2 N-Fc fusion protein vaccine is administered to patients who have been infected by the SARS-CoV-2 virus and have contracted COVID-19, to limit the scope of the infection and to ameliorate the disease. In examples, the novel SARS-CoV-2 N-Fc fusion protein is expected to stimulate the subject to produce both humoral immunity and cell-mediated immunity, generating high levels of IgG and IgM antibodies which bind the N protein of the native virus which inhibits the viral RNA replication and blocks the virus transmission in the body. In examples, the novel SARS-CoV-2 N-Fc fusion protein is expected to produce high levels of interferon gamma (IFN- γ) which play an important role in inducing the immune response by promoting macrophage activation, mediating antiviral and antibacterial immunity, enhancing antigen presentation, orchestrating activation of the innate immune system, coordinating lymphocyte-endothelium interaction, regulating Th1/Th2 balance, and controlling cellular proliferation and apoptosis.

[0062] In an example, a pharmaceutical composition of a novel SARS-CoV-2 N-Fc fusion protein is administered as a prophylactic COVID-19 vaccine for individuals that have not been infected by the SARS-CoV-2 virus, resulting in the individual producing their own pool of anti-SARS-CoV-2 antibodies and immunity.

Equivalents and Definitions

[0063] As used herein, the articles “a” and “an” refer to one or more than one, e.g., to at

least one, of the grammatical object of the article. The use of the words “a” or “an” when used in conjunction with the term “comprising” herein may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.” As used herein, the phrase “and/or,” when used in a list of two or more items, means that any one of the listed items can be employed by itself or any combination of two or more of the listed items can be employed. For example, if a composition is described as containing or excluding components A, B, and/or C, the composition can contain or exclude A alone; B alone; C alone; A and B in combination; A and C in combination; B and C in combination; or A, B, and C in combination.

[0064] As used herein, “about” and “approximately” generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements.

[0065] As used herein, an amount of a molecule, compound, conjugate, or substance effective to treat a disorder (e.g., a disorder described herein), “therapeutically effective amount,” or “effective amount” refers to an amount of the molecule, compound, conjugate, or substance which is effective, upon single or multiple dose administration(s) to a subject, in treating a subject, or in curing, alleviating, relieving or improving a subject with a disorder (e.g., a disorder described herein) beyond that expected in the absence of such treatment.

[0066] As used herein, the term “analog” refers to a compound or conjugate (e.g., a compound or conjugate as described herein, e.g., nucleocapsid) having a chemical structure similar to that of another compound or conjugate but differing from it in at least one aspect.

[0067] As used herein, the term “antigen” refers to any substance that causes a patient’s immune system to produce antibodies against it. An antigen may be a substance from the environment, such as chemicals, bacteria, viruses, or pollen, or an antigen may also form inside the body. An example of an antigen is the SARS-CoV-2 virus.

[0068] As used herein, the term “antibody” or “antibody molecule” refers to an immunoglobulin molecule (Ig), or immunologically active portions of an immunoglobulin (Ig) molecule, i.e., a molecule that contains an antigen binding site that specifically binds, e.g., immunoreacts with, an antigen. As used herein, the term “antibody domain” refers to a variable or constant region of an immunoglobulin. It is documented in the art that human antibodies comprise several classes, for example IgA, IgM, or IgG in the case of mammals (e.g., humans and dogs). Classes of mammalian IgG immunoglobulins can be further classified into different isotypes, such as IgGA, IgGB, IgGC and IgGD for dogs and IgG1, IgG2, IgG3, and IgG4 for humans. Those skilled in the art will recognize that immunoglobulin isotypes of a given immunoglobulin class will comprise different amino acid sequences, structures, and functional properties from one another (e.g., different binding affinities to Fc(gamma) receptors or ACE2 receptor). “Specifically binds” or “immunoreacts with” means that the antibody reacts with one

or more antigenic determinants of the desired antigen and has a lower affinity for other polypeptides, e.g., does not react with other polypeptides.

[0069] As used herein, the term “dimer” refers to a protein or a fusion protein comprising two polypeptides linked covalently. In embodiments, two identical polypeptides are linked covalently (e.g., via disulfide bonds) forming a “homodimer” (diagrammatically represented in FIG. 1, which is an illustration of an insulin-Fc fusion protein for reference, and FIG. 2, which is an illustration of a novel SARS-CoV-2 N-Fc fusion protein). Referring to FIG. 1 in more detail, the insulin polypeptide (comprising an insulin B-chain analog connected via a C-chain peptide to an insulin A-chain analog) may have one or more amino acid mutations from native insulin. The insulin peptide is connected via a linker to an Fc fragment. Disulfide bonds (the total number of disulfide bonds in actuality may be greater or less than the number shown in FIG. 1) create a homodimer from two identical Fc fusion proteins. Referring to FIG. 2 in more detail, a novel SARS-CoV-2 N-Fc fusion protein may comprise a nucleocapsid protein fragment which may comprise a portion of the full SARS-CoV-2 nucleocapsid protein. The nucleocapsid protein fragment may have one or more amino acid mutations from the native SARS-CoV-2 nucleocapsid protein. The nucleocapsid protein fragment is connected to an Fc fragment using a peptide linker. Disulfide bonds create a homodimer from two identical novel SARS-CoV-2 N-Fc fusion proteins (the total number of disulfide bonds in actuality may be greater or less than the number shown in FIG. 2). The novel SARS-CoV-2 N-Fc fusion protein homodimer may be encoded by a single nucleic acid molecule, wherein the homodimer is made recombinantly inside a cell by first forming novel SARS-CoV-2 N-Fc fusion protein monomers and by then assembling two identical novel SARS-CoV-2 N-Fc fusion protein monomers into the homodimer upon further processing inside the cell.

[0070] As used herein, the terms “multimer,” “multimeric,” or “multimeric state” refer to non-covalent, associated forms of Fc fusion protein dimers that may be in equilibrium with Fc fusion protein dimers or may act as permanently aggregated versions of Fc fusion protein dimers (e.g., dimers of Fc fusion protein homodimers, trimers of Fc fusion protein homodimers, tetramers of Fc fusion protein homodimers, or higher order aggregates containing five or more Fc fusion protein homodimers). It may be expected that multimeric forms of Fc fusion proteins may have different physical, stability, or pharmacologic activities from that of fusion protein homodimers.

[0071] As used herein, a SARS-CoV-2 nucleocapsid-Fc fusion protein and a novel SARS-CoV-2 N-Fc fusion protein (which terms may be interchangeably used) refers to a human immunoglobulin Fc domain that is linked to a SARS-CoV-2 nucleocapsid fragment or an analog thereof, which is useful in generating antibodies that specifically bind the N protein of SARS-

CoV-2. For ease of reference, the term nucleocapsid, unless otherwise dictated by the context, encompasses protein residues consisting of fragments of the SARS-CoV-2 nucleocapsid protein that retain the activity of the nucleocapsid protein. As used herein, the general terms “fusion protein” and “Fc fusion protein” refer to a protein comprising more than one part, for example from different sources (e.g., different proteins, polypeptides, cells, etc.), that are covalently linked through peptide bonds. Fc fusion proteins are covalently linked by (i) connecting the genes that encode for each part into a single nucleic acid molecule and (ii) expressing in a host cell (e.g., HEK cell or CHO cell) the protein for which the nucleic acid molecule encodes. The fully recombinant synthesis approach is preferred over methods in which the therapeutic protein and Fc fragments are synthesized separately and then chemically conjugated. The chemical conjugation step and subsequent purification process increase the manufacturing complexity, reduce product yield, and increase cost.

[0072] As used herein, the term “bioactivity,” “activity,” “biological activity,” “potency,” “bioactive potency,” or “biological potency” refers to the extent to which an Fc fusion protein binds to or activates a cell receptor and/or exerts the production or reduction of native or foreign substances. As used herein, “in vitro activity” or “receptor activity” refers to the affinity with which an Fc fusion protein binds to the cell receptor and is typically measured by the concentration of an Fc fusion protein that causes the Fc fusion protein to reach half of its maximum binding (i.e., EC50 value). For example, the “bioactivity” of a novel SARS-CoV-2 N-Fc fusion protein refers to the extent to which the novel SARS-CoV-2 N-Fc fusion protein induces the production of anti-SARS-CoV-2 antibodies in a cellular assay or in a target subject.

[0073] As used herein, the term “biosynthesis,” “recombinant synthesis,” or “recombinantly made” refers to the process by which an Fc fusion protein is expressed within a host cell by transfecting the cell with a nucleic acid molecule (e.g., vector) encoding the Fc fusion protein (e.g., where the entire Fc fusion protein is encoded by a single nucleic acid molecule). Exemplary host cells include mammalian cells, e.g., HEK293 cells or CHO cells. The cells can be cultured using standard methods in the art and the expressed Fc fusion protein may be harvested and purified from the cell culture using standard methods in the art.

[0074] As used herein, the term “cell surface receptor” refers to a molecule such as a protein, generally found on the external surface of the membrane of a cell and which interacts with soluble molecules, e.g., molecules that circulate in the blood supply. In some embodiments, a cell surface receptor may include a host cell receptor (e.g., an ACE2 receptor) or an Fc receptor which binds to an Fc fragment or the Fc region of an antibody (e.g., an Fc(gamma) receptor, for example Fc(gamma) receptor I, or an Fc neonatal receptor, for example FcRn). As used herein, “in vitro activity” or “Fc(gamma) receptor activity” or “Fc(gamma) receptor binding” or “FcRn

receptor activity” or “FcRn binding” refers to the affinity with which an Fc fusion protein binds to the Fc receptor (e.g. Fc(gamma) receptor or FcRn receptor) and is typically measured by the concentration of an Fc fusion protein that causes the Fc fusion protein to reach half of its maximum binding (i.e., EC50 value) as measured on an assay (e.g., an enzyme-linked immunosorbent assay (ELISA) assay) using OD 450 nm values as measured on a microplate reader.

[0075] As used herein, the term “immunogenic” or “immunogenicity” refers to the capacity for a given molecule (e.g., an Fc fusion protein of the present invention) to provoke the immune system of a target subject such that after administration of the molecule, the subject develops antibodies capable of binding all or specific portions of the molecule (i.e., anti-drug antibodies or ADA). As used herein, the terms “neutralizing,” “neutralizing antibodies,” or “neutralizing anti-drug antibodies” refer to the capacity for antibodies to interfere with all or a portion of the Fc fusion protein’s biological activity in the target subject. For example, in the case of a novel SARS-CoV-2 N-Fc fusion protein molecule (or a pharmaceutical composition thereof) administered to humans, the immunogenicity refers to antibodies that bind to the SARS-CoV-2 nucleocapsid portion of the molecule since the hIgG-Fc portion of the molecule is endogenous to humans and therefore unlikely to elicit anti-hIgG-Fc antibodies. Likewise, antibodies generated by the administration of a novel SARS-CoV-2 N-Fc fusion protein molecule (or a pharmaceutical composition thereof) are neutralizing when those anti-SARS-CoV-2 antibodies inhibit the binding between the SARS-CoV-2 N protein host cells, which is directly related to the bioactivity of the SARS-CoV-2 RBD in the subject.

[0076] As used herein, the term “monomer” refers to a protein or a fusion protein comprising a single polypeptide. In embodiments, the “monomer” is a protein or a fusion protein, e.g., a single polypeptide, comprising a nucleocapsid fragment polypeptide and an Fc fragment polypeptide, wherein the nucleocapsid fragment and Fc fragment polypeptides are joined by peptide bonds to form the single polypeptide. In embodiments, the monomer is encoded by a single nucleic acid molecule.

[0077] As used herein and as illustrated in FIG. 1 and FIG. 2, “N-terminus” refers to the start of a protein or polypeptide that is initiated by an amino acid containing a free amine group that is the alpha-amino group of the amino acid (e.g., the free amino that is covalently linked to one carbon atom that is located adjacent to a second carbon atom, wherein the second carbon atom is part of the carbonyl group of the amino acid). As used herein and as illustrated in FIG. 1 and FIG. 2, “C-terminus” refers to the end of a protein or polypeptide that is terminated by an amino acid containing a carboxylic acid group, wherein the carbon atom of the carboxylic acid group is located adjacent to the alpha-amino group of the amino acid.

[0078] As used herein, the term “carrier” is used herein to refer to diluents, excipients, vehicles, and the like, in which the Fc fusion protein(s) may be dispersed, emulsified, or encapsulated for administration. Suitable carriers will be pharmaceutically acceptable. As used herein, the term “pharmaceutically acceptable” means not biologically or otherwise undesirable, in that it can be administered to a subject without excessive toxicity, irritation, or allergic response, and does not cause unacceptable biological effects or interact in a deleterious manner with any of the other components of the composition in which it is contained. A pharmaceutically-acceptable carrier would naturally be selected to minimize any degradation of the compound or other agents and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art. Pharmaceutically-acceptable ingredients include those acceptable for veterinary use as well as human pharmaceutical use and will depend on the route of administration. Any carrier compatible with the excipient(s) and the Fc fusion protein(s) can be used.

[0079] As used herein, “pharmacodynamics” or “PD” generally refers to the biological effects of an Fc fusion protein in a subject. As an example, herein, the PD of a novel SARS-CoV-2 N-Fc fusion protein refers to the measure of the anti-SARS-CoV-2 antibody titers over time in a subject after the administration of the novel SARS-CoV-2 N-Fc fusion protein.

[0080] As used herein, “pharmacokinetics” or “PK” generally refers to the characteristic interactions of an Fc fusion protein and the body of the subject in terms of its absorption, distribution, metabolism, and excretion. As an example, herein, the PK refers to the concentration of a novel SARS-CoV-2 N-Fc fusion protein in the blood or serum of a subject at a given time after the administration of the novel SARS-CoV-2 N-Fc fusion protein. As used herein, “half-life” refers to the time taken for the concentration of Fc fusion protein in the blood or serum of a subject to reach half of its original value as calculated from a first order exponential decay model for drug elimination. Fc fusion proteins with greater “half-life” values demonstrate greater duration of action in the target subject.

[0081] The terms “sequence identity,” “sequence homology,” “homology,” or “identical” in amino acid or nucleotide sequences as used herein describes that the same nucleotides or amino acid residues are found within the variant and reference sequences when a specified, contiguous segment of the nucleotide sequence or amino acid sequence of the variant is aligned and compared to the nucleotide sequence or amino acid sequence of the reference sequence. Methods for sequence alignment and for determining identity between sequences are known in the art, including the use of Clustal Omega, which organizes, aligns, and compares sequences for similarity, wherein the software highlights each sequence position and compares across all sequences at that position and assigns one of the following scores: an “*” (asterisk) for sequence

positions which have a single, fully conserved residue, a “:” (colon) indicates conservation between groups of strongly similar properties with scoring greater than 0.5 in the Gonnet PAM 250 matrix, and a “.” (period) indicates conservation between groups of weakly similar properties with scoring less than or equal to 0.5 in the Gonnet PAM 250 matrix, a “-” (dash) indicates a sequence gap, meaning that no local homology exists within a particular set of comparisons within a certain range of the sequences, and an empty space “ ” indicates little or no sequence homology for that particular position across the compared sequences.

[0082] With respect to optimal alignment of two nucleotide sequences, the contiguous segment of the variant nucleotide sequence may have additional nucleotides or deleted nucleotides with respect to the reference nucleotide sequence. Likewise, for purposes of optimal alignment of two amino acid sequences, the contiguous segment of the variant amino acid sequence may have additional amino acid residues or deleted amino acid residues with respect to the reference amino acid sequence. In some embodiments, the contiguous segment used for comparison to the reference nucleotide sequence or reference amino acid sequence will comprise at least 6, 10, 15, or 20 contiguous nucleotides, or amino acid residues, and may be 30, 40, 50, 100, or more nucleotides or amino acid residues. Corrections for increased sequence identity associated with inclusion of gaps in the variant’s nucleotide sequence or amino acid sequence can be made by assigning gap penalties. Methods of sequence alignment are known in the art.

[0083] In embodiments, the determination of percent identity or “homology” between two sequences is accomplished using a mathematical algorithm. For example, the percent identity of an amino acid sequence is determined using the Smith-Waterman homology search algorithm using an affine 6 gap search with a gap open penalty of 12 and a gap extension penalty of 2, BLOSUM matrix 62. In embodiments, the percent identity of a nucleotide sequence is determined using the Smith-Waterman homology search algorithm using a gap open penalty of 25 and a gap extension penalty of 5. Such a determination of sequence identity can be performed using, for example, the DeCypher Hardware Accelerator from TimeLogic.

[0084] As used herein, the term “homology” is used to compare two or more proteins by locating common structural characteristics and common spatial distribution of, for instance, beta strands, helices, and folds. Accordingly, homologous protein structures are defined by spatial analyses. Measuring structural homology involves computing the geometric-topological features of a space. One approach used to generate and analyze three-dimensional (3D) protein structures is homology modeling (also called comparative modeling or knowledge-based modeling) which works by finding similar sequences on the basis of the fact that 3D similarity reflects 2D similarity. Homologous structures do not imply sequence similarity as a necessary

condition.

[0085] As used herein, the terms “subject” and “patient” are intended to include mice, non-human primates (NHP), rabbits, canines, and humans. Exemplary canine subjects include dogs having a disease or a disorder, e.g., diabetes or another disease or disorder described herein, or normal subjects. Exemplary human subjects include individuals that have a disease, e.g., COVID-19, including variants of COVID-19 or SARS-CoV-2 infection, or another virus, have previously had a disease or disorder described herein, or normal subjects.

[0086] As used herein, the term “titer” or “yield” refers to the amount of a fusion protein product (e.g., an Fc fusion protein described herein) resulting from the biosynthesis (e.g., in a mammalian cell, e.g., in a HEK293 cell or CHO cell) per volume of the cell culture. The amount of product may be determined at any step of the production process (e.g., before or after purification), but the yield or titer is always stated per volume of the original cell culture. As used herein, the term “product yield” or “total protein yield” refers to the total amount of Fc fusion protein expressed by cells and purified via at least one affinity chromatography step (e.g., Protein A or Protein G) and includes monomers of Fc fusion protein, homodimers of Fc fusion protein, and higher-order molecular aggregates of homodimers of Fc fusion protein. As used herein, the term “percent homodimer” or “%homodimer” refers to the proportion of a fusion protein product (e.g., an Fc fusion protein described herein) that is the desired homodimer. As used herein, the term “homodimer titer” refers to the product of the %homodimer and the total protein yield after Protein A purification step reported per volume of the cell culture.

[0087] As used herein, the terms “treat” or “treating” or “treatment” of a subject having a disease or a disorder refers to an intervention performed with the intention of preventing the development or altering the pathology of infection. Accordingly, “treatment” refers to both therapeutic treatment and prophylactic or preventative measures. A therapeutic agent may directly decrease the pathology of infection or render the infection more susceptible to treatment by other therapeutic agents or, for example the host’s immune system. Improvement after treatment may be manifested as a decrease or elimination of such symptoms. Thus, the compositions are useful in treating an infection by preventing the development of observable clinical symptoms from infection, and/or reducing the incidence or severity of clinical symptoms and/or effects of the infection, and/or reducing the duration of the infection/symptoms/effect. Treating a subject having a disease or disorder may refer to a subject having a disease or a disorder refer to subjecting the subject to a regimen, for example the administration of a fusion protein such as an Fc fusion protein described herein, or a pharmaceutical composition of a fusion protein such as an Fc fusion protein described herein, such that at least one symptom of the disease or disorder is cured, healed, alleviated, relieved,

altered, remedied, ameliorated, or improved. Treating includes administering an amount effective to alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease or disorder, or the symptoms of the disease or disorder. Treating includes administering an amount effective to generate antibodies to a disease or disorder in a normal subject or a subject that has previously had the disease or disorder. The treatment may inhibit deterioration or worsening of a symptom of a disease or disorder.

[0088] As used herein, “prophylactic vaccine” refers to a treatment that introduces an antigen into a patient with the goal that the patient’s immune system will create antibodies for the antigen and increase or improve the subject’s immune response to the associated illness or virus. In other words, a vaccinated subject will have a higher degree of resistance to illness or disease from the associated virus as compared to a non-vaccinated subject. This resistance may be evident by a decrease in severity or duration of symptoms of illness, decrease or elimination of viral shedding, and in some case the prevention of observable symptoms of infection in the vaccinated subject. In embodiments, a patient treated with a prophylactic vaccine does not have antibodies for the antigen prior to the treatment with the prophylactic vaccine (otherwise stated, the patient is “antibody naïve”).

[0089] As used herein, “therapeutic vaccine” refers to a treatment that introduces an antigen into a patient that already has the associated illness or virus, with the goal that the patient’s immune system will create antibodies for the antigen enabling the patient’s body to fight harder against the illness or virus that it already has.

[0090] As used herein, “booster vaccine” refers to an extra administration of a vaccine after the patient has previously received an initial administration of a vaccine, or after a patient has acquired antibodies through having had and recovered from the associated illness or virus. In some examples, an additional dose of a vaccine is needed periodically to “boost” the immunity of a patient to an illness or virus causing antigen by increasing the patient’s antigen antibody titer.

[0091] As used herein, when referring to an amino acid in some portion of the SARS-CoV-2 nucleocapsid, for example a SARS-CoV-2 nucleocapsid fragment, a cited amino acid position is referenced as the position of the amino acid in the SARS-CoV-2 nucleocapsid of SEQ ID NO: 10. As an example, a reference to a mutation of an amino acid at position 41 of a SARS-CoV-2 nucleocapsid fragment refers to the amino acid at the 41st position in SEQ ID NO: 10 even when the SARS-CoV-2 nucleocapsid fragment comprises only a portion of SEQ ID NO: 10, for example a portion of SEQ ID NO: 10 beginning at the 41st amino acid.

[0092] As used herein, “nucleocapsid fragment” refers to a portion of a novel SARS-CoV-2 N-Fc fusion protein that comprises some portion of the nucleocapsid protein of the SARS-

CoV-2 virus given in SEQ ID NO: 10. In examples, the nucleocapsid fragment is linked to a human Fc fragment or analog thereof, as illustrated in FIG. 2.

Rationale for Fc Fusion Protein Vaccines

[0093] As discussed above, the recombinant protein-based subunit vaccine approach has an advantage of safety and multiple-booster dosing relative to inactivated or live-attenuated virus and nucleic acid vector-based vaccine formats, in addition to allowing for the selective use of the most dominant epitopes to generate potent neutralizing Ab titers. Furthermore, such a protein-based vaccine is more cost-effectively manufactured in large quantities and is stable at mild temperatures, allowing for easier transportation and storage. However, given the challenges of a recombinant SARS-CoV-2 spike protein or nucleocapsid subunit vaccine to induce a strong protective immune response in an immunologically naïve human population, the antigen must be modified and/or formulated with additional immune-enhancing features to overcome the activation thresholds of naïve T and B cells.

Experimental Experience with Insulin-Fc Fusion Proteins in Canines

[0094] One example of a fusion protein formed by linking a therapeutic protein to an immunoglobulin Fc domain is an insulin-Fc fusion protein. This construct has been used to provide ultra-long acting basal insulin therapy for diabetic subjects. The combination of an insulin analog as the therapeutic protein with an Fc domain via a peptide linker has been shown to achieve significantly longer activity in vivo, in the order of days. An example of ultra-long acting insulin-Fc fusion proteins for use in treating diabetes in cats and dogs is described in WO2020006529A1. In an example from WO2020006529A1, an exemplary insulin analog is: FVNQHLCGSHLVEALELVCGERGFHYGGGGGGSGGGGGIVEQCCTSTCSLDQLENYC (SEQ ID NO: 4)

and an exemplary linker, used to link the therapeutic protein (i.e., the insulin analog) to the Fc domain is: GGGGGQGGGGQGGGGQGGGGG (SEQ ID NO: 3).

[0095] Insulin-Fc fusion protein molecules for a given species (e.g., dog, cat, or human) suitable for ultra-long acting treatment for diabetes should be manufacturable in mammalian cells, for example human embryonic kidney (HEK, e.g. HEK293) cells, with an acceptable titer of the desired homodimer product (e.g., greater than 50 mg/L homodimer titer from transiently transfected HEK cells, greater than 75 mg/L from transiently transfected HEK cells, greater than 100 mg/L from transiently transfected HEK cells, etc.). Experience has demonstrated that homodimer titers less than 50 mg/L will not likely result in commercial production homodimer titers in Chinese hamster ovary (CHO) cells that meet the stringently low manufacturing cost

requirements for veterinary products.

[0096] The insulin-Fc fusion proteins for dogs described in WO2020006529A1 (incorporated by reference herein) and herein were manufactured in HEK cells according to Example 10 or in CHO cells according to Example 11. The insulin-Fc fusion proteins were purified according to Example 12. Using the conventional purification method, only the compounds comprising the canine IgGA and the canine IgGB immunoglobulin Fc fragment showed any appreciable protein yields. The structure of the insulin-Fc fusion proteins was confirmed by non-reducing and reducing CE-SDS according to Example 13 and the sequence was confirmed by LC-MS with glycan removal according to Example 14. The purity (as assessed by the percent homodimer of the fusion protein yield) was measured according to Example 15. The canine IgGA version of the insulin-Fc fusion protein was highly aggregated with low levels of bioactivity, whereas the canine IgGB version of the insulin-Fc fusion protein exhibited a low degree of aggregation (i.e., high % homodimer), a high titer of the desired homodimer (i.e., a homodimer titer greater than 50 mg/L), and appreciable levels of long-duration glucose lowering bioactivity in dogs. Therefore, the canine IgGB (SEQ ID NO: 2) immunoglobulin Fc fragment is the preferred Fc fragment for insulin-Fc fusion proteins used in dogs.

DCPKCPAPEMLGGPSVFIFPPKPKDTLLIARTPEVTCVVVDLDPEDPEVQISWFVDGKQ
MQTAKTQPREEQFNQTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTISKAR
GQAHQPSVYVLPSPREELSKNTVSLTCLIKDFFPPDIDVEWQSNQEQEPESKYRTTPPQ
LDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG (SEQ ID NO:
2)

[0097] An exemplary canine ultra-long acting insulin-Fc fusion protein comprising the insulin analog of SEQ ID NO: 4 with the canine native IgGB fragment of SEQ ID NO: 2 via the peptide linker of SEQ ID NO: 3 is:

FVNQHLCGSHLVEALELVCGERGFHYGGGGGGSGGGGGIVEQCCTSTCSLDQLENYC
GGGGGQGGGGQGGGGQGGGGGDCPKCPAPEMLGGPSVFIFPPKPKDTLLIARTPEVT
CVVVDLDPEDPEVQISWFVDGKQMKTAKTQPREEQFNQTYRVVSVLPIGHQDWLKG
KQFTCKVNNKALPSPIERTISKARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFFPPD
IDVEWQSNQEQEPESKYRTTPPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEA
LHNHYTQESLSHSPG (SEQ ID NO: 5)

[0098] The binding of the insulin-Fc fusion protein of SEQ ID NO: 5 to the Fc(gamma) Receptor I (RI) was assessed according to Example 16. Since canine receptor I was not commercially available, human Fc(gamma) receptor I (i.e., rhFc(gamma) receptor I) was used as a surrogate mammalian receptor. The OD values proportional to the binding of rhFc(gamma)

receptor I to SEQ ID NO: 5 were plotted against log concentrations of rhFc (gamma) receptor I added to each, to generate binding curves using GraphPad Prism software. The results shown in FIG. 3 illustrate that the OD450 values increase with increased doses of the insulin-Fc fusion protein of SEQ ID NO: 5 for all Fc(gamma) receptors.

[0099] The in vivo pharmacodynamics (PD) after periodic administrations of the insulin-Fc fusion protein of SEQ ID NO: 5 manufactured in HEK cells according to Example 10 was evaluated according to Example 18 or Example 19. The test population consisted of six beagle dogs with diabetes that was chemically induced using alloxan-streptozotocin, each weighing approximately 10 kg. The anti-insulin antibody (AIA) titers were measured weekly over 8 weeks for the six beagle dogs with chemically induced diabetes that were subjects in the lab test for SEQ ID NO: 5 according to Example 18. FIG. 4 shows titers of AIA for the six beagles with chemically induced diabetes over a series of eight weekly doses of the insulin-Fc fusion protein of SEQ ID NO: 5. FIG. 5 shows percentage change in the titers of AIA from Day 0 of the trial for the six beagles with chemically induced diabetes over a series of eight weekly doses of the insulin-Fc fusion protein of SEQ ID NO: 5. The data demonstrates that the beagles' AIA titers did not substantially increase over the eight administered doses of the insulin-Fc fusion protein of SEQ ID NO: 5.

[0100] Based on these positive lab test results with the chemically induced diabetic beagles, field trials with actual client-owned, naturally occurring diabetic dogs of varying ages, breeds, and extent of diabetes disease were initiated according to Example 18 or Example 19. The client dogs in the field trial had all been receiving insulin treatment with a known veterinary or human insulin product up to the point of the trial initiation and were given SEQ ID NO: 5 according to Protocol 1 as described in Example 18 or Protocol 2 as described in Example 19.

[0101] The AIA titer was again measured weekly over the course of the treatment according to Example 19. Unexpectedly, in contrast to the results obtained in the chemically induced diabetic beagles, several (8/20) client dogs in this "wild" patient population demonstrated a marked increase in anti-insulin antibodies. A normalized AIA titer of 0.15 was considered the minimum measurement for the client dog to be considered immunogenic to SEQ ID NO: 5. For client dogs that had a non-zero AIA titer at the start of the treatment, if the AIA more than doubled after being treated with the insulin-Fc fusion protein of SEQ ID NO: 5, the insulin-Fc fusion protein of SEQ ID NO: 5 was considered to be immunogenic in that particular client dog. FIG. 6 is a plot of normalized AIA titer for each of the client dogs in which the insulin-Fc fusion protein of SEQ ID NO: 5 was considered immunogenic as measured weekly during the duration of their treatment (according to Protocol 1 as described in Example 18 or Protocol 2 as described in Example 19) followed for the treatment is indicated for each dog). In each of the dogs shown

in FIG. 6, the AIAs neutralized the therapeutic effect of the insulin-Fc fusion protein of SEQ ID NO: 5, rendering it no longer capable of controlling blood glucose levels in the client-owned diabetic dogs. The observed immunogenicity was additionally unexpected because the insulin analog portion of the insulin-Fc fusion protein is a near-native peptide for the dogs. Furthermore, the IgGB Fc fragment portion of the insulin-Fc fusion protein is a native canine Fc fragment. The results indicate that the specific activity of the dog IgGB Fc fragment was capable of inducing a pronounced and lasting increase in antibody titers specific to the therapeutic protein region of the fusion protein (i.e., the insulin). Other than demonstrating a significant increase in neutralizing AIA titers, the dogs otherwise remained healthy through repeated dosing and did not experience any signs of anaphylaxis or cytokine storms associated with the treatment.

[0102] In some cases, when the client dog began to show high levels of AIAs, the dosing of the insulin-Fc fusion protein of SEQ ID NO: 5 was discontinued, at which point the AIA titer began to decrease (see for example Dog 2 on FIG. 4). FIG. 7 is a plot of normalized AIA titer for example Dog 2 over a period of 12 weeks of once-weekly dosing. It can be seen that the AIA titer began to measurably increase after the 4th dose (Day 21) and the AIA titer began to steeply increase after the 6th dose (Day 35). The dosing was stopped after the 8th dose (Day 49), and no drug was administered on Day 56 or Day 63. FIG. 7 illustrates that the AIA titer growth immediately slowed and then the AIA titer began to fall after Day 56. The dosing regime was resumed on Day 70, with doses on Day 70 and again on Day 77. The AIA titer growth after the resumption of dosing on Day 70 matched or exceeded the maximum AIA titer growth in the 7 weeks up to when the dosing was stopped, illustrating that the titer of anti-drug antibodies was unexpectedly restored in an appreciably shorter time than the build-up over the initial series of doses. This robust recall response may be indicative of a responsive memory immune cell population.

[0103] Another observation from the field trial of client owned diabetic dogs was that there was an apparent difference in the dogs treated with the insulin-Fc fusion protein of SEQ ID NO: 5 made in CHO cells according to Example 11, and the insulin-Fc fusion protein of SEQ ID NO: 5 made in HEK cells according to Example 10, with the CHO-made insulin-Fc fusion protein of SEQ ID NO: 5 showing a markedly higher prevalence of anti-drug antibodies, as shown in FIG. 8.

[0104] Each IgG fragment contains a conserved asparagine (N)-glycosylation site in the CH2 domain of each heavy chain of the Fc region. Herein, the notation used to refer to the conserved N-glycosylation site is “cNg” (shown in FIG. 1 and in FIG. 2). In therapeutic monoclonal antibodies, the glycosylation is at the conserved amino acid N297 in the CH2 region (shown in FIG. 1 and FIG. 2). For an insulin-Fc fusion protein, the absolute position of the cNg

site from the N-terminus of the B-chain of the insulin-Fc fusion protein varies depending on the length of the insulin polypeptide, the length of the linker, and any omitted amino acids in the Fc fragment prior to the cNg site. Herein, the notation used to refer to the absolute position of the cNg site in a given insulin-Fc fusion protein sequence (as measured counting from the N-terminus of the B-chain of the insulin-Fc fusion protein) is “NB(number)”. For example, if the cNg site is found at the 151st amino acid position as counted from the N-terminus of the B-chain, the absolute position of this site is referred to as cNg-NB151. As a further example, if the cNg site is found at the 151st amino acid position as counted from the N-terminus of the B-chain, and the asparagine at this site is mutated to serine, this mutation is noted as “cNg- NB151-S”.

[0105] One possible difference between fusion proteins recombinantly manufactured in HEK cells according to Example 10 and fusion proteins recombinantly manufactured in CHO cells according to Example 11 is the composition of the oligosaccharides that attach at the cNg site. Given that the canine IgGB isotypes interact with Fc(gamma) receptors, there may be a risk of unwanted immunogenicity after repeated injections. One method for reducing the Fc(gamma) interaction involves deglycosylating or preventing the glycosylation of the Fc fragment during synthesis in the host cell. Creation of antibodies is clearly undesirable for treatment of a chronic disease such as diabetes as the antibodies neutralize the therapeutic value of the drug. Accordingly, this led to attempting to create a non-glycosylated canine insulin-Fc fusion protein. One way to remove the attached glycan from a synthesized insulin-Fc fusion protein is to mutate the cNg site to prevent the attachment of glycans altogether during production in the host cell. Herein, the notation used to describe a cNg mutation is cNg-(substituted amino acid). For example, if the asparagine at the cNg site is mutated to serine, this mutation is notated as “cNg-S”.

[0106] A canine insulin-Fc fusion protein was designed, comprising the IgGB Fc fragment of SEQ ID NO: 2 with cNg-S at position 73 (bold residue below), the linker of SEQ ID NO: 3, and the following insulin analog:

FVNQHLCGSHLVEALALVCGERGFHYGGGGGGSGGGGGIVEQCCTSTCSLDQLENY
C (SEQ ID NO: 6)

[0107] The resulting insulin-Fc fusion protein is shown below:

FVNQHLCGSHLVEALALVCGERGFHYGGGGGGSGGGGGIVEQCCTSTCSLDQLENY
CGGGGGQGGGGQGGGGQGGGGDCPKCPAPEMLGGPSVFIFPPKPKDTLLIARTPEV
TCVVVDLDPEDPEVQISWFDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLK
GKQFTCKVNNKALPSPIERTISKARGQAHQPSVYVLPPSREELSKNTVSLTCLIKDFFPP
DIDVEWQSNGQQEPESKYRTTPQLDEGSGYFLYSKLSVDKSRWQRGDTFICAVMHE

ALHNHYTQESLSHSPG (SEQ ID NO: 7)

[0108] The insulin-Fc fusion protein of SEQ ID NO: 7 was manufactured in HEK cells according to Example 10 or in CHO cells according to Example 11. The insulin-Fc fusion protein was purified according to Example 12. The structure of the insulin-Fc fusion protein was confirmed by non-reducing and reducing CE-SDS according to Example 13 and the sequence was confirmed by LC-MS with glycan removal according to Example 14. The purity (as assessed by the percent homodimer of the fusion protein yield) was measured according to Example 15.

[0109] This fusion protein demonstrated desirable in vitro and in vivo properties similar to SEQ ID NO: 5. As illustrated in FIG. 3, the only difference is that the Fc(gamma) RI binding affinity for the insulin-Fc fusion protein of SEQ ID NO: 7 was significantly reduced compared to that of the insulin-Fc fusion protein of SEQ ID NO: 5. A field trial in five diabetic client dogs of varying ages, breeds, and extent of diabetes disease was initiated. The five client dogs in the field trial had all been receiving insulin treatment with a known veterinary or human insulin product to the point of the trial initiation and were given the insulin-Fc fusion protein of SEQ ID NO: 7 on a once-a-week basis.

[0110] The AIA titer was again measured weekly or as frequently as possible over the course of the treatment according to Example 19. As compared to the dogs receiving the insulin-Fc fusion protein of SEQ ID NO: 5, none of the client dogs in this “wild” patient population demonstrated insulin anti-drug antibodies when dosed with the non-glycosylated insulin-Fc fusion protein of SEQ ID NO: 7. A normalized AIA titer of 0.15 was considered the minimum measurement for the client dog to be considered immunogenic to SEQ ID NO: 7. For client dogs that had a non-zero AIA titer at the start of the treatment, if the AIA more than doubled after being treated with the insulin-Fc fusion protein of SEQ ID NO: 7, the client dog was considered to be immunogenic. FIG. 9 is a plot of the normalized AIA titers for each of the client dogs as measured over the duration of their treatment, demonstrating that none of the five client dogs in this “wild” patient population demonstrated insulin anti-drug antibodies when dosed with the non-glycosylated insulin-Fc fusion protein of SEQ ID NO: 7. As shown in FIG. 10, this is in contrast to the dogs receiving the insulin-Fc fusion protein of SEQ ID NO: 5, where twelve dogs total demonstrated insulin anti-drug antibodies compared to none of the dogs receiving the non-glycosylated insulin-Fc fusion protein of SEQ ID NO: 7.

[0111] Taken together, the results demonstrate that, unexpectedly, outside a laboratory animal population, certain Fc fusion proteins have the potential of inducing high titers of antibodies against the therapeutic peptide or protein component, and that this response may be re-induced rapidly and robustly upon subsequent presentation of the therapeutic peptide or

protein component. Furthermore, these preliminary data indicate that the induction of anti-therapeutic protein or peptide antibodies is more likely in individuals who have already developed an immune response to that particular therapeutic protein or peptide. These results are in contrast to numerous published results that show the potential for Fc fusion proteins to induce immune tolerance against the fused therapeutic peptide or protein, and that haptens like DNP, nucleosides or peniciloyl groups, when chemically coupled to IgG carriers, were highly tolerogenic hapten-carrier conjugates.

[0112] For a therapeutic protein such as insulin which is used to treat a chronic disease (i.e., diabetes), anti-insulin antibodies render the therapy useless. Nevertheless, the aforementioned results led to the unique insight of how one might effectively design an Fc fusion protein to induce antibodies against, for example, a viral antigen to neutralize its activity. Based on the field experiments, the desired Fc fusion protein should be, at a minimum, native to the target subject (e.g., a human Fc or hFc for a human subject, a dog Fc or dFc for a dog subject), properly glycosylated at the Fc-cNg site, and capable of binding the Fc(gamma)I receptor. These findings present an opportunity to develop a novel therapeutic Fc-fusion protein against, for example, the novel coronavirus SARS-CoV-2.

Novel SARS-CoV-2 Fc Fusion Proteins

[0113] The outbreak of COVID-19 represents a serious threat to public health. There is an urgent need for safe and effective solutions to reduce or prevent against infection by its causative agent, the SARS-CoV-2 virus. The surface glycoprotein including the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein (SP) has been identified, and it has been found that the SARS-CoV-2 SP/RBD binds strongly to human and bat angiotensin-converting enzyme 2 (ACE2) receptors. The SARS-CoV-2 SP/RBD being a foreign antigen, a fusion protein comprising this antigen and a glycosylated human immunoglobulin Fc fragment (herein referred to as a SARS-CoV-2-RBD-hIgG-Fc fusion protein or SP/RBD-Fc fusion protein) is a promising approach to create a fusion protein that can amplify existing antibody titers in a patient or induce new antibody titers in patients with no or low immune response against SARS-CoV-2. However, this presents challenges due to variants of the spike protein which have evolved over time, making existing vaccines that target extinct variants less effective or ineffective in preventing COVID-19. Methods for making and using Fc fusion proteins for use in a prophylactic or booster vaccine which is efficacious for causing patients to create anti-virus antibodies to the SARS-CoV-2 virus in the face of new mutations in the SARS-CoV-2 spike protein meets this urgent need and would have significant public health value.

[0114] The goal therefore is to create an Fc fusion protein comprising some portion of the

SARS-CoV-2 nucleocapsid protein (or an analog thereof) and a human Fc fragment (e.g., human IgG1 or hIgG1) containing a site or residue with a tendency towards glycosylation in order to create a manufacturable conjugate that presents the antigen (SARS-CoV-2 nucleocapsid) in a novel manner to cause a patient to produce anti-SARS-CoV-2 antibodies rapidly at high titers.

[0115] A novel SARS-CoV-2 N-Fc fusion protein comprising a bivalent analog of the SARS-CoV-2 nucleocapsid protein recombinantly fused to a human IgG1 Fc moiety as shown in FIG. 2 would facilitate the focused delivery of the nucleocapsid antigen to local APCs that internalize the nucleocapsid novel SARS-CoV-2 N-Fc fusion protein via Fc(gamma) receptors.

[0116] The complete nucleocapsid protein for SARS-CoV-2 is shown below (GenBank: QHD43416.1):

MSDNGPQNQRNAPRITFGGPSDESTGSNQNGERSGARSKQRRPQGLPNNTASWFTALT
QHGKEDLKFRGQGVPIINTNSSPDDQIGYYRRATRRIRGGDGKMKDLSRWYFYLLG
TGPEAGLPYGANKDGIWVATEGALNTPKDHIGTRNPANNAIIVLQLPQGTTLPKGFY
AEGSRGGSQASSRSSRSRNSSRNSTPGSSRGTSARMAGNGGDAALALLLLDRLNQL
ESKMSGKGQQQQGQTVTKKSAAEASKKPRQKRTATKAYNVTQAFGRRGPEQTQGN
FGDQELIRQGTDYKHWPQIAQFAPSASAFFGMSRIGMEVTPSGTWLTYTGAIKLDDK
DPNFKDQVILLNKHIDAYKTFP (SEQ ID NO: 10)

[0117] Previous work with insulin-Fc fusion proteins, such as is described in WO2018107117A1 and WO2020006529A1, has demonstrated that the choices of the protein sequence, the linker sequence, and the composition of the Fc domain can all potentially influence protein yields, purity, and bioactivity.

[0118] In choosing the viral protein for the novel SARS-CoV-2 N-Fc fusion protein it is conceivable that one could choose a subset of the virus that includes some portion of the nucleocapsid. The viral protein for the novel SARS-CoV-2 N-Fc fusion protein may comprise all or a portion of the SARS-CoV-2 nucleocapsid protein. The viral protein for the novel SARS-CoV-2 N-Fc fusion protein may comprise all or a portion of the non-nucleocapsid portions of the SARS-CoV-2 virus, for example portions of the spike protein, portions of the M protein, or portions of the E protein of SARS-CoV-2. In examples, the viral protein for the novel SARS-CoV-2 N-Fc fusion protein comprises all or a portion of the SARS-CoV-2 nucleocapsid and all or a portion of the non-nucleocapsid portions of the SARS-CoV-2 virus. In examples, one or more amino acids in the nucleocapsid fragment of the novel SARS-CoV-2 N-Fc fusion protein may be mutated from their native state.

[0119] Based on experience manufacturing insulin-Fc fusion proteins, different viral protein designs will result in different protein yields of the novel SARS-CoV-2 N-Fc fusion protein. For example, one could choose larger or shorter portions of the nucleocapsid protein sequence of

SEQ ID NO: 10 and optionally mutate certain amino acids, to produce the desired viral portion for the Fc fusion protein. The resulting protein yield when the selected viral protein is attached to an Fc fragment can be experimentally determined. Furthermore, the length and composition of the linker connecting the selected viral protein to the Fc fragment will similarly have an impact on the protein yield, as will the choice of the Fc fragment and the portion of the Fc fragment hinge region that is linked to the viral protein.

[0120] FIG. 2 shows an illustration of an exemplary novel SARS-CoV-2 N-Fc fusion protein according to the present disclosure. The novel SARS-CoV-2 N-Fc fusion protein may comprise a peptide linker. In examples, some amino acids in the nucleocapsid fragment of the novel SARS-CoV-2 N-Fc fusion protein are mutated from their native state. In examples, the therapeutic protein comprising a fragment of the SARS-CoV-2 nucleocapsid protein is located on the N-terminal side of the Fc fragment. The novel SARS-CoV-2 N-Fc fusion protein comprises domains in the following orientation from N- to C-termini: (N-terminus)--therapeutic protein--peptide linker--Fc fragment--(C-terminus) (e.g., (N-terminus)--nucleocapsid protein--peptide linker--Fc fragment--(C-terminus)). The nucleocapsid fragment of the novel SARS-CoV-2 N-Fc fusion protein shown in FIG. 2 may or may not express well as part of a fusion protein recombinantly manufactured in host cells (i.e., manufactured in HEK cells according to Example 1.)

[0121] In all descriptions that follow, a cited amino acid position is referenced to the position of the amino acid in the SARS-CoV-2 nucleocapsid of SEQ ID NO: 10. The complete SARS-CoV-2 nucleocapsid protein is composed of 419 amino acids. The sequence and domain architecture of the SARS-CoV-2 nucleocapsid protein has been analyzed using IUPred2A. (*Baker, N. A., Sept, D., Joseph, S., Holst, M. J. & McCammon, J. A.* Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc. Natl Acad. Sci. USA* 98, 10037–10041 (2001). The SARS-CoV-2 nucleocapsid protein consists of an N-terminal domain (NTD), an RNA binding domain (RNABD), a linker domain, a dimerization domain, and a C-terminal domain (CTD). (*Cubuk, J., Alston, J.J., Incicco, J.J. et al.* The SARS-CoV-2 nucleocapsid protein is dynamic, disordered, and phase separates with RNA. *Nat Commun* 12, 1936 (2021)).

[0122] As a first attempt in creating a novel SARS-CoV-2 N-Fc fusion protein, a fragment of the viral nucleocapsid comprising the N-terminal domain (NTD), the RNA binding domain (RNABD), the linker domain and the dimerization domain was selected (SEQ ID NO: 10) resulting in a nucleocapsid fragment of 364 amino acids.

[0123] The nucleocapsid fragment (SEQ ID NO: 10) was linked using the peptide linker SGGGSGGGS (SEQ ID NO: 14) to a human IgG1 Fc fragment comprising the following

sequence:

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK
TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK
TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG (SEQ
ID NO: 1)

[0124] The human IgG1 fragment of SEQ ID NO: 1 illustrates that the N-terminal lysine on the native human IgG1 fragment was eliminated in an attempt to improve manufacturing yield and purity. In addition, the asparagine at the cNg site on the human IgG1 fragment was conserved to preserve the glycan attachment during fusion protein production in host cells.

[0125] The resultant SARS-CoV-2 N-Fc fusion protein is given below:

MSDNGPQNQRNAPRITFGGPSDESTGSNQNGERSGARSKQRRPQGLPNNTASWFTALT
QHGKEDLKFRGQGVPIINTNSSPDDQIGYYRRATRRIRGGDGKMKDLSRWYFYLLG
TGPEAGLPYGANKDGIIWVATEGALNTPKDHIGTRNPANNAIIVLQLPQGTTLPKGFY
AEGSRGGSQASSRSSRSRNSSRNSTPGSSRGTSARMAGNGGDAALALLLDRLNQL
ESKMSGKGQQQGGQTVTKSAAEASKKPRQKRTATKAYNVTQAFGRRGPEQTQGN
FGDQELIRQGTDYKHWPQIAQFAPSASAFFGMSRIGMEVTPSGTWLTYTGAIKLDDK
DPNFKDQVILLNKHIDAYKTFPSGGSGGGSDKHTHTCPPCPAPELLGGPSVFLFPPKPK
DTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV
LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKN
QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ
QGNVFCFSVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 17)

[0126] The SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 17 was manufactured in HEK293 cells according to Example 1. Unexpectedly, this resulted in extremely low protein yield of only 14mg/L.

[0127] Research has shown that the N-terminal domain of the nucleocapsid protein is disordered, flexible, and transiently interacts with the RNABD domain of the nucleocapsid protein. (*Cubuk, J., Alston, J.J., Incicco, J.J. et al.*) It was hypothesized that inclusion of the entire NTD was contributing to the poor protein yield of SEQ ID NO: 17. In a second attempt to create a SARS-CoV-2 N-Fc fusion protein, the majority of the N-terminal domain of the nucleocapsid protein fragment of SEQ ID NO: 10 was removed, specifically the SARS-CoV-2 nucleocapsid protein of SEQ ID NO: 10 was shortened and the first 40 amino acids (amino acids at positions 1-40 of SEQ ID NO: 10) were eliminated. The resulting SARS-CoV-2 nucleocapsid protein fragment comprised amino acids 41 to 364 of SEQ ID NO: 10 and is shown below:

RPQGLPNNTASWFTALTQHGKEDLKFRGQGVPIINTNSSPDDQIGYYRRATRRIRGGD

GKMKDLSRWYFYFYLGTGPEAGLPYGANKDGIWVATEGALNTPKDHIGTRNPANN
AAIVLQLPQGTTLPKGFYAEGRGGSQASSRSSRSRNSSRNSTPGSSRGTSARMAGN
GGDAALALLLLDRLNQLESKMSGKGQQQQGQTVTKKSAAEASKKPRQKRTATKAY
NVTQAFGRRGPEQTQGNFGDQELIRQGTDYKHWPQIAQFAPSASAFFGMSRIGMEVT
PSGTWLTYTGAIKLDDKDPNFKDQVILLNKHIDAYKTFP (SEQ ID NO: 11)

[0128] A side-by-side comparison of the SARS-CoV-2 nucleocapsid fragment of SEQ ID NO: 10 and the shortened nucleocapsid fragment of SEQ ID NO: 11 was performed using Clustal Omega and is shown in FIG. 14. “*” represents complete homology across all sequences at a given sequence position. A “:” (colon) indicates conservation between groups of strongly similar properties with scoring greater than 0.5 in the Gonnet PAM 250 matrix. A “-” (dash) indicates a sequence gap, meaning that no local homology exists within a particular set of comparisons within a certain range of the sequences.

[0129] The nucleocapsid fragment of SEQ ID NO: 11 was linked via the peptide linker SGGSGSGGS (SEQ ID NO: 14) to a human IgG1 Fc fragment comprising the following sequence:

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK
TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK
TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG (SEQ
ID NO: 1)

[0130] As was the case for the SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 17, the N-terminal lysine on the human IgG1 fragment was eliminated as shown above in SEQ ID NO: 1 in an attempt to improve manufacturing yield and purity. In addition, the asparagine at the cNg site on the human IgG1 fragment was conserved to preserve the glycan attachment during fusion protein production in host cells. The resultant SARS-CoV-2 N-Fc fusion protein is given below:
RPQGLPNNTASWFTALTQHGKEDLKFPGRGQVPINTNSSPDDQIGYYRRATRRIRGGD
GKMKDLSRWYFYFYLGTGPEAGLPYGANKDGIWVATEGALNTPKDHIGTRNPANN
AAIVLQLPQGTTLPKGFYAEGRGGSQASSRSSRSRNSSRNSTPGSSRGTSARMAGN
GGDAALALLLLDRLNQLESKMSGKGQQQQGQTVTKKSAAEASKKPRQKRTATKAY
NVTQAFGRRGPEQTQGNFGDQELIRQGTDYKHWPQIAQFAPSASAFFGMSRIGMEVT
PSGTWLTYTGAIKLDDKDPNFKDQVILLNKHIDAYKTFPSGGSGSGGSDKTHTCPPCP
APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNA
KTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK TISKAKGQPR
EPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG
SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 18)

[0131] The SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 18 was manufactured in HEK293 cells according to Example 1. In spite of the removal of the N-terminal domain in an attempt to prevent its transient interactions with the RNABD domain of the nucleocapsid protein fragment, unexpectedly, this also gave rise to a low protein yield titer of 18mg/L.

[0132] It was hypothesized that the overall length of the SARS-CoV-2 Fc fusion protein molecule was contributing to the low protein yield. Research has also shown that the central linker of the SARS-CoV-2 nucleocapsid is highly dynamic and there is minimal interaction between the nucleocapsid and the dimerization domain. However, in the complete absence of the dimerization domain and CTD domain there is speculation that the central linker domain can either self-interact or interact with the RNABD domain. (*Cubuk, J., Alston, J.J., Incicco, J.J. et al.*) Instead of removing the dimerization domain and CTD domain and keeping the entire central linker domain, a portion of the linker domain was retained. Specifically, the complete linker domain comprises amino acids 174 to 243, with amino acid 1 being the first amino acid of SEQ ID NO: 10. In a further attempt to produce a novel SARS-CoV-2 N-Fc fusion protein with acceptable manufacturing yield, the portion of the linker domain from amino acid 174 to amino acid 208 was retained, removing the 38 amino acids at the C-terminus end of the linker domain, resulting in the nucleocapsid fragment of SEQ ID NO: 12, shown below:

RPQGLPNNTASWFTALTQHGKEDLKFPGRGQVPINTNSSPDDQIGYYRRATRRIRGGD
GKMKDLSPRWYFYLLGTGPEAGLPYGANKDGIWVATEGALNTPKDHIGTRNPANN
AAIVLQLPQGTTLPKGFYAEGRGGSQASSRSSRSRNSSRNSTPGSSRGTSPPA (SEQ ID
NO: 12)

[0133] A side-by-side comparison of the SARS-CoV-2 nucleocapsid fragment of SEQ ID NO: 10 and the further shortened nucleocapsid fragment of SEQ ID NO: 12 was performed using Clustal Omega and is shown in FIG. 15. “*” represents complete homology across all sequences at a given sequence position. A “:” (colon) indicates conservation between groups of strongly similar properties with scoring greater than 0.5 in the Gonnet PAM 250 matrix. A “-” (dash) indicates a sequence gap, meaning that no local homology exists within a particular set of comparisons within a certain range of the sequences.

[0134] The nucleocapsid fragment of SEQ ID NO: 12 was linked via the peptide linker SGGGSGGGS (SEQ ID NO: 14) to a human IgG1 Fc fragment comprising the following sequence:

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK
TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK
TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG (SEQ

ID NO: 1)

[0135] As was the case for the SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 17 and SEQ ID NO: 18, the N-terminal lysine on the human IgG1 fragment was eliminated as shown above in SEQ ID NO: 1 in an attempt to improve manufacturing yield and purity. In addition, the asparagine at the cNg site on the human IgG1 fragment was conserved to preserve the glycan attachment during fusion protein production in host cells. The resultant novel SARS-CoV-2 N-Fc fusion protein is given below:

RPQGLPNNTASWFTALTQHGKEDLKFPQGQVPINTNSSPDDQIGYYRRATRRIRGGD
GKMKDLSRWYFYLLGTGPEAGLPYGANKDGIIWVATEGALNTPKDHIGTRNPANN
AAIVLQLPQGTTLPKGFYAEGRGGSQASSRSSRSRNRNSTPGSSRGTSPPASGGGS
GGGSDKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA
PIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN
NYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
(SEQ ID NO: 19)

[0136] The novel SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 19 was manufactured in HEK293 cells according to Example 1. The further shortened novel SARS-CoV-2 N-Fc fusion protein gave rise to a significant improvement in manufacturing yield, resulting in a protein titer of 163 mg/L.

[0137] Given the decreased efficacy of vaccines leveraging the SARS-CoV-2 spike protein region in the presence of mutations, it was noted that the nucleocapsid fragment of SEQ ID NO: 12 (shown below) included two of the amino acids that are mutations in the N-protein that is present in the alpha, gamma and omicron SARS-CoV-2 variants, specifically R203 and G204, that native form of which are highlighted in bold below, are mutated in these variants.

RPQGLPNNTASWFTALTQHGKEDLKFPQGQVPINTNSSPDDQIGYYRRATRRIRGGD
GKMKDLSRWYFYLLGTGPEAGLPYGANKDGIIWVATEGALNTPKDHIGTRNPANN
AAIVLQLPQGTTLPKGFYAEGRGGSQASSRSSRSRNRNSTPGSS**RG**TSPA (SEQ ID
NO: 12)

[0138] In an attempt to address any potential decrease in efficacy in the alpha, gamma and omicron SARS-CoV-2 variants, the nucleocapsid fragment of SEQ ID NO: 12 was modified at these two amino acids, where the arginine (R) at position 203 was modified to lysine (K) and the glycine (G) at position 203 was modified to arginine (R), again highlighted in bold below. This resulted in the nucleocapsid fragment of SEQ ID NO: 13, which is shown below.

RPQGLPNNTASWFTALTQHGKEDLKFPQGQVPINTNSSPDDQIGYYRRATRRIRGGD
GKMKDLSRWYFYLLGTGPEAGLPYGANKDGIIWVATEGALNTPKDHIGTRNPANN

AAIVLQLPQGTTLPKGFYAEGSRGGSQASSRSSRSRNSSRNSTPGSSKRTSPA (SEQ ID NO: 13)

[0139] In this nucleocapsid fragment, as with SEQ ID NO: 12, the portion of the linker domain from amino acid 174 to amino acid 208 was retained, removing the 38 amino acids at the C-terminus end of the linker domain, resulting in the nucleocapsid fragment.

[0140] A side-by-side comparison of the SARS-CoV-2 nucleocapsid fragments of SEQ ID NO: 10, SEQ ID NO: 12 and SEQ ID NO: 13 with the mutations was performed using Clustal Omega and is shown in FIG. 16. "*" represents complete homology across all sequences at a given sequence position. A ":" (colon) indicates conservation between groups of strongly similar properties with scoring greater than 0.5 in the Gonnet PAM 250 matrix. A "-" (dash) indicates a sequence gap, meaning that no local homology exists within a particular set of comparisons within a certain range of the sequences.

[0141] The nucleocapsid fragment of SEQ ID NO: 13 was linked via the peptide linker SGGGSGGGS (SEQ ID NO: 14) to a human IgG1 Fc fragment comprising the following sequence:

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK
TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK
TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 1)

[0142] As was the case for the SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 17 and SEQ ID NO: 18, the N-terminal lysine on the human IgG1 fragment was eliminated as shown above in SEQ ID NO: 1 in an attempt to improve manufacturing yield and purity. In addition, the asparagine at the cNg site on the human IgG1 fragment was conserved to preserve the glycan attachment during fusion protein production in host cells. The resultant novel SARS-CoV-2 N-Fc fusion protein is given below:

RPQGLPNNTASWFTALTQHGKEDLKFPGRGQVPINTNSSPDDQIGYYRRATRRIRGGD
GKMKDLSRWYFYLLGTGPEAGLPYGANKDGIWVATEGALNTPKDHIGTRNPANN
AAIVLQLPQGTTLPKGFYAEGSRGGSQASSRSSRSRNSSRNSTPGSSKRTSPASGGGS
GGGSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA
PIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN
NYKTTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
(SEQ ID NO: 20)

[0143] The novel SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 20 was manufactured

in HEK293 cells according to Example 1. The novel SARS-CoV-2 N-Fc fusion protein with the variant amino acid mutations on the central linker portion of the nucleocapsid fragment gave rise to a protein titer of 117 mg/L, which is acceptable according to the design goal (protein yield of greater than 100 mg/L). However, this yield is not as high as the novel SARS-CoV-2 N-Fc fusion protein with the non-mutated central linker portion of nucleocapsid fragment.

[0144] In an attempt to maintain the high yields obtained by removing the dimerization portion and part of the central linker portion of the SARS-CoV-2 nucleocapsid protein in the novel SARS-CoV-2 N-Fc fusion protein construct of SEQ ID NO: 19, a further nucleocapsid fragment was used, in which the entire central linker portion was eliminated, leaving the complete RNABD of the nucleocapsid protein with a small portion of the N-terminal domain (NTD). The resulting nucleocapsid fragment is shown below as SEQ ID NO: 8.

RSGARSKQRRPQGLPNNTASWFTALTQHGKEDLKFRGQGVPIINTNSSPDDQIGYYR
RATRRIRGGDGKMKDLSRWYFYLLGTGPEAGLPYGANKDGIWVATEGALNTPKD
HIGTRNPANNAIVLQLPQGTTLPKGFYAE (SEQ ID NO: 8)

[0145] A side-by-side comparison of the SARS-CoV-2 nucleocapsid fragment of SEQ ID NO: 10 and SEQ ID NO: 8 was performed using Clustal Omega and is shown in FIG. 17. “*” represents complete homology across all sequences at a given sequence position. A “:” (colon) indicates conservation between groups of strongly similar properties with scoring greater than 0.5 in the Gonnet PAM 250 matrix. A “-” (dash) indicates a sequence gap, meaning that no local homology exists within a particular set of comparisons within a certain range of the sequences.

[0146] The nucleocapsid fragment of SEQ ID NO: 8 was linked via the peptide linker SGGGSGGGS (SEQ ID NO: 14) to a human IgG1 Fc fragment comprising the following sequence:

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK
TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK
TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG (SEQ
ID NO: 1)

[0147] The N-terminal lysine on the human IgG1 fragment was eliminated as shown above in SEQ ID NO: 1 in an attempt to improve manufacturing yield and purity. In addition, the asparagine at the cNg site on the human IgG1 fragment was conserved to preserve the glycan attachment during fusion protein production in host cells. The resultant novel SARS-CoV-2 N-Fc fusion protein is given below:

RSGARSKQRRPQGLPNNTASWFTALTQHGKEDLKFRGQGVPIINTNSSPDDQIGYYR
RATRRIRGGDGKMKDLSRWYFYLLGTGPEAGLPYGANKDGIWVATEGALNTPKD

HIGTRNPANNAIIVLQLPQGTTLPKGFYAESGGSGGGSDKHTCPCPAPELLGGPS
VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS
RDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPVLDSGDSFFLYSKLTV
DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 15)

[0148] The novel SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 15 was manufactured in HEK293 cells according to Example 1. The novel SARS-CoV-2 Fc fusion protein with the removed central linker portion of the nucleocapsid fragment gave rise to a protein titer of 140 mg/L, which is an improvement from the novel SARS-CoV-2 N-Fc fusion protein which included the mutated central linker portion of nucleocapsid fragment (SEQ ID NO: 20). The novel SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 15 was manufactured in HEK293 cells in a considerably larger batch (0.5L instead of 0.03L) according to Example 1. In this larger batch, the novel SARS-CoV-2 Fc fusion protein with the central linker portion of the nucleocapsid fragment removed gave rise to a protein titer of 198 mg/L, which is a further improvement from the novel SARS-CoV-2 N-Fc fusion protein which included the mutated central linker portion of nucleocapsid fragment (SEQ ID NO: 20).

[0149] A further attempt to create a novel SARS-CoV-2 N-Fc fusion protein with a nucleocapsid fragment with the full N-terminal domain (NTD) and the RNABD domain only was performed, as retaining the NTD portion may be beneficial. In this novel SARS-CoV-2 N-Fc fusion protein, the nucleocapsid fragment of SEQ ID NO: 9, shown below, was used.

MSDNGPQNQRNAPRITFGGSPDSTGSNQNGERSGARSKQRRPQGLPNNTASWFTALT
QH GKEDLK FPRGQGVPI NTSSPDDQIGYYRRATRRIRGGDGKMKDLSRWYFYLLG
TGPEAGLPYGANKDGIIWVATEGALNTPKDHIGTRNPANNAIIVLQLPQGTTLPKGFY
AE (SEQ ID NO: 9)

[0150] A side-by-side comparison of the SARS-CoV-2 nucleocapsid fragment of SEQ ID NO: 9 and SEQ ID NO: 8 was performed using Clustal Omega and is shown in FIG. 18. “*” represents complete homology across all sequences at a given sequence position. A “:” (colon) indicates conservation between groups of strongly similar properties with scoring greater than 0.5 in the Gonnet PAM 250 matrix. A “-” (dash) indicates a sequence gap, meaning that no local homology exists within a particular set of comparisons within a certain range of the sequences.

[0151] The nucleocapsid fragment of SEQ ID NO: 9 was linked via the peptide linker SGGSGGGG (SEQ ID NO: 14) to a human IgG1 Fc fragment comprising the following sequence:

DKHTCPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK

TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK
TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG (SEQ
ID NO: 1)

[0152] The N-terminal lysine on the human IgG1 fragment was eliminated as shown above in SEQ ID NO: 1 in an attempt to improve manufacturing yield and purity. In addition, the asparagine at the cNg site on the human IgG1 fragment was conserved to preserve the glycan attachment during fusion protein production in host cells. The resultant novel SARS-CoV-2 N-Fc fusion protein is given below:

MSDNGPQNQRNAPRITFGGSDSTGSNQNGERSGARSKQRRPQGLPNNTASWFTALT
QH GKEDLKFP RQGVPINTNSSPDDQIGYYRRATRIRGGDGKMKDLSRWYFYLLG
TGPEAGLPYGANKDGIIWVATEGALNTPKDHIGTRNPANNAIIVLQLPQGTTLPKGFY
AESGGGSGGGSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH
EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE
SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQ
KSLSLSPG (SEQ ID NO: 16)

[0153] The novel SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 16 was manufactured in HEK293 cells according to Example 1. The novel SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 16 gave rise to an acceptable protein titer of 173 mg/L.

[0154] Four candidate novel SARS-CoV-2 N-Fc fusion proteins therefore met the design goal for protein yield of greater than 100 mg/L: SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 19 and SEQ ID NO: 20. The novel SARS-CoV-2 N-Fc fusion proteins of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 19 and SEQ ID NO: 20 recombinantly manufactured in HEK cells according to Example 1 were purified according to Example 2. The Fc fusion protein structures of the novel SARS-CoV-2 N-Fc fusion proteins of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 19 and SEQ ID NO: 20 are confirmed according to Example 3, and sequence identification is performed according to Example 4. To obtain the homodimer titer of each of the manufactured novel SARS-CoV-2 N-Fc fusion proteins, the %homodimer was measured according to Example 5 and the homodimer titer was calculated by multiplying the %homodimer by the protein titer of the recombinantly manufactured fusion protein. The homodimer titers of the novel SARS-CoV-2 N-Fc fusion proteins of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 19 and SEQ ID NO: 20 are shown below together with the protein yields in Table 1.

Table 1: Protein titer, homodimer % and homodimer yield of SARS-CoV-2 N-Fc fusion proteins.				
Name	Description	Titer (mg/L)	Homodimer %	Homodimer Yield (mg/L)
SEQ ID NO: 15	Nucleocapsid fragment (32-174) (30mL batch)	140	100	140
SEQ ID NO: 15	Nucleocapsid fragment (32-174) (500 mL batch)	198.2	92.6	183.5
SEQ ID NO: 16	Nucleocapsid fragment (1-174), (30mL batch)	173	99.6	172.3
SEQ ID NO: 19	Nucleocapsid fragment (41-208) (30mL batch)	163	97.6	159.1
SEQ ID NO: 20	Nucleocapsid fragment (41-208), R203K, G204R (30mL batch)	117	98.8	115.6

[0155] The Fc(gamma) Receptor I binding of the SARS-CoV-2 N-Fc fusion proteins of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 19 and SEQ ID NO: 20 manufactured in HEK293 cells according to Example 1 is measured according to Example 6. As described in Example 6, the OD450 measurements for Human Fc(gamma) Receptor I, Fc(gamma) Receptor IIA, Fc(gamma) Receptor IIB, Fc(gamma) Receptor III, FcRn, and ACE2 receptor binding are expected to increase as a function of the concentration of the SARS-CoV-2 N-Fc fusion protein.

[0156] A side-by-side comparison of the nucleocapsid frame domains of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 12 and SEQ ID NO: 13 was performed using Clustal Omega and is shown in FIG. 19. “*” represents complete homology across all sequences at a given sequence position. A “:” (colon) indicates conservation between groups of strongly similar properties with scoring greater than 0.5 in the Gonnet PAM 250 matrix. A “-” (dash) indicates a sequence gap, meaning that no local homology exists within a particular set of comparisons within a certain range of the sequences, while “.”, “.” or spaces refer to conservative, moderate, or very different amino acid mutations across the sequences at a given sequence position respectively.

[0157] To highlight the amino acid mutations in the nucleocapsid regions, a side-by-side comparison of the nucleocapsid frame domains of SEQ ID NO: 12 and SEQ ID NO: 13 was performed using Clustal Omega and is shown in FIG. 20. “*” represents complete homology across all sequences at a given sequence position. A “:” (colon) indicates conservation between groups of strongly similar properties with scoring greater than 0.5 in the Gonnet PAM 250 matrix. A “-” (dash) indicates a sequence gap, meaning that no local homology exists within a

particular set of comparisons within a certain range of the sequences, while “:”, “.” or spaces refer to conservative, moderate, or very different amino acid mutations across the sequences at a given sequence position respectively.

SARS-CoV-2 N-Fc Fusion Proteins for Use as a Primary Vaccine

[0158] A SARS-CoV-2 N-Fc fusion protein may be used as a primary vaccine. In one or more embodiments, the SARS-CoV-2 N-Fc fusion protein is provided in a pharmaceutical composition. Injection of any protein can induce an immune response, the magnitude and type of which is highly dependent on the “status” of the respective immune system. For example, injection of a foreign antigen (Ag) relative to a self Ag will induce a greater immune response in an immune system that maintains central and peripheral tolerance mechanisms. Moreover, foreign Ag administration to an immune system that has been primed to previous exposure to the respective Ag (e.g., a viral infection) will lodge a more rapid and elevated immune response relative to that of an Ag-naïve system. The immunological basis of this priming is two-fold: 1) an Ag-naïve immune system has naïve B and T lymphocytes that have a much higher threshold of activation than do the Ag-primed “memory” cells of a Ag-primed immune system, such that the antigen-presenting cells (APCs) that present Ag require much less Ag to activate primed memory T cells, and 2) due to expansion of memory T cells during the Ag priming exposure, there are inherently greater numbers of such cells upon re-exposure to an injected Ag. Note that dominant APCs are dendritic cells (DCs) and macrophages that present Ag in complex with Major Histocompatibility Complex (MHC) molecules on their surface to T cell Ag receptors. FIG. 11 is a schematic diagram depicting example modes in which an antigen may interact with an antigen presenting cell, e.g., a dendritic cell.

[0159] APCs can influence both the “magnitude” and “type” of response to Ag. B cells participate in the immune response directly by humoral immunity (antibody production) and also participate in the T-cell immune response as specific APCs that selectively capture and present antigens to T cells. Both these B-cell functions are achieved through activation of the surface B cell receptor (BCR), which is essentially a membrane bound antibody that binds specifically to a particular antigen. Multivalent soluble antigens such as the Fc-fusion homodimer containing the specific antigen can be recognized by BCRs and activate them. Thus, the SARS-CoV-2 N-Fc fusion protein homodimers can activate B cells through antigen-specific BCR activation leading to an increase in antibody production, and, through B-cell mediated APC activity, also result in an increase in T cell recognition and reactivity directed specifically against the RBD epitopes. Thus, these fusion proteins activate both humoral and cellular immunity after administration. More particularly, the induced Th1-type (cellular) immunity

activates the body's cell-killing machinery like cytotoxic T cells, NK cells, and macrophages which target cells that are already infected with virus. Concurrently, in Th2-type immunity, the T helper cells stimulate B cells to proliferate and differentiate into plasma cells that secrete antigen-specific antibodies. These antibodies help control infection by either (i) neutralization of virus by binding of antibodies to virus surface RBD moieties required for cell entry, wherein antibodies also help clear antibody-bound virus via Fc-directed phagocytosis. (ii) antibody directed cellular toxicity (ADC) which occurs when the antibodies bind antigen presented on the surface of infected cells and direct NK cells to destroy them, or (iii) potentially neutralizing virus internal antigens that become exposed inside cells, wherein the antibody and exposed internal virus milieu come into contact, e.g. inside cells.

Adjuvants

[0160] In some examples, the Th1 cell response is required to clear most viral and bacterial infections, in which virus-like or bacterial-like substances (non-Ag in nature) condition APCs to express key cytokines and surface co-stimulatory molecules that, during Ag presentation, drive T cells to become the Th1 type. In fact, this APC activation is the conceptual basis of many immune enhancing substances called adjuvants. Dominant APCs are dendritic cells (DCs) and macrophages that present Ag in complex with Major Histocompatibility Complex (MHC) molecules on their surface to T cell Ag receptors. These APCs can influence both the "magnitude" and "type" of response to Ag. Some adjuvants are designed to trick the immune system into reacting to the injected vaccine Ag as if it were part of an on-going infection (i.e., infectious agents provide such natural viral or bacterial adjuvant substances). Therefore, adjuvants activate APCs for greater Ag-presentation capabilities necessary to overcome the high activation threshold of naïve T cells, in addition to shaping their development into the Th1 response to effectively clear the respective infection. Note that such T cells provide critical help to B cells that specifically bind the respective Ag to produce Ag-specific antibody (Ab) titers.

[0161] The Fc fusion protein used as a primary vaccine may be co-administered with an adjuvant to enhance or otherwise alter the immune response in the target subject. Adjuvants activate APCs for greater Ag-presentation capabilities which are necessary to overcome the high activation threshold of naïve T cells, in addition to shaping their development into the Th1 response to effectively clear the respective infection. In examples, known adjuvants may be used in a pharmaceutical composition of the SARS-CoV-2 N-Fc fusion protein to enhance the induction of anti-SARS-CoV-2 antibodies. Known adjuvants include adjuvants used for respiratory virus infections including trivalent or monovalent influenza vaccines, pandemic H1N1, H5N1, and SARS-CoV vaccines during the last decade in human clinical studies.

[0162] Examples of adjuvants that may be employed in the pharmaceutical compositions disclosed herein include but are not limited to oil-in-water, amorphous aluminum hydroxyphosphate sulfate (AAHS), aluminum hydroxide, aluminum phosphate, potassium aluminum sulfate (Alum), Freund's adjuvant (complete and/or incomplete), squalene, AS02, AS03, AS04, MF59, AS01B, QS-21, CpG 1018, ISCOMS, Montanide™ ISA-51, Montanide™ ISA-720, polylactide co-glycolide (PLG), monophosphoryl lipid A (MPL), Detox, AGP [RC-529], DC_Chol, OM-174 (lipid A derivative), CpG motifs (synthetic oligonucleotides containing immunostimulatory CpG motifs), modified LT and CT, hGM-CSF, hIL-12, Immudaptin, Inert vehicles, such as gold particles as well as various experimental adjuvants from sources such as Advax (Australia) such as AddaVax (Invivogen) or other Advax-based vaccine adjuvants.

[0163] In some examples, the selected adjuvant may be MF59 (Novartis) and AS-03 (GlaxoSmithKline). A custom formulation of MF59 (Novartis) or an equivalent such as AddaVax (Invivogen) or other Advax-based vaccine adjuvants from Vaxine Pvt Ltd. (Australia) may be used in a pharmaceutical composition of the SARS-CoV-2 N-Fc fusion protein. In preferred embodiments, the SARS-CoV-2 N-Fc fusion protein is co-administered with the Montanide™ ISA-720 adjuvant to enhance or otherwise alter the immune response in the target subject. Many different adjuvants used for respiratory virus infections are tested extensively in seasonal trivalent flu vaccines and with pandemic H1N1 and H5N1 vaccines (Protein Sciences) in Australia and recently in NIH supported human influenza vaccine trials conducted by Sanofi Pasteur in the United States and may be used in a pharmaceutical composition of the SARS-CoV-2 N-Fc fusion protein.

[0164] In one or more embodiments, the SARS-CoV-2 N-Fc fusion protein formulation is prepared onsite for administration. In one aspect, the SARS-CoV-2 N-Fc fusion protein is mixed with an adjuvant onsite under sterile mixing conditions. In one aspect, the SARS-CoV-2 N-Fc fusion protein and adjuvant are thoroughly mixed and/or emulsified to prepare a homogenous emulsion for administration to the subject. The adjuvanted formulation of the SARS-CoV-2 N-Fc fusion protein or a pharmaceutical composition thereof is administered to a patient by subcutaneous (s.c.) injection or intramuscular (i.m.) injection, as the s.c. or i.m. injection sites are more likely to induce a strong antibody response due to there being more dendritic cells (DCs) in the subcutaneous and intramuscular spaces.

[0165] As described above, in some cases, it may be advantageous to use an adjuvant in the pharmaceutical composition in order to increase the quantity of anti-SARS-CoV-2 antibody titers as measured according to Example 7 and/or the virus-neutralizing capacity of the anti-SARS-CoV-2 antibody titers as measured according to Example 9. The use of an adjuvant may

be especially advantageous in antibody naïve patients who do not possess an underlying immune response to the virus or the RNABD. Furthermore, an adjuvant may be advantageous in older subjects who experience altered immune competence with increasing age, so-called immunosenescence, which is the result of changes at multiple levels of the immune system over time. Once a patient has measurable antibodies, upon re-challenge with the SARS-CoV-2 virus, the patient will exhibit very rapid development of anti-SARS-CoV-2 antibodies to mount their own defense against COVID-19.

Primary SARS-CoV-2 N-Fc Fusion Vaccines Evaluated in Mice

[0166] The efficacy of an exemplary SARS-CoV-2 N-Fc fusion protein of this disclosure or a pharmaceutical composition thereof may be initially evaluated in mice immunization studies for their capacity to induce high-titer anti-nucleocapsid protein IgG titers according to the procedure in Example 9. BALB/c mice are a relevant animal model that has been extensively used for preclinical immunogenicity assessment of vaccines. This strain generates robust Ab responses when immunized with adjuvanted and non-adjuvanted vaccine candidates. Moreover, mouse-specific reagents are widely available for evaluating the kinetics and characteristics of a variety of immune responses to vaccination, including relevant Ab isotypes and T cell responses (e.g., Th1 vs. Th2 responses). Therefore, the BALB/c mouse model is selected to evaluate the immunogenicity of SARS-CoV-2 N-Fc fusion protein vaccines with respect to Ag dose, potentiation by adjuvants, routes of administration, and dosing frequency required to achieve optimal Ab responses.

[0167] Briefly, target mice (e.g., BALB/c mice) are injected three times at predetermined intervals (e.g., on Day 0, Day 21, and Day 46) with an exemplary SARS-CoV-2 N-Fc fusion protein (with or without Montanide™ ISA 720 adjuvant) or pharmaceutical compositions thereof, and serum is collected at regular intervals (every 7 days beginning at Day 14).

[0168] After administering one or more than one treatment of the SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 15 to N=7 BALB/c mice according to Example 8, anti-SP/RBD SARS-CoV-2 IgG antibody titers were measured according to Example 7. As expected, and as illustrated in FIG. 12, as the exemplary SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 15 does not contain any portion of the SP/RBD of SARS-CoV-2, no measurable anti-SARS-CoV-2 antibody titers are present post-treatment.

[0169] The serum anti-SARS-CoV-2 nucleocapsid protein antibody titers are measured according to the procedure in Example 9.

[0170] At a dose level of 10 µg of the SARS-CoV-2 N-Fc fusion protein without adjuvant, the SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 15 induced measurable anti-SARS-CoV-

2 nucleocapsid protein antibody titers beginning around 40 days after the first injection on Day 0. It is expected that at a dose level of 10 µg of the SARS-CoV-2 N-Fc fusion protein without adjuvant, the SARS-CoV-2 N-Fc fusion proteins of SEQ ID NO: 16, SEQ ID NO: 19 and SEQ ID NO: 20 will induce measurable anti-SARS-CoV-2 nucleocapsid protein antibody titers beginning around 40 days after the first injection on Day 0.

[0171] As previously discussed, adjuvants activate APCs for greater Ag-presentation capabilities which are necessary to overcome the high activation threshold of naïve T cells, in addition to shaping their development into the Th1 response to effectively clear the respective infection. When the SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 15 was combined with Montanide™ ISA 720 adjuvant (30%/70% v/v), the anti-SARS-CoV-2 nucleocapsid protein antibody titers beginning around 40 days after the first injection on Day 0 were greater compared to the SARS-CoV-2 N-Fc fusion protein without adjuvant. It is expected that when the SARS-CoV-2 N-Fc fusion proteins of SEQ ID NO: 16, SEQ ID NO: 19 and SEQ ID NO: 20 are combined with Montanide™ ISA 720 adjuvant (30%/70% v/v), the anti-SARS-CoV-2 nucleocapsid protein antibody titers beginning around 40 days after the first injection on Day 0 will be greater compared to the SARS-CoV-2 N-Fc fusion protein without adjuvant. These data strongly supported the selection of Montanide™ ISA 720 as the lead adjuvant candidate for this vaccine development program.

[0172] The kinetic response, that is the duration of response, to dose levels varying from 1 µg to 100 µg after 1, 2, and 3 doses is expected to demonstrate increasing anti-SARS-CoV-2 nucleocapsid protein antibody titers at all dose levels up to at least 56 days post vaccination.

SARS-CoV-2 N-Fc Fusion Proteins for Use as a Booster Vaccine

[0173] In examples, an exemplary SARS-CoV-2 N-Fc fusion protein of this disclosure, for example the SARS-CoV-2 N-Fc fusion proteins of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 19 or SEQ ID NO: 20 may be used as a booster vaccine. Administration of the fusion protein to subjects that already have low but measurable antibody levels to the SARS-CoV-2 antigen to amplify their antibody titers increases their antiviral protection. N-protein (nucleocapsid) fragment variants are synthesized to maximize antigenicity while the Fc region prolongs antigen residence time. Without wishing to be bound to any particular theory of mechanism, it is believed that during the longer in vivo residence time, the naturally glycosylated human Fc fragment will help bind Fc(gamma) receptors on antigen-presenting cells (APCs), which will in turn cause greater presentation of the SARS-CoV-2 nucleocapsid analog antigen to T-cells and/or B-cells (as depicted in FIG. 11), which is expected to produce a strong immune response to the SARS-CoV-2 nucleocapsid antigen. Specifically, the APCs

internalize the SARS-CoV-2 nucleocapsid antigen via Fc(gamma) receptors, and then process and present nucleocapsid fragments to CD4+ Th cells that in turn promote ("help") B cell activation and anti-SARS-CoV-2 nucleocapsid IgG (i.e., Ab) production. Antigen-presenting cells may be, for example, dendritic cells (DCs), monocytes or macrophages that can internalize the molecules of the SARS-CoV-2 N-Fc fusion proteins of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 19 or SEQ ID NO: 20 via Fc-receptor mediated phagocytosis (e.g., through the Fc region of the SARS-CoV-2 N-Fc fusion protein binding to the Fc(gamma) receptors in immune cells as depicted in FIG. 11). Fc-mediated uptake of the SARS-CoV-2 N-Fc fusion protein by, for example, a subset of DCs (e.g., cDC₂s) promotes the development of anti-SARS-CoV-2 T helper 2 (Th2) cells through secretion of IL-10 and IL-33. Anti-SARS-CoV-2 Th2 cells activate anti-SARS-CoV-2 B-cells, for example by cross linking their antigen receptors to allow the B-cells to attract the Th2 cells. B-cell antigen receptor (BCR) mediated uptake binds the SARS-CoV-2 RNABD of the SARS-CoV-2 N-Fc fusion protein molecules, then delivers the SARS-CoV-2 antigen to intracellular sites where it is degraded and returned to the B-cell surface as peptides bound to MHC class II molecules. The peptide MCH class II complex can be recognized by the SARS-CoV-2-specific helper T cells stimulating them to make proteins that in turn cause the B-cell to proliferate and its progeny to differentiate into B cells that secrete anti-SARS-CoV-2 antibodies. The SARS-CoV-2 N-Fc fusion proteins of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 19 or SEQ ID NO: 20 may directly expose the SARS-CoV-2 RNABD (nucleocapsid) fragment to antigen producing cells for a protracted period of time due to the presence of the Fc fragment. Furthermore, and as previously described, the glycosylated Fc fragment in the SARS-CoV-2 N-Fc fusion proteins of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 19 or SEQ ID NO: 20 is expected to induce a very strong immune response directed to the therapeutic or antigen portion of the fusion protein. These properties in combination significantly increase the amount of anti-viral antibodies while also decreasing the amount of antigen necessary to produce the required immune response.

[0174] In examples, a therapy comprising treatment of a patient with the SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 19 or SEQ ID NO: 20 or a pharmaceutical composition thereof, may be administered as a booster vaccine to recovered patients of COVID-19 that are already antibody-positive to SARS-CoV-2, as a means to amplify their antibody titers and affinity so that when these treated patients are subsequently confronted with the virus, they will have sufficient immunity to prevent infection and/or serious symptoms related to infection with the SARS-CoV-2 virus. Furthermore, a therapy comprising a treatment of a patient with the SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 19 or SEQ ID NO: 20 or a pharmaceutical composition thereof, may be

administered as a booster vaccine to subjects that have been previously immunized with a vaccine against the SARS-CoV-2 virus as a means to amplify their antibody titers and affinity specifically against the SARS-CoV-2 N-Fc fusion protein nucleocapsid. Such a therapy is critical in cases where vaccines are not 100% effective and/or the where induced antibody titers wane over time. In examples, the SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 19 or SEQ ID NO: 20 or a pharmaceutical composition thereof may be administered to a patient by subcutaneous injection (s.c.) or intramuscularly (i.m.), as the s.c. or i.m. injection sites are more likely to induce a strong antibody response due to there being more dendritic cells (DCs) in the subcutaneous and intramuscular spaces.

Fc Fusion Protein Production

[0175] In embodiments, a fusion protein can be expressed by a cell as described in more detail in the Examples section.

Expression and Purification

[0176] A SARS-CoV-2 N-Fc fusion protein can be expressed recombinantly, e.g., in a eukaryotic cell, e.g., mammalian cell or non-mammalian cell. Exemplary mammalian cells used for expression include HEK cells (e.g., HEK293 cells) or CHO cells. CHO cells can be subdivided into various strains or subclasses, (e.g., CHO DG44, CHO-M, CHO-SE™ and CHO-K1), and some of these cell strains may be genetically engineered for optimal use with a particular type of nucleic acid molecule (e.g., a vector comprising DNA) or a particular cell growth media composition as described in the Examples section. Cells may be transfected with a nucleic acid molecule (e.g., vector) encoding the SARS-CoV-2 N-Fc fusion protein (e.g., where the entire SARS-CoV-2 N-Fc fusion protein is encoded by a single nucleic acid molecule). HEK293 cells may be transfected with a vector that encodes for the SARS-CoV-2 N-Fc fusion protein, but this process only results in temporary expression of the SARS-CoV-2 N-Fc fusion protein for a period of time (e.g., 3 days, 4 days, 5, days, 7 days, 10 days, 12 days, 14 days, or more) before the host cell stops expressing appreciable levels of the SARS-CoV-2 N-Fc fusion protein (i.e., transient transfection). HEK293 cells that are transiently transfected with nucleic acid sequences encoding for SARS-CoV-2 N-Fc fusion proteins often allow for more rapid production of recombinant proteins which facilitates making and screening multiple SARS-CoV-2 N-Fc fusion protein candidates. CHO cells may be transfected with a vector that is permanently incorporated into the host cell DNA and leads to consistent and permanent expression (i.e., stable transfection) of the SARS-CoV-2 N-Fc fusion protein as long as the cells are cultured appropriately. CHO cells and CHO cell lines that are stably transfected with nucleic acids encoding for SARS-CoV-2 N-Fc fusion proteins often take longer to develop, but they

often produce higher protein yields and are more amenable to manufacturing low-cost products (e.g., products for use in the veterinary pharmaceutical market). Cells and cell lines can be cultured using standard methods in the art.

[0177] In examples, the SARS-CoV-2 N-Fc fusion protein may be purified or isolated from the cells (e.g., by lysis of the cells). The SARS-CoV-2 N-Fc fusion protein is secreted by the cells and may be purified or isolated from the cell culture media in which the cells were grown. Purification of the SARS-CoV-2 N-Fc fusion protein can include using column chromatography (e.g., affinity chromatography) or using other separation methods based on differences in size, charge, and/or affinity for certain molecules. Purification of the SARS-CoV-2 N-Fc fusion protein involves selecting or enriching for proteins containing an Fc fragment, e.g., by using Protein A beads or a Protein A column that cause proteins containing an Fc fragment to become bound with high affinity at neutral solution pH to the Protein A covalently conjugated to the Protein A beads. The bound SARS-CoV-2 N-Fc fusion protein may then be eluted from the Protein A beads by a change in a solution variable (e.g., a decrease in the solution pH). Other separation methods such as ion exchange chromatography and/or gel filtration chromatography can also be employed alternatively or additionally. Purification of the SARS-CoV-2 N-Fc fusion protein may further comprise filtering or centrifuging the protein preparation, diafiltration, ultrafiltration, and filtration through porous membranes of various sizes, as well as final formulation with excipients.

[0178] The purified SARS-CoV-2 N-Fc fusion protein can be characterized, e.g., for purity, protein yield, structure, and/or activity, using a variety of methods, e.g., absorbance at 280 nm (e.g., to determine protein yield), size exclusion or capillary electrophoresis (e.g., to determine the molecular weight, percent aggregation, and/or purity), mass spectrometry (MS) and/or liquid chromatography (LC-MS) (e.g., to determine purity and/or glycosylation), and/or ELISA (e.g., to determine extent of binding, e.g., affinity, to a SARS-CoV-2 antibody or ACE2). Exemplary methods of characterization are also described in the Examples section.

[0179] The protein yield of a SARS-CoV-2 N-Fc fusion protein after production in transiently transfected HEK cells and protein A purification may be greater than 5 mg/L, 10 mg/L, or 20 mg/L, or more preferably greater than 50 mg/L (e.g., greater than 60 mg/L, greater than 70 mg/L, greater than 80 mg/L, greater than 90 mg/L, greater than 100 mg/L). The %homodimer of a SARS-CoV-2 N-Fc fusion protein after production in transiently transfected HEK cells and protein A purification is greater than 70% (e.g., greater than 80%, greater than 85%, greater than 90%, greater than 95%, greater than 96%, greater than 97%, greater than 98%, greater than 99%). The homodimer titer of a SARS-CoV-2 N-Fc fusion protein after production in transiently transfected HEK cells and protein A purification, calculated as the product

between the SARS-CoV-2 N-Fc fusion protein yield and the %homodimer, may be greater than 50 mg/L (e.g., greater than 60 mg/L, greater than 70 mg/L, greater than 80 mg/L, greater than 90 mg/L, greater than 100 mg/L).

Pharmaceutical Compositions and Routes of Administration

[0180] The amount and concentration of the SARS-CoV-2 N-Fc fusion protein in the pharmaceutical compositions, as well as the quantity of the pharmaceutical composition administered to a subject, can be selected based on clinically relevant factors, such as medically relevant characteristics of the subject (e.g., age, weight, gender, other medical conditions, and the like), the solubility of compounds in the pharmaceutical compositions, the potency and activity of the compounds, and the manner of administration of the pharmaceutical compositions.

[0181] Formulations of the present disclosure include those suitable for parenteral administration. The phrases “parenteral administration” and “administered parenterally” as used herein means modes of administration other than enteral and topical administration, usually by intravenous, intramuscular, or subcutaneous injection.

[0182] Examples of suitable aqueous and non-aqueous carriers that may be employed in the pharmaceutical compositions of the disclosure include water, saline, ethanol, salts, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate, buffering agents, such as potassium and/or sodium phosphates, pH buffers, such as hydrochloric acid and/or sodium hydroxide, and the like. Proper fluidity can be maintained, for example, by the use of coating or emulsifier materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants, e.g., Tween-like surfactants. In some examples, the pharmaceutical composition (e.g., as described herein) comprises a Tween-like surfactant, e.g., polysorbate-20, Tween-20 or Tween-80. In some examples, the pharmaceutical composition (e.g., as described herein) comprises a Tween-like surfactant, e.g., Tween-80, at a concentration between about 0.001% and about 2%, or between about 0.005% and about 0.1%, or between about 0.01% and about 0.5%.

[0183] The SARS-CoV-2 N-Fc fusion protein may be administered as a bolus, infusion, or an intravenous push, or administered through syringe injection, pump, pen, needle, or indwelling catheter. The SARS-CoV-2 N-Fc fusion protein may be administered by a subcutaneous bolus injection. In examples, the SARS-CoV-2 N-Fc fusion protein or a pharmaceutical composition thereof is administered to a patient by subcutaneous injection (s.c.) or intramuscularly (i.m.), as the s.c. or i.m. injection sites are more likely to induce a strong antibody response due to there being more dendritic cells (DCs) in the subcutaneous and intramuscular spaces. Methods of

introduction may also be provided by rechargeable or biodegradable devices. Various slow-release polymeric devices have been developed and tested in vivo in recent years for the controlled delivery of drugs, including proteinaceous biopharmaceuticals. A variety of biocompatible polymers (including hydrogels), including both biodegradable and non-degradable polymers, can be used to form an implant for the sustained release of a compound at a particular target site. Additional pharmaceutically-acceptable ingredients for use in the compositions include buffering agents, salts, stabilizing agents, diluents, preservatives, antibiotics, isotonic agents, and the like.

Dosages

[0184] In use, a therapeutically-effective amount of the SARS-CoV-2 N-Fc fusion protein is administered to a subject in need thereof. Administration of the SARS-CoV-2 N-Fc fusion protein elicits an immune response in the subject, and more specifically an immune response against coronavirus infection, more specifically SARS-CoV-2 or variants. The immune response will be demonstrated by a lack of observable clinical symptoms, or reduction of clinical symptoms normally displayed by an infected subject, reduced viral shedding, faster recovery times from infection, and/or reduced duration of infection. In another embodiment, a method of activating an immune cell at a site of infection or disease is provided comprising administering a therapeutically-effective amount of the SARS-CoV-2 N-Fc fusion protein to a mammal. In another aspect, a method of increasing antibody production in a subject is provided comprising administering a therapeutically-effective amount of the SARS-CoV-2 N-Fc fusion protein to a mammal.

[0185] It will be appreciated that therapeutic and prophylactic methods described herein are applicable to humans as well as any suitable warm-blooded animal, including, without limitation, dogs, cats, and other companion animals, as well as, rodents, primates, horses, cattle, sheep, pigs, etc. The methods can be also applied for clinical research and/or study.

[0186] As used herein, the phrase “effective amount” or “therapeutically effective amount” is meant to refer to a therapeutic or prophylactic amount of the SARS-CoV-2 N-Fc fusion protein that would be appropriate for an embodiment of the present disclosure, that will elicit the desired therapeutic or prophylactic effect or response, including alleviating some or all of such symptoms of infection or reducing the predisposition to the infection, when administered in accordance with the desired treatment regimen. One of skill in the art recognizes that an amount may be considered therapeutically “effective” even if the condition is not totally eradicated or prevented, but it or its symptoms and/or effects are improved or alleviated partially in the subject. The therapeutically effective dosage of SARS-CoV-2 N-Fc fusion peptide may vary depending on the size and species of the subject, and according to the mode of

administration.

[0187] Actual dosage levels of the SARS-CoV-2 N-Fc fusion protein can be varied so as to obtain an amount of the active ingredient that is effective to achieve the desired therapeutic response for a particular subject. The selected dosage level will depend upon a variety of factors including the activity of the particular fusion protein employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular fusion protein employed, the age, sex, weight, condition, general health and prior medical history of the subject being treated, and like factors well known in the medical arts. In general, a suitable dose of an SARS-CoV-2 N-Fc fusion protein will be the amount that is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above.

[0188] The immunogenic formulation is provided, in various aspects, in unit dosage form for ease of administration and uniformity of dosage. "Unit dosage form" as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated, each unit containing a predetermined quantity of the SARS-CoV-2 N-Fc fusion protein calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms is dictated by and are directly dependent on the unique characteristics of the excipient(s) and therapeutic agent(s) and the particular biological effect to be achieved. In one or more embodiments, the formulation is provided in a kit of components for administration of the SARS-CoV-2 N-Fc fusion protein to the subject. In one or more embodiments, a pharmaceutical composition comprising the SARS-CoV-2 N-Fc fusion protein dispersed in a suitable carrier is provided in a unit dosage form (e.g., vial). In one or more embodiments, the kit further comprises a discrete unit dosage form (e.g., vial) containing an adjuvant and/or other carrier system for onsite mixing of the SARS-CoV-2 N-Fc fusion protein for administration. In one or more embodiments, the kit comprises one or more emulsifying needles and syringes for onsite mixing of the immunogenic formulation for administration. In one or more embodiments, the kit comprises one or more dosing syringes for administering the prepared immunological composition to the subject. In one or more embodiments, the kit further comprises instructions for preparing the immunogenic composition and/or administering the immunogenic composition.

[0189] In examples following the procedures described above and in the Examples that follow, it was shown that a dose levels of 10 µg of SARS-CoV-2 N-Fc fusion protein induced significant anti-nucleocapsid Ab titers 42 days after a first injection on Day 0 and a second injection on Day 21 in mice. It is expected that dose levels of 10 µg and 30 µg will be effective

in rabbits and non-human primates.

[0190] The present disclosure contemplates formulation of a SARS-CoV-2 N-Fc fusion protein in any of the aforementioned pharmaceutical compositions and preparations. Furthermore, the present disclosure contemplates administration via any of the foregoing routes of administration. One of skill in the art can select the appropriate formulation, dose level and route of administration based on the condition being treated and the overall health, age, and size of the patient being treated.

EXAMPLES

[0191] The present technology is further illustrated by the following Examples. It is to be understood, however, that these examples are provided by way of illustration and nothing therein should be taken as a limitation upon the overall scope of the technology.

General Examples for Synthesis, Purification and Validation of SARS-CoV-2 N-Fc Fusion Proteins

Example 1: Synthesis and Methods of Making of a SARS-CoV-2 N-Fc Fusion Protein in HEK293 Cells.

[0192] SARS-CoV-2 N-Fc fusion proteins were synthesized as follows. A gene sequence of interest was constructed using proprietary software (LakePharma, Belmont, CA) and was cloned into a high expression mammalian vector. HEK293 cells were seeded in a shake flask 24 hours before transfection and were grown using serum-free chemically defined media. A DNA expression construct that encodes the SARS-CoV-2 N-Fc fusion protein of interest was transiently transfected into a suspension of HEK293 cells using the (LakePharma, Belmont, CA) standard operating procedure for transient transfection. After 20 hours, the cells were counted to determine the viability and viable cell count, and the titer was measured by FortéBio® Octet® (Pall FortéBio LLC, Fremont, CA). Additional readings were taken throughout the transient transfection production run. The culture was harvested on or after Day 5.

Example 2: Purification of a SARS-CoV-2 N-Fc Fusion Protein Manufactured in HEK293 Cells.

[0193] Purification of a SARS-CoV-2 N-Fc fusion protein was performed as follows. Conditioned media supernatants containing the secreted SARS-CoV-2 N-Fc fusion protein were harvested from the HEK293 production runs and were clarified by centrifugation. The supernatant containing the desired SARS-CoV-2 N-Fc fusion protein was run over a Protein A column, washed and eluted using a low pH gradient. Afterwards, the eluted fractions containing the desired protein were pooled and buffer exchanged into 200 mM HEPES, 100 mM NaCl, 50

mM NaOAc, pH 7.0 buffer. A final filtration step was performed using a 0.2 μ m membrane filter. The final protein concentration was calculated from the solution optical density at 280 nm. Further optional purification by ion-exchange chromatography (e.g., using an anion exchange bead resin or a cation exchange bead resin), gel filtration chromatography, or other methods were performed as necessary.

Example 3: SARS-CoV-2 N-Fc Fusion Protein Structure Confirmation by Non-Reducing and Reducing CE-SDS.

[0194] Capillary electrophoresis sodium dodecyl sulfate (CE-SDS) analysis is performed in a LabChip® GXII (Perkin Elmer, Waltham, MA) on a solution of a purified SARS-CoV-2 N-Fc fusion protein dissolved in 200 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH 7.0 buffer, and the electropherogram is plotted. Under non-reducing conditions, the sample is run against known molecular weight (MW) protein standards, and the eluting peak represents the ‘apparent’ MW of the fusion protein homodimer.

[0195] Under reducing conditions (e.g., using beta-mercaptoethanol to break disulfide bonds of the SARS-CoV-2 N-Fc fusion protein homodimer), the apparent MW of the resulting SARS-CoV-2 N-Fc fusion protein monomer is compared against half the molecular weight of the SARS-CoV-2 N-Fc fusion protein homodimer as a way of determining that the structural purity of the SARS-CoV-2 N-Fc fusion protein is likely to be correct.

Example 4: SARS-CoV-2 N-Fc Fusion Protein Sequence Identification by LC-MS with Glycan Removal.

[0196] To obtain an accurate estimate of the SARS-CoV-2 N-Fc fusion protein mass via mass spectroscopy (MS), the sample is first treated to remove naturally occurring glycan that might interfere with the MS analysis. 100 μ L of a 2.5 mg/mL SARS-CoV-2 N-Fc fusion protein dissolved in 200 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH 7.0 buffer solution is first buffer exchanged into 0.1 M Tris, pH 8.0 buffer containing 5 mM EDTA using a Zeba desalting column (Pierce, ThermoFisher Scientific, Waltham, MA). 1.67 μ L of PNGase F enzyme (Prozyme N-glycanase) is added to this solution to remove N-linked glycan present in the fusion protein (e.g., glycan linked to the side chain of the asparagine located at the cNg-N site), and the mixture is incubated at 37°C overnight in an incubator. The sample is then analyzed via LC-MS (NovaBioassays, Woburn, MA) resulting in a molecular mass of the molecule which corresponds to the desired homodimer without the glycan. This mass is then further corrected since the enzymatic process used to cleave the glycan from the cNg-asparagine also deaminates the asparagine side chain to form an aspartic acid, and in doing so the enzymatically treated homodimer gains 2 Da overall, corresponding to a mass of 1 Da for each chain present in the

homodimer. Therefore, the actual molecular mass is the measured mass minus 2 Da to correct for each of the enzymatic modifications of the SARS-CoV-2 N-Fc fusion protein structure in the analytical sample.

Example 5: %Homodimer by Size-Exclusion Chromatography for a SARS-CoV-2 N-Fc Fusion Protein.

[0197] Size-exclusion chromatography (SEC-HPLC) of SARS-CoV-2 N-Fc fusion proteins was carried out using a Waters 2795HT HPLC (Waters Corporation, Milford, MA) connected to a 2998 Photodiode array at a wavelength of 280 nm. 100 µL or less of a sample containing a SARS-CoV-2 N-Fc fusion protein of interest was injected into a MAbPac SEC-1, 5 µm, 4 × 300 mm column (ThermoFisher Scientific, Waltham, MA) operating at a flow rate of 0.2 mL/min and with a mobile phase comprising 50 mM sodium phosphate, 300 mM NaCl, and 0.05% w/v sodium azide, pH 6.2. The MAbPac SEC-1 column operates on the principle of molecular size separation. Therefore, larger soluble SARS-CoV-2 N-Fc aggregates (e.g., multimers of SARS-CoV-2 N-Fc fusion protein homodimers) eluted at earlier retention times, and the non-aggregated homodimers eluted at later retention times. In separating the mixture of homodimers from aggregated multimeric homodimers via analytical SEC-HPLC, the purity of the SARS-CoV-2 N-Fc fusion protein solution in terms of the percentage of non-aggregated homodimer was ascertained.

Example 6: In vitro Fc(Gamma), FcRn, and ACE2 Receptors Binding Affinity for a SARS-CoV-2 N-Fc Fusion Protein.

[0198] The binding of a SARS-CoV-2 N-Fc fusion protein to Fc(gamma) receptors at pH 7.4 is conducted using an ELISA assay as follows. Human Fc(gamma) receptors I, IIa, IIb, III and the FcRn receptor are used as mammalian receptors. A SARS-CoV-2 N-Fc fusion protein is diluted to 10 µg/mL in sodium bicarbonate buffer at pH 9.6 and coated on Maxisorp (Nunc) microtiter plates overnight at 4°C, after which the microplate strips are washed 5 times with PBST (PBS/0.05% Tween-20) buffer and blocked with Superblock blocking reagent (ThermoFisher). Serial dilutions of biotinylated rhFc(gamma) receptors (recombinant human Fc(gamma)RI, Fc(gamma)RIIa, Fc(gamma)RIIb, Fc(gamma)RIII, FcRn; R&D Systems) are prepared in PBST/10% Superblock buffer from 6000 ng/mL to 8.2 ng/mL and loaded at 100 µL/well onto the microplate strips coated with the SARS-CoV-2 N-Fc fusion protein. Recombinant human ACE2 receptor is purchased from R&D systems and biotinylated using known conjugation procedures and then prepared in PBST/10% Superblock buffer from 6000 ng/mL to 8.2 ng/mL and loaded at 100 µL/well onto the microplate strips coated with the SARS-

CoV-2 N-Fc fusion protein. The microtiter plate is incubated for 1 hour at room temperature after which the microplate strips are washed 5 times with PBST and then loaded with 100 μ L/well of streptavidin-HRP diluted 1:10000 in PBST/10% Superblock buffer. After incubating for 45 min, the microplate strips are washed again 5 times with PBST. TMB is added to reveal the bound Fc(gamma), FcRn, or ACE2 receptors proteins and stopped with ELISA stop reagent (Boston Bioproducts). The plate is read in an ELISA plate reader at 450 nm, and the OD values (proportional to the binding of each rhFc(gamma), FcRn, or ACE2 receptor to the SARS-CoV-2 N-Fc fusion protein) are plotted against log concentrations of each rhFc(gamma) receptor or FcRn or ACE2 receptor added to each well to generate binding curves using GraphPad Prism software.

General Examples for In vivo Serology Assays for Evaluating SARS-CoV-2 N-Fc Fusion Protein Action in Serum

Example 7: In vivo Quantitative ELISA for Evaluating SARS-CoV-2 RBD IgG Antibody Titer in Vaccinated Mouse Serum.

[0199] A quantitative SARS-CoV-2 SP/RBD-specific IgG ELISA was used to measure anti-SP/RBD IgG Ab titers in vaccinated mouse serum and plasma samples, including heat-inactivated serum or heat-inactivated plasma. The ELISA method used a recombinant SP/RBD immobilized on plastic wells of a 96-well microtiter ELISA plate (as is shown in FIG. 21) as the capture antigen that binds anti-SP/RBD-specific antibodies (IgG) in serum samples when incubated in the microplate wells.

[0200] Study serum samples diluted at 1:100-1:500 in Sample Dilution Buffer (SDB; contains a mixture of 10% Superblock (Thermo) in PBS/0.05% Tween 20) are added to the SP/RBD coated wells in duplicate or singled and incubated for one hour. After washing the plates with PBS/0.05% Tween 20 (PBST) buffer to remove all unbound molecules, HRP-conjugated anti-mouse IgG secondary antibody (1:40,000 diluted) is added as the detection reagent and incubated for 45 minutes. Following washes with PBST buffer, trimethylbenzidine (TMB) reagent is added to each well that was catalyzed by the HRP enzyme and incubated for 10-20 minutes. This causes a colorimetric change that is proportional to the amount of bound HRP-antibody conjugate. The enzyme substrate reaction is then stopped by the addition of Stop Reagent (1% H₂SO₄) and the color intensity (optical density, OD) of each well was measured using a spectrophotometric microplate reader at 450 nm wavelength.

[0201] The standard curve was generated using serial dilutions of a purified mouse IgG sample of known quantity i.e., μ g/mL) bound directly to the plastic well (i.e., the well does not contain bound-SP/RBD or any serum sample) and developed the same as described above for

serum samples. The quantity of SP/RBD-specific antibodies (i.e., titer value) for each serum sample is expressed as $\mu\text{g/mL}$ units derived from the standard curve using a 4-parameter curve fit model via an appropriate software (e.g., SoftMaxPro or Gen 5).

Example 8: In vivo General Preclinical Evaluation of the Effectiveness of SARS-CoV-2 N-Fc Formulations in Inducing an Anti-SP/RBD IgG Ab Titer or an Anti-Nucleocapsid Protein IgG Titer Response in Mice.

[0202] In vivo studies to evaluate the effectiveness of a SARS-CoV-2 N-Fc fusion protein formulation was carried out in mice as follows.

[0203] BALB/c mice (n=7/group) (Jackson Laboratories, Bar Harbor, ME) were acclimatized for at least 7 days before being weighed and then assigned into study groups for dosing. Mice were randomly assigned into each study group. The mice were kept 5 animals per cage in automatically ventilated racks and were given standard irradiated feed and filtered water ad libitum. Mice were ear tagged individually for identification and the study cages were labeled with animal IDs, study name, and study group identification, and the study records including individual and group dosing sheets, blood/serum collection sheets, weight measurement, and health observation records per standard operating procedures (SOPs) and specific study protocols.

[0204] Mice were administered up to three subcutaneous doses of 10 μg /dose of the SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 15 with or without Montanide™ ISA 720 (30%/70% v/v), synthesized in transiently transfected HEK293 cells according to Example 1. Doses were given on Day 0, Day 21, and Day 46. Mice were observed for one to three hours after dose administration for any immediate reactions and then daily for general health. All mice were non-terminally bled via submandibular venipuncture at Day 0 prior to the first immunization and then at Day 14, Day 21, Day 28, Day 35, Day 42, Day 55, Day 70, Day 83, and Day 105 to obtain serum samples for SARS-CoV-2 nucleocapsid IgG Ab titer assessment. The collected blood was allowed to clot, and the serum was separated by centrifuging micro-vacutainer tubes and aliquoted and frozen for antibody analysis by ELISA according to the methods described in Example 7 or Example 9.

Example 9: ELISA to Measure In vitro IgG Antibodies Against SARS-CoV-2 Nucleocapsid Protein (e.g., N-protein) in Human Serum After Treatment With the SARS-CoV-2 N-Fc Fusion Protein of SEQ ID NO: 15.

[0205] The SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 15 was synthesized according

to Example 1 and purified according to Example 2. The fusion protein structure is confirmed by non-reducing and reducing CE-SDS according to Example 3 and the fusion protein sequence identification is confirmed by LC-MS with glycan removal according to Example 4. According to the procedure of Example 8, groups of N=7 BALB/c mice (Jackson Laboratories) were administered via subcutaneous injection the SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 15 with or without Montanide™ ISA 720 adjuvant on Day 0, Day 21, and Day 46.

[0206] A COVID-19 N-Protein Human IgG ELISA Kit (Abcam# ab274339) is an in vitro indirect ELISA for the quantitative measurement of human IgG antibody against SARS-CoV-2 Nucleocapsid protein (e.g., N protein) in human serum. Standard 96-well plates (12 strips with 8 wells/strip as shown in FIG. 21) were coated with the SARS-CoV-2 N protein, which combines with the corresponding antibody present in a human serum sample and Positive Control which was used as calibration curve for interpretation purposes. The wells were washed, mouse IgG secondary antibody (1:40,000 diluted) was added to mouse samples. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted to the wells. The wells were again washed, a TMB substrate solution was added to the wells and the color develops in proportion to the amount of COVID19 N protein human/mouse IgG antibody bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color was measured at 450 nm. The Positive Controls were from an inactivated serum sample which contains SARS-COV-2 N protein human IgG antibody.

[0207] Anti-nucleocapsid protein IgG titers in mice that were administered the SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 15 were measured on Day 14 and Day 21 (post 1 dose), on Day 28 and Day 42 (post 2 doses) and on Day 55, Day 70, Day 83 and Day 105 (post 3 doses). As shown in FIG. 13, the SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 15 induced significant anti-nucleocapsid protein IgG titers when measured on Day 42, Day 55, Day 70 and Day 83. The levels of anti-nucleocapsid protein IgG titers measured on Day 42, Day 55, Day 70 and Day 83 induced by the SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 15 with Montanide™ ISA 720 adjuvant were measurably increased, indicating that the adjuvant produced a strong enhancing effect when delivered with the SARS-CoV-2 N-Fc fusion protein of SEQ ID NO: 15, supporting its dose-sparing characteristics and selection as the clinical lead adjuvant.

General Examples for Synthesis, Purification and Validation of Canine Insulin-Fc Fusion Proteins

Example 10: Synthesis and Methods of Making an Insulin-Fc Fusion Protein in HEK293 Cells.

[0208] Insulin-Fc fusion proteins were synthesized as follows. A gene sequence of interest

was constructed using proprietary software (LakePharma, Belmont, CA) and was cloned into a high expression mammalian vector. HEK293 cells were seeded in a shake flask 24 hours before transfection and were grown using serum-free chemically defined media. A DNA expression construct that encodes the insulin-Fc fusion protein of interest was transiently transfected into a suspension of HEK293 cells using the (LakePharma, Belmont, CA) standard operating procedure for transient transfection. After 20 hours, the cells were counted to determine the viability and viable cell count, and the titer was measured by FortéBio® Octet® (Pall FortéBio LLC, Fremont, CA). Additional readings were taken throughout the transient transfection production run. The culture was harvested on or after Day 5.

Example 11: Synthesis and Methods of Making an Insulin-Fc Fusion Protein in CHO Cells.

[0209] A CHO cell line was originally derived from CHO-K1 (LakePharma, Belmont, CA), and the endogenous glutamine synthetase (GS) genes were knocked out by recombinant technology using methods known in the art. Stable expression DNA vectors were designed and optimized for CHO expression and GS selection and incorporated into a high expression mammalian vector (LakePharma, Belmont, CA). The sequence of each completed construct was confirmed prior to initiating scale up experiments. The suspension-adapted CHO cells were cultured in a humidified 5% CO₂ incubator at 37°C in a chemically defined media (CD OptiCHO; Invitrogen, Carlsbad, CA). No serum or other animal-derived products were used in culturing the CHO cells.

[0210] Approximately 80 million suspension-adapted CHO cells, growing in CD OptiCHO media during the exponential growth phase, were transfected by electroporation using MaxCyte® STX® system (MaxCyte, Inc., Gaithersburg, MD) with 80 µg DNA to create a stable CHO cell line for each insulin-Fc fusion protein (DNA construct contains the full-length sequence of the insulin-Fc fusion protein). After twenty-four hours, the transfected cells were counted and placed under selection for stable integration of the insulin-Fc fusion genes. The transfected cells were seeded into CD OptiCHO selection media containing between 0-100 µM methionine sulfoximine (MSX) at a cell density of 0.5×10^6 cells/mL in a shaker flask and incubated at 37°C with 5% CO₂. During a selection process, the cells were spun down and resuspended in fresh selection media every 2-3 days until the CHO stable pool recovered its growth rate and viability. The cell culture was monitored for growth and titer.

[0211] The cells were grown to 2.5×10^6 cells per mL. At the time of harvest for cell banking, the viability was above 95%. The cells were then centrifuged, and the cell pellet was resuspended in the CD OptiCHO media with 7.5% dimethyl sulfoxide (DMSO) to a cell count of 15×10^6 cells per mL per vial. Vials were cryopreserved for storage in liquid nitrogen.

[0212] A small-scale-up production was performed using the CHO cells as follows. The cells were scaled up for production in CD OptiCHO growth medium containing 100 μ M MSX at 37°C and fed every 2-4 days as needed, with CD OptiCHO growth medium supplemented with glucose and additional amino acids as necessary for approximately 14-21 days. The conditioned media supernatant harvested from the stable pool production run was clarified by centrifuge spinning. The protein was run over a Protein A (MabSelect, GE Healthcare, Little Chalfont, United Kingdom) column pre-equilibrated with binding buffer. Washing buffer was then passed through the column until the OD280 value (NanoDrop, Thermo Scientific) was measured to be at or near background levels. The insulin-Fc fusion protein was eluted using a low pH buffer, elution fractions were collected, and the OD280 value of each fraction was recorded. Fractions containing the target insulin-Fc fusion protein were pooled and optionally further filtered using a 0.2 μ M membrane filter.

[0213] The cell line was optionally further subcloned to monoclonality and optionally further selected for high titer insulin-Fc-fusion protein-expressing clones using the method of limiting dilution, a method known to those skilled in the art. After obtaining a high titer, monoclonal insulin-Fc fusion protein-expressing cell line, production of the insulin-Fc fusion protein was accomplished as described above in growth medium without MSX, or optionally in growth medium containing MSX, to obtain a cell culture supernatant containing the recombinant, CHO-made, insulin-Fc fusion protein. The MSX concentration was optionally increased over time to exert additional selectivity for clones capable of yielding higher product titers.

Example 12: Purification of an Insulin-Fc Fusion Protein.

[0214] Purification of an insulin-Fc fusion protein was performed as follows. Conditioned media supernatants containing the secreted insulin-Fc fusion protein were harvested from the transiently or stably transfected HEK production runs and were clarified by centrifugation. The supernatant containing the desired insulin-Fc fusion protein was run over a Protein A column and eluted using a low pH gradient. Afterwards, the eluted fractions containing the desired protein were pooled and buffer exchanged into 200 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH 7.0 buffer. A final filtration step was performed using a 0.2 μ m membrane filter. The final protein concentration was calculated from the solution optical density at 280 nm. Further optional purification by ion-exchange chromatography (e.g., using an anion exchange bead resin or a cation exchange bead resin), gel filtration chromatography, or other methods was performed as necessary.

Example 13: Insulin-Fc Fusion Protein Structure Confirmation by Non-reducing and Reducing CE-SDS.

[0215] Capillary electrophoresis sodium dodecyl sulfate (CE-SDS) analysis was performed in a LabChip® GXII (Perkin Elmer, Waltham, MA) on a solution of a purified insulin-Fc fusion protein dissolved in 200 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH 7.0 buffer, and the electropherogram was plotted. Under non-reducing conditions, the sample was run against known molecular weight (MW) protein standards, and the eluting peak represented the 'apparent' MW of the insulin-Fc fusion protein homodimer.

[0216] Under reducing conditions (e.g., using beta-mercaptoethanol to break disulfide bonds of the insulin-Fc fusion homodimer), the apparent MW of the resulting insulin-Fc fusion protein monomer is compared against half the molecular weight of the insulin-Fc fusion protein homodimer as a way of determining that the structural purity of the insulin-Fc fusion protein is likely to be correct.

Example 14: Insulin-Fc Fusion Protein Sequence Identification by LC-MS with Glycan Removal.

[0217] To obtain an accurate estimate of the insulin-Fc fusion protein mass via mass spectroscopy (MS), the sample was first treated to remove naturally occurring glycan that might interfere with the MS analysis. 100 µL of a 2.5 mg/mL insulin-Fc fusion protein dissolved in 200 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH 7.0 buffer solution was first buffer exchanged into 0.1 M Tris, pH 8.0 buffer containing 5 mM EDTA using a Zeba desalting column (Pierce, ThermoFisher Scientific, Waltham, MA). 1.67 µL of PNGase F enzyme (Prozyme N-glycanase) was added to this solution in order to remove N-linked glycan present in the insulin-Fc fusion protein (e.g., glycan linked to the side chain of the asparagine located at the cNg-N site), and the mixture was incubated at 37°C overnight in an incubator. The sample was then analyzed via LC-MS (NovaBioassays, Woburn, MA) resulting in a molecular mass of the molecule which corresponds to the desired homodimer without the glycan. This mass was then further corrected since the enzymatic process used to cleave the glycan from the cNg-asparagine also deaminates the asparagine side chain to form an aspartic acid, and in doing so the enzymatically treated homodimer gains 2 Da overall, corresponding to a mass of 1 Da for each chain present in the homodimer. Therefore, the actual molecular mass was the measured mass minus 2 Da to correct for the enzymatic modification of the insulin-Fc fusion protein structure in the analytical sample.

Example 15: %Homodimer by Size-Exclusion Chromatography for an Insulin-Fc Fusion Protein.

[0218] Size-exclusion chromatography (SEC-HPLC) of insulin-Fc fusion proteins was carried out using a Waters 2795HT HPLC (Waters Corporation, Milford, MA) connected to a 2998 Photodiode array at a wavelength of 280 nm. 100 μ L or less of a sample containing an insulin-Fc fusion protein of interest was injected into a MAbPac SEC-1, 5 μ m, 4 $\times$ 300 mm column (ThermoFisher Scientific, Waltham, MA) operating at a flow rate of 0.2 mL/min and with a mobile phase comprising 50 mM sodium phosphate, 300 mM NaCl, and 0.05% w/v sodium azide, pH 6.2. The MAbPac SEC-1 column operates on the principle of molecular size separation. Therefore, larger soluble insulin-Fc aggregates (e.g., multimers of insulin-Fc fusion protein homodimers) eluted at earlier retention times, and the non-aggregated homodimers eluted at later retention times. In separating the mixture of homodimers from aggregated multimeric homodimers via analytical SEC-HPLC, the purity of the insulin-Fc fusion protein solution in terms of the percentage of non-aggregated homodimer was ascertained.

Example 16: In vitro Fc(gamma) Receptor I Binding Affinity Assay for an Insulin-Fc Fusion Protein.

[0219] The binding of insulin-Fc fusion proteins to the Fc(gamma) receptor I at pH 7.4 was conducted using an ELISA assay as follows. Since canine Fc(gamma) receptor I was not commercially available, human Fc(gamma) receptor I (i.e., rhFc(gamma) receptor I) was used as a surrogate mammalian receptor. Insulin-Fc compounds were diluted to 10 μ g/mL in sodium bicarbonate buffer at pH 9.6 and coated on Maxisorp (Nunc) microtiter plates overnight at 4°C, after which the microplate strips were washed 5 times with PBST (PBS/0.05% Tween-20) buffer and blocked with Superblock blocking reagent (ThermoFisher). Serial dilutions of biotinylated rhFc(gamma) receptor I (recombinant human Fc(gamma)R-I; R&D Systems) were prepared in PBST/10% Superblock buffer from 6000 ng/mL to 8.2 ng/mL and loaded at 100 μ L/well onto the microplate strips coated with insulin-Fc fusion protein. The microtiter plate was incubated for 1 hour at room temperature after which the microplate strips were washed 5 times with PBST and then loaded with 100 μ L/well of streptavidin-HRP diluted 1:10000 in PBST/10% Superblock buffer. After incubating for 45 min, the microplate strips were washed again 5 times with PBST. TMB was added to reveal the bound Fc(gamma) receptor I proteins and stopped with ELISA stop reagent (Boston Bioproducts). The plate was read in an ELISA plate reader at 450 nm, and the OD values (proportional to the binding of rhFc(gamma) receptor I to insulin-Fc protein) were plotted against log concentrations of rhFc(gamma) receptor I added to each well to generate binding curves using GraphPad Prism software.

Example 17: In vivo Pharmacodynamics (PD) After Periodic Administrations of an Insulin Fc-Fusion Protein in Client Owned Canines.

[0220] A bioactive insulin-Fc fusion homodimer construct was synthesized according to Example 10 or Example 11 and purified according to Example 12 was assessed for its effects on fasting blood glucose levels as follows.

[0221] Protocol 1 is an unmasked, self-controlled, single arm, pilot field efficacy study treating client-owned dogs, diagnosed with diabetes mellitus, with an insulin-Fc fusion protein. The effect of the drug is assessed by comparing glycemic control (based on clinical signs, fructosamine levels, and interstitial glucose concentrations using a continuous glucose monitoring unit (CGMS)) while on a standard insulin therapy (for one week) vs. treatment with an escalating dose of an insulin-Fc fusion protein over 8 weeks. Doses are administered subcutaneously starting at 0.1 mg/kg and then increased each successive week up to a maximum of 0.5 mg/kg based on CGMS results and clinical signs. The Protocol 1 Study Timeline used for some dogs is given in Table 2.

Table 2: Protocol 1 Study Timeline	
Visit 1 (Day -7)	Screening (CBC/Chem/UA/PLI/fructosamine [6mL of blood, 3 mL of urine]/abdominal ultrasound/chest radiographs). Sedation might be required for imaging studies.
Visit 2 (Day 0)	Sensor placement (+/- sedation). Continue insulin treatment as previously prescribed at home for 6 days. Discontinue insulin on the night of Day 6 (do not administer PM insulin on Day 6 and AM insulin on Day 7).
Visit 3 (Day 7)	Blood sample (1 mL for PK), 0.1 mg/kg insulin-Fc fusion protein injection (after BG>300mg/dL), hospitalize for 2-3 days for observation and treatment of hypoglycemia if BG < 50.
Visit 4 (Day 14)	Sensor placement (+/- sedation). Blood sample (1 mL for PK), insulin-Fc fusion protein injection. Consider dose increase based on CGMS.
Visit 5 (Day 21)	Blood sample (1 mL for PK), insulin-Fc fusion protein injection. Consider dose increase based on CGMS.
Visit 6 (Day 28)	Sensor placement (+/- sedation). Blood sample (1 mL for PK), insulin-Fc fusion protein injection. Consider dose increase based on CGMS.
Visit 7 (Day 35)	Insulin-Fc fusion protein injection. Consider dose increase based on CGMS. Blood sample (fructosamine, CBC/Chem/UA, 6 mL of blood total).
Visit 8	Sensor placement (+/- sedation). Blood sample (1 mL for PK), insulin-Fc fusion

(Day 42)	protein injection. Consider dose increase based on CGMS.
Visit 9 (Day 49)	Blood sample (1 mL for PK), insulin-Fc fusion protein injection. Consider dose increase based on CGMS.
Visit 10 (Day 56)	Sensor placement (+/- sedation). Blood sample (1 mL for PK), insulin-Fc fusion protein injection. Consider dose increase based on CGMS.
Visit 11 (Day 63)	Blood sample (fructosamine, CBC/Chem/UA, 6 mL of blood total), +/- sedation. At the night of visit 11, resume insulin therapy as before the study (with commercially available insulin that the dog was on prior to the study).

[0222] Protocol 2 is an unmasked, self-controlled, single arm, pilot field efficacy study treating client-owned dogs, diagnosed with diabetes mellitus, with an insulin-Fc fusion protein. The effect of the drug is assessed by comparing glycemic control (based on clinical signs, fructosamine levels, and interstitial glucose concentrations using a continuous glucose monitoring unit (CGMS)) while on a standard insulin therapy (for one week) vs. treatment with an escalating dose of insulin-Fc fusion protein over 5 weeks. Doses are administered subcutaneously starting at 0.1 mg/kg and then increased each successive week up to a maximum of 0.5 mg/kg based on CGMS results and clinical signs. The Protocol 2 Study Timeline used for some dogs is given in Table 3.

Table 3: Protocol 2 Study Timeline	
Visit 1 (Day -7)	Health Screening (physical exam/thorough review of diabetes history/CBC/Chem/UA/PLI/fructosamine [6 mL of blood, 3 mL of urine].
Visit 2 (Day 0)	Sensor placement (+/- sedation). Baseline serum sample and BG from blood sample. Continue insulin treatment as previously prescribed at home for 6 days. Discontinue insulin on the night of Day 6 (do not administer PM insulin on Day 6 or AM insulin on Day 7).
Visit 3 (Day 7)	Blood sample (1 mL for PK), 0.1 mg/kg insulin-Fc fusion protein injection, hospitalize for 1-2 days for observation and treatment of hypoglycemia (if BG < 50 mg/dL).
Visit 4 (Day 14)	Sensor placement (+/- sedation). Blood sample (1 mL for PK), insulin-Fc fusion protein injection. Consider dose increase based on FGMS.
Visit 5 (Day 21)	Sensor placement (+/- sedation). Blood sample (1 mL for PK), insulin-Fc fusion protein injection. Consider dose increase based on FGMS.
Visit 6 (Day 28)	Sensor placement (+/- sedation). Blood sample (PK), insulin-Fc fusion protein injection. Consider dose increase based on FGMS.

Visit 7	Insulin-Fc fusion protein injection. Consider dose increase based on FGMS.
(Day 35)	Blood sample (fructosamine, CBC/Chem/UA, 6 mL of blood total).

[0223] After completion of either of the above protocols, there is an optional “home use” evaluation for up to one year. During this phase, the veterinarians treat each dog on a case-by-case basis as they would with any other patient on conventional insulin. Clinical signs and interstitial glucose concentrations using a continuous glucose monitoring unit (CGMS) are monitored throughout this phase of the study. In addition, blood samples are collected as frequently as possible to evaluate fructosamine levels, blood chemistry and blood cell counts, and test for the presence of anti-drug and anti-insulin antibodies.

Example 18: In vivo Anti-Insulin Antibody (AIA) Titer Measurement after Periodic Administrations of an Insulin-Fc Fusion Protein in Canines – Protocol 1.

[0224] Maxisorp ELISA Plates (Nunc) were coated with purified RHI diluted in coating buffer (pH=9.6 Carbonate-Biocarbonate buffer) at 30 µg/mL overnight at 4°C. Plates were then washed 5x with PBST (PBS + 0.05% Tween 20) and blocked for ≥ 1 hour (or overnight) with SuperBlock blocking solution (ThermoFisher). For calculating the AIA in dog IgG units, strips were directly coated with 1:2 serial dilutions of dog IgG (Jackson ImmunoResearch) in pH=9.6 Carb-Biocarb coating buffer at concentrations between 300-4.69 ng/mL overnight at 4°C and were used to create a 7 point pseudo-standard curve. The standards strip plates were also washed and blocked with SuperBlock blocking solution for ≥ 1 hour (or overnight).

[0225] Test serum samples were diluted to ≥ 1:100 (typically tested as 1:200) in PBST/SB/20%HS sample dilution buffer (PBS+0.1% Tween 20+10% SuperBlock+20% horse serum) and added to RHI coated strips 100 µL/well in duplicates. Duplicate strips of dog IgG coated standard strips were also added to each plate and filled with PBST/SB (PBS+0.1% Tween 20+10% SuperBlock) buffer 100 µL/well. Plates were incubated for 1 hour at room temperature. Following incubation, plates were washed 5 times with PBST. For detection of AIAs, HRP conjugated Goat anti-Dog IgG F(ab')₂ (Jackson ImmunoResearch), which was cross-reacted to dog IgG, was diluted in PBST/SB to 1:10,000 and added 100µL/well to both sample and standard wells and incubated for 45 minutes at room temperature in the dark. Plates were washed 5 times with PBST and developed by the adding 100 µL/well TMB substrate (Invitrogen) for 15-20 minutes at room temperature in the dark. Color development is then stopped by addition of 100 µL/well of ELISA Stop Solution (Boston Bioproducts) and absorbance is read at 450nm using a SpectraMax plate reader within 30 minutes. Anti-drug antibody concentration was determined by interpolating the OD values in the 4-PL pseudo-standard curve using the SoftMax

Pro software.

Example 19: In vivo Anti-Insulin Antibody (ALA) Titer Measurement after Periodic Administrations of Insulin-Fc Fusion Protein in Canines – Protocol 2.

[0226] The general procedure is as follows: The serum with labeled antigen is incubated with and without cold insulin overnight. Antibody-bound labeled antigens are precipitated with protein-A/G Sepharose in a 96-well plate format, with each serum tested in duplicate. The 96-well plates are washed to remove unbound labeled antigens. Each well is counted with a 96-well plate beta counter. The results are expressed as an index that adjusts the delta counts per minute (cpm) of the test serum for the delta cpm of positive and negative control sera in a particular assay.

[0227] The specific procedure involves the preparation of two buffers as follows: Buffer 1 (150 mM NaCl, 20 mM Tris-HCl, 1%BSA, 0.15%Tween-20, 0.1%Sodium Azide pH 7.4) and Buffer 2 (same as Buffer 1 except for 0.1%BSA instead of 1%BSA). Each serum sample is spun down to remove fibrin clots when necessary. Then the stock solution of radiolabeled insulin is prepared by dissolving 10 μ Ci of 125I-insulin powder in 1 mL of 5%BSA in PBS. The “hot” insulin antigen solution is prepared using 3040 μ L of Buffer 1 and 160 μ L of the stock radiolabeled insulin solution. The “cold inhibited” insulin antigen solution is prepared using 2784 μ L of Buffer 1, 160 μ L of the stock radiolabeled insulin solution, and 256 μ L of Humulin® solution (Eli Lilly, IN). All solutions are kept on ice prior to use. 6 μ L of each serum sample is mixed with 30 μ L of the “hot” insulin antigen solution, and 6 μ L of each serum sample is mixed with 30 μ L of the “cold inhibited” insulin antigen solution in a PCR tube. The resulting mixtures are incubated overnight at 4°C. The plate is then coated with BSA by adding 150 μ L of Buffer 1 to each well followed by overnight incubation at room temperature under an aluminum foil cover followed by washing and removal of the wash buffer. The Protein-A/G Sepharose mixture is prepared in two parts. The Protein-A Sepharose solution is prepared in Buffer 1 at a 62.5% concentration by volume. The Protein-G Sepharose solution is prepared in Buffer 1 at 40% concentration by volume. Finally, the Protein-A/G Sepharose mixture is prepared by mixing the Protein-A and Protein-G Sepharose solutions at a 4:1 ratio (final concentration: 50% Protein-A/8% Protein-G Sepharose). To perform the assay, 50 μ L of the Protein A/G-Sepharose mixture and 30 μ L of the overnight incubated serum solutions are added to each well in duplicate. The plate is mixed on a plate shaker for 45 minutes at 40C and then washed seven times using a Millipore plate washer device with 200 μ L of wash buffer per well and then placed in a 37°C incubator for 15 minutes to dry. 50 μ L of scintillation cocktail (Microscint-20) is added to each well and the plate is counted using a 96-well plate counter to determine the cpm for each well.

EQUIVALENTS

[0228] In the claims, articles such as “a,” “an,” and “the” may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include “or” between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The disclosure includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The disclosure includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process.

[0229] Furthermore, the disclosure encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, and descriptive terms from one or more of the listed claims are introduced into another claim. For example, any claim that is dependent on another claim can be modified to include one or more limitations found in any other claim that is dependent on the same base claim. Where elements are presented as lists, e.g., in Markush group format, each subgroup of the elements is also disclosed, and any element(s) can be removed from the group. It should be understood that, in general, where the disclosure, or aspects of the disclosure, is/are referred to as comprising particular elements and/or features, certain embodiments of the disclosure or aspects of the disclosure consist, or consist essentially of, such elements and/or features. For purposes of simplicity, those embodiments have not been specifically set forth in haec verba herein. It is also noted that the terms “comprise(s),” “comprising,” “contain(s),” and “containing” are intended to be open and the use thereof permits the inclusion of additional elements or steps. Where ranges are given, endpoints are included. Furthermore, unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or sub-range within the stated ranges in different embodiments of the disclosure, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

[0230] Additional advantages of the various embodiments of the technology will be apparent to those skilled in the art upon review of the disclosure herein and the working examples below. It will be appreciated that the various embodiments described herein are not necessarily mutually exclusive unless otherwise indicated herein. For example, a feature described or depicted in one embodiment may also be included in other embodiments, but is not necessarily included. Thus, the present invention encompasses a variety of combinations and/or integrations of the specific embodiments described herein.

[0231] The present description also uses numerical ranges to quantify certain parameters relating to various embodiments of the invention. It should be understood that when numerical ranges are provided, such ranges are to be construed as providing literal support for claim limitations that only recite the lower value of the range as well as claim limitations that only recite the upper value of the range. For example, a disclosed numerical range of about 10 to about 100 provides literal support for a claim reciting "greater than about 10" (with no upper bounds) and a claim reciting "less than about 100" (with no lower bounds).

We claim:

1. A fusion protein comprising a nucleocapsid domain and an Fc fragment, wherein the nucleocapsid domain and the Fc fragment are connected by a peptide linker, wherein the nucleocapsid domain comprises the following sequence:

RSGARSKQRRPQGLPNNTASWFTALTQHGKEDLKFPRGQGVPI NTNSSPDDQIGY
YRRATTRRIRGGDGKMKDLSRWYFYLTGTGPEAGLPYGANKDGIWVATEGALN
TPKDHIGTRNPANNAIIVLQLPQGTTLPKGFYAE (SEQ ID NO: 8).

2. The fusion protein of claim 1, wherein the Fc fragment comprises the following sequence:

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
APIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNG
QPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK
SLSLSPG (SEQ ID NO: 1).

3. The fusion protein of claim 1, wherein the linker comprises the following sequence:
SGGGSGGG (SEQ ID NO: 14).

4. The fusion protein of claim 1, wherein the fusion protein comprises the following sequence:

RSGARSKQRRPQGLPNNTASWFTALTQHGKEDLKFPRGQGVPI NTNSSPDDQIGY
YRRATTRRIRGGDGKMKDLSRWYFYLTGTGPEAGLPYGANKDGIWVATEGALN
TPKDHIGTRNPANNAIIVLQLPQGTTLPKGFYAESGGGSGGGSDKTHTCPPCPAPE
LLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAK
TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP
REPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLD
SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG (SEQ ID
NO: 15).

5. The fusion protein of claim 1, wherein the fusion protein is a homodimer.
6. The fusion protein of claim 1, wherein the Fc fragment is glycosylated.

7. An immunogenic composition comprising a fusion protein according to claim 1 and a pharmaceutically-acceptable carrier.
8. The immunogenic composition of claim 7, further comprising an adjuvant.
9. The immunogenic composition of claim 8, where the adjuvant is Montanide™ ISA-720.
10. The immunogenic composition of claim 8, wherein said fusion protein is emulsified with said adjuvant.
11. A method for increasing antibody production in a subject against an antigenic agent, the method comprising administering a therapeutically effective amount of a fusion protein according to claim 1 to said subject.
12. The method of claim 11, wherein the subject has a measurable antibody titer against said antigenic agent prior to administration of the fusion protein.
13. The method of claim 11, wherein the subject is antibody naïve prior to administration of the fusion protein.
14. The method of claim 11, wherein the fusion protein is administered via injection.
15. The method of claim 11, wherein the fusion protein is administered subcutaneously or intramuscularly.
16. The method of claim 11, wherein the fusion protein is co-administered with an adjuvant.
17. The method of claim 16, further comprising pre-mixing said fusion protein with said adjuvant before said administration.
18. The method of claim 17, wherein said pre-mixing comprises emulsifying said adjuvant and said fusion protein to yield an emulsion, and administering said emulsion to said subject.

19. A method of inducing an immune response in a subject against viral infection, said method comprising administering a therapeutically effective amount of a fusion protein according to claim 1 to said subject.
20. The method of claim 19, wherein the subject has a measurable antibody titer against said viral infection prior to administration of the fusion protein.
21. The method of claim 19, wherein the subject is antibody naïve prior to administration of the fusion protein.
22. The method of claim 19, wherein the fusion protein is administered via injection.
23. The method of claim 22, wherein the fusion protein is administered subcutaneously or intramuscularly.
24. The method of claim 19, wherein the fusion protein is co-administered with an adjuvant.
25. The method of claim 24, further comprising pre-mixing said fusion protein with said adjuvant before said administration.
26. The method of claim 25, wherein said pre-mixing comprises emulsifying said adjuvant and said fusion protein to yield an emulsion, and administering said emulsion to said subject.
27. A method of producing a fusion protein according to claim 1, said method comprising transiently transfecting a nucleic acid encoding for the fusion protein into a HEK293 cell, wherein the transfected HEK293 cell expresses the fusion protein, and wherein the yield of the purified or isolated fusion protein is greater than 100 mg/L in any of the foregoing expression systems.
28. A cell engineered to express a fusion protein according to claim 1.
29. A cDNA encoding a fusion protein according to claim 1.

30. A fusion protein comprising the sequence of SEQ ID NO: 15 or pharmaceutical composition thereof for use in treatment and/or prophylaxis of a viral infection from SARS-CoV-2 virus.