(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 September 2021 (16.09.2021)

WIPO | PCT

(10) International Publication Number
WO 2021/183665 A1

(51) International Patent Classification:
C12N 15/62 (2006.01) *C12N 7/00* (2006.01)
C07K 14/005 (2006.01) *A61K 39/215* (2006.01)
C12N 15/81 (2006.01) *A61P 31/14* (2006.01)

(21) International Application Number:
PCT/US2021/021737

(22) International Filing Date:
10 March 2021 (10.03.2021)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

62/988,328	11 March 2020 (11.03.2020)	US
63/009,960	14 April 2020 (14.04.2020)	US
63/010,010	14 April 2020 (14.04.2020)	US
16/883,263	26 May 2020 (26.05.2020)	US
63/059,975	01 August 2020 (01.08.2020)	US
63/064,157	11 August 2020 (11.08.2020)	US
63/117,460	24 November 2020 (24.11.2020)	US
63/117,922	24 November 2020 (24.11.2020)	US
63/117,847	24 November 2020 (24.11.2020)	US
63/118,697	26 November 2020 (26.11.2020)	US
63/135,380	08 January 2021 (08.01.2021)	US
17/198,164	10 March 2021 (10.03.2021)	US

(71) Applicant: **NANT HOLDINGS IP, LLC** [US/US]; 9920 Jefferson Boulevard, Culver City, California 90232 (US).

(72) Inventors: **SOON-SHIONG, Patrick**; 9920 Jefferson Boulevard, Culver City, California 90232 (US). **SIELING, Peter**; 9920 Jefferson Boulevard, Culver City, California 90232 (US). **SHIN, Annie**; 9920 Jefferson Boulevard, Culver City, California 90232 (US). **SENDER, Leonard**; 9920 Jefferson Boulevard, Culver City, California 90232 (US). **SAFRIT, Jeffrey**; 9920 Jefferson Boulevard, Culver City, California 90232 (US). **RICE, Adrian**; 9920 Jefferson Boulevard, Culver City, California 90232 (US). **RABIZADEH, Shahrooz**; 9920 Jefferson Boulevard, Culver City, California 90232 (US). **NIAZI, Kayvan**; 9920 Jefferson Boulevard, Culver City, California 90232 (US). **GEISSERT, Lisa**; 9920 Jefferson Boulevard, Culver City,

(54) Title: TREATMENT OF COVID-19 AND METHODS THEREFOR

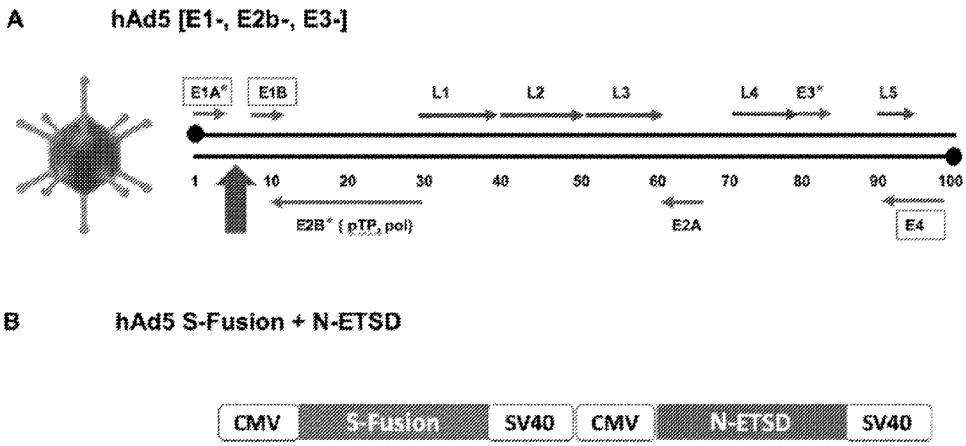

FIG.25

(57) Abstract: A vaccine composition to induce immunity against a coronavirus in a subject comprises a recombinant nucleic acid that encodes N-ETSD, a modified nucleocapsid protein that includes an endosomal targeting sequence, and/or that encodes S-Fusion, a modified spike protein that has improved surface expression. The vaccine may be formulated as a recombinant nucleic acid, recombinant yeast, and/or recombinant virus such as an adenovirus and can be administered via injection and/or mucosal delivery.

[Continued on next page]

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California 90232 (US). **GABITZSCH, Elizabeth**; 9920 Jefferson Boulevard, Culver City, California 90232 (US).

(74) **Agent: FESSENMAIER, Martin** et al.; Umberg Zipser LLP, 1920 Main Street, Suite 750, Irvine, California 92614 (US).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

TREATMENT OF COVID-19 AND METHODS THEREFOR

[0001] This application claims priority to our co-pending US Provisional patent applications with the serial numbers 62/988,328, filed 11 Mar 2020, 63/009,960, filed 14 Apr 2020, 63/010,010, filed 14 Apr 2020, 63/059,975, filed 1 Aug 2020, 63/064,157, filed 11 Aug 2020, 63/117,460, filed 24 Nov 2020, 63/117,847, filed 24 Nov 2020, 63/117,922, filed 24 Nov 2020, 63/118,697, filed 26 Nov 2020, 63/135,380, filed 8 Jan 2021, and US non-provisional patent application 16/883,263, filed 26 May 2020, and US non-provisional patent application with the title "Anti COVID-19 Therapies targeting nucleocapsid and spike proteins", filed concurrently herewith and having reference number 102538.0080US3, all of which are incorporated by reference herein.

Sequence Listing

[0002] The content of the ASCII text file of the sequence listing named 102690-0041PCT_REV003_ST25.txt, which is 172 KB in size was created on 3/4/2021 and electronically submitted via EFS-Web along with the present application and is incorporated by reference in its entirety.

Field

[0003] The field of the present disclosure is vaccine compositions and methods to generate immunity against coronaviruses, and particularly as it relates to SARS-CoV-2.

Background

[0004] The background description includes information that may be useful in understanding the present disclosure. It is not an admission that any of the information provided herein is prior art or relevant to the presently claimed invention, or that any publication specifically or implicitly referenced is prior art.

[0005] All publications and patent applications herein are incorporated by reference to the same extent as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Where a definition or use of a term in an incorporated reference is inconsistent or contrary to the definition of that term provided herein, the definition of that term provided herein applies and the definition of that term in the reference does not apply.

[0006] While SARS-CoV2 diagnostic tests have become available in relatively short time, numerous attempts to treat the disease have so far shown mixed or inconclusive results. Most typically, patients with severe symptoms are treated to maintain respiration/blood oxygenation. The COVID19 mortality rate is significant, particularly in elderly, immune compromised individuals, and individuals with heart disease, lung disease, or diabetes. Despite improvements in acute care, it has become apparent that containment of the disease is critically important as social distancing and other public health mitigation measures can provide only moderate relief.

[0007] To that end, numerous candidate anti-SARS-CoV2 vaccine compositions target one or more proteins of the virus (see *e.g.*, *FIMMU* 2020, 11:602256). For example, Sinovac and Sinopharm are currently testing inactivated virus vaccine preparations. Cansino Biologics, Janssen Pharma, Oxford University, and Garnaleya have developed vaccines based on a non-replicating adenoviral vector that encodes one or more viral proteins. Novamax produced a protein subunit-based vaccine. More recently, RNA-based vaccines from Moderna and Pfizer have been approved in several jurisdictions. Most of these vaccines induce at least some (typically non-sterile) immunity against infection leading to disease, but it is unclear whether protection is effective over several months and/or if sufficient immune memory protects an inoculated individual over extended periods. In addition, it is unclear whether such vaccines generate clinically meaningful T cell-based responses.

[0008] While certain vaccines have become available for use, vaccine distribution and administration on a rapid and global scale has encountered substantial difficulties and slowed down global distribution. In addition, logistical requirements and need of a medical professional restrict administration to specific vaccination infrastructure.

[0009] To overcome such difficulties, solid dosage forms of vaccines are desired. However, such dosage forms (*e.g.*, powders, tablets, capsules) require disintegration and release of the active ingredient(s) at the physiologically relevant location. The dosage form loaded with the active ingredient must be stable during transport and storage from the time of production until administration. Mucosal or oral delivery of a vaccine would be highly desirable, because such administration assists in generating IgA-, IgE-, and IgM-class antibodies, which are important for immunity against mucosal or intestinal infection. Unfortunately, such vaccines are not available.

[0010] Even though various vaccine compositions and methods to induce immunity against SARS-CoV-2 are known in the art, all suffer from several drawbacks. Therefore, there remains a need for improved vaccine compositions and methods.

Summary

[0011] Disclosed herein are various vaccine compositions and methods therefor in which a recombinant modified nucleocapsid protein and/or a modified spike protein of a coronavirus induces immunity against the coronavirus in a subject. Most preferably, the recombinant proteins are encoded in a recombinant nucleic acid that can be delivered in a lipid formulation, as part of a recombinant virus, and/or as part of a recombinant yeast or yeast lysate.

[0012] Also disclosed herein is a recombinant nucleic acid that comprises a first portion encoding a severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein (N) fused to an endosomal targeting sequence (N-ETSD), wherein the first portion is functionally coupled to one or more regulatory elements that enable N-ETSD expression, and a second portion encoding a SARS virus spike protein (S), wherein the second portion is each functionally coupled to one or more regulatory elements that enable S expression.

[0013] In some embodiments, the SARS virus is SARS-CoV-2, and/or the endosomal targeting sequence of the N-ETSD is encoded at a 5'-end and/or a 3'-end of the first portion. In further embodiments, the second portion encodes S optimized for surface expression. The first and second portions can be arranged in a bicistronic sequence. For example, the N-ETSD may have an amino acid sequence that has at least 90% identity to amino acid sequence SEQ ID NO:1. The first portion may, in certain embodiments, have nucleotide sequence SEQ ID NO:2. In another example, the S protein or S-fusion protein may have an amino acid sequence that has at least 90% identity to amino acid sequence SEQ ID NO:3 (e.g., SEQ ID NO:3) or at least 90% identity to SEQ ID NO:4 (e.g., SEQ ID NO:4). The second portion may, in certain embodiments, have nucleotide sequence SEQ ID NO:5 or SEQ ID NO:6.

[0014] In still further embodiments, the recombinant nucleic acid may further comprise a third portion that encodes a co-stimulatory molecule or an immune stimulatory cytokine. The recombinant nucleic acid may also be integrated into a viral or yeast expression vector (*e.g.*, an adenoviral expression vector having an E1 gene region deletion and an E2b gene region deletion,

and/or a yeast expression vector for *Saccharomyces cerevisiae*). Most typically, but not necessarily, the nucleic acid is a deoxyribonucleic acid (DNA).

[0015] A recombinant replication defective adenovirus is described herin that comprises an E1 gene deletion, an E2b gene deletion, and a recombinant nucleic acid that includes a first portion encoding a SARS coronavirus N-ETSD. The first portion is functionally coupled to one or more regulatory elements that enable N-ETSD expression. The adenovirus also includes a second portion encoding a SARS S protein. The second portion is each functionally coupled to one or more regulatory elements that enable S expression.

[0016] In further embodiments, the adenovirus may further comprise a third portion encoding a co-stimulatory molecule or an immune stimulatory cytokine, and/or the recombinant adenovirus may have an E3 and/or E4 gene region deletion.

[0017] Alternatively or additionally, a recombinant yeast is disclosed herein that comprises a recombinant nucleic acid that includes a first portion encoding a SARS coronavirus N-ETSD, wherein the first portion is functionally coupled to one or more regulatory elements that enable N-ETSD expression, and a second portion encoding a SARS S protein, wherein the second portion is each functionally coupled to one or more regulatory elements that enable S expression. The yeast may be *S. cerevisiae*. In certain embodiments, the yeast may be lysed.

[0018] In yet further embodiments, disclosed herein is a vaccine composition that includes the recombinant nucleic acids presented herein, and the recombinant nucleic acid may be encapsulated in a lipid nanoparticle.

[0019] In still other embodiments, a vaccine composition is disclosed herein that comprises aragonite particles admixed with a recombinant replication defective adenovirus as presented herein, wherein the recombinant replication defective adenovirus is lyophilized. Most typically, the aragonite particles have an average particle size between 100 nm and 1 mm.

[0020] Recombinant nucleic acids are presented herein for generating a vaccine against SARS virus and/or for inducing immunity against SARS virus. Likewise, disclosed herein is a recombinant replication defective adenovirus or recombinant yeast for inducing immunity against SARS virus.

[0021] Various objects, features, aspects and advantages of the subject matter disclosed herein will become more apparent from the following detailed description of preferred embodiments, along with the accompanying drawing figures in which like numerals represent like components.

Brief Description of The Drawing

[0022] FIG.1 schematically illustrates various crystalline forms of calcium carbonate.

[0023] FIG.2 shows three photographs (right to left: 1, 2, 3) of bivalent human adenovirus serotype 5 COVID-Spike and Nucleocapsid antigen vaccine (hAD5-COVID-S/N) in a non-coated aragonite capsule (Sample #6) in 0.1 M hydrochloric acid (HCL) with observed wrinkling, swelling, or a hole in capsule as indicated: 1): 2 minutes post HCL acid exposure; 2): 2 hours post HCL acid exposure; and 3) 2 hours post HCL acid exposure and dried.

[0024] FIG.3 shows three photographs (right to left: 1, 2, 3) of hAD5-COVID-S/N in a non-coated aragonite capsule (Samples #7 or #8) in 0.1 M HCL with observed swelling, twisting, or a hole in capsule as indicated: 1): Sample #7 at 2 hours post HCL acid exposure; 2): Sample #8 at 2 hours post HCL acid exposure; 3) At 2 hours post HCL acid exposure and dried.

[0025] FIG.4 shows two photographs (right to left: 1, 2) of hAD5-COVID-S/N in a non-coated lactose capsule (Samples #3 or #4) in 0.1 M HCL with observed swelling of capsule as indicated: 1): Sample #3 at 2 hours post HCL acid exposure; 2): Sample #4 at 2 hours post HCL acid exposure.

[0026] FIG.5 shows two photographs (right to left: 1, 2) of hAD5-COVID-S/N in a coated aragonite capsule (Samples #1 or #5) in 0.1 M HCL with observed swelling of capsule as indicated: 1): Sample #1 at 2 hours post HCL acid exposure; 2): Sample #5 at 2 hours post HCL acid exposure.

[0027] FIG.6 shows Infectious Units per gram (IFU/g) (y-axis) for indicated hAD5-COVID-S/N Capsule Type, as indicated.

[0028] FIG.7 shows the percentage (%) of Virus Recovery (y-axis) for each hAD5-COVID-S/N Capsule Type as indicated.

[0029] FIG.8 shows IFU/g for each hAD5-COVID-S/N Capsule Type and corresponding pH as indicated.

[0030] FIG.9 shows the percentage (%) of Virus Recovery for each hAD5-COVID-S/N Capsule Type, as indicated.

[0031] FIG.10 shows Percent Virus Recovered for each hAD5-COVID-S/N Capsule Type as indicated, with acid treatment indicated for those with shading.

[0032] FIG.11 shows IFU/g for each hAD5-COVID-S/N Capsule Type as indicated, with acid treatment indicated for those with shading.

[0033] FIG.12 depicts a conceptual illustration of an ideal vaccine that will elicit durable and effective immunity across multiple pathways.

[0034] FIG.13 depicts results of an exemplary expression experiment where SARS-CoV2 N is overexpressed in *S. cerevisiae*.

[0035] FIG.14 shows ELISA results detecting IgG seroreactivity against SARS-CoV-2 spike in sera samples drawn from immunized macaques.

[0036] FIG.15 depicts serum inhibiting SARS-CoV-2 infectivity. Panel A shows sera from vaccinated Group 1 macaques inhibiting SARS-CoV-2 infectivity *in vitro*. Panel B shows sera from vaccinated Group 2 macaques. The dotted line indicates 20% inhibition.

[0037] FIG.16 depicts non-human primate (NHP) nasopharyngeal viral load over time. Panel A shows viral load (qPCR) in nasal swabs from macaques following SC+SC+oral vaccination. Panel B shows viral load (qPCR) in nasal swabs following SC+oral+oral vaccination.

[0038] FIG.17 depicts the viral load in NHP over time in the lungs. Panel A shows viral load (qPCR) in BAL from Group 1 macaques following SC+SC+oral vaccination, Panel B shows viral load (qPCR) in BAL from Group 1 macaques following SC+oral+oral vaccination.

[0039] FIG.18 depicts IgG & IgM seroreactivity against SARS-CoV-2 spike in sera samples from human patients immunized with various experimental anti- SARS-CoV-2 vaccines.

[0040] FIG.19 shows ELISpot results from Th1 N-responsive patients.

[0041] FIG.20 shows ELISpot results from patient 4 (N-unresponsive) and patient 10 (weakly Th1 N-responsive).

[0042] FIG.21 depicts exemplary results for humoral responses and neutralizing capability of sera from hAd5 S-Fusion + N-ETSD vaccinated NHP. Anti-spike IgG levels (ELISA; OD 450nm) are shown for (A) individual SC Oral-Oral NHP along with the (B) geometric mean and (C) inhibition in the surrogate assay. These data are also shown for SC-SC-Oral NHP: (D) individual anti-S IgG, (E) geometric mean, and (F) inhibition in the surrogate assay. Inhibition above 20% (dashed line) with a sera dilution of 1:30 is correlated with neutralization of SARS-CoV-2 infection. NHP received vaccination on Days 0, 14, & 28 (black arrows).

[0043] FIG.22 depicts viral load (gRNA) in nasal passages and lung of SC-Oral-Oral and SC-SC-Oral vaccinated NHP post-challenge. (A) Individual viral gRNA (RT qPCR) and (B) the geometric mean for nasal swab samples; and (C) gRNA and (D) the geometric mean for bronchoalveolar lavage (BAL) samples from SC-Oral-Oral NHP. (E) Individual gRNA and (F) the geometric mean for nasal swab samples; and (G) gRNA and (H) the geometric means for BAL samples from SC-SC-Oral NHP. SARS-CoV-2 challenge was on Day 56 (black arrows). The level of detection (LOD; dashed line) was 54 gene copies/mL (GC/mL) for gRNA and 119 GC/mL for sgRNA. For values below the LOD, half the LOD value (or 27 GC/mL for gRNA and 59 GC/mL for sgRNA) was used for graphing of individual values and calculation of the geometric mean.

[0044] FIG.23 depicts viral replication (sgRNA) in nasal passages and lung in SC-Oral-Oral and SC-SC-Oral vaccinated NHP post-challenge. (A) Individual viral sgRNA (RT qPCR) and (B) the geometric mean for nasal swab samples; and (C) sgRNA and (D) the geometric mean for bronchoalveolar lavage (BAL) samples from SC-Oral-Oral NHP. (E) Individual sgRNA and (F) the geometric mean for nasal swab samples; and (G) sgRNA and (H) the geometric means for BAL samples from SC-SC-Oral NHP. SARS-CoV-2 challenge was on Day 56 (black arrows). The LOD (dashed line) was 54 GC/mL for gRNA and 119 GC/mL for sgRNA. For values below the LOD, half the LOD value was used for graphing of individual values and calculation of the geometric mean.

[0045] FIG.24 depicts T-cell responses to vaccination and neutralization capability of sera post-SARS-CoV-2 challenge. (A) Interferon-g (IFN-γ) and (B) interleukin-4 (IL-4) secretion by PBMC-derived T cells from SC-Oral-Oral vaccinated NHP in response to spike (S) and nucleocapsid (N) peptides as determined by ELISpot as well as (C) the ratio IFN-γ/IL-4 ratio are shown. Ratios of 'infinity' due to undetectable IL-4 are represented as open circles. Cells pulsed with PMA-ionomycin were used as positive controls. Data graphed with mean and SEM. (D) MN_{50}

(dilution factor which SARS-CoV-2 infection of Vero E6 cells is inhibited by 50%) throughout the course of the study is shown; an unpaired, two-tailed Student's t-test was used to compare MN_{50} for vaccinated and placebo NHP on Day 70. Nasal gRNA (E) and sgRNA (F) on Days 57, 63, & 70, as well as lung gRNA (G) and sgRNA on Days 57 & 63 are presented. The same data are shown for SC-SC-Oral NHP, including T-cell responses (I-J), MN_{50} (L), nasal gRNA (M) and sgRNA (N), as well as lung gRNA (O) and sgRNA (P). The level of detection for gRNA was 54 GC/mL and for sgRNA was 119 GC/mL. Half the LOD was used for graphing of data below the LOD.

[0046] FIG.25 schematically depicts the hAd5 platform and the hAd5 S-Fusion + N-ETSD construct. Panel A shows the human adenovirus serotype 5 vaccine platform with E1, E2b, and E3 regions deleted (*). The vaccine construct is inserted in the E1 regions (arrow). Panel B shows the dual-antigen vaccine comprises both S-Fusion and N-ETSD under control of cytomegalovirus (CMV) promoters and with C-terminal SV40 poly-A sequences delivered by the hAd5 [E1-, E2b-, E3-] platform.

[0047] FIG.26 depicts exemplary constructs for cloning into an adenovirus.

[0048] FIG.27 is a western blot showing *in vitro* construct expression and detection of S and N.

[0049] FIG.28 depicts additional exemplary constructs for cloning into an adenovirus.

[0050] FIG.29 depicts antibody response to N with a Th1 phenotype. Humoral Immune Responses T_H1 vs T_H2 associated isotype analysis is shown.

[0051] FIG.30 depicts cell mediated immunity (CMI) response to N focus phenotype – IFN-γ and IL-2 ELISpot.

[0052] FIG.31 depicts enhanced cell surface expression of RBD with S Fusion and with S Fusion+N combination constructs compared to S-WT.

[0053] FIG.32 depicts antigen recognition by recovered COVID-19 patient plasma. Antigens include RBD-ETSD and fusion S / N-ETSD constructs.

[0054] FIG.33 depicts the SARS-CoV-2 virus, spike, the hAd5 [E1-, E2b-, E3-] vector and vaccine candidate constructs. (a) Trimeric S protein is displayed on the viral surface; the N protein is associated with the viral RNA. (b) RBD is within the S1 region, followed by other functional

regions, the transmembrane domain (TM) and the C-terminus (CT), which is within the virus. (c) The second-generation human adenovirus serotype 5 (hAd5) vector used has the E1, E2b, and E3 regions deleted. Constructs are shown for (d) S wild type (S-WT), (e) S-RBD with the Enhanced T-cell Stimulation Domain (S RBD-ETSD), (f) S-Fusion, (g) N-ETSD, and (h) bivalent hAd5 S-Fusion + N-ETSD; LP – Leader peptide.

[0055] FIG.34 depicts HEK293T transfection with hAd5 S-Fusion + ETSD, resulting in enhanced RBD surface expression. Flow cytometric analysis of an anti-RBD antibody with construct-transfected cells reveals no detectable RBD surface expression in either S-WT or (b) S-WT + N-ETSD transfected cells. Surface RBD expression was high for S RBD- ETSD and S RBD-ETSD + N-ETSD (c, d). Expression was low in (e) S-Fusion transfected cells. Cell surface expression of the RBD was high in (f) S-Fusion + N-ETSD transfected cells, particularly at day 1 and 2. (g) No expression was detected the N-ETSD negative control. Y-axis scale is normalized to mode (NM).

[0056] FIG.35 depicts immunoblot analysis of S expression. Cell surface RBD expression with (a) hAd5 S-WT, S-Fusion, and (c) S-Fusion + N-ETSD in HEK 293T cells shows high correlation with (d) expression of S in immunoblots of HEK 293T cell lysates probed using anti-full length (S2) antibody. Y-axis scale is normalized to mode (NM).

[0057] FIG.36 depicts binding of recombinant ACE2-Fc HEK293T cell-surface expressed RBD after transfection confirms native protein folding. Flow cytometric analysis of binding between recombinant ACE2- Fc, with which the spike RBD interacts *in vivo* to initiate infection, and cell-surface antigens expressed after transfection of HEK293T cells with (a) hAd5 S-WT, (b) hAd5 S-Fusion, (c) hAd5 S-Fusion + N-ETSD, (d) hAd5 S RBD-ETSD, or (e) hAd5 S RBD-ETSD + N-ETSD constructs reveals the highest binding is seen for both ACE-Fc and an anti-RBD specific antibody (f-j) after transfection with the bivalent S-Fusion + N-ETSD. Both S RBD-ETSD-containing constructs also showed binding. Y-axis scale is normalized to mode (NM).

[0058] FIG.37 depicts N expressed from hAd5 N-ETSD is localized to the endosomal/lysosomal compartment. In HeLa cells infected with N-ETSD, (a) N (red) co-localizes with the endosomal marker CD71 (b) as indicated by the arrow in (c). In transfected HeLa cells, (d) N-ETSD also co-localizes with the lysosomal marker Lamp1, whereas (e) N wild type (N-WT) does not, showing instead diffuse cytoplasmic distribution.

[0059] FIG.38 depicts ICS detection of cytokine-expressing splenocytes from hAd5 S-Fusion + N-ETSD inoculated Day 28 CD-1 mice in response to peptide pools. (a) The highest $CD8\beta^+$ splenocyte IFN-γ response was in hAd5 S-Fusion + N-ETSD-inoculated mouse splenocytes exposed to S peptide pool 1 (S- pep pool 1); splenocytes from these mice also expressed IFN-γ in response to the N peptide pool (N-pep pool). (b) CD4+ splenocytes from hAd5 S-Fusion + N-ETSD-inoculated mice only expressed IFN-γ in response to the N peptide pool. (c) IFN-γ TNF-α responses of $CD8\beta^+$ splenocytes from hAd5 S-Fusion + N-ETSD-inoculated mice were very similar to those in (a); as were (d) CD4+ splenocytes to the N peptide pool to those in (b). N = 5 mice per group. All data sets graphed as the mean with SEM and all statistics performed using the Mann-Whitney test where *<0.05, **<0.01, ***<0.001, and ****<0.0001.

[0060] FIG.39 depicts anti-spike and anti-nucleocapsid antibody responses in sera from hAd5 S-Fusion + N-ETSD vaccinated mice. Based on absorbance, there was significant production of both (a) anti-S antibodies and (c) anti-nucleocapsid antibodies. (b, d) The ng equivalents are shown. Sera diluted 1:30 for anti-spike and 1:90 for anti-nucleocapsid antibodies. Data graphed as the mean and SEM. Statistical analysis was performed using an unpaired two-tailed Student's t-test where *<0.05, **<0.01, ***<0.001, and ****<0.0001.

[0061] FIG.40 depicts cPass and Vero E6 cell SARS-CoV-2 confirm neutralization by antibodies. (a) In the cPass assay, inhibition of S RBD interaction with ACE2 was significant at both 1:20 and 1:60 dilutions of serum from hAd5 S-Fusion + N-ETSD vaccinated mice. (b) The results in the Vero E6 cell SARS-CoV-2 viral infection for mice that showed S-specific antibodies by ELISA also showed high neutralization for mice and very high neutralization for pooled sera (G4 pool, blue line) even compared to COVID-19 convalescent serum. G4 pool – mice with S-specific antibodies; M1, M2, M3, M4 – mouse ID; +C – convalescent serum; and media – media only negative control.

[0062] FIG.41 depicts isotypes for anti-spike and anti-nucleocapsid antibodies. Panels A and C show that IgG2a and IgG2b isotype anti-spike and anti-nucleocapsid antibodies were significantly increased for hAd5 S-Fusion + N- ETSD mice compared to hAd5 Null mice. Panels B and D shows the ng equivalents for antibody isotypes. Data graphed as the mean and SEM. Statistical analysis was performed using an unpaired two-tailed Student's t-test where *<0.05, **<0.01, ***<0.001, and ****<0.0001.

[0063] FIG.42 depicts ELISpot of secreted cytokines. (a) IFN-γ secretion by hAd5 S-Fusion + N-ETSD splenocytes was significantly higher than hAd5 Null in response to both S peptide pool 1 and the N peptide pool; but (b) IL-4 was only secreted with hAd5 S-Fusion + N-ETSD in response to the N peptide pool (one high outlier in hAd5 null removed). N = 5 mice per group. All data sets graphed as the mean with SEM and all statistics performed using the Mann-Whitney test where *<0.05, **<0.01, ***<0.001, and ****<0.0001.

[0064] FIG.43 depicts ratios for T-cell and humoral responses reveal Th1 predominance. (a) The ratio of total Th1 (IFN-γ) to Th2 (IL-4) spot-forming units is shown for responses to the combined S pools and to the N pool. (b) The Th1/Th2 ratio for antibodies against S and N is shown. For both (a) and (b) the dashed line indicates a ratio of 1 or a balance of Th1 and Th2 (no predominance).

[0065] FIG.44 schematically illustrates the hAd5 vector, SARS-CoV-2, spike, and constructs. (A) The human adenovirus serotype 5 with E1, E2b, and E3 regions deleted (hAd5 [E1-, E2b-, E3-]) is shown. (B) The SARS-CoV-2 virus displays spike (S) protein as a trimer on the viral surface. S protein comprises the N-terminal (NT), the S1 region including the RBD, the S2 and TM regions, and the C-terminal (CT); other function regions not labeled. (C) Spike wild type (SWT), (D) spike fusion (S-Fusion), (E) nucleocapsid without ETSD and predominantly cytoplasmic localization (N); (F) N with the Enhanced T-Cell Stimulation Domain (N-ETSD), and (G) the bivalent S-Fusion + N-ETSD constructs are shown.

[0066] FIG.45 depicts photomicrographs establishing that N-ETSD localizes to endosomes, lysosomes, and autophagosomes. MoDCs were infected with Ad5 N-ETSD or N without ETSD and were co-labeled with anti-flag (N, N-ETSD here have a flag tag) and anti-CD71 (endosomal marker), anti-Lamp 1 (lysosomal marker), or anti-LC3a/b antibodies. (A) N-ETSD, (B) CD71, and (C) overlay. (D) N, (E) CD71, and (F) overlay. (G) NETSD, (H) Lamp-1, and (I) overlay. (J) N, (K) Lamp-1, and (L) overlay. (M) N-ETSD, (N) LC3a/b, and (O) overlay. N/N-ETSD is red, other markers green, co-localization indicated by yellow arrows, and white arrows indicate lymphocytes.

[0067] FIG.46 demonstrates that patient plasma antibodies recognize SARS-CoV-2 antigens expressed by MoDCs after hAd5 S-Fusion + N-ETSD infection. (A) MoDCs from two normal individuals were infected with hAd5 vaccines overnight, then exposed to previously infected patient plasma from a single individual; antibody binding to the DC cell surface was detected by flow cytometry. The flow histograms for hAd5 S-WT, S-Fusion, S-Fusion + N-ETSD, and Null are shown for MoDCs from two sources, (B) MoDC1 and (C) MoDC2. (D) The DMFI (difference

in MFI for binding to uninfected and infected cells) is graphed for hAd5 S-WT, S-Fusion, S-Fusion + N-ETSD and Null infected MoDCs..

[0068] FIG.47 depicts exemplary results for T cell responses (of previously SARS-CoV-2 infected patient and virus-naive T-cell) to MoDCs pulsed with SARS-CoV-2 peptides. T cells from all four previously SARS-CoV-2 infected patients (Pt) show significant IFN-γ responses to S1, S2, and N peptide pool-pulsed MoDCs as compared to 'none'. T cells from virus-naïve (unexposed, UnEx) control individuals showed far lower responses. Statistical analysis performed using One-way ANOVA and Tukey's post-hoc analysis for samples from each patient compared only to 'none' where * p<= 0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Data graphed as the mean and SEM; n = 3-4.

[0069] FIG.48 establishes that peptide-pulsed MoDCs from patients previously infected with SARS-CoV-2 stimulate autologous patient T cells to secrete IFN-γ. (A) MoDCs derived from previously infected SARSCoV-2 patients Pt4 and Pt 3 were pulsed with SARS-CoV-2 peptide mixes (S1, S2 or N) overnight and then incubated with autologous CD4+ (A, B) or CD8+ (C, D) T cells. IFN-γ levels were determined by ELISpot. Statistical analysis performed using One-way ANOVA and Dunnett's post-hoc multiple comparison analysis to compare each peptide pool to Veh (shown above the bar) or between peptide pools (above line) where *p<0.05, **p<0.01 and ****p<0.00001. Data graphed as mean and SEM; n = 3.

[0070] FIG.49 demonstrates that IFN-γ secretion by T cells from previously SARS-CoV-2 infected patients is greater in response to MoDC expression of N-ETSD compared to N. (A) Experimental design. (B-D) Secretion of IFN-γ by autologous CD3+ T cells in response to hAd5-N-ETSD- and hAd5 Nexpressing MoDCs is shown. (E-G) Secretion of IL-4 by CD3+ cells in response to infected MoDCs is shown with the same scales as IFN-γ for each. IFN-γ secretion by (H-J) CD4+ and (KM) CD8+ T cells in response to hAd5-N-ETSD compared to Null are shown. Statistical analysis performed using One-way ANOVA and Tukey's post-hoc multiple comparison analysis, where *p<=0.05; **p<0.01, ***p<0.001 and ****p<0.0001. Comparison to Null shown above bars, comparison between N-ETSD and N above lines. Data graphed as mean and SEM; n = 3-4.

[0071] FIG.50 shows that previously SARS-CoV-2 infected patient T-cell responses to the bivalent vaccine and its individual components reveal distinct antigen specificity of T-cell populations. (A-C) CD3+ T cell IFN-γ responses for three patients. (D-F) CD3+ T cell IL-4

responses. (G-I) CD4+ IFN-γ responses. (J-L) CD8+ IFN-γ responses. Statistical analysis was performed using One-way ANOVA and Tukey's post-hoc multiple comparison analysis to compare each antigen-containing construct to the Null construct, where *$p<0.05$ and ****$p<0.00001$. Comparison to Null only above bars; comparison between antigen-expressing vaccines above lines. Data graphed as mean and SEM; n = 3-4.

Detailed Description

[0072] Various vaccine compositions and methods of inducing immunity against the SARS-CoV-2 virus and closely related viruses and mutant forms of the SARS-CoV-2 virus not only promote generation of therapeutically effective antibodies but that also elicit a robust T cell response. These vaccine compositions can be administered via different routes, including intramuscular, subcutaneous, oral, and mucosal routes (alone or in combination), and may even be used as oral boost after currently known RNA-based vaccines.

[0073] In particularly contemplated embodiments, a recombinant construct comprises a modified nucleocapsid protein and/or a modified spike protein. Preferably, the modified nucleocapsid protein comprises a trafficking sequence to so route the modified nucleocapsid protein to the endosomal/lysosomal subcellular compartments, thereby taking advantage of a key antigen presentation pathway to stimulate CD4+ T cells which in turn license dendritic cells to activate naïve CD8+ cytotoxic T cells. Likewise, it is preferred that the modified spike protein has a modification that enhances surface expression of the modified spike protein to thereby render an immune response more robust against the spike antigen. Indeed, while the vaccines disclosed herein (e.g., hAd5 [E1-,E2b-] vaccines) are exemplified using the N antigen tagged with the ETSD peptide, in principle any antigen can be profitably redirected to the endosomal/lysosomal subcellular compartment. Exemplary antigens to be tagged with ETSD for use in this manner in adenoviral or yeast vaccine vectors include CEA, human epidermal growth factor receptor 1 (HER1), HER2/neu, HER3, HER4, prostate-specific antigen (PSA), PSMA, folate receptor alpha, WT1, p53, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, BAGE, DAM-6, DAM-10, GAGE-1, GAGE-2, GAGE-8, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7B, NA88-A, NY-ESO-1, MART-1, MC1R, Gp100, PSM, Tyrosinase, TRP-1, TRP-2, ART-4, CAMEL, CEA, Cyp-B, BRCA1, Brachyury, Brachyury (TIVS7-2, polymorphism), Brachyury (IVS7 T/C polymorphism), T Brachyury, hTERT, hTRT, iCE, MUC1, MUC1 (VNTR polymorphism), MUC1c, MUC1n, MUC2, PRAME, P15, RU1, RU2, SART-1,

SART-3, AFP, β-catenin/m, Caspase-8/m, CDK-4/m, ELF2M, GnT-V, G250, HSP70-2M, HST-2, KIAA0205, MUM-1, MUM-2, MUM-3, Myosin/m, RAGE, SART-2, TRP-2/INT2, 707-AP, Annexin II, CDC27/m, TPI/mbcr-abl, ETV6/AML, LDLR/FUT, Pml/RARα, HPV E6, HPV E7, and TEL/AML1.

[0074] "N-ETSD" refers to a modified nucleocapsid protein of the SARS-CoV-2 virus that includes an endosomal targeting sequence. An exemplary N-ETSD has an amino acid sequence of SEQ ID NO:1 and a nucleotide sequence of SEQ ID NO:2.

[0075] "S-HA" or "Spike" or "S" refers to a spike protein of the SARS-CoV-2 virus that has an HA tag. An exemplary S-HA has an amino acid sequence of SEQ ID NO:3 and a nucleotide sequence of SEQ ID NO:5.

[0076] "S-Fusion" or "spike-fusion" refers to a modified spike protein of the SARS-CoV-2 virus that has increased surface expression. An exemplary S-Fusion has an amino acid sequence of SEQ ID NO:4 and a nucleotide sequence of SEQ ID NO:6.

[0077] "N" or "N-wt" or "nucleocapsid" refers to the nucleocapsid protein of the SARS-CoV-2 virus. An exemplary N protein has an amino acid sequence of SEQ ID NO:7.

[0078] "ETSD" refers to an endosomal targeting sequence. An exemplary ETSD has an amino acid sequence of SEQ ID NO:8.

[0079] "ACE2" refers to the Angiotensin-converting enzyme 2. An exemplary (human) ACE2 has an amino acid sequence of SEQ ID NO:9.

[0080] "Soluble ACE2 protein" refers to a mutant and truncated form of ACEs that is soluble under physiological conditions. An exemplary soluble ACEs has an amino acid sequence of SEQ ID NO:10.

Modified Spike and Nucleocapsid Constructs and Methods

[0081] Disclosed herein are recombinant viruses and yeasts. The viruses and yeasts disclosed herein may be useful for a variety of purposes, such as treating and/or preventing a coronavirus disease. In one aspect, disclosed herein is a replication defective adenovirus, wherein the adenovirus comprises an E1 gene region deletion; an E2b gene region deletion; an E3 gene region deletion, a nucleic acid encoding a coronavirus 2 (CoV2) nucleocapsid protein, a CoV2

nucleocapsid protein fused to an endosomal targeting sequence (N-ETSD), and a nucleic acid encoding a CoV2 spike protein sequence optimized for cell surface expression (S- Fusion).

[0082] In one embodiment, the N-ETSD polypeptide may comprises a sequence with at least 80% identity to SEQ ID NO:1. In other embodiments, the identity value is at least 85%. In still other embodiments, the identity value is at least 90%. In some embodiments, the identity value is at least 95%. In some embodiments, the identity value is at least 99%. In some embodiments, the identity value is 100%. It is further contemplated that the N-ETSD fusion protein contains a linker between the N-ETSD domain and the nucleocapsid protein. For example, this linker may be a 16 amino acid linker having the sequence $(G_3S)_4$. In certain embodiments, methods are disclosed herein for enhancing the immunogenicity of an intracellular antigen, the methods comprising tagging the antigen with ETSD and expressing the tagged antigen in an antigen-presenting cell (*e.g.*, a dendritic cell).

[0083] In some embodiments, the fusion protein comprising N-ETSD and CoV-2 nucleocapsid protein may be encoded by a nucleic acid sequence having at least 80% identity to SEQ ID NO:2. In some embodiments, the identity value is at least 85%. In some embodiments, the identity value is at least 90%. In some embodiments, the identity value is at least 95%. In some embodiments, the identity value is at least 99%. In some embodiments, the identity value is 100%.

[0084] The CoV-2 spike protein is contemplated to have at least 85% identity to SEQ ID NO:3. In some embodiments, the identity value is at least 85%. In some embodiments, the identity value is at least 90%. In some embodiments, the identity value is at least 95%. In some embodiments, the identity value is at least 99%. In some embodiments, the identity value is 100%. The nucleic acid encoding the CoV-2 spike protein has at least 85% identity to SEQ ID NO:5. In some embodiments, the identity value is at least 85%. In some embodiments, the identity value is at least 90%. In some embodiments, the identity value is at least 95%. In some embodiments, the identity value is at least 99%. In some embodiments, the identity value is 100%.

[0085] The CoV-2 spike fusion protein is contemplated to have at least 85% identity to SEQ ID NO:4. In some embodiments, the identity value is at least 85%. In some embodiments, the identity value is at least 90%. In some embodiments, the identity value is at least 95%. In some embodiments, the identity value is at least 99%. In some embodiments, the identity value is 100%. The nucleic acid encoding the CoV-2 spike fusion protein has at least 85% identity to SEQ ID NO:6. In some embodiments, the identity value is at least 85%. In some embodiments, the identity

value is at least 90%. In some embodiments, the identity value is at least 95%. In some embodiments, the identity value is at least 99%. In some embodiments, the identity value is 100%.

[0086] In a second aspect of this disclosure, provided herein is a recombinant yeast comprising a nucleic acid encoding a protein selected from the group consisting of a coronavirus 2 (CoV-2) nucleocapsid protein, a CoV2 N-ETSD protein, a CoV2 spike protein, a CoV2 spike-fusion protein, and a combination thereof. Moreover, each of these encoded proteins may be further modified as described in more detail below. Preferably, the recombinant yeast is *Saccharomyces cerevisiae.*

[0087] In some embodiments of this second aspect, the CoV-2 nucleocapsid protein or variant thereof comprises a sequence with at least 80% identity to SEQ ID NO:1 or SEQ ID NO:7. In other embodiments, the identity value is at least 85%. In still other embodiments, the identity value is at least 90%. In some embodiments, the identity value is at least 95%. In some embodiments, the identity value is at least 99%. In some embodiments, the identity value is 100%.

[0088] In some embodiment of this second aspect, the CoV-2 spike protein or spike fusion protein comprises a sequence with at least 80% identity to SEQ ID NO:3 or SEQ ID NO:4. In other embodiments, the identity value is at least 85%. In still other embodiments, the identity value is at least 90%. In some embodiments, the identity value is at least 95%. In some embodiments, the identity value is at least 99%. In some embodiments, the identity value is 100%.

[0089] In some embodiments, the nucleic acid encoding the CoV-2 spike protein or spike fusion protein comprises a sequence with at least 80% identity to SEQ ID NO:5 or SEQ ID NO:6. In other embodiments, the identity value is at least 85%. In still other embodiments, the identity value is at least 90%. In some embodiments, the identity value is at least 95%. In some embodiments, the identity value is at least 99%. In some embodiments, the identity value is 100%.

[0090] The adenoviruses and yeasts disclosed herein may further comprise a nucleic acid encoding a trafficking sequence, a co-stimulatory molecule, and/or an immune stimulatory cytokine. The co-stimulatory molecule is selected from the group consisting of CD80, CD86, CD30, CD40, CD30L, CD40L, ICOS-L, B7-H3, B7-H4, CD70, OX40L, 4-1BBL, GITR-L, TIM-3, TIM-4, CD48, CD58, TL1A, ICAM-1, and LFA3. The immune stimulatory cytokine may be selected from the group consisting of IL-2, IL-12, IL-15, nogapendekin alfa-imbakicept, IL-21, IPS1, and LMP1. Additionally or alternatively, the vaccines disclosed herein may also encode SARS-CoV-2 M

protein, with or without an ETSD tag. Additionally or alternatively, the adenovirus and/or yeast may be administered in combination with one or more immune stimulatory cytokines (*e.g.*, IL-2, IL-12, IL-15, nogapendekin alfa-imbakicept, IL-21, IPS1, & LMP1). By "in combination" in this context is intended that the immune stimulatory cytokine(s) is/are administered within 24 hrs of the adenovirus and/or yeast. That is to say, the adenovirus and/or yeast may be administered to a patient (e.g., a patient over 50 years of age), and then within the following 24 hrs, one or more immune stimulatory cytokines (*e.g.*, IL-2, IL-12, IL-15, nogapendekin alfa-imbakicept, IL-21, IPS1, & LMP1) may be administered to the same patient. Additionally or alternatively, one or more immune stimulatory cytokines (*e.g.*, IL-2, IL-12, IL-15, nogapendekin alfa-imbakicept, IL-21, IPS1, & LMP1) may be administered to a patient (e.g., a patient over 50 years of age), and then within the following 24 hrs, the adenovirus and/or yeast may be administered to the same patient.

[0091] Beyond 40 years of age, patients' ability to mount T cell responses to vaccines gradually declines. Therefore, administration of an adenovirus and/or yeast as described herein in combination with one or more immune stimulatory cytokines (*e.g.*, IL-2, IL-12, IL-15, nogapendekin alfa-imbakicept, IL-21, IPS1, & LMP1) may be particularly useful in elderly patients. As used herein, "elderly" coveys patients over 50 years of age, *e.g.*, patients over 55 years of age, over 60 years of age, over 65 years of age, over 70 years of age, over 75 years of age, over 80 years of age, over 85 years of age, or over 90 years of age.

[0092] Most preferably, the recombinant virus is administered via subcutaneous or subdermal injection. However, in other contemplated aspects, administration may also be intravenous injection. Alternatively, or additionally, antigen presenting cells may be isolated or grown from cells of the patient, infected *in vitro*, and then transfused to the patient.

[0093] In one aspect of any of the embodiments described above or elsewhere herein, the composition is formulated in a pharmaceutically acceptable excipient suitable for administration to a subject.

[0094] It is still further contemplated that the recombinant viruses and yeasts contemplated herein may further comprises a sequence that encodes at least one of a co-stimulatory molecule, an immune stimulatory cytokine, and a protein that interferes with or down-regulates checkpoint inhibition. For example, suitable co-stimulatory molecules include CD80, CD86, CD30, CD40, CD30L, CD40L, ICOS-L, B7-H3, B7-H4, CD70, OX40L, 4-1BBL, GITR-L, TIM-3, TIM-4,

CD48, CD58, TL1A, ICAM-1, and/or LFA3, while suitable immune stimulatory cytokine include IL-2, IL-12, IL-15, IL-15 super agonist (N803), IL-21, IPS1, and/or LMP1, and/or suitable proteins that interfere include antibodies against or antagonists of CTLA-4, PD-1, TIM1 receptor, 2B4, and/or CD160.

[0095] It should be appreciated that all of the above noted co-stimulatory genes are well known in the art, and sequence information of these genes, isoforms, and variants can be retrieved from various public resources, including sequence data bases accessible at the NCBI, EMBL, GenBank, RefSeq, etc. Moreover, while the above exemplary stimulating molecules are preferably expressed in full length form as expressed in human, modified and non-human forms are also deemed suitable so long as such forms assist in stimulating or activating T-cells. Therefore, muteins, truncated forms and chimeric forms are expressly contemplated herein.

[0096] The immunotherapeutic compositions disclosed herein may be either "prophylactic" or "therapeutic". When provided prophylactically, the compositions of the present disclosure are provided in advance of the development of, or the detection of the development of, a coronavirus disease, with the goal of preventing, inhibiting or delaying the development of the coronavirus disease; and/or generally preventing or inhibiting progression of the coronavirus disease in an individual. Therefore, prophylactic compositions can be administered to individuals that appear to be coronavirus disease free (healthy, or normal, individuals), or to individuals who has not yet been detected of coronavirus. Individuals who are at high risk for developing a coronavirus disease, may be treated prophylactically with a composition of the instant disclosure.

[0097] When provided therapeutically, the immunotherapy compositions are provided to an individual who is diagnosed with a coronavirus disease, with the goal of ameliorating or curing the coronavirus disease; increasing survival of the individual; preventing, inhibiting, reversing or delaying development of coronavirus disease in the individual.

[0098] In yet another embodiment, disclosed herein is a vaccine composition comprising the adenovirus or yeast as disclosed above, and wherein the composition is formulated for injection. The vaccine composition may be used for inducing immunity against CoV-2 in a patient in need thereof, by administering to the patient the vaccine composition.

[0099] Also disclosed herein are methods for preventing and/or treating coronavirus diseases, and especially COVID-19. Preferably, the method includes using a viral or yeast vector that encodes

the wild-type or modified form of a nucleocapsid protein and/or the wild-type or modified form of a spike protein of the coronavirus in an immunogenic composition that is administered to a subject individual. The virus and/or yeast vaccine, thus administered, would infect the individual with CoV-2 the wild-type or modified form of the nucleocapsid or spike protein. With that in place, the individual would have an immune response against it, and be vaccinated. Notably, as the nucleocapsid protein and the spike protein are relatively conserved polypeptides, immune responses can be elicited for a variety of members of the coronavirus family.

[00100] Where the recombinant vector is an adenovirus, the adenoviral vector may be modified to encode the wild-type or modified form of the nucleocapsid protein, and/or spike protein. Similarly, in case of yeast, the yeast vector may also be modified to encode the wild-type or modified form of the nucleocapsid protein, and/or the spike protein. As is shown in more detail below, positive immune responses were obtained on cell mediated immunity upon administration of immunogenic compositions comprising the viral and/or yeast vectors in patients in need thereof. Thus, in one embodiment, the present disclosure contemplates creating the coronaviral spikes to be expressed on the yeast surface. In such embodiment, the yeast is acting as an avatar coronavirus to stimulate B cells, which then results in humoral immunity.

[00101] As disclosed herein is a next generation bivalent human adenovirus serotype 5 (hAd5) vaccine capable of inducing immunity in patients with pre-existing adenovirus immunity, comprising both an S sequence optimized for cell surface expression (S- Fusion) and a conserved nucleocapsid (N) antigen that is designed to be transported to the endosomal subcellular compartment, with the potential to generate durable immune protection. As further described herein, such bivalent vaccine has been found to be optimized for immunogenicity as evidenced by the following findings:

1) The optimized S-Fusion displayed improved S receptor binding domain (RBD) cell surface expression compared to S-WT where little surface expression was detected;

2) The expressed RBD from S-Fusion retained conformational integrity and recognition by ACE2-Fc;

3) The viral N protein modified with an enhanced T-cell stimulation domain (ETSD) localized to endosomal/lysosomal subcellular compartments for MHC I/II presentation; and

4) These optimizations to S and N (S-Fusion and N-ETSD) generated enhanced de novo antigen-specific B cell and CD4+ and CD8+ T-cell responses in antigen-naive pre-clinical models.

[00102] Both the T-cell and antibody immune responses to S and N components demonstrated a T-helper 1 (Th1) bias. The antibody responses were neutralizing as demonstrated by independent SARS-CoV-2 neutralization assays. Thus, in one embodiment, the next generation bivalent hAd5 S-Fusion+N-ETSD vaccine provides robust, durable cell-mediated and humoral immunity against SARS-CoV-2 infection. Moreover, and as also further described in more detail below, the vaccine construct may be administered orally, intranasally, or sublingually. Thus, in one embodiment, the instant disclosure also provides beyond injectable formulations (*e.g.*, SC or IM) vaccine constructs in oral, intranasal, and sublingual formulation to induce mucosal immunity in addition to cell-mediated and humoral immunity. Viewed from another perspective, substantial immunity can be generated by injection, oral/mucosal administration, alone or in combination. In one embodiment, the COVID-19 vaccine disclosed herein generates long-term T and B cell memory.

Coronaviruses and vaccines therefor

[00103] Coronaviruses are found in avian and mammalian species. They resemble each other in morphology and chemical structure: for example, the coronaviruses of humans and cattle are antigenically related. There is no evidence, however, that human coronaviruses can be transmitted by animals. In animals, various coronaviruses invade many different tissues and cause a variety of diseases in humans. One such disease was Severe acute respiratory syndrome (SARS) coronavirus disease that spread to several countries in Asia, Europe and North America in late 2002/early 2003. Another such disease is the novel Coronvirus Disease of 2019 (COVID 19) that has spread to several countries in the world. In December of 2019, reports emerged from Wuhan, China concerning a new infectious respiratory disease with high morbidity and mortality1-3 that displayed human-to-human transmission. The causative agent was rapidly identified as a novel coronavirus and was designated SARS-coronavirus 2 (SARS-CoV-2). The disease it causes is referred to as COVID- 19 and has rapidly become a worldwide pandemic that has disrupted socioeconomic life and resulted in more than 32 million infections and more than 1,100,000 deaths worldwide as of late October 2020.

[00104] COVID 19 usually begins with a fever greater than 38° C. Initial symptoms can also include cough, sore throat, malaise and mild respiratory symptoms. Within two days to a week, patients may have trouble breathing. Patients in more advanced stages of COVID 19 develop either

pneumonia or respiratory distress syndrome. Public health interventions, such as surveillance, travel restrictions and quarantines, are being used to contain the spread of COVID 19. It is unknown, however, whether these draconian containment measures can be sustained with each appearance of the COVID 19 in humans. Furthermore, the potential of this new and sometimes lethal CoV as a bio-terrorism threat is obvious.

[00105] Coronavirus virions are spherical to pleomorphic enveloped particles. The envelope is studded with projecting glycoproteins, and surrounds a core consisting of matrix protein enclosed within which is a single strand of positive-sense RNA (Mr 6×10^6) associated with nucleocapsid protein. In that regard, it should be noted that the terms "nucleocapsid protein," "nucleoprotein," and "nucleocapsid" are used interchangeably throughout this disclosure. The coronavirus nucleocapsid (N) is a structural protein found in all coronaviruses, including COVID 19. The nucleocapsid protein forms complexes with genomic RNA, interacts with the viral membrane protein during virion assembly and plays a critical role in enhancing the efficiency of virus transcription and assembly.

[00106] Another protein found throughout all coronavirus virions is the viral spike (S) protein. Coronaviruses are large positive-stranded RNA viruses typically with a broad host range. Like other enveloped viruses, CoV enter target cells by fusion between the viral and cellular membranes, and that process is mediated by the viral spike (S) protein.

[00107] SARS-CoV-2 is an enveloped positive sense, single-strand RNA β coronavirus primarily composed of four structural proteins: spike (S), nucleocapsid (N), membrane (M), and envelope, as well as the viral membrane and genomic RNA. Of these, S is the largest and N the most prevalent. The S glycoprotein is displayed as a trimer on the viral surface (**FIG.33**, Panel A), whereas N is located within the viral particle. A schematic of the S primary structure is shown in **FIG.33**, Panel B. The sequence of SARS-CoV-2 was published and compared to that of previous coronaviruses. This was soon followed by reports on the crystal structure of the S protein. The virus uses the S protein to enter host cells by interaction of the S receptor binding domain (S RBD) with angiotensin- converting enzyme 2 (ACE2), an enzyme expressed on a variety of cell types in the nose, mouth, gut, and lungs, as well as other organs, and importantly on the alveolar epithelial cells of the lung where infection is predominantly manifested. As represented in **FIG.33**, Panel B, the S RBD is found within the S1 region of the spike polypeptide.

[00108] The methods and compositions disclosed herein target the nucleoprotein and the spike protein that is conserved in all types of coronaviruses. In one embodiment, the present disclosure provides a vaccine formulation comprising a recombinant entity, wherein the recombinant entity comprises a nucleic acid that encodes a nucleocapsid protein of coronavirus 2 (CoV2) or modified form thereof and/or wherein the recombinant entity encodes a spike protein of CoV2 or modified form thereof. The vaccine formulation may be useful for treating a disease, such as a coronavirus mediated disease or infection. Thus, in another embodiment, a method for treating a coronavirus disease is contemplated for a patient in need thereof. Such method will preferably include a step of administering to the subject an immunotherapy composition comprising a recombinant entity, wherein the recombinant entity comprises a nucleic acid that encodes a nucleocapsid protein of coronavirus 2 (CoV2) or a modified form thereof and/or a nucleic acid that encodes a spike protein of coronavirus 2 (CoV2) or a modified form thereof. The coronavirus contemplated herein may be coronavirus disease 2019 (COVID-19) and/or severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

[00109] For example, the present disclosure provides a method for treating coronavirus disease 2019 (COVID-19) and/or severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), in a patient in need thereof, comprising: administering to the subject a first immunotherapy composition comprising a recombinant virus, wherein the recombinant virus comprises a nucleic acid that encodes a nucleocapsid protein of coronavirus 2 (CoV2) or modified form thereof, administering to the subject a second immunotherapy composition comprising a recombinant yeast, wherein the recombinant yeast comprises a nucleic acid that encodes a spike protein of CoV2. The first and second immunotherapy compositions may be administered concurrently or sequentially to the patient.

[00110] Viewed form a different perspective, contemplated herein is a viral vector (*e.g.*, recombinant adenovirus genome, optionally with a deleted or non-functional E2b gene) that comprises a nucleic acid that encodes (a) at least a nucleocapsid protein or modified form thereof; and (b) at least one spike protein or modified form thereof. The viral vector may further encode one or more co-stimulatory molecules. Most typically, the nucleic acid will also include a trafficking signal to direct a peptide product encoded by the nucleic acid to the cytoplasm, the endosomal compartment, or the lysosomal compartment, and the peptide product may also comprise a sequence portion that enhances intracellular turnover of the peptide product.

[00111] The majority of current SARS-CoV-2 vaccines under development target S because of the potential to neutralize the ability of the virus to bind host cells by production of antibodies against the RBD. Support for RBD as a key antigen was recently confirmed, and it was reported that in 44 hospitalized COVID-19 patients, RBD-specific IgG responses and neutralizing antibody titers are detectable in all patients by 6 days post-PCR confirmation of infection, and that the two are correlated. In addition to humoral responses, S epitopes are also frequent targets of COVID-19 recovered patient T cells, providing further justification for inclusion of S in prophylactic immunization strategies.

[00112] Despite the urgent need for rapid development of SARS-CoV-2 vaccines, reliance on any one antigen cargo or immunological pathway as occurring in the monovalent vaccines under development is not without risk. Evaluation of nearly 4000 SARS-CoV-2 genomic sequences has identified numerous mutations in S with the D614G variant emerging recently as a potentially more infectious strain six months after identification of the original virus.

[00113] In designing the vaccine disclosed herein, to overcome the risk of the emergence of new strains of the virus with mutations in S and to provide additional antigens against which responses can be elicited, an optimized N sequence was added. The N protein is a highly conserved and antigenic SARS-CoV- 2-associated protein that has been studied previously as an antigen in coronavirus vaccine design for SARS-CoV. N associates with viral RNA within the virus and has a role in viral RNA replication, virus particle assembly, and release. SARS-CoV-2 N is a highly antigenic protein and nearly all patients infected with SARS-CoV-2 have antibody responses to N. Furthermore, another study reported that most, if not all, COVID-19 survivors tested were shown to have N-specific CD4+ T-cell responses.

[00114] Currently, there is keen focus on generation of humoral responses to vaccines with, arguably, less attention being paid to T-cell responses. The natural history of SARS-CoV-2 infection would suggest, however, that a robust T-cell response to vaccination is at least as important as the production of antibodies and should be a critical consideration for COVID-19 vaccine efficacy.

[00115] First, the humoral and T-cell responses are highly correlated, with titers of neutralizing antibodies being proportional to T-cell levels, suggesting the T response is necessary for an effective humoral response. It is well established that the activation of CD4+ T helper cells enhances B-cell production of antibodies. Second, virus-specific CD4+ and CD8+ T cells are not

only widely detected in COVID-19 patients, based on findings from patients recovered from the closely- related SARS-CoV, but such T cells persist for at least 6–17 years, suggesting that T cells may be an important part of long-term immunity. These T-cell responses were predominantly to N, and it has been reported that in all 36 convalescent COVID-19 patients in their study, the presence of CD4+ and CD8+ T cells recognizing multiple regions of the N protein could be demonstrated. Examination of blood from 23 individuals who had recovered from SARS- CoV and found that the memory T cells acquired 17 years ago also recognized multiple proteins of SARS-CoV-2. These findings emphasize the importance of designing a vaccine with the highly conserved nucleocapsid present in both SARS-CoV and SARS-CoV-2. Third, recovered patients exposed to SARS-CoV-2 have been found without seroconversion, but with evidence of T-cell responses. The T-cell based responses become even more critical given the finding in at least one study that neutralizing antibody titers decline in some COVID-19 patients after about 3 months.

[00116] In one embodiment, the vaccines disclosed herein result in the generation of T-cell in addition to humoral responses. A bivalent vaccine comprising many antigens, S RBD as displayed by inclusion of full-length S including SD1, S1 and S2 epitopes, along with N, was contemplated and shown to be more effective in eliciting both T-cell and antibody-based responses than a construct with either antigen alone by presenting both unique and conserved SARS-CoV-2 antigenic sites to the immune system. The importance of both S and N was highlighted by identifying that both S and N antigens as *a priori* potential B and T-cell epitopes for the SARS-CoV virus show close similarity to SARS-CoV-2 that are predicted to induce both T and B cell responses.

[00117] An additional consideration for design of an effective vaccine is the likelihood of antigen presentation on the surface of a recombinant protein-expressing cell, and expression in a conformation that recapitulates natural virus infection. First, because wild type N does not have a signaling domain that directs it to endosomal processing and ultimately MHC class II complex presentation to CD4+ T cells, the wild type N sequence is not optimal for induction of a vigorous CD4+ T-cell responses, a necessity for both cell-mediated and B cell memory. To overcome this limitation, an Enhanced T-cell Stimulation Domain (ETSD) to N allows the necessary processing and presentation. One preferred ETSD polypeptide has an amino acid sequence of SEQ ID NO:8. Of course, it should be appreciated that the sequence can be modified while maintaining the desired activity. Accordingly, an ETSD sequence may have at least 85%, or at least 90%, or at least 95, or at least 98% identity to SEQ ID NO:8. Second, to display the highly antigenic RBD region of S

on the cell surface, an optimized the wild type S protein into an 'S-Fusion' sequence increases the likelihood of native folding, increased stability, and proper cell surface expression of the RBD. Thus, in one embodiment, the vaccine construct comprises an S-Fusion sequence and an N-ETSD sequence.

[00118] The vaccine platform utilized here is a next-generation recombinant human adenovirus serotype 5 (hAd5) vector with deletions in the E1, E2b, and E3 gene regions (hAd5 [E1-, E2b-, E3-]). This hAd5 [E1-, E2b-, E3-] vector (**FIG.33**, Panel C) is primarily distinguished from other first- generation [E1-, E3-] recombinant Ad5 platforms by having additional deletions in the early gene 2b (E2b) region that remove the expression of the viral DNA polymerase (pol) and in pre terminal protein (pTP) genes, and its propagation in the E.C7 human cell line. Removal of these E2b regions confers advantageous immune properties by minimizing immune responses to Ad5 viral proteins such as viral fibers, thereby eliciting potent immune responses to specific antigens in patients with pre-existing adenovirus (Ad) immunity. As a further benefit of these deletions, the vector has an expanded gene-carrying/cloning capacity compared to the first generation Ad5 [E1-, E3-] vectors. This next generation hAd5 [E1-, E2b-, E3-] vaccine platform, in contrast to Ad5 [E1-, E3-]-based platforms, does not promote activities that suppress innate immune signaling, thereby allowing for improved vaccine efficacy and a superior safety profile independent of previous Ad immunity. Since these deletions allow the hAd5 platform to be efficacious even in the presence of existing Ad immunity, this platform enables relatively long- term antigen expression without significant induction of anti-vector immunity. It is therefore also possible to use the same vector/construct for homologous prime-boost therapeutic regimens unlike first-generation Ad platforms which face the limitations of pre-existing and vaccine-induced Ad immunity. Importantly, this next generation Ad vector has demonstrated safety in over 125 patients with solid tumors. In these Phase I/II studies, CD4+ and CD8+ antigen-specific T cells were successfully generated to multiple somatic antigens (CEA, MUC1, brachyury) even in the presence of pre-existing Ad immunity.

[00119] The instant disclosure provides findings of confirmed enhanced cell-surface expression and physiologically relevant folding of the expressed S RBD from S-Fusion by ACE2-Fc binding. The N-ETSD protein was successfully localized to the endosomal/lysosomal subcellular compartment for MHC presentation and consequently generated both CD4+ and CD8+ T-cell responses. Immunization of CD-1 mice with the hAd5 S Fusion + N-ETSD vaccine elicited both humoral and cell-mediated immune responses to vaccine antigens. CD8+ and CD4+ T-cell

responses were noted for both S and N. Statistically significant IgG responses were seen for antibody generation against S and N. Potent neutralization of SARS-CoV-2 by sera from hAd5 S Fusion + N-ETSD-immunized mice was confirmed by two independent SARS-CoV-2 neutralization assays: the cPass assay measuring competitive inhibition of RBD binding to ACE2,44 and in the live SARS-CoV-2 virus assay with infected Vero E6 cells. Analysis of T-cell responses as well as humoral responses to S and N were skewed toward a Th1-specific response.

[00120] Taken together, these findings illustrate that hAd5 S-Fusion + N-ETSD vaccine compositions would be particularly effective against the SARS-CoV-2.

Recombinant viruses

[00121] With respect to recombinant viruses it is contemplated that all known manners of making recombinant viruses are deemed suitable for use herein, however, especially preferred viruses are those already established in therapy, including adenoviruses, adeno-associated viruses, alphaviruses, herpes viruses, lentiviruses, etc. Among other appropriate choices, adenoviruses are particularly preferred.

[00122] Moreover, it is further generally preferred that the virus is a replication deficient and non-immunogenic virus. For example, suitable viruses include genetically modified alphaviruses, adenoviruses, adeno-associated viruses, herpes viruses, lentiviruses, etc. However, adenoviruses are particularly preferred. For example, genetically modified replication defective adenoviruses are preferred that are suitable not only for multiple vaccinations but also vaccinations in individuals with preexisting immunity to the adenovirus (see *e.g.*, WO 2009/006479 and WO 2014/031178, which are incorporated by reference in its entirety). In some embodiments, the replication defective adenovirus vector comprises a replication defective adenovirus 5 vector. In some embodiments, the replication defective adenovirus vector comprises a deletion in the E2b region. In some embodiments, the replication defective adenovirus vector further comprises a deletion in the El region. In that regard, it should be noted that deletion of the E2b gene and other late proteins in the genetically modified replication defective adenovirus to reduce immunogenicity. Moreover, due to these specific deletions, such genetically modified viruses were replication deficient and allowed for relatively large recombinant cargo.

[00123] For example, WO 2014/031178 describes the use of such genetically modified viruses to express CEA (colorectal embryonic antigen) to provide an immune reaction against colon

cancer. Moreover, relatively high titers of recombinant viruses can be achieved using genetically modified human 293 cells as has been reported (*e.g.*, *J Virol.* 1998 Feb; 72(2): 926–933).

[00124] El -deleted adenovirus vectors Ad5 [E1-] are constructed such that a trans gene replaces only the El region of genes. Typically, about 90% of the wild-type Ad5 genome is retained in the vector. Ad5 [E1-] vectors have a decreased ability to replicate and cannot produce infectious virus after infection of cells not expressing the Ad5 El genes. The recombinant Ad5 [E1-] vectors are propagated in human cells allowing for Ad5 [E1-] vector replication and packaging. Ad5 [E1-] vectors have a number of positive attributes; one of the most important is their relative ease for scale up and cGMP production. Currently, well over 220 human clinical trials utilize Ad5 [E1-] vectors, with more than two thousand subjects given the virus *sc*, *im*, or *iv*. Additionally, Ad5 vectors do not integrate; their genomes remain episomal. Generally, for vectors that do not integrate into the host genome, the risk for insertional mutagenesis and/or germ-line transmission is extremely low if at all. Conventional Ad5 [E1-] vectors have a carrying capacity that approaches 7kb.

[00125] One obstacle to the use of first generation (El -deleted) Ad5 -based vectors is the high frequency of pre-existing anti-adeno virus type 5 neutralizing antibodies. Attempts to overcome this immunity is described in WO 2014/031178, which is incorporated by reference herein. Specifically, a novel recombinant Ad5 platform has been described with deletions in the early 1 (El) gene region and additional deletions in the early 2b (E2b) gene region (Ad5 [E1-, E2b-]). Deletion of the E2b region (that encodes DNA polymerase and the pre-terminal protein) results in decreased viral DNA replication and late phase viral protein expression. E2b deleted adenovirus vectors provide an improved Ad-based vector that is safer, more effective, and more versatile than First Generation adenovirus vectors.

[00126] In a further embodiment, the adenovirus vectors contemplated for use in the present disclosure include adenovirus vectors that have a deletion in the E2b region of the Ad genome and, optionally, deletions in the El, E3 and, also optionally, partial or complete removal of the E4 regions. In a further embodiment, the adenovirus vectors for use herein have the El and/or the preterminal protein functions of the E2b region deleted. In some cases, such vectors have no other deletions. In another embodiment, the adenovirus vectors for use herein have the El, DNA polymerase and/or the preterminal protein functions deleted.

[00127] The term "E2b deleted", as used herein, refers to a specific DNA sequence that is mutated in such a way so as to prevent expression and/or function of at least one E2b gene product. Thus, in certain embodiments, "E2b deleted" is used in relation to a specific DNA sequence that is deleted (removed) from the Ad genome. E2b deleted or "containing a deletion within the E2b region" refers to a deletion of at least one base pair within the E2b region of the Ad genome. Thus, in certain embodiments, more than one base pair is deleted and in further embodiments, at least 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, or 150 base pairs are deleted. In another embodiment, the deletion is of more than 150, 160, 170, 180, 190, 200, 250, or 300 base pairs within the E2b region of the Ad genome. An E2b deletion may be a deletion that prevents expression and/or function of at least one E2b gene product and therefore, encompasses deletions within exons of encoding portions of E2b-specific proteins as well as deletions within promoter and leader sequences. In certain embodiments, an E2b deletion is a deletion that prevents expression and/or function of one or both of the DNA polymerase and the preterminal protein of the E2b region. In a further embodiment, "E2b deleted" refers to one or more point mutations in the DNA sequence of this region of an Ad genome such that one or more encoded proteins is non-functional. Such mutations include residues that are replaced with a different residue leading to a change in the amino acid sequence that result in a nonfunctional protein.

[00128] As will be readily appreciated, the desired nucleic acid sequences (for expression from virus infected cells) are under the control of appropriate regulatory elements well known in the art. In view of the above, it should be appreciated that compositions and methods presented are not only suitable for directing virally expressed antigens specifically to one or another (or both) MHC systems, but will also provide increased stimulatory effect on the CD8+ and/or CD4+ cells via inclusion of various co-stimulatory molecules (*e.g.*, ICAM-1 (CD54), ICOS-L, LFA-3 (CD58), and at least one of B7.1 (CD80) and B7.2 (CD86)), and via secretion or membrane bound presentation of checkpoint inhibitors.

[00129] With respect to viral expression and vaccination systems it is contemplated that all therapeutic recombinant viral expression systems are deemed suitable for use herein so long as such viruses are capable to lead to expression of the recombinant payload in an infected cell.

[00130] Regardless of the type of recombinant virus it is contemplated that the virus may be used to infect patient (or non-patient) cells *ex vivo* or *in vivo*. For example, the virus may be injected subcutaneously or intravenously, or may be administered intranasally or via inhalation to

so infect the patient's cells, and especially antigen presenting cells. Alternatively, immune competent cells (*e.g.*, NK cells, T cells, macrophages, dendritic cells, etc.) of the patient (or from an allogeneic source) may be infected *in vitro* and then transfused to the patient. Alternatively, immune therapy need not rely on a virus but may be effected with nucleic acid transfection or vaccination using RNA or DNA, or other recombinant vector that leads to the expression of the neoepitopes (*e.g.*, as single peptides, tandem mini-gene, etc.) in desired cells, and especially immune competent cells. Such nucleic acids will typically be delivered in association with a lipid formulation to protect the nucleic acid from degradation and to facilitate uptake of the nucleic acid into a target cell.

[00131] As noted above, the desired nucleic acid sequences (for expression from virus infected cells) are under the control of appropriate regulatory elements well known in the art. For example, suitable promoter elements include constitutive strong promoters (*e.g.*, SV40, CMV, UBC, EF1A, PGK, CAGG promoter), but inducible promoters are also deemed suitable for use herein, particularly where induction conditions are typical for a tumor microenvironment. For example, inducible promoters include those sensitive to hypoxia and promoters that are sensitive to TGF-β or IL-8 (e.g., via TRAF, JNK, Erk, or other responsive elements promoter). In other examples, suitable inducible promoters include the tetracycline-inducible promoter, the myxovirus resistance 1 (Mx1) promoter, etc.

[00132] The replication defective adenovirus comprising an E1 gene region deletion, an E2b gene region deletion, and a nucleic acid encoding a coronavirus 2 (CoV2) nucleocapsid protein and/or a CoV2 spike protein, as disclosed herein may be administered to a patient in need for inducing immunity against CoV2. Routes and frequency of administration of the therapeutic compositions described herein, as well as dosage, may vary from individual to individual, and the severity of the disease, and may be readily established using standard techniques. In some embodiments, the administration comprises delivering 4.8-5.2 x 10^{11} replication defective adenovirus particles, or 4.9-5.1 x 10^{11} replication defective adenovirus particles, or 4.95-5.05 x 10^{11} replication defective adenovirus particles, or 4.99-5.01 x 10^{11} replication defective adenovirus particles.

[00133] The administration of the virus particles can be through a variety of suitable paths for delivery. One preferred route contemplated herein is by injection, such as intracutaneous injection,

intramuscular injection, intravenous injection or subcutaneous injection. In some embodiments, a subcutaneous delivery may be preferred.

Recombinant Yeasts

[00134] With respect to yeast expression and vaccination systems, it is contemplated that all known yeast strains are deemed suitable for use herein. However, it is preferred that the yeast is a recombinant *Saccharomyces* strain that is genetically modified with a nucleic acid construct encoding a protein selected from the group consisting of coronavirus 2 (CoV2) nucleocapsid protein, CoV2 spike protein, and a combination thereof, to thereby initiate an immune response against the CoV2 viral disease. In one aspect of any of the embodiments of the disclosure described above or elsewhere herein, the yeast vehicle is a whole yeast. The whole yeast, in one aspect is killed. In one aspect, the whole yeast is heat inactivated. In one preferred embodiment, the yeast is a whole, heat-inactivated yeast from *Saccharomyces cerevisiae*.

[00135] The use of a yeast based therapeutic compositions are disclosed in the art. For example, WO 2012/109404 discloses yeast compositions for treatment of chronic hepatitis B infections.

[00136] It is noted that any yeast strain can be used to produce a yeast vehicle of the present disclosure. Yeasts are unicellular microorganisms that belong to one of three classes: *Ascomycetes*, *Basidiomycetes* and *Fungi Imperfecti*. One consideration for the selection of a type of yeast for use as an immune modulator is the pathogenicity of the yeast. In preferred embodiments, the yeast is a non-pathogenic strain such as *Saccharomyces cerevisiae* as non-pathogenic yeast strains minimize any adverse effects to the individual to whom the yeast vehicle is administered. However, pathogenic yeast may also be used if the pathogenicity of the yeast can be negated using pharmaceutical intervention.

[00137] For example, suitable genera of yeast strains include *Saccharomyces*, *Candida*, *Cryptococcus, Hansenula, Kluyveromyces, Pichia, Rhodotorula, Schizosaccharomyces* and *Yarrowia*. In one aspect, yeast genera are selected from *Saccharomyces, Candida, Hansenula, Pichia or Schizosaccharomyces*, and in a preferred aspect, *Saccharomyces* is used. Species of yeast strains that may be used include *Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Candida albicans, Candida kefyr, Candida tropicalis, Cryptococcus laurentii, Cryptococcus neoformans, Hansenula anomala, Hansenula polymorpha, Kluyveromyces fragilis,*

Kluyveromyces lactis, Kluyveromyces marxianus var. lactis, Pichia pastoris, Rhodotorula rubra, Schizosaccharomyces pombe, and *Yarrowia lipolytica*.

[00138] It should further be appreciated that a number of these species include a variety of subspecies, types, subtypes, etc. that are intended to be included within the aforementioned species. In one aspect, yeast species used in the instant disclosure include *S. cerevisiae, C. albicans, H. polymorpha, P. pastoris and S. pombe. S. cerevisiae* is useful due to it being relatively easy to manipulate and being "Generally Recognized As Safe" or "GRAS" for use as food additives (GRAS, FDA proposed Rule 62FR18938, Apr. 17, 1997). Therefore, particularly contemplated herein is a yeast strain that is capable of replicating plasmids to a particularly high copy number, such as a *S. cerevisiae* cir strain. The *S. cerevisiae* strain is one such strain that is capable of supporting expression vectors that allow one or more target antigen(s) and/or antigen fusion protein(s) and/or other proteins to be expressed at high levels. In addition, any mutant yeast strains can be used, including those that exhibit reduced post-translational modifications of expressed target antigens or other proteins, such as mutations in the enzymes that extend N-linked glycosylation.

[00139] Expression of contemplated peptides/proteins in yeast can be accomplished using techniques known to those skilled in the art. Most typically, a nucleic acid molecule encoding at least one protein is inserted into an expression vector such manner that the nucleic acid molecule is operatively linked to a transcription control sequence to be capable of effecting either constitutive or regulated expression of the nucleic acid molecule when transformed into a host yeast cell. As will be readily appreciated, nucleic acid molecules encoding one or more proteins can be on one or more expression vectors operatively linked to one or more expression control sequences. Particularly important expression control sequences are those which control transcription initiation, such as promoter and upstream activation sequences.

[00140] Any suitable yeast promoter can be used in the methods and compositions of the present disclosure and a variety of such promoters are known to those skilled in the art and have generally be discussed above. Promoters for expression in *Saccharomyces cerevisiae* include promoters of genes encoding the following yeast proteins: alcohol dehydrogenase I (ADH1) or II (ADH2), CUP1, phosphoglycerate kinase (PGK), triose phosphate isomerase (TPI), translational elongation factor EF-1 alpha (TEF2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; also referred to as TDH3, for triose phosphate dehydrogenase), galactokinase (GAL1), galactose-1-phosphate

uridyl-transferase (GAL7), UDP-galactose epimerase (GAL10), cytochrome c1 (CYC1), Sec7 protein (SEC7) and acid phosphatase (PHO5), including hybrid promoters such as ADH2/GAPDH and CYC1/GAL10 promoters, and including the ADH2/GAPDH promoter, which is induced when glucose concentrations in the cell are low (e.g., about 0.1 to about 0.2 percent), as well as the CUP1 promoter and the TEF2 promoter. Likewise, a number of upstream activation sequences (UASs), also referred to as enhancers, are known. Upstream activation sequences for expression in *Saccharomyces cerevisiae* include the UASs of genes encoding the following proteins: PCK1, TPI, TDH3, CYC1, ADH1, ADH2, SUC2, GAL1, GAL7 and GAL10, as well as other UASs activated by the GAL4 gene product, with the ADH2 UAS being used in one aspect. Since the ADH2 UAS is activated by the ADR1 gene product, it may be preferable to overexpress the ADR1 gene when a heterologous gene is operatively linked to the ADH2 UAS. Transcription termination sequences for expression in Saccharomyces cerevisiae include the termination sequences of the alpha-factor, GAPDH, and CYC1 genes. Transcription control sequences to express genes in methyltrophic yeast include the transcription control regions of the genes encoding alcohol oxidase and formate dehydrogenase.

[00141] Likewise, transfection of a nucleic acid molecule into a yeast cell according to the present disclosure can be accomplished by any method by which a nucleic acid molecule administered into the cell and includes diffusion, active transport, bath sonication, electroporation, microinjection, lipofection, adsorption, and protoplast fusion. Transfected nucleic acid molecules can be integrated into a yeast chromosome or maintained on extrachromosomal vectors using techniques known to those skilled in the art. As discussed above, yeast cytoplast, yeast ghost, and yeast membrane particles or cell wall preparations can also be produced recombinantly by transfecting intact yeast microorganisms or yeast spheroplasts with desired nucleic acid molecules, producing the antigen therein, and then further manipulating the microorganisms or spheroplasts using techniques known to those skilled in the art to produce cytoplast, ghost or subcellular yeast membrane extract or fractions thereof containing desired antigens or other proteins. Further exemplary yeast expression systems, methods, and conditions suitable for use herein are described in US20100196411A1, US2017/0246276, or US 2017/0224794, and US 2012/0107347.

[00142] So produced recombinant viruses and yeasts may then be individually or in combination used as a therapeutic vaccine in a pharmaceutical composition, typically formulated as a sterile injectable composition with a virus of between 10^4-10^{13} virus or yeast particles per dosage unit, or more preferably between10^9-10^{12} virus or yeast particles per dosage unit.

Alternatively, virus or yeast may be employed to infect patient cells *ex vivo* and the so infected cells are then transfused to the patient. However, alternative formulations are also deemed suitable for use herein, and all known routes and modes of administration are contemplated herein.

[00143] In further contemplated embodiments, second generation hAd5 [E1-, E2b-, E3-] based vaccines disclosed herein overcome pre-existing Anti-Ad5 immunity. To avoid the Ad immunization barrier and circumvent the adverse conditions for first generation Ad5 [E1- E3-] vectors, an advanced 2nd generation human adenoviral (hAd5) vector was constructed having two (2) additional deletions in the E2b region, removing the DNA polymerase and the preterminal protein genes [E1-, E2b-, E3-]. (Former names of the adenovirus vector were Ad5, ETBX in literature)

[00144] E2b-deleted hAd5 vectors have up to a 12-14 kb gene-carrying capacity as compared to the 7-kb capacity of first generation Ad5 [E1-] vectors, providing space for multiple genes if needed. hAd5 [E1-, E2b-, E3-] based recombinant vectors are produced using the human E.C7 cell line. Deletion of the E2b region also confers advantageous immune properties on these novel Ad vectors, eliciting potent immune responses to specific, non-viral antigens while minimizing the immune responses to Ad viral proteins.

[00145] hAd5 [E1-, E2b-, E3-] vectors induce a potent cell mediated immune (CMI) response, as well as Abs against the vectored antigens even in the presence of Ad immunity. hAd5 [E1-, E2b-, E3-] vectors also have reduced adverse reactions as compared to Ad5 [E1-] vectors, in particular the appearance of hepatotoxicity and tissue damage. In one embodiment, the reduced inflammatory response against hAd5 [E1-, E2b-, E3-] vector viral proteins and the resulting evasion of pre-existing Ad immunity increases the capability for the hAd5 [E1-, E2b-, E3-] vectors to infect dendritic cells (DC), resulting in greater immunization of the vaccine. In addition, increased infection of other cell types provides high levels of antigen presentation needed for a potent CD8+ and CD4+ T cell responses, leading to memory T cell development. In one embodiment, hAd5 [E1-, E2b-, E3-] vectors are superior to Ad5 [E1-] vectors in immunogenicity and safety and will be the best platform to develop a COVID-19 vaccine in a rapid and efficient manner. In one embodiment, a prophylactic vaccine is tested against COVID-19 by taking advantage of this new hAd5 vector system that overcomes barriers found with other Ad5 systems and permits the immunization of people who have previously been exposed to Ad5.

[00146] Track Record of Rapid Vaccine Development Utilizing Second Generation Human (hAd5) Adenovirus Platform During Pandemic Treats: H1N1 Experience in 2009. To address emerging pathogen threats, especially in times of pandemic, it is critical that modernized vaccine technologies be deployed. These technologies will utilize the power of genomic sequencing, rapid transfection in well-established vaccine vectors to rapidly identify constructs with high immunogenicity.

[00147] Vaccines against emerging pathogens such as the 2009 H1N1 pandemic virus can benefit from current technologies such as rapid genomic sequencing to construct the most biologically relevant vaccine. A novel platform (hAd5 [E1-, E2b-, E3-]) has been utilized to induce immune responses to various antigenic targets. This vector platform expressed hemagglutinin (HA) and neuraminidase (NA) genes from 2009 H1N1 pandemic viruses. Inserts were consensuses sequences designed from viral isolate sequences and the vaccine was rapidly constructed and produced. Vaccination induced H1N1 immune responses in mice, which afforded protection from lethal virus challenge. In ferrets, vaccination protected from disease development and significantly reduced viral titers in nasal washes. H1N1 cell mediated immunity as well as antibody induction correlated with the prevention of disease symptoms and reduction of virus replication. The hAd5 [E1-, E2b-, E3-] has thus demonstrated the capability for the rapid development of effective vaccines against infectious diseases.

[00148] For at least these reasons, it is generally preferred that contemplated vaccine compositions when based on an adenoviral vector, will utilize a recombinant hAd5 [E1-, E2b-, E3-] platform to generate recombinant nucleic acids for therapeutic use in human.

Example 1: Selected hAd5 vaccine constructs and results

[00149] Disclosed herein are constructs that have been constructed and tested, and in particular a hAd5-COVID-19 vaccine construct E1-, E2b-, E3- hAd5 vector with SARS-CoV-2 (S/N) protein insert (**FIG.26**). This construct has been tested in preclinical experiments, including *in vitro* expression (**FIG.27**) and small animal immunogenicity. Multiple COVID-19 constructs include RBD-alone, S1-alone, S1-fusion proteins, and combinations of RBD, S1 and S1 fusions with N. Preliminary *in-vitro* studies demonstrate that these constructs (**FIG.28**) recognize convalescent serum antibodies and could serve as alternative vaccines.

[00150] Rationale for Inclusion of Nucleocapsid (N) in hAd5 Constructs for COVID-19: The nucleocapsid (N) protein of SARS-CoV-2 is highly conserved and highly expressed. Previous research with the related coronavirus that causes SARS demonstrated that N protein is immunogenic, when integrated with intracellular trafficking constructs. To date, all vaccine strategies in development involve developing immunogenicity against spike (S) protein. However, very recent evidence in patients who recovered from COVID-19 demonstrates Th1 immunity generated against the nucleocapsid (N). Additional reports further confirmed that in the predictive bioinformatics model, T and B cell epitopes were highest for both spike glycoprotein and nucleoprotein. The present disclosure confirms that by combining S with N, long-term cell-mediated immunity with a Th1 phenotype can be induced. Indeed, significant potential exists for this combination vaccine to serve as a long-term "universal" COVID-19 vaccine in light of mutations undergoing in S and the finding that the structural N protein is highly conserved in the coronavirus family.

Example 2: Immunogenicity Studies (Small Animal Model):

[00151] Homologous prime-boost immunogenicity in BALB-c mice. Mice have been treated with 1, 2 or 3 doses of the hAd5 COVID-19 vaccine and serum and splenocyte samples are being tested for SARS-CoV-2 antigen-specific immune responses. Serum is tested for anti-spike and anti-nucleocapsid antibody responses by ELISA. Splenocytes is tested for spike- and nucleocapsid-specific cell mediated immune responses by ELISPOT and intracellular cytokine simulation assays.

[00152] The results show promising immunogenic activity. In one embodiment, hAd5 [E1-,E2b-, E3-] N-ETSD, a vaccine containing SARS-CoV-2 nucleocapsid plus an enhanced T cell stimulation domain (ETSD), alters T cell responses to nucleocapsid. Mice were immunized subcutaneously (SC) with a dose of 1010 VP twice at 7-day intervals. Blood was collected at several time points and spleen was collected upon sacrifice in order to perform immunogenicity experiments. Splenocytes were isolated and tested for cell mediated immune (CMI) responses. The results showed that SARS-CoV-2 nucleocapsid antigen specific CMI responses were detected by ELISpot and flow cytometry analyses in the spleens of all the mice immunized with hAd5 [E1-, E2b-, E3-] N-ETSD vaccine but not vector control (hAd5 [E1-, E2b-, E3-] null) immunized mice. In addition, antibody responses were detected in all the mice immunized with hAd5 [E1-, E2b-,

E3-]-N-ETSD vaccine but not vector control (Ad5 [E1-, E2b-, E3-]-null) immunized mice (**FIG.29** and **FIG.30**).

Example 3: Enhanced RBD Cell Surface Expression:

[00153] Further evidence of the potential enhancing immunogenicity value of N when combined with S was the surprising finding of enhanced surface expression of the RBD protein in 293 cells transfected with the N-ETSD+S construct as seen in **FIG.31**. Expression and presentation of RBD appears to be highly important as evidenced by the recent report by others who showed that rare but recurring RBD-specific antibodies with potent antiviral activity were found in all individuals tested who had recovered from COVID-19 infections. This finding of enhanced expression of RBD when N is combined with S-Fusion was corroborated in studies using plasma from a patient recovered from COVID-19 infection (**FIG.32**). The alternative construct of RBD-ETSD could serve as an alternative vaccine.

[00154] In summary, on the basis of enhanced expression and exposure of the RBD protein with S Fusion and S Fusion + N construct, both were tested in the hAd5 vector. Furthermore, on the basis of recent clinical data from patients recovered from COVID-19, as well as the corroborating preclinical data that the N construct induces long lasting $CD4^+$ and Th1 cell-mediated immunity, this combination of S Fusion + N construct could provide long-lasting immunity beyond short term neutralizing antibodies.

Example 4: Immunogenicity Testing of Candidate COVID-19 Vaccine Constructs

[00155] Two adenovirus-based COVID-19 vaccine constructs will be tested in preclinical experiments, including *in vitro* expression; small animal immunogenicity, and non-human primate immunogenicity and efficacy.

[00156] Constructs description: Two (2) second generation hAd5-based COVID-19 vaccine constructs were evaluated. First is a hAd5 vector with SARS-CoV-2 with spike protein insert (see FIG.26). Second is E1-, E2b-, E3- hAd5 vector with SARS-CoV-2 wild type spike protein (S) insert and Nucleocapsid protein (N) insert containing an Endosomal-targeting domain sequence (ETSD) in the same vector backbone.

[00157] Immunogenicity Studies: Homologous prime-boost immunogenicity in mice was examined by treating Mice with 1, 2 or 3 doses of the adenovirus vaccine candidates listed in

FIG.26 and serum and splenocyte samples will be tested for SARS-CoV-2 antigen-specific immune responses. Serum is being tested for anti-spike and anti-nucleocapsid antibody responses by ELISA. Splenocytes will be tested for spike- and nucleocapsid-specific cell mediated immune responses by ELISPOT and intracellular cytokine simulation assays. Data from these studies are disclosed throughout this disclosure.

[00158] SARS-CoV-2 Virus Neutralization Studies: Serum from the mice immunized during the course of the immunogenicity studies described above is used will be sent to a testing lab for SARS-CoV-2 neutralization studies to be performed in their ABSL-3 facility. Serum will be tested for COVID 19 virus neutralizing activity by mixing various dilutions of serum with COVID 19 virus, incubating the mixture, and then exposing the mixture to Vero cells to detect cytopathic effect (CPE).The last dilution that prevents CPE will be considered the endpoint neutralizing titer.

[00159] Immunogenicity and Efficacy Evaluation in Non-Human Primates: Rhesus macaques will be treated with three doses of the adenovirus vaccine candidates listed in **FIG.26**. SARS-CoV-2 antigen-specific immune responses will be monitored in serum and PBMCs by ELISA, ELISPOT and ICS throughout the course of the therapy. Four weeks after the final vaccination, animals will be challenged with SARS-CoV-2 and monitored for disease hallmarks and virus shedding.

[00160] Example 5: Phase Ib Clinical trial testing of hAd5 [E1-, E 2b-, E3-] CoV-2 vaccine.

[00161] Study Design: This is a Phase 1b open-label study in adult healthy subjects. This clinical trial is designed to assess the safety, reactogenicity, and immunogenicity of the hAd5-COVID-19-S and hAd5-COVID-19-S/N vaccines. The hAd5-COVID-19-S and hAd5-COVID-19-S/N vaccines are hAd5 [E1-, E2b-, E3-] vector-based targeting vaccines encoding the SARS-CoV-2 Spike (S) protein alone or together with the SARS-CoV-2 nucleocapsid (N) protein. The hAd5 [E1-, E2b-, E3-] vector is the platform technology for targeted vaccines that has demonstrated safety in over 125 patients with cancer to date at doses as high as 5×10^{11} virus particles per dose. Co-administration of three different hAd5 [E1-, E2b-, E3-] vector-based vaccines on the same day at 5×10^{11} virus particles per dose each (1.5×10^{12} total virus particles) has also been demonstrated to be safe.

[00162] COVID-19 infection causes significant morbidity and mortality in a worldwide population. The hAd5-COVID-19-S and hAd5-COVID-19-S/N vaccines are designed to induce both a humoral and cellular response even in individuals with pre-existing adenoviral immunity.

Thus, the potential exists for the hAd5-COVID-19-S and hAd5-COVID-19-S/N to induce anti-COVID-19 immunity and prevent or lessen the health impact of COVID-19 infection in healthy subjects.

[00163] Phase 1b Safety Analysis: In the initial safety analysis of phase 1b, a total of 40 healthy subjects will be divided into 4 dosing cohorts (cohorts 1A, 1B, 2A, 2B; n = 10 for each cohort):

- Cohort 1A - hAd5-COVID-19-S at 5×10^{10} viral particles (VP) per dose (n = 10),
- Cohort 1B - hAd5-COVID-19-S at 1×10^{11} VP per dose (n = 10),
- Cohort 2A - hAd5-COVID-19-S/N at 5×10^{10} VP per dose (n = 10),
- Cohort 2B - hAd5-COVID-19-S/N at 1×10^{11} VP per dose (n = 10).

[00164] Each subject will receive a subcutaneous (SC) injection of hAd5-COVID-19-S or hAd5-COVID-19-S/N on Day 1 and Day 22 (*i.e.*, 2 doses). This dosing schedule is consistent with hAd5 [E1-, E2b-, E3-] vector-based vaccines currently in clinical trials. Cohorts 1-2 will enroll in parallel and may be opened at the same time or in a staggered manner depending upon investigational product supply. Subjects in cohorts 1A and 2A will complete the low-dose vaccination regimen first. After all subjects in cohorts 1A and 2A have completed at least a single dose and follow-up assessments during the toxicity assessment period through study day 8, enrollment will proceed if the Safety Review Committee (SRC) and at least one qualified infectious disease physician, independent of the Sponsor and trial, confirms absence of safety concerns. Subjects will then be enrolled in higher-dose cohorts 1B and 2B and vaccinated. For all subjects, follow-up study visits will occur at days 8, 22, 29, 52, and at months 3, 6, and 12 following the final vaccination. Additional follow up for safety information will occur via telephone contact as noted in the Schedule of Events. The primary objectives of the initial safety phase 1b are to evaluate preliminary safety and reactogenicity of the hAd5-COVID-19-S and hAd5-COVID-19-S/N vaccines. The secondary objectives are to evaluate the extended safety and immunogenicity of the hAd5-COVID-19-S and hAd5-COVID-19-S/N vaccines.

Example 6: Expanded Phase 1b: Safety and Immunogenicity for Construct Selection

[00165] Phase 1b expansion will proceed if the SRC determines it is safe to do so based on a review of safety data from the phase 1b safety assessment. In phase 1b expansion, a total of 60 healthy subjects will be divided into 4 dosing cohorts (cohorts 1A, 1B, 2A, 2B; n = 15 for each cohort):

- Cohort 1A - hAd5-COVID-19-S at 5×10^{10} VP per dose (n = 15)

- Cohort 1B - hAd5-COVID-19-S at 1×10^{11} VP per dose (n = 15)
- Cohort 2A - hAd5-COVID-19-S/N at 5×10^{10} VP per dose (n = 15)
- Cohort 2B - hAd5-COVID-19-S/N at 1×10^{11} VP per dose (n = 15)

[00166] Each subject will receive a SC injection of hAd5-COVID-19-S or hAd5-COVID-19-S/N on Day 1 and Day 22 (*i.e.*, 2 doses). For all subjects, follow-up study visits will occur at days 8, 22, 29, 52, and at months 3, 6, and 12 following the final vaccination. Additional follow up for safety information will occur via telephone contact as noted in the Schedule of Events. The primary objective of the expanded phase 1b is to select the most immunogenic construct between hAd5-COVID-19-S and hAd5-COVID-19-S/N and dose level as determined by changes in humoral and cellular immunogenicity indexes. The secondary objectives are to assess safety and reactogenicity of hAd5-COVID-19-S and hAd5-COVID-19-S/N.

[00167] Embodiments of the present disclosure are further described in the following examples. The examples are merely illustrative and do not in any way limit the scope of the invention as claimed.

Example 7: The hAd5 [E1-, E2b-, E3-] platform and constructs

[00168] For the examples presented here, the next generation hAd5 [E1-, E2b-, E3-] vector was used (**FIG.33**, Panel C) to create viral vaccine candidate constructs. As shown in **FIG. 33**, Panels D-H, a variety of constructs were created: **FIG. 33**, Panel D: S WT: S protein comprising 1273 amino acids and all S domains: extracellular (1-1213), transmembrane (1214-1234), and cytoplasmic (1235-1273) (Unitprot P0DTC2); **FIG. 33**, Panel E: S RBD-ETSD: S Receptor Binding Domain with an Enhanced T-cell Stimulation Domain (ETSD); **FIG. 33**, Panel F: S Fusion: S optimized to enhance surface expression and display of RBD; **FIG. 33**, Panel G: N-ETSD: The nucleocapsid (N) sequence with the ETSD; and **FIG. 33**, Panel H: Bivalent S-Fusion + N-ETSD; S-WT + N-ETSD and S RBD-ETSD + N-ETSD constructs were also produced but are not shown.

Example 8: Enhanced HEK 293T cell-surface expression of RBD following transfection with Ad5 S- Fusion + N-ETSD

[00169] As shown in **FIG.34**, anti-RBD-specific antibodies did not detect RBD on the surface of HEK 293T cells transfected with hAd5 S-WT (**FIG.34**, Panel A) or hAd5 S-WT + N-ETSD (Fig. 9b) constructs, while hAd5 S-Fusion alone was slightly higher (**FIG.34**, Panel E). As

expected, both constructs with RBD, hAd5 RBD-ETSD and RBD-ETSD + N-ETSD, showed high binding of anti-RBD antibody (**FIG.34**, Panels C and D). Notably, high cell-surface expression of RBD was detected after transfection with bivalent hAd5 S-Fusion + N-ETSD (**FIG.34**, Panel F). These findings support the proposition that an hAd5 S-Fusion + N-ETSD construct, containing a high number and variety of antigens provided by both full-length, optimized S with proper folding and N leads to enhanced expression and cell surface display of RBD in a vaccine construct.

Example 9: Immunoblot correlation of enhanced S expression with hAd5 S-Fusion + N-ETSD

[00170] Immunoblot analysis of S expression correlated with enhanced S expression (**FIG.35**), showing again that the bivalent hAd5 S-Fusion + N-ETSD construct enhances expression of S compared to S-Fusion alone. **FIG.35** depicts immunoblot analysis of S expression. Cell surface RBD expression with (a) hAd5 S-WT, S-Fusion, and (c) S-Fusion + N-ETSD in HEK 293T cells shows high correlation with (d) expression of S in immunoblots of HEK 293T cell lysates probed using anti-full length (S2) antibody. Y-axis scale is normalized to mode (NM).

Example 10: Confirmation of native folding of enhanced surface RBD following hAd5 S-Fusion + N-ETSD transfection

[00171] Determination of the binding of recombinant ACE2-Fc was performed to confirm the native, physiologically relevant folding of the S RBD after expression from the hAd5 S-Fusion +N-ETSD vaccine candidate. S RBD binds ACE2 during the course of SARS-CoV-2 infection and an effective neutralizing antibody prevents this interaction and thus infection. Such a neutralizing antibody is more likely to be effective if raised in response to S presented in the correct conformation. In addition to enhancement of cell surface expression, the optimized S allows for proper protein folding. It was found that compared to either hAd5 S-WT or hAd5 S-Fusion (**FIG.36**, Panels A and B, respectively), ACE2-Fc binding to S RBD expressed from the hAd5 S-Fusion + N-ETSD was clearly enhanced (**FIG.36**, Panel C). Anti-RBD antibody binding studies (**FIG.36**, Panels F-J) performed with the same experiment, confirmed the enhanced surface expression findings noted by ACE2-Fc binding. The hAd5 S-Fusion + N-ETSD vaccine candidate was elected for clinical trials based on these findings of conformationally correct and enhanced S RBD expression, which is important for production of neutralizing antibodies.

Example 11: hAd5 N-ETSD successfully directs N to an endosomal/lysosomal compartment

[00172] The ETSD design successfully translocated N to the endosomal subcellular compartment. After infection of HeLa cells with N-ETSD, N co-localized with the endosomal marker 45 transferrin receptor (CD71), as shown in **FIG.37**, Panel C, and also co-localized with the lysosomal marker Lamp1 (**FIG.37**, Panel D), demonstrating that N-ETSD is translocated throughout the endosomal pathway to lysosomes, enabling processing for MHC II presentation. N-wild type (N-WT), compared to N-ETSD, shows diffuse cytoplasmic distribution and does not co-localize with the lysosomal marker (**FIG.37**, Panel E). These findings confirm the role of the ETSD in directing N to an endosomal/lysosomal compartment that will result in increased MHC II presentation and CD4+ activation by N.

Example 12: *In Vivo* hAd5 S-Fusion + N-ETSD Vaccine Immunogenicity Studies

[00173] Based on the evidence that S-Fusion + N-ETSD resulted in enhanced expression of physiologically-relevant RBD and that N-ETSD successfully translocated to the endosomal / lysosomal compartment, the bivalent hAd5 S-Fusion + N-ETSD vaccine was chosen for inoculation of 7-week old female CD-1 mice. The unique properties of this construct would result in the generation of both CD8+ and CD4+ T-cell responses and neutralizing antibodies. As described in Methods, mice received an initial injection on Day 0 and a second injection on Day 21. Sera were collected on Day 0 and at the end of the study on Day 28 for antibody and neutralization analyses. Splenocytes were also collected on Day 28 for intracellular cytokine staining (ICS) and ELISpot analyses. All age- and gender-matched animals assigned to the study appeared normal with no site reactions and no loss of body weight throughout the dosing were seen, consistent with previous observations with the hAd5 [E1-, E2b-, E3-] platform

Example 13: hAd5 S-Fusion + N-ETSD generates both CD8β+ and CD4+ T-cell responses

[00174] CD8+ activation by both S and N: CD8β+ splenocytes from hAd5 S-Fusion + N-ETSD vaccinated mice exposed to S peptide pool 1 (containing RBD and S1) show IFN-γ expression that is significantly higher compared to hAd5 null mice (**FIG.38**, Panel A); splenocytes from these mice also expressed intracellular IFN-γ in response to the N peptide pool. Evaluation of simultaneous IFN-γ/TNF- α expression from CD8 β + splenocytes (**FIG.38**, Panel C) mirrored those for IFN-γ expression alone. These results indicate that both S and N activate CD8+ T cells.

[00175] CD4+ activation by N: Although CD8+ cytotoxic T cells mediate killing of virus infected cells, CD4+ T cells are required for sustained cytotoxic T lymphocyte (CTL) activity. Thus, CD4+ T cells in the vaccinated animals was evaluated. In contrast to CD8 β + splenocytes, only the N peptide pool stimulated CD4+ splenocytes from hAd5 S-Fusion + N-ETSD-inoculated mice to express IFN-γ (**FIG.38**, Panel B) or IFN-γ/TNF-α (**FIG.38**, Panel D) at levels that were substantially higher than hAd5 Null control. The contribution by N of CD4+ T-cell responses is vital to an effective immune response to the candidate vaccine.

Example 14: hAd5 S-Fusion + N-ETSD generates antibody responses to both S and N antigens

[00176] The primary objective of coronavirus vaccines currently in development are neutralizing antibodies against spike. In mice vaccinated with the bivalent vaccine there was significant production of both anti-S (**FIG.39**, Panel A) and anti-N (**FIG.39**, Panel C) antibodies in the sera from CD-1 mice vaccinated with hAd5 S-Fusion + N-ETSD at Day 28 in the study. Compared to anti-S antibodies, anti-N antibodies were higher in sera, given the dilution factor for sera was 1:90 for anti-N antibody analysis and 1:30 for anti-S antibody analysis.

[00177] A standard curve of IgG was generated, then absorbance values were converted into mass equivalents for both anti-S and anti-N antibodies (**FIG.39**, Panels B and D). These values were used to calculate that hAd5 S-Fusion + NETSD vaccination generated a geometric mean value of 5.8 μg S-specific IgG and 42 μg N-specific IgG per mL of serum, therefore the relative μg amount of anti-N antibodies is higher than that for anti-S antibodies and reflects the strong contribution of N to anti-SARS-CoV-2 antibody production.

Example 15: hAd5 S-Fusion + N-ETSD vaccine generates potent neutralizing antibodies as assessed by both cPass and live virus neutralization assays

[00178] Neutralizing antibody activity was evaluated using a cell free assay (cPass) as well as live virus infection *in vitro*. As seen in **FIG.40**, Panel A, the cPass assay showed inhibition of S RBD:ACE2 binding for all mice and ~100% inhibition for two mice at both dilutions of 1:20 and 1:60. The Vero E6 neutralization assay results are shown for the four mice that showed S-specific antibodies by ELISA. The high persistent neutralization seen even at the high dilution factors suggests the intriguing possibility that the bivalent, multi-antigen, multi-epitope generation by hAd5 S-Fusion + N-ETSD vaccine, could result in synergies of neutralizing immune responses (**FIG.40**, Panel B); at epitopes in addition to those associated with RBD-ACE2 binding. As can be

seen in **FIG.40**, Panel B, the value for 50% neutralization (IC50) is present at 1:10,000 serum dilution for the G4 pool of sera from mice that showed S-specific antibodies, ten times higher than the convalescent serum with a dilution of 1:1,000. The potent neutralization, confirmed by two assays, supports the predicted efficacy of the hAd5 S-Fusion + ETSD vaccine candidate and its advancement to clinical trials.

Example 16: hAd5 S-Fusion + N-ETSD generates Th1 dominant responses both in humoral and T-cell immunity

[00179] Antibody Th1 dominance in response to N and S: IgG2a, IgG2b, and IgG3 represent Th1 dominance; while IgG1 represents Th2 dominance. For both anti-S (**FIG.41**, Panel A) and anti-N (**FIG.41**, Panel C) antibodies in sera from hAd5 S-Fusion + N-ETSD vaccinated mice, IgG2a and IgG2b isotypes were predominant and significantly higher compared to the hAd5 Null control. These data show the Th1 dominance of antibody production in response to the hAd5 S-Fusion + N-ETSD vaccine.

[00180] T-cell Th1 dominance in response to N and S: IFN-γ production correlates with CTL activity 47 (Th1 dominance), whereas, IL-4 causes delayed viral clearance 48 (Th2 dominance). A ratio of IFN-γ to IL-4 of 1 is balanced and a ratio greater than 1 is demonstrative of Th1 dominance. ELISpot from animals immunized with the bivalent S plus N vaccine showed IFN-γ secretion was significantly higher for hAd5 S-Fusion + N-ETSD than for hAd5 Null splenocytes in response to both S peptide pool 1 and the N peptide pool (**FIG.42**, Panel A), but IL-4 was only secreted at significantly higher levels for hAd5 S-Fusion + N-ETSD in response to the N peptide pool (**FIG.42**, Panel B).

[00181] The Th1-type predominance is also seen when the ratio of IFN-γ to IL-4 based on spot forming units in response to the combined S peptide pools and the N peptide pool, is considered (**FIG.43**, Panel A). Th1 predominance was seen again in humoral responses, where the ratio based on ng equivalence of Th1 related antibodies (IgG2a, IgG2b, and IgG3) to Th2 related antibodies (IgG1) for both anti-S and anti-N antibodies is greater than 1 in all mice (**FIG.43**, Panel B).

[00182] This Th1 dominant profile of the hAd5 S-Fusion + N-ETSD vaccine candidate provides further justification for hAd5 S-Fusion + N-ETSD to be the lead candidate for clinical testing.

[00183] The hAd5 S-Fusion + N-ETSD vaccine was designed to overcome the risks of an S-only vaccine and elicit both T-cell immunity and neutralizing antibodies, leveraging the vital role

T cells play in generating long-lasting antibody responses and in directly killing infected cells. Both CD4+ and CD8+ T cells are multifunctional, and induction of such multifunctional T cells by vaccines correlated with better protection against infection. Enhanced CD4+ T-cell responses and Th1 predominance resulting from expression of an S antigen optimized for surface display and an N antigen optimized for endosomal/lysosomal subcellular compartment localization and thus MHC I and II presentation, led to increased dendritic cell presentation, cross-presentation, B cell activation, and ultimately high neutralization capability. Furthermore, the potent neutralization capability at high dilution seen for the pooled sera from hAd5 S-Fusion + N-ETSD vaccinated mice, combined with Th1 dominance of antibodies generated in response to both S and N antigens, supports the objective of this vaccine design.

[00184] Contemporaneous MHC I and MHC II presentation of an antigen by the antigen presenting cell activates CD4+ and CD8+ T cells simultaneously and is optimal for the generation of memory B and T cells. A key finding of the construct is that N-ETSD is directed to the endosomal/lysosomal compartment. There N-ETSD elicits a CD4+ response, a necessity for induction of memory T cells and helper cells for B cell antibody production. Others have also reported on the importance of lysosomal localization for eliciting the strongest T-cell IFN-γ and CTL responses, compared to natural N.50,51

[00185] The T-cell responses to the S and N antigens expressed by hAd5 S-Fusion + N-ETSD were polycytokine, including IFN-γ and TNF-a, consistent with successful antimicrobial immunity in bacterial and viral infections. Post-vaccination polycytokine T-cell responses have been shown to correlate with vaccine efficacy, including those with a viral vector. Highly relevant here, polycytokine T-cell responses to SARS-CoV-2 N protein are consistent with recovered COVID-19 patients, suggesting that the bivalent hAd5 S-Fusion + N-ETSD vaccine will provide vaccine subjects with greater protection against SARS-CoV-2.

[00186] In contrast to N, the S protein, here expressed as S-Fusion with confirmed enhanced RBD cell-surface expression and conformational integrity as evidenced by high ACE2-Fc binding, generated predominantly CD8+ T cells. Our results confirmed the vaccine design goal, showing that S-Fusion induced elevated levels of antigen-specific T-cell responses against S compared to S-WT. To ensure MHC presentation to both MHC I (for CD8+ T-cell activation) and MHC II (for CD4+ T-cell activation), it is necessary to vaccinate with both S and N antigens optimized to produce this coordinated response.

[00187] The neutralization data with live SARS-CoV-2 virus demonstrated the potency of the antibody response generated following vaccination with hAd5 S-Fusion + N-ETSD, with evidence of high neutralization even at a high dilution factor. In addition, a striking synergistic effect of pooled sera was evident, with potent neutralization even greater than control convalescent serum at $\geq$ 1:1,000 dilution.

[00188] The hAd5 S-Fusion + N-ETSD construct described above is delivered by a next generation hAd5 [E1-, E2b-, E3-] platform wherein the E2b deletion (pol) alone enables prolonged transgene production and allows homologous vaccination (prime and the boost formulation is the same) in the presence of pre-existing adenoviral immunity.38 In addition to the generation of cellular and humoral immunity by the subcutaneous injection of hAd5 S-Fusion + N-ETSD, the same vaccine in an oral or sublingual formulation may also induce IgA mucosal immunity.

Example 17: Methods and constructs

The hAd5 [E1-, E2b-, E3-] platform and constructs

[00189] For studies herein, the 2nd generation hAd5 [E1-, E2b-, E3-] vector was used (**FIG.44**, Panel A) to create viral vaccine candidate constructs. hAd5 [E1-, E2b-, E3-] backbones containing SARS-CoV-2 antigen expressing inserts and virus particles were produced as previously described. In brief, high titer adenoviral stocks were generated by serial propagation in the E1- and E2b-expressing E.C7 packaging cell line, followed by CsCl2 purification, and dialysis into storage buffer (2.5% glycerol, 20 mM Tris pH 8, 25 mM NaCl) by ViraQuest Inc. (North Liberty, IA). Viral particle counts were determined by sodium dodecyl sulfate disruption and spectrophotometry at 260 and 280 nm and viral titers were determined using the Adeno-X™ Rapid Titer Kit (Takara Bio). The constructs created included:

[00190] S-WT: S protein comprising 1273 amino acids and all S domains: extracellular (1-1213), transmembrane (1214-1234), and cytoplasmic (1235-1273) (Unitprot P0DTC2); S RBD-ETSD: S Receptor Binding Domain (S RBD) with an ETSD (SEQ ID NO:11); N-ETSD: Nucleocapsid (N) with ETSD; S-WT + N-ETSD: S-WT with an Enhanced T-cell Stimulation Domain (ETSD); S-RBD-ETSD + N-ETSD; S-Fusion: S optimized to enhance surface expression and display of RBD; and Bivalent S-Fusion + N-ETSD;

Transfection of HEK 293T cells with hAd5 constructs

[00191] To determine surface expression of the RBD epitope by vaccine candidate constructs, we transfected HEK 293T cells with hAd5 construct DNA and quantified surface RBD by flow cytometric detection using anti-RBD antibodies. There were seven constructs tested: S-WT, S-WT+ N-ETSD, S RBD-ETSD, S RBD-ETSD + N-ETSD, S-Fusion, S-Fusion + N-ETSD, and N-ETSD. HEK 293T cells (2.5 x 10^5 cells/well in 24 well plates) were grown in DMEM (Gibco Cat# 11995-065) with 10% FBS and 1X PSA (100 units/mL penicillin, 100 µg/mL streptomycin, 0.25 ug/mL Amphotericin B) at 37°C. Cells were transfected with 0.5 µg of hAd5 plasmid DNA using a JetPrime transfection reagent (Polyplus Catalog # 89129-924) according to the manufacturer's instructions. Cells were harvested 1, 2, 3, and 7 days post transfection by gently pipetting cells into medium and labeled with an anti-RBD monoclonal antibody (clone D003 Sino Biological Catalog # 40150-D003) and F(ab')2-Goat anti-Human IgG-Fc secondary antibody conjugated with R-phycoerythrin (ThermoFisher Catalog # H10104). Labeled cells were acquired using a Thermo-Fisher Attune NxT flow cytometer and analyzed using Flowjo Software.

Immunocytochemical labeling of hAd5 infected HeLa cells

[00192] To determine subcellular localization of N after infection or transfection of HeLa cells with hAd5 N-wild type (WT) or hAd5 N-ETSD (each with a flag tag to allow labeling), 48 hours after infection or transfection cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.4% Triton X100, in PBS) for 15 min. at room temperature. To label N, cells were then incubated with an anti-flag monoclonal (Anti-Flag M2 produced in mouse, Sigma cat# F1804) antibody at 1:1000 in phosphate buffered saline with 3% BSA overnight at 4°C, followed by washes in PBS and a 1 hour incubation with a goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 555 (Life Technologies, Cat# A32727) at 1:500. For co-localization studies, cells were also incubated overnight at 4oC with a sheep anti-Lamp1 Alexa Fluor 488- conjugated (lysosomal marker) antibody (R&D systems, Cat# IC7985G) at 1:10 or a rabbit anti- CD71 (transferrin receptor, endosomal marker) antibody (ThermoFisher Cat# PA5-83022) at 1:200. After removal of the primary antibody, two washes in PBS and three 3 washes in PBS with 3% BSA, cells were incubated with fluor-conjugated secondary antibodies when applicable at 1:500 (Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, Life technologies, A-11034) for 1 hour at room temperature. After brief washing, cells were mounted with Vectashield Antifade mounting medium with DAPI (Fisher Scientific,

Cat#NC9524612) and immediately imaged using a Keyence all-in-one Fluorescence microscope camera and Keyence software.

Immunoblot analysis of S antigen expression

[00193] HEK 293T cells transfected with hAd5 S-WT, S-Fusion, or S-Fusion + N-ETSD constructs were cultured and transfected as described in the main manuscript and harvested 3 days after transfection in 150 mL RIPA lysis buffer with 1X final Protease Inhibitor cocktail (Roche). After protein assay, equivalent amounts of total protein were loaded into and run on a 4 to 12% gradient polyacrylamide gel (type) and transferred to nitrocellulose membranes using semi-dry transfer apparatus. Anti-Spike S2 (SinoBiological Cat #40590-T62) was used as the primary antibody and IRDye® 800CW Goat anti-Rabbit IgG (H + L) (Li-Cor, 925-32211) as the secondary antibody using the Ibind Flex platform. Antibody-specific signals were detected with an infrared Licor Odyssey instrument.

ACE2-IgG1Fc binding to hAd5 transfected HEK 293T cells

[00194] HEK 293T cells were cultured at 37°C under conditions described above for transfection with hAd5 S-WT, S-Fusion, S-Fusion + N-ETSD, S RBD-ETSD, or S RBD-ETSD + N-ETSD and were incubated for 2 days and harvested for ACE2-Fc binding analysis. Recombinant ACE2-IgG1Fc protein was produced using Maxcyte transfection in CHO-S cells that were cultured for 14 days. ACE2-IgG1Fc was then purified using a MabSelect SuRe affinity column on AKTA Explorer.

[00195] Purified ACE2-IgG1Fc was dialyzed into 10 mM HEPES, pH7.4, 150 mM NaCl and concentrated to 2.6 mg/mL. For binding studies, the ACE2-IgG1Fc was used at a concentration of 1 μg/mL for binding. Cells were incubated with ACE2-Fc for 20 minutes and, after a washing step, were then labeled with a PE conjugated F(ab')2-goat anti-human IgG Fc secondary antibody at a 1:100 dilution, incubated for 20 minutes, washed and acquired on flow cytometer. Histograms are based on normalized mode (NM) of cell count – count of cells positive for signal in PE channel.

Vaccination of CD-1 mice with the hAd5 S-Fusion + N-ETSD vaccine candidate

[00196] CD-1 female mice (Charles River Laboratories) 7 weeks of age were used for immunological studies performed at the vivarium facilities of Omeros Inc. (Seattle, WA). After an initial blood draw, mice were injected with either hAd5 Null (a negative control) or vaccine

candidate hAd5 S- Fusion + N-ETSD on Day 0 at a dose of 1 $\times 10^{10}$ viral particles (VP). There were 5 mice per group. Mice received a second vaccine dose on Day 21 and on Day 28, blood was collected via the submandibular vein from isoflurane-anesthetized mice for isolation of sera and then mice were euthanized for collection of spleen and other tissues.

Splenocyte collection and Intracellular cytokine staining (ICS)

[00197] Spleens were removed from each mouse and placed in 5 mL of sterile medium of RPMI (Gibco Cat # 22400105), HEPES (Hyclone Cat# SH30237.01), 1X Pen/Strep (Gibco Cat # 15140122), and 10% FBS (Gibco Cat # 16140-089). Splenocytes were isolated within 2 hours of collection. ICS for flow cytometric detection of CD8β+ and CD4+ T-cell-associated IFN-γ and IFN-γ/TNFα+ production in response to stimulation by S and N peptide pools.

[00198] Stimulation assays were performed using 10^6 live splenocytes per well in 96-well U-bottom plates. Splenocytes in RPMI media supplemented with 10% FBS were stimulated by the addition of peptide pools at 2 μg/mL/peptide for 6 h at 37°C in 5% CO2, with protein transport inhibitor, GolgiStop (BD) added two hours after initiation of incubation. Stimulated splenocytes were then stained for lymphocyte surface markers CD8β and CD4, fixed with CytoFix (BD), permeabilized, and stained for intracellular accumulation of IFN-γ and TNF-α. Fluorescent-conjugated antibodies against mouse CD8β antibody (clone H35-17.2, ThermoFisher), CD4 (clone RM4-5, BD), IFN-γ (clone XMG1.2, BD), and TNF-α (clone MP6-XT22, BD) and staining was performed in the presence of unlabeled anti-CD16/CD32 antibody (clone 2.4G2). Flow cytometry was performed using a Beckman-Coulter Cytoflex S flow cytometer and analyzed using Flowjo Software.

ELISpot assay

[00199] ELISpot assays were used to detect cytokines secreted by splenocytes from inoculated mice. Fresh splenocytes were used on the same day, as were cryopreserved splenocytes containing lymphocytes. The cells (2-4 x 10^5 cells per well of a 96-well plate) were added to the ELISpot plate containing an immobilized primary antibodies to either IFN-γ or IL-4 (BD), and were exposed to various stimuli (e.g. control peptides, target peptide pools/proteins) comprising 2 μg/mL peptide pools or 10 μg/mL protein for 36-40 hours. After aspiration and washing to remove cells and media, extracellular cytokine was detected by a secondary antibody to cytokine conjugated to biotin (BD). A streptavidin/horseradish peroxidase conjugate was used detect the

biotin-conjugated secondary antibody. The number of spots per well, or per 2-4 x 10^5 cells, was counted using an ELISpot plate reader.

ELISA for detection of antibodies

[00200] For antibody detection in sera from inoculated mice, ELISAs specific for spike and nucleocapsid antibodies, as well as for IgG subtype (IgG1, IgG2a, IgG2b, and IgG3) antibodies were used. A microtiter plate was coated overnight with 100 ng of either purified recombinant SARS-CoV-2 S-FTD (full-length S with fibritin trimerization domain, constructed and purified by ImmunityBio, Inc., 9920 Jefferson Blvd, Culver City, CA 90232), SARS-CoV-2 S RBD (Sino Biological, Beijing, China; Cat # 401591- V08B1-100) or purified recombinant SARS-CoV-2 nucleocapsid (N) protein (Sino Biological, Beijing, China; Cat # 40588-V08B) in 100 µL of coating buffer (0.05 M Carbonate Buffer, pH 9.6). The wells were washed three times with 250 µL PBS containing 1% Tween 20 (PBST) to remove unbound protein and the plate was blocked for 60 minutes at room temperature with 250 µL PBST. After blocking, the wells were washed with PBST, 100 µL of diluted serum samples were added to wells, and samples incubated for 60 minutes at room temperature. After incubation, the wells were washed with PBST and 100 µL of a 1/5000 dilution of anti-mouse IgG HRP (GE Health Care; Cat # NA9310V), or anti-mouse IgG1 HRP (Sigma; Cat # SAB3701171), or anti- mouse IgG2a HRP (Sigma; Cat # SAB3701178), or anti-mouse IgG2b HRP (Sigma; catalog# SAB3701185), or anti-mouse IgG3 HRP conjugated antibody (Sigma; Cat # SAB3701192) was added to wells. For positive controls, a 100 µL of a 1/5000 dilution of rabbit anti-N IgG Ab or 100 µL of a 1/25 dilution of mouse anti-S serum (from mice immunized with purified S antigen in adjuvant) were added to appropriate wells. After incubation at room temperature for 1 hour, the wells were washed with PBS-T and incubated with 200 µL o-phenylenediamine-dihydrochloride (OPD substrate (Thermo Scientific Cat # A34006) until appropriate color development. The color reaction was stopped with addition of 50 µL 10% phosphoric acid solution (Fisher Cat # A260-500) in water and the absorbance at 490 nm was determined using a microplate reader (SoftMax® Pro, Molecular Devices).

Calculation of relative µg amounts of antibodies

[00201] A standard curve of IgG was generated, and absorbance values were converted into mass equivalents for both anti-S and anti-N antibodies. hAd5 S-Fusion + N-ETSD vaccination generated a geometric mean value of 5.8 µg S-specific IgG and 42 µg N-specific IgG per milliliter of serum.

cPassTM Neutralizing Antibody Detection

[00202] The GenScript cPass™ for detection of neutralizing antibodies was used according to the manufacturer's instructions.44 The kit detects circulating neutralizing antibodies against SARS- CoV-2 that block the interaction between the S RBD with the ACE2 cell surface receptor. It is suitable for all antibody isotypes and appropriate for use with in animal models without modification.

Vero E6 cell neutralization assay

[00203] All aspects of the assay utilizing virus were performed in a BSL3 containment facility according to the ISMMS Conventional Biocontainment Facility SOPs for SARS-CoV-2 cell culture studies. Vero e6 kidney epithelial cells from Cercopithecus aethiops (ATCC CRL-1586) were plated at 20,000 cells/well in a 96-well format and 24 hours later, cells were incubated with antibodies or heat inactivated sera previously serially diluted in 3-fold steps in DMEM containing 2% FBS, 1% NEAAs, and 1% Pen-Strep; the diluted samples were mixed 1:1 with SARS-CoV-2 in DMEM containing 2% FBS, 1% NEAAs, and 1% Pen-Strep at 10,000 TCID 50/mL for 1 hr. at 37°C, 5% CO2. This incubation did not include cells to allow for neutralizing activity to occur prior to infection. The samples for testing included sera from the four mice that showed > 20% inhibition of ACE2 binding in cPass, pooled sera from those four mice, sera from a COVID-19 convalescent patient, and media only. For detection of neutralization, 120 µL of the virus/sample mixture was transferred to the Vero E6 cells and incubated for 48 hours before fixation with 4% PFA. Each well received 60 µL of virus or an infectious dose of 600 TCID50. Control wells including 6 wells on each plate for no virus and virus-only controls were used. The percent neutralization was calculated as 100-((sample of interest-[average of "no virus"])/[average of "virus only"])*100) with a stain for CoV-2 Np imaged on a Celigo Imaging Cytometer (Nexcelom Bioscience).

Vaccine Characterization

[00204] The majority of current prophylactic anti-SARS-CoV-2 vaccines under development are designed to prevent further disease-related mortality and morbidity by targeting the viral spike (S) protein with the goal of generating neutralizing antibody responses in recipients prior to viral exposure. However, recent characterization of COVID-19 patient immune responses to SARS-CoV-2 indicates that other immune cells such as T cells are critical to clearing infection and

producing long-term immunity to coronavirus infections. Both CD4+ and CD8+ T cells underpin durable humoral responses because CD4+ T cells, while not effector cells like CD8+ T cells, are critical to the generation of robust and long-lasting immunity afforded by antibody-secreting plasma cells and the elimination of infected cells by memory cytotoxic CD8+ T cells.The presently disclosed dual-antigen candidate vaccine more broadly activates the immune system to combat SARS-CoV-2, by the inclusion of a modified viral nucleocapsid (N) antigen, a potent CD4+ and CD8+ T cell target, along with an optimized S protein (S-Fusion) to stimulate humoral responses.

[00205] The human adenovirus serotype 5 (hAd5) E1, E2b, and E3 region-deleted [E1-, E2b-, E3-] vaccine platform (**FIG.44**, Panel A) is superior to the adenovirus platforms used in other COVID-19 vaccines currently in clinical trials because it is effective in the presence of pre-existing adenovirus immunity and has a reduced likelihood of generating a vector-targeted host immune response, thus can be used as both the prime and boost. Using this platform, the vaccine comprises the optimized S surface protein, S-Fusion, to increase cell-surface display and humoral responses; as well as the highly conserved and antigenic N protein found within the viral particle, here with subcellular compartment targeting sequences for enhanced antigen presentation. This strategy will be safe and robust in eliciting humoral and T cell responses to SARS-CoV-2. The addition of N addresses the risk of loss of vaccine efficacy for S-only monovalent vaccines due to the emergence of mutations in S in the population over time. In contrast, N is highly conserved with a lower risk of mutation, while also being highly immunogenic. It is a known target antigen for natural immunity, with antibodies and T cells against N being found in the majority of persons recovered from SARS-CoV-2 and similar virus SARS-CoV infections. In the present vaccine, the Enhanced T cell Stimulation Domain (ETSD) directs N protein to the endosomal-lysosomal subcellular compartment after translation to support MHC class II presentation for T helper cell activation and promotion of CD8+ T cell activation through dendritic cell licensing. Despite the risk of emerging mutations, S remains a key antigen for vaccination due to its role in infection. Spike, displayed as a trimer on the viral surface (**FIG.44**, Panel B), has a Receptor Binding Domain (RBD) that interacts with host angiotensin-converting enzyme 2 (ACE2) to facilitate entry into the host cells and propagation, thus antibodies against S are key to neutralization of infection. Antibodies against S RBD are commonly found in patients recovered from COVID19 51 as are antibodies against other S epitopes. In the bivalent vaccine construct, S-Fusion is S optimized by addition of a fusion linker to display the S RBD in a physiologically relevant form on the cell surface with the goal of improving generation of anti-S RBD antibodies that will be virus neutralizing.

[00206] The presently disclosed bivalent hAd5 S-Fusion + N-ETSD vaccine generates excellent T-cell responses. Vaccines currently in clinical trials focus on generating humoral responses as a means to neutralize infection. However, given that antibodies, even if successfully generated and sufficiently neutralizing, may wane over time, the T cell response becomes critical. If T cell responses are absent due to virus-induced lymphopenia, even in the presence of abundant neutralizing antibodies, an infected person is at risk for developing acute symptoms of the disease. While it cannot be excluded that S (and other viral proteins) can induce T-cell responses, the evidence in the literature support a key role for N. Not only have T cell responses to N been found in the majority of patients recovered from COVID-19, these responses to N in patients exposed to very similar virus SARS-CoV are remarkably durable. Compelling evidence of the importance of N in natural T cell immunity can be found in the recent report of Ferretti et al. who found, using an unbiased genome-wide screen for the precise peptide sequences recognized by memory CD8+ T cells of COVID-19 patients, that only 3 of the 29 shared epitopes were from the spike protein, whereas the highest density of epitopes was located in the nucleocapsid protein. Thus, in the hAd5 S-Fusion + N-ETSD vaccine, N is expected to not only elicit a humoral response, but also a T-cell response that better recapitulates disease-limiting natural immunity.

[00207] In an initial report, hAd5 S-Fusion + N-ETSD vaccine provided enhanced cell-surface expression of S RBD that was readily recognized by ACE2, reflecting its conformational integrity. N-ETSD with endosomal/lysosomal localization for enhanced antigen presentation generated both neutralizing antibody and CD4+/CD8+ T-cell-mediated responses with Th1 predominance in inoculated mice. The present disclosure extends those findings using plasma and monocyte-derived dendritic cells (MoDCs) from previously SARS-CoV-2 infected patients to confirm native S antigen expression. We elucidate N-ETSD localization in antigen-presenting MoDCs, showing that it localizes to endosomes, lysosomes, and autophagosomes. Protein processing through this subcellular pathway enhances MHC class II presentation and increases peptide recycling to also enable MHC class I presentation. By localizing the nucleocapsid protein to the late lysosome-autophagosome compartment both CD4+ and CD8+ SARS-CoV-2 specific memory T cells are recalled from patients previously infected with SARS-CoV-2. Moreover, in these immune-response recall studies, *in vitro*, hAd5-infected MoDCs presenting S-Fusion and N-ETSD elicit a predominant Th1 response from autologous memory T cells of previously SARS-CoV-2 infected patients. N in particular drives the CD8+ T cell responses in *in vitro* recall studies. Recapitulation of natural infection and immunity, to the degree it can be achieved by vaccination, by the hAd5 S-Fusion + N-ETSD vaccine makes it a prime candidate for clinical testing of its ability to protect

individuals from SARS-CoV-2 infection and COVID-19 and this second-generation vaccine construct has now entered into Phase I clinical trials.

Example 18: The hAd5 [E1-, E2b-, E3-] platform and constructs

[00208] For studies here, the next generation hAd5 [E1-, E2b-, E3-] vector was used (**FIG.44**, Panel A) to create viral vaccine candidate constructs. A variety of constructs were created: **FIG.44**, Panel C: S WT: S protein comprising 1274 amino acids and all S domains: extracellular (1-1213), transmembrane (1214-1234), and cytoplasmic (1235-1273) (Unitprot P0DTC2); **FIG.44**, Panel D: S-Fusion: S optimized to enhance surface expression and display of RBD; **FIG.44**, Panel E: N (N without ETSD): Nucleocapsid (wild type) sequences with tags for immune detection, but without ETSD modification, and predominantly cytoplasmic localization. **FIG.44**, Panel F. N with the Enhanced T cell Stimulation Domain (N-ETSD): Nucleocapsid (wild type) with ETSD to direct lysosomal/endosomal localization and tags for immune detection; and **FIG.44**, Panel G: The Bivalent hAd5 S-Fusion + N-ETSD vaccine.

[00209] Nucleocapsid antigen engineered with an Enhanced T cell Stimulation Domain (ETSD) directs N to endosomes, lysosomes and autophagosomes in MoDCs, driving enhanced CD4+ T-cell activation:

[00210] The hAd5 bivalent vaccine construct includes sequences designed to target N to MHC class II antigen loading compartments. To further investigate factors that affect antigen presentation in MoDCs, MoDCs from healthy subjects were infected with hAd5 N-ETSD or hAd5 N and localization was determined by immunocytochemistry. N-ETSD showed localization to discrete vesicles, some coincident with CD71, a marker of recycling endosomes (**FIG.45**, Panels A-C), and LAMP-1, a marker for late endosome/lysosomes (**FIG.45**, Panels G-I), whereas N was expressed diffusely and uniformly throughout the cytoplasm (**FIG.45**, Panels D-F and J-L). Lysosomes fuse with autophagosomes to enhance peptide processing and MHC class II presentation. N-ETSD also displayed some co-localization with the autophagosome marker (**FIG.45**, Panels M-O). Protein processing in autophagosomes plays a key role in MHC-mediated antigen presentation in DCs, providing a potential mechanism of enhanced CD4+ T cells induced by N-ETSD in the vaccine construct. Evidence of this T cell interaction with a MoDC infected with N-ETSD translocated to autophagosomes (and, it is assumed, also endosomes and lysosomes) is seen in this phase-contrast microscopy of the N-ETSD and LC3a/b co-labeled cells, which reveals the elongated DC morphology in contrast to the spherical morphology of undifferentiated

lymphocytes. Lymphocytes, also distinguished by the absence of infection by hAd5 N-ETSD (lymphocytes lack the hAd5 receptor), were also seen to interact with N-ETSD-expressing MoDCs.

[00211] Validation of SARS-CoV-2 antibody and cell-mediated immune responses from previously SARS-CoV-2 infected patients and virus-naïve patients for memory T cell recall studies:

[00212] For the studies described below, plasma samples were collected from four individuals convalescing from SARS-CoV-2 infection as confirmed by antibody assays and patient history as described below. The presence of anti-Spike IgG, and of neutralizing antibodies by both the cPass 66 and live virus assays, were confirmed in all patient samples. Samples were also collected from four virus-naïve individuals and were used as controls. In additional studies to validate immune responses to SARS-CoV-2 antigens, the binding of previously SARS-CoV-2 infected patient and virus-naïve control individual plasma to human embryonic kidney (HEK) 293T cells transfected with either hAd5 S-Fusion alone or hAd5 S-Fusion + N-ETSD was assessed. This binding reflects the presence of antibodies in plasma that recognize antigens expressed by the hAd5 vectored vaccines. Quantification of histograms showed little or no binding of virus-naïve plasma antibodies to cells expressing either construct, and the highest binding of plasma antibodies from a previously SARSCoV-2 infected patient to cells expressing the bivalent S-Fusion + N-ETSD construct. This could be due to either the enhanced cell surface expression of S found in hAd5 S-Fusion + N-ETSD infected HEK 293T cells as compared to hAd5 S-Fusion alone or expression of both S and N antigens.

Example 19: N-ETSD optimizes spike antigen expression: binding of plasma antibodies from previously infected SARS-CoV-2 patients is enhanced for hAd5 S-Fusion + N-ETSD infected MoDCs compared to hAd5 S-Fusion or hAd5 S-WT.

[00213] The studies herein focus on the responses of T cells from previously SARS-CoV-2 infected patients to hAd5 vaccine construct-infected autologous MoDCs. DCs are powerful antigen presenting cells for processing and presenting complex antigens acquired through infection or phagocytosis to elicit a T-cell response. Therefore, in addition to the assessment of binding of patient plasma antibodies to hAd5 vaccine expressing HEK 293T cells, MoDCs from two healthy individuals were infected overnight with hAd5 S-WT, hAd5 S-Fusion, hAd5 S-Fusion + N-ETSD, or hAd5 Null then evaluated gene expression using plasma from a previously SARS-CoV-2

infected patient (**FIG.46**, Panel A). For both MoDC sources, the highest binding of plasma antibodies from a previously infected patient to the MoDCs was seen after hAd5 S-Fusion + N-ETSD infection (**FIG.46**, Panels B-D), providing further evidence that antigen expression is optimized in the hAd5 S-Fusion + N-ETSD bivalent vaccine. This finding in a highly relevant *in vitro* system of human MoDCs and plasma is not only an important confirmation of results from testing with HEK 293T cells and commerciallyavailable anti-S RBD antibodies, it represents a potential method to screen plasma for SARS-CoV-2 antigen reactivity.

Example 20: SARS-CoV-2 peptide pool immune reaction:

[00214] T cells from previously infected SARS-CoV-2 patients secrete significant levels of interferon-g (IFN-γ) in response to S1, S2, and N SARSCoV-2 peptide pools compared to T cells from virus-naïve controls To demonstrate the reactivity of T cells from four previously infected SARS-CoV-2 patients versus virus-naive T cells from four unexposed individuals, T cells from each group were cultured with autologous MoDCs pulsed with peptide mixes spanning the sequences of N and S proteins. T cells from previously infected SARS-CoV-2 patients but not unexposed subjects secreted IFNg in response to SARS-CoV-2 antigens (**FIG.47**), validating selective reactivity of T cells from patients previously infected with SARS-CoV-2.

Example 21: SARS-CoV-2 peptide pool immune reaction: CD4+ T cells from previously infected SARSCoV-2 patients recognize S and N peptide pool antigens, but CD8+ T cells display greater recognition of N peptide antigens.

[00215] CD4+ T cells of the two patient samples tested responded to both the S and N peptide pools, with a higher response to N by Pt3 (**FIG.48**, Panel B). In contrast, CD8+ T cells from both patients responded to N with high significance, but not to S1 or S2 peptide pools (**FIG.48**, Panels C and D). These data are consistent with published studies demonstrating T-cell responses against multiple antigens, including S and N in previously infected SARS-CoV-2 patients.

Example 22: Autologous MoDCs infected with endo/lysosome-directed nucleocapsid-ETSD elicit higher levels of IFN-γ secretion from CD4+ and CD8+ T cells from previously infected SARS-CoV-2 patients compared to cytoplasmic nucleocapsid protein (hAd5 N).

[00216] To evaluate the immune significance of endo/lysosome-localized N-ETSD versus cytoplasmic N, MoDCs were infected with hAd5 constructs (Null, N-ETSD or N) then incubated with autologous CD3+ and CD4+- or CD8+-selected T cells (**FIG.49**, Panel A). CD3+ T cells

from previously infected SARS-CoV-2 patients showed significantly greater IFN-γ secretion in response to NETSD than both Null and cytoplasmic N in the two patients where N-ETSD and N were compared (**FIG.49**, Panels C and D). There were relatively few interleukin-4 (IL-4) secreting CD3+ T cells for all patients (**FIG.49**, Panels E-G). Both CD4+ and CD8+ selected T-cell populations showed significantly greater IFN-γ responses to N-ETSD than Null (**FIG.49**, Panels H-M) and in two of three patients, CD4+ and CD8+ T cells showed greater recognition of N-ETSD compared to N. The high IFN-γ and low IL4 responses indicate a predominant Th1 cytokine response to N/N-ETSD. Cell expression of NETSD and N were equivalent in 293T HEK cells, suggesting the reason for the elevated T-cell response to N-ETSD was likely processing and MHC loading of the epitopes.

Example 23: Th1 dominant SARS-CoV-2 specific CD4+ and CD8+ memory T-cell recall to nucleocapsid and spike antigens is induced by hAd5 S-Fusion + N-ETSD infection of autologous MoDCs from previously SARS-CoV- 2 infected patients.

[00217] N-ETSD is more effective than N in eliciting patient T-cell cytokine responses. For total T cells (CD3+), IFN-γ responses were similar for S-Fusion + N-ETSD and N-ETSD with responses to S-Fusion being relatively low (**FIG.50**, Panels A-C). The number of IL-4 secreting T cells was very low for all (**FIG.50**, Panels D-F). Based on the increased expression of S in the bivalent vaccine compared to monovalent S-Fusion, the increased T-cell response could be explained by either T cells recognizing increased S or the presence of N. Importantly, these T-cell responses were characterized by a predominance of IFN-γ (Th1) relative to IL-4 (Th2). CD4+ T cells from all three patients showed significantly greater recognition of all three constructs compared to Null (**FIG.50**, Panels G-I). While there were greater responses to specific constructs in some individuals, overall the responses to S-Fusion, S-Fusion + N-ETSD and N-ETSD were similar. CD8+ T cells from all three patients recognized the bivalent and N-ETSD vaccines at a significantly higher level than Null; in only two of three patients did CD8+T cells recognize S-Fusion to a significant degree above Null (**FIG.50**, Panels J-L). These data indicate that T cells from previously infected SARS-CoV-2 patients have reactivity and immune memory recall to both of the vaccine antigens (S and N) in the vaccine vector.

[00218] One feature of S-Fusion is the higher expression of S RBD compared to S-WT. This was a goal of the vaccine design based on findings from earlier studies of S cryo-electron micrograph structures that suggested RBD epitopes would be largely unavailable for immune

detection. A further advantage accrues from combining S with N in the bivalent vaccine, through the ability of N to enhance immune detection of S, a phenomenon that has been observed by others for gene expression in general. The vaccine-expressed N protein traffics to the endosomal/lysosomal subcellular compartments, a key antigen presenting pathway to stimulate CD4+ T cells so that they can license dendritic cells to activate naïve CD8+ CTL. N-ETSD localizes to endosomes and lysosomes, as well as to autophagosomes, in MoDCs. Both endosomal and lysosomal targeting are desirable for enhanced antigen presentation and CD4+ T-cell activation. Lysosomes can fuse acidic autophagosomes, facilitating protein processing; this has important implications for effective immune stimulation by modulation of MHC class II presentation. T cells of previously infected SARS-CoV-2 patients more readily recognized N-ETSD than N. The data presented here strongly support the potential of enhanced efficacy of a vaccine construct specifically expressing the modified N-ETSD.

[00219] T cells are critical for elimination of SARS-CoV infection. 36,74-78 Here, hAd5 expressed S and N elicited strong antigen-specific IFN-γ, but virtually no IL-4 secretion from T cells of previously infected SARS-CoV-2 patients, pointing to Th1 dominance. Antiviral Th1 cytokine responses eliminate a variety of viruses from infected hosts 79 including the virus closely related to SARSCoV-2, SARS-CoV. 80 These data are also consistent with the studies in preclinical models. Importantly, the data suggests that both S and N are targets of CD4+ T cells that help both antibody production from B cells and CD8+ T cell memory, which together function to kill virus infected targets. The recognition of these vaccine antigens by the T-cell subsets are consistent with immune control of the pathogen. Intriguingly, the hAd5 S-Fusion + N-ETSD T-cell biased vaccine has the potential to not only provide protection for uninfected patients, but also to be utilized as a therapeutic for already infected patients to induce rapid clearance of the virus by activating T cells to kill the virus-infected cells, thereby reducing viral replication and lateral transmission. Importantly, the T cell recall of N-ETSD was shown to be Th1 dominant as shown by the vigorous interferon-g response and the low IL-4 response.

Methods and constructs used above

Example 25: The hAd5 [E1-, E2b-, E3-] platform and constructs

For studies herein, the 2nd generation hAd5 [E1-, E2b-, E3-] vector was used (**FIG.44**, Panel A) to create viral vaccine candidate constructs. hAd5 [E1-, E2b-, E3-] backbones containing SARSCoV-2 antigen expressing inserts and virus particles were produced as previously described.

In brief, high titer adenoviral stocks were generated by serial propagation in the E1- and E2b expressing E.C7 packaging cell line, followed by CsCl2 purification, and dialysis into storage buffer (2.5% glycerol, 20 mM Tris pH 8, 25 mM NaCl) by ViraQuest Inc. (North Liberty, IA). Viral particle counts were determined by sodium dodecyl sulfate disruption and spectrophotometry at 260 and 280 nm. Viral titers were determined using the Adeno-X™ Rapid Titer Kit (Takara Bio). The constructs created included: i. S-WT: S protein comprising 1273 amino acids and all S domains: extracellular (1-1213), transmembrane (1214-1234), and cytoplasmic (1235-1273) (Unitprot P0DTC2); ii. S Fusion: S optimized to enhance surface expression and display of RBD; iii. N: Nucleocapsid (N) wild type sequence protein containing tags for immune detection; iv. N-ETSD: N with an Enhanced T-cell Stimulation Domain (ETSD) together with tags for immune detection; and v. Bivalent S-Fusion + N-ETSD;

[00220] Collection of plasma and peripheral blood mononuclear cells from patients with confirmed previous SARS-CoV-2 infection and from virus-naïve volunteers: Blood was collected with informed consent via venipuncture from volunteers who had either not been exposed (UNEX) to SARS-CoV-2 as confirmed by ELISA and multiple negative SARSCoV-2 tests or who had recovered from COVID-19 as indicated by recent medical history and a positive SARS-CoV-2 antibody test (Patients, Pt). A third source of whole blood was apheresis of healthy subjects from a commercial source (HemaCare). Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation and plasma was collected after density gradient centrifugation.

[00221] Monocyte-derived dendritic cells (MoDC) were differentiated from PBMC using GM-CSF (200U/ml) and IL-4 (100U/ml) as previously described86. Briefly, monocytes were enriched by adherence on plastic, while the non-adherent cells were saved and frozen as a source of lymphocytes, specifically T cells. Adherent cells were differentiated into dendritic cells (3-5 d in RPMI containing 10% FBS), then frozen in liquid nitrogen for later use. T cells were enriched from the non-adherent fraction of PBMC using MojoSort (BioLegend CD3 enrichment). CD4+ and CD8+ T cells were enriched using analogous kits from the same manufacturer. Efficiency of the cell separations was evaluated by flow cytometry.

[00222] Infection of MoDCs with hAd5 N-WT or N-ETSD and labeling with anti-N, anti-CD71, antiLAMP-1, and Anti-LC3a/b antibodies: Freshly thawed MoDCs were plated on 4-well Lab-Tek II CC2 Chamber Slides, using 3×10^4 cells per well and transduction performed at MOI

5000 one hour after plating using hAd5 N-ETSD or hAd5 N. Slides were incubated o/n at 37 C, fixed in 4% paraformaldehyde for 15 minutes, then permeabilized with 1% Triton X100, in PBS) for 15 min. at room temperature. To label N, cells were then incubated with an anti-flag monoclonal (Anti-Flag M2 produced in mouse) antibody at 1:1000 in phosphate buffered saline (PBS) with 3% BSA, 0.5% Triton X100 and 0.01% saponin overnight at 4 C, followed by three washes in PBS and a 1 hour incubation with a goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 555 (Life Technologies) at 1:500. For co-localization studies, cells were also incubated overnight at 4 C with a rabbit anti-CD71 (transferrin receptor, recycling/sorting endosomal marker) antibody (ThermoFisher) at 1:200; sheep anti-Lamp1 Alexa Fluor 488-conjugated (lysosomal marker) antibody (R&D systems) at 1:10; or a rabbit monoclonal anti human LC3a/b (Light Chain 3, autophagy marker) antibody (Cell Signaling Tech #12741S) used at 1:100. After removal of the primary antibody, two washes in PBS and three washes in PBS with 3% BSA, cells were incubated with fluor-conjugated secondary antibodies when applicable at 1:500 (Goat anti-Rabbit IgG (H+L) secondary antibody, Alexa Fluor 488; 1:500 dilution) for 1 hour at room temperature. After brief washing, cells were mounted with Vectashield Antifade mounting medium with DAPI (Fisher Scientific) and immediately imaged using a Keyence all-in-one Fluorescence microscope camera and Keyence software.

[00223] Binding of plasma antibodies from previously SARS-CoV-2 infected patients to antigens expressed by vaccine-infected MoDCs: Binding of plasma antibodies from previously infected subjects to antigens expressed on the surface of MoDCs was determined by differentiation of MoDCs from peripheral blood mononuclear cells (PBMC) to DC, infection of the MoDCs, incubation with previously infected patient plasma, and detection of binding to infected and uninfected MoDCs by flow cytometry.

[00224] MoDCs were infected (0.5 x 106/well in 12 well plates) at MOI 5000 using hAd5 S-WT, SFusion, S-Fusion + N-ETSD or a 'Null' construct that expresses green fluorescent protein (GFP). One day after infection, the MoDCs were detached using EDTA (0.5 mM), gently pipetted and transferred for incubation with previously infected patient plasma at 1:100 dilution from a single patient (Pt4) at (4°C) for 30 minutes, and plasma antibodies detected on the MoDC surface by goat anti-human IgG (phycoerythrin conjugated). Cells were acquired as described above for flow cytometric analyses. Data were graphed as the DMFI, that is, the difference in binding between infected and uninfected MoDCs.

[00225] Previously SARS-CoV-2 infected patient and virus-naïve individual T cell and selected CD4+ and CD8+ T cell secretion of IFN-γ in response to MoDCs pulsed with SARS-CoV-2 peptide antigen pools

[00226] The ability of T cells from previously infected patients used in these studies to recognize SARS-CoV-2 antigens *in vitro* was validated and then similar analyses were performed for selected CD4+ and CD8+ T cells. Briefly, MoDC (2 x 104) were pulsed with SARS-CoV-2 peptide antigens (1μg/ml, PepMix S comprising the S1 and S2 pools PM-WCPV-S-1; and N PM-WCPVNCAP-1, both JPT Peptide Technologies) then autologous T cells (1 x 10^5), enriched from the non-adherent fraction of PBMC using MojoSort (BioLegend CD3 enrichment) were added in enriched RPMI (10% human AB serum). Cells were cultured in a microtiter plate (Millipore) containing an immobilized primary antibody to target IFN-γ, overnight (37°C), then IFN-γ spot forming cells enumerated by ELISpot. For ELISpot detection, after aspiration and washing to remove cells and media, IFN-γ was detected by a secondary antibody to cytokine conjugated to biotin. A streptavidin/horseradish peroxidase conjugate was used detect the biotin-conjugated secondary antibody. The number of spots per well (1 x 10^5 cells), was counted using an ELISpot plate reader. IL-4 was measured by ELISpot using a kit (MabTech) with wells precoated with antiIL-4 antibody and following the manufacturer's instructions. Remaining steps for IL-4 detection were identical to those for IFN-γ, but with alkaline phosphatase detection rather than peroxidase.

[00227] Determination of previously SARS-CoV-2 infected patient-derived T-cell reactivity in response to autologous hAd5 vaccine-infected MoDCs: MoDCs were infected with hAd5 S-Fusion, S-Fusion + N-ETSD, N-ETSD, N or GFP/Null constructs and incubated overnight at 37°C. The infected MoDCs were cultured with CD3+, CD4+, or CD8+ T-cells from the same individuals overnight. Antigen specific T-cell responses were enumerated using ELISpot as described above.

AdV/Yeast Combination and Yeast Lysate Formulations

[00228] In addition to viral constructs expressing N-ETSD and/or S-Fusion (or proteins with similarity to N-ETSD and/or S-Fusion), the N-ETSD and/or S-Fusion (or proteins with similarity to N-ETSD and/or S-Fusion) can also be expressed in yeast, either on yeast cells that present the antigen(s) or within yeast cells that may be prepared as lysates to so further enhance immunogenicity as is discussed in more detail below.

[00229] **FIG.12** depicts a conceptual illustration of an ideal vaccine that will elicit durable and effective immunity across multiple pathways. As can be readily seen, a vaccine composition is preferably (but not necessarily) composed of a yeast vaccine composition and a viral vaccine composition, optionally in concert with a macrophage polarizing agent such as RP182 and an immune stimulatory cytokine or cytokine analog (*e.g.*, N-803).

[00230] With regard to the yeast component it is most typically preferred that the yeast is a recombinant yeast that expressed from a recombinant nucleic acid one or more antigens of interest, and possibly further DAMP or PAMP or STING pathway signals. The so produced recombinant yeast is typically heat inactivated, and after heat-inactivation the yeast is lysed. Most preferably, the yeast is lysed using pressure homogenization and the homogenate is then clarified, preferably via filtration. However, it should be appreciated that other methods of lysing are also deemed suitable, including enzymatic or chemical lysing of the cells wall, sonication, flash-freezing/thawing, etc. Likewise, clarification of the lysate may also include centrifugation, settling, flocculation, etc.

[00231] As will be readily appreciated, the recombinant antigen and other recombinant proteins can be expressed from any suitable promotor using known expression cassettes. For example, **FIG.13** depicts results of an exemplary expression experiment where the SARS CoV nucleocapsid protein (N) is overexpressed in *Saccharomyces cerevisiae*. As can be seen, significant quantities of the recombinant protein were expressed in the yeast.

[00232] Using a mouse model, lysed tarmogen (yeast with recombinantly expressed antigen) was up to 150-fold more immunogenic than intact tarmogen with regard to induction of T cell responses (based on numerous antigen specific T cell activation studies in hundreds of mice). Advantageously, the lysed yeast has a favorable safety profile and 10,000 10 YU doses can be prepared in 2 hours one small-scale pressure homogenizer. Intact yeast are Th1-Th17 inducing and trigger IFNγ, IL2, IL12, IL6, TNFα, GM-CSF, while yeast lysates clearly induced strong Th1 effects and diminished Th17. Therefore, intact or lysed yeast will induce coronavirus-specific antibodies (see *e.g.*, doi.org/10.1016/j.jaut.2005.01.008, doi.org/10.1155/2016/4131324, or sfamjournals.onlinelibrary.wiley.com/doi/pdf/10.1111/lam.12188).

Formulations for Oral/Mucosal Administration

[00233] In further embodiments, disclosed herein are compositions of and methods for producing a vaccine composition using aragonite to form a solid dosage form (*e.g.*, powder, tablet, or capsule) that is stable during storage, easily administered (*e.g.*, oral administration), and dissolves (*e.g.*, releases the antigenic or pre-antigenic vaccine molecule(s)) after passing through the stomach of the subject receiving the vaccine.

[00234] In particular, the present disclosure is directed to an aragonite composition made of a plurality of aragonite particles loaded with vaccine active ingredient(s) rendering a solid dosage vaccine in the form of a powder, tablet, or capsule. More specifically, the vaccine composition may include a powder form (*e.g.*, lyophilized) recombinant expression construct for expressing a corresponding antigen to the relevant infection/disease that has been blended with and thereby loaded on, the surface of the plurality of aragonite particles. In exemplary embodiments, the vaccine composition immunizes against a coronavirus. Preferably, the recombinant expression construct is an adenovirus construct expressing at least one antigenic coronavirus protein or protein fragment.

[00235] Notably, the use of aragonite in the presently contemplated solid dosage form allows for cost effective manufacturing and easy administration of a stable vaccine composition. As such, the presently contemplated vaccine tablet or capsule can be mass produced and easily transported. Furthermore, the solid dosage form allows for oral administration which for most persons can be self-administered without the need for a healthcare professional. The tablet forms may also be made with additional excipients and/or additives (*e.g.*, flavors and gelatins) to form a lozenge.

[00236] Aragonite (*e.g.*, oolitic aragonite) is one of the purest forms of naturally precipitated calcium carbonate. With reference to **FIG.1**, aragonite has a crystalline morphology of orthorhombic, bipyramidal, characteristically needle-shaped crystals, and as such is distinct from calcite and vaterite. Aragonite can be processed to recrystallize and/or reform in various shapes, such that it can be used for various purposes that take advantage of the mechanical and chemical properties of the calcium carbonate minerals. Aragonite particles as disclosed herein are solid matter having a regular (*e.g.*, spherical, or ovoid) or irregular shape. As used herein, aragonite particles have an average particle size of between 100 nm to 1 mm. Methods for milling aragonite particles are described in US 2020/0308015, the entire contents of which are herein incorporated by reference. For example, methods for milling aragonite particles are disclosed of 2.0 to 3.5

micron size with a clean top size. A clean top size means that very few particles are larger than the 3.5 micron size when produced using the disclosed milling method with a classifier set at 2.0 to 3.5 micron or 2.5 to 3.5 micron size range. Accordingly, aragonite particles as disclosed herein using the methods of U.S. 2020/0308015 have a cleaner top size than conventional GCC.

[00237] Aragonite's adsorption capacity is a function of three parameters: (1) surface charge (also known as "ζ (zeta) potential"); (2) surface area/void ratio; and (3) particle solubility. By accurately measuring these three parameters, one can determine what materials will adsorb to aragonite particle surfaces under given conditions. Notably, the zeta potential of aragonite increases the stability of surfactants such as glycerol and sorbitol.

[00238] Furthermore, aragonite has a naturally high number of measurable pores in particles with diameters less than 2 nm (*i.e.*, a high "microporosity"). *See, e.g.*, EP 2719373. As such, the aragonite platform grips active ingredient particles strongly together allowing for the loaded aragonite to be formulated in a solid dosage form—*e.g.*, powder, tablets, or capsules.

[00239] Advantageously, untreated aragonite has a neutral pH (7.8 to 8.2), a natural hydrophilic nature, electron charge (zeta potential), and already created nitrogenous pairing with amino acids and proteins. Without being bound by any one theory, these advantageous properties of aragonite render aragonite metastable under ambient conditions. More specifically, aragonite particles naturally include approximately 2-3% amino acid content, the majority of which are aspartic acid (approximately 25 to 30%) and glutamic acid (approximately 8 to 10%) rendering the aragonite surface hydrophilic. *See, e.g.*, Mitterer, 1972, *Geochimic et Cosmochimica Acta*, 36: 1407-1422. Accordingly, in some embodiments a vaccine composition (*e.g.*, recombinant adenovirus) is coupled directly to the natural, untreated surface of aragonite particles.

[00240] Currently, calcium carbonate utilized in the marketplace is processed as or from ground calcium carbonate (GCC), precipitated calcium carbonate (PCC) (synthesized), and/or limestone production. The product produced is a commodity grade with different attributes. To get a clean particle sized distribution (PSD) top size and low retain, most companies utilize a wet grinding process by either high solids or low solids. As used herein, aragonite refers to naturally occurring aragonite having a crystalline morphology of orthorhombic, bipyramidal, and characteristically needle-shaped crystals that is distinct from GCC, PCC, and limestone. For example, ball milled aragonite using the system and methods disclosed in U.S. 2020/0308015, can produce an aragonite particle of 2.0 to 3.5 micron size with a clean top size. A clean top size means that very few

particles are larger than the 3.5 micron size when produced using this system and method with a classifier set at 2. to 3.5 micron size range or 2.0 to 3.5 micron size range. For example, for aragonite produced in this set range using the disclosed system, only <0.0005% are retained on a 325 mesh and only slightly more <0.0007% are retained on a 500 mesh, as compared to a GCC product having the same median (D50) particle size distribution (PSD). Accordingly, aragonite produced using the contemplated system and methods have a cleaner top size than conventional GCC.

[00241] Advantageously, the solid dosage form made of aragonite provides a solid vaccine form capable of being ingested by oral administration. The presently disclosed solid form having an enteric coating is ingested and the antigenic molecules loaded in the inner core are not released until after passing through the stomach, thereby allowing for absorption of the antigenic or pre-antigenic molecules into the bloodstream and delivery to immune cells. As used herein, antigenic molecule refers to the desired vaccine active ingredient(s) blended with and loaded on the surface of aragonite particles. These antigenic molecules may be antigenic in the form loaded on the aragonite particles or they may be a molecule or molecules (*e.g.*, an expression vector) that are capable of producing (*e.g.*, expressing) at least one antigenic protein or fragment. In this way, the active ingredient or vaccine active ingredient as disclosed herein is referred to as an antigenic molecule which includes both antigenic molecules and pre-antigenic molecules, unless specified otherwise.

[00242] In exemplary embodiments, the aragonite vaccine composition includes: i) an inner core made of aragonite and the specific antigenic molecules; ii) an outer core of aragonite that completely surrounds the inner core such that the entire outer surface of the inner core is in contact with only the outer core and no surface of the inner core is exposed; and iii) a coating covering all the outer surface of the outer core. Preferably the inner core is made of aragonite particles having a diameter of at least 2 um or greater thereby providing a surface area for capturing and loading the specific antigenic molecules thereon. Preferably, the outer core does not include any antigenic molecules and is made of 90% to 100% aragonite. More preferably, the outer core comprises only 100% aragonite. The outer coating of the solid composition may be any suitable coating (*e.g.*, enteric coating) that is stable in the highly acidic, low pH environment of the stomach (*e.g.*, at a pH of approximately 3) and dissolves in the higher pH of the small intestine (*e.g.*, at a pH of approximately 7 to 9). Suitable examples of enteric coatings include biopolymer dispersions such as methacrylic acid, ethyl acrylate, and/or a plasticizer/stabilizer (*e.g.*, triethyl citrate (TEC)).

Additionally, an anti-tacking agent may also be combined with the enteric coating—*e.g.*, glycerol monostearate.

[00243] In specific embodiments, the inner core is made of aragonite particles having a diameter of at least 2 um or greater (*e.g.*, 2 to 3.5 um) that have been blended with a lyophilized powder of the antigenic molecules. In some embodiments, additional excipients are blended with the aragonite and antigenic molecules. For example, dimethyl glycine and/or methylsulfonylmethane (MSM) may be combined with the lyophilized powder of antigenic molecules and blended with the aragonite particles.

[00244] In further exemplary embodiments, the lyophilized antigenic molecules comprise a lyophilized recombinant expression vector having nucleic acids corresponding to (*i.e.*, encoding) at least one antigenic protein or optimized protein or fragment thereof of the SARS-coronavirus 2 (SARS-CoV-2 or CoV2). For example, the contemplated solid dosage form vaccine composition disclosed herein may encode an antigen of at least one of the nucleoprotein (N) protein and the spike (S) protein of the coronavirus 2 virus (CoV2), both of which are conserved in all types of coronaviruses. In one embodiment, the antigen encoding molecules are a lyophilized recombinant entity, wherein the recombinant entity comprises a nucleic acid that encodes the nucleocapsid protein of CoV2 or a fragment thereof, and/or wherein the recombinant entity encodes the spike protein of CoV2 or fragment thereof. The vaccine formulation may be useful for treating a disease, such as a coronavirus mediated disease or infection. Thus, in another embodiment, disclosed is a method for treating a coronavirus disease, in a patient in need thereof, wherein the method includes: administering to the subject the solid dosage form vaccine composition comprising the recombinant entity comprising a nucleic acid that encodes at least the CoV2 N protein or fragment thereof, and preferably encodes both the CoV2 N protein or fragment thereof and the CoV2 S protein or fragment thereof. The coronavirus contemplated herein may be coronavirus disease 2019 (COVID-19) and/or severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

[00245] In further exemplary embodiments, the contemplated solid dosage form vaccine composition disclosed herein comprises aragonite blended with a lyophilized recombinant entity made of a bivalent human adenovirus serotype 5 (hAd5) expression vector. The hAd5 is capable of inducing immunity in patients with pre-existing adenovirus immunity and expresses antigens for producing antibodies that target the coronavirus 2 spike (S) protein and/or nucleocapsid (N) protein. For example, the hAd5 CoV2 may encode a modified nucleocapsid protein having 85%,

86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO:1. In more specific embodiments, the hAd5 includes both an S sequence optimized for cell surface expression (S- Fusion) and a conserved nucleocapsid (N) antigen designed to be transported to the endosomal subcellular compartment, with the potential to generate durable immune protection against CoV2, as disclosed in U.S. Application No. 16/883,263, the entire contents of which are herein incorporated by reference. For example, the hAd5 CoV2 may encode a S-Fusion or S-HA protein having 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO:4 or SEQ ID NO:3, respectively.

[00246] Advantageously, the bivalent hAD5 vaccine provides: (i) optimized S-Fusion having improved S receptor binding domain (RBD) cell surface expression as compared to S-WT (wild type) where little surface expression was detected; (ii) the expressed RBD from S-Fusion retained conformational integrity and recognition by ACE2-Fc; (iii) the viral N protein modified with an enhanced T-cell stimulation domain (ETSD) localized to endosomal/lysosomal subcellular compartments for MHC I/II presentation; and (iv) these optimizations to S and N (S-Fusion and N-ETSD) generated enhanced *de novo* antigen-specific B cell and CD4+ and CD8+ T-cell responses in antigen-naive pre-clinical models as is shown in more detail below.

[00247] In preferred embodiments, the lyophilized bivalent hAd5 vaccine comprises a replication defective adenovirus having an E1, an E2b, and an E3 gene region deletion along with a nucleic acid encoding a coronavirus 2 (CoV2) nucleocapsid (N) protein fused to an endosomal targeting sequence (N-ETSD), and a nucleic acid encoding a CoV2 spike (S) protein sequence optimized for cell surface expression (S-Fusion). Typically, the nucleic acid encoding the CoV2 N-ETSD protein in the hAd5 adenovirus has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 2. More typically, the CoV2 N-ETSD protein encoded in the hAd5 adenovirus has a least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 1. Typically, the nucleic acid encoding the CoV2 S-HA or S-Fusion protein in the hAd5 adenovirus has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 5 or SEQ ID NO: 6, respectively. More typically, the CoV2 S-HA or S-Fusion protein encoded in the hAd5 adenovirus has 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 3 or SEQ ID NO: 4.

[00248] In additional embodiments, the lyophilized bivalent hAd5 vaccine may also include a nucleic acid encoding a trafficking sequence, a co-stimulatory molecule, and/or an immune stimulatory cytokine. Examples of the encoded co-stimulatory molecule include one or more of CD80, CD86, CD30, CD40, CD30L, CD40L, ICOS-L, B7-H3, B7-H4, CD70, OX40L, 4-1BBL, GITR-L, TIM-3, TIM-4, CD48, CD58, TL1A, ICAM-1, and LFA3. Examples of the immune stimulatory cytokine include one or more of IL-2, IL-12, IL-15, IL-15 super agonist (N803), IL-21, IPS1, and LMP1.

[00249] Viewed form a different perspective, contemplated herein is solid dosage form of a corona virus vaccine composition made of aragonite particles blended with a lyophilized form a viral vector (*e.g.*, a lyophilized powder composition comprising a recombinant adenovirus genome, optionally with a deleted or non-functional E2b gene) that comprises a nucleic acid that encodes (a) at least a wild-type or modified nucleocapsid protein; and/or (b) at least one wild-type or modified spike protein. The viral vector may further comprise co-stimulatory molecule. Typically, the nucleic acid encodes a trafficking signal to direct a peptide product encoded by the nucleic acid to the cytoplasm, the endosomal compartment, or the lysosomal compartment, and the peptide product will further comprise a sequence portion that enhances intracellular turnover of the peptide product.

[00250] As discussed, the manufacture and preparation of a solid dosage vaccine composition is possible because aragonite allows for direct coupling of the lyophilized vaccine active ingredients. Accordingly, contemplated methods for making the disclosed solid dosage form vaccine include forming the inner core of aragonite and the vaccine active ingredients by loading the vaccine active ingredients (*e.g.*, by mixing) the milled aragonite particles as disclosed herein and in U.S. 2020/0308015, the entire contents of which are herein incorporated by reference. Any suitable method of blending the lyophilized vaccine active ingredients with the aragonite particles may be used. Loading or mixing of milled aragonite particles (*e.g.*, having a D50 PSD of 2 um to 3.5 um) with the lyophilized vaccine active ingredients may be carried out by any conventional methodology. The mixing of the aragonite with the active ingredients includes mixing dry weights of both the aragonite and the lyophilized active ingredients into a closed container suitable for rotation/inversion. For example, inversion or rotation of the aragonite and active ingredients may be carried out for about 5 minutes up to 30 minutes. For example, the vaccine composition may be loaded onto the solid dosage form in a mixer (*e.g.*, tumbling mixer) or a blender. The amount of aragonite may vary depending on the amount of lyophilized active ingredients and a determined

titer of the active ingredients for inducing an immune response. For example, an effective dose of lyophilized bivalent hAd5 vaccine as disclosed herein may be about 1 x 10^9 IU per capsule (or tablet). Accordingly, a lyophilized bivalent hAd5 vaccine composition having a titer of 2.21 x 10^7 IU/mg, requires about 45.25 mg of lyophilized bivalent hAd5 powder per capsule. In some embodiments, the weight of the solid single capsule may be of between about 300 mg to 600 mg. In a specific embodiment, for a 550 mg capsule, approximately 40 to 60 mg of lyophilized active ingredient (*e.g.*, lyophilized bivalent hAD5 expression both N and S CoV2 proteins) is mixed with 490 to 510 mg aragonite. For an active ingredient having a titer of 2.21 x 10^7 IU/mg, 45.25 mg of the lyophilized active ingredient may be blended with about 504.75 mg aragonite. The tablet or caplet formation of the mixture may be carried out using any suitable encapsulation method and/or kit known in the art. For example, the Capsule Machine Filler (Item # CPM1001/2081677).

[00251] In preferred embodiments, the contemplated method for making a solid dose vaccine tablet or capsule also includes making an outer core of aragonite that encompasses (*e.g.*, completely encloses) the inner core. Typically, the outer core is made of mostly (*e.g.*, at least 90%) aragonite and more typically, the outer core is made of at least 99% aragonite.

[00252] In order to deliver the antigenic molecules to the bloodstream in a tablet or capsule for oral administration, the antigenic molecules must remain in the inner core encompassed by the outer core until they have passed through the stomach and are available for absorption by in the intestines (*e.g.*, the small intestines). Accordingly, the outer core is coated with an enteric coating that is stable in the low pH of the stomach (*e.g.*, at a pH of approximately 3) and dissolves in the higher pH of the intestines (*e.g.*, at a pH of approximately 7 to 9). The outer coating of the solid composition may be any suitable enteric coating. Examples of enteric coating include methacrylic acid and/or ethyl acrylate polymers in triethylcitrate (TEC). Methods for applying an enteric coating are well known in art. For example, a coating device or apparatus may be used. Specific examples of a coating device include the ProCoater (manufactured by Torpac).

[00253] Coated (C) and non-coated (NC) capsules made of aragonite particles or lactose mixed with hAD5-COVID-S/N were exposed to acid (HCl) to determine the acid permeability of the various capsules. For example, **FIG.2** shows three photographs (right to left: 1, 2, 3) of bivalent human adenovirus serotype 5 COVID-Spike and Nucleocapsid antigen vaccine (hAD5-COVID-S/N) in a non-coated aragonite capsule (Sample #6) in 0.1 M hydrochloric acid (HCL) with observed wrinkling, swelling, or a hole in capsule as indicated: 1): 2 minutes post HCL acid

exposure; 2): 2 hours post HCL acid exposure; and 3) 2 hours post HCL acid exposure and dried. **FIG.3** shows three photographs (right to left: 1, 2, 3) of hAD5-COVID-S/N in a non-coated aragonite capsule (Samples #7 or #8) in 0.1 M HCL with observed swelling, twisting, or a hole in capsule as indicated: 1): Sample #7 at 2 hours post HCL acid exposure; 2): Sample #8 at 2 hours post HCL acid exposure; 3) At 2 hours post HCL acid exposure and dried. **FIG.4** shows two photographs (right to left: 1, 2) of hAD5-COVID-S/N in a non-coated lactose capsule (Samples #3 or #4) in 0.1 M HCL with observed swelling of capsule as indicated: 1): Sample #3 at 2 hours post HCL acid exposure; 2): Sample #4 at 2 hours post HCL acid exposure. **FIG.5** shows two photographs (right to left: 1, 2) of hAD5-COVID-S/N in a coated aragonite capsule (Samples #1 or #5) in 0.1 M HCL with observed swelling of capsule as indicated: 1): Sample #1 at 2 hours post HCL acid exposure; 2): Sample #5 at 2 hours post HCL acid exposure. Clearly, the coated capsule was stable in acid.

[00254] The sample capsules were further assayed for infectious units per gram (IFU/gram) and percent of virus recovery as shown in **FIGS.6-11.** More particularly, **FIG.6** shows Infectious Units per Gram (IFU/Gram) (y-axis) for indicated hAD5-COVID-S/N Capsule Type, as indicated. **FIG.7** shows the percentage (%) of Virus Recovery (y-axis) for each hAD5-COVID-S/N Capsule Type as indicated. **FIG.8** shows IFU/Gram for each hAD5-COVID-S/N Capsule Type and corresponding pH as indicated. **FIG.9** shows the percentage (%) of Virus Recovery for each hAD5-COVID-S/N Capsule Type, as indicated. **FIG.10** shows Percent Virus Recovered for each hAD5-COVID-S/N Capsule Type as indicated, with acid treatment indicated for those with shading. **FIG.11** shows Infectious Units/gram for each hAD5-COVID-S/N Capsule Type as indicated, with acid treatment indicated for those with shading. The results are also summarized in **Table 2** below.

Table 2. hAd5-COVID-S+N Capsules (1:10 ratio virus:compounding agent, 1×10^9 IFU/capsule)

Capsule particulars	Exposure 0.1*M* HCl	Mass recovered (mg)	pH post resuspension	Resuspension vol. (mL)
Aragonite (coated) 1	2 hrs	357	8.81	3.57
Aragonite (non-coated) 2	None	499	N/A	4.99
Aragonite (non-coated) 3	None	495	N/A	4.95
Aragonite (non-coated) 4	None	500.5	N/A	5.005
Aragonite (coated) 5	2 hrs	424.4	8.84	4.244
Aragonite (non-coated) 6	2 hrs	545	7.78	5.45
Aragonite (non-coated) 7	2 hrs	629	7.79	6.29
Aragonite (non-coated) 8	2 hrs	859	7.0	4.295
Lactose (non-coated) 1	None	507	N/A	5.07
Lactose (non-coated) 2	None	513.5	N/A	5.135

Lactose (non-coated) 3	2 hrs	N/A	N/A	N/A
Lactose (non-coated) 4	2 hrs	802.3	2.14	4.011
Capsule particulars	Titer (IFU/mL)	IFUs/capsule	% Recovery	IFUs/g recovered
Aragonite (coated) 1	3.90×10^{7}	1.39×10^{8}	13.90	3.90×10^{8}
Aragonite (non-coated) 2	7.60×10^{7}	3.79×10^{8}	37.90	7.60×10^{8}
Aragonite (non-coated) 3	1.00×10^{8}	4.95×10^{8}	49.50	1.00×10^{9}
Aragonite (non-coated) 4	4.10×10^{7}	2.05×10^{8}	20.50	4.10×10^{8}
Aragonite (coated) 5	4.70×10^{7}	1.99×10^{8}	19.90	4.70×10^{8}
Aragonite (non-coated) 6	3.20×10^{7}	1.74×10^{8}	17.40	3.20×10^{8}
Aragonite (non-coated) 7	2.90×10^{7}	1.82×10^{8}	18.20	2.90×10^{8}
Aragonite (non-coated) 8	4.00×10^{7}	1.72×10^{8}	17.20	2.00×10^{8}
Lactose (non-coated) 1	3.60×10^{7}	1.83×10^{8}	18.30	3.60×10^{8}
Lactose (non-coated) 2	3.70×10^{7}	1.90×10^{8}	19.00	3.70×10^{8}
Lactose (non-coated) 3	N/A	N/A	N/A	N/A
Lactose (non-coated) 4	4.10×10^{7}	1.64×10^{8}	16.40	2.5×10^{8}

[00255] In alternative embodiments, the contemplated dosage form includes an inner core of aragonite impregnated with (*i.e.,* coupled with) carbon dioxide (CO2) prior to the addition of the vaccine composition. *See, e.g.,* EP 2719373 and US 2020/0155458. In additional embodiments, the contemplated dosage form includes aragonite with a biocompatible polymer and/or a disintegrating agent mixed and processed with the aragonite prior to the addition of the vaccine composition. Typically, aragonite is impregnated with CO2 and mixed with both a biocompatible polymer and a disintegrating agent prior to the addition of the vaccine composition. More typically, aragonite is impregnated with CO2, mixed with a biocompatible polymer and a disintegrating agent, and formed (*e.g.,* compressed) into a solid form prior to the addition of the vaccine composition. *See, e.g.,* EP 2719373 and US 2020/0155458.

[00256] In additional embodiments, as mentioned herein, the aragonite may be coupled with carbon dioxide (CO2) and mixed with at least one biocompatible polymer. Typically, the weight ratio of CO2-coupled aragonite to the biocompatible polymer is from about 95:5 to 5:95. In additional embodiments, the biocompatible polymer is a hot melt extruded biocompatible polymer. Exemplary biocompatible polymers include polylactic acid (PLA), polyethylene, polystyrene, polyvinylchloride, polyamide 66 (nylon), polycaprolactame, polycaprolactone, acrylic polymers, acrylonitrile butadiene styrene, polybenzimidazole, polycarbonate, polyphenylene oxide/sulfide, polypopylene, teflon, polylactic acid, aliphatic polyester such as polyhydroxybutyrate, poly-3-hydroxybutyrate (P3HB), polyhydroxyvalerate, polyhydroxybutyrate-polyhydroxyvalerate copolymer, poly(3-hydroxybutyrate-co-3-hydroxyvalerate), polyglyconate, poly(dioxanone) and mixtures thereof. Preferably, the biocompatible polymer resin is PLA.

[00257] Additional excipients may also be added to the inner core and/or the outer core of the solid dosage form as determined by the manufacturing and packaging needs. Additional excipients may include ion exchange resins, gums, chitin, chitosan, clays, gellan gum, crosslinked polacrillin copolymers, agar, gelatine, dextrines, acrylic acid polymers, carboxymethylcellulose sodium/calcium, hydroxpropyl methyl cellulose phthalate, shellac or mixtures thereof, lubricants, inner-phase lubricants, outer-phase lubricants, impact modifiers, plasticizers, waxes, stabilizers, pigments, coloring agents, scenting agents, taste masking agents, flavoring agents, sweeteners, mouth-feel improvers, binders, diluents, film forming agents, adhesives, buffers, adsorbents, odor-masking agents and mixtures thereof.

[00258] In alternative embodiments, the aragonite particle surface may be treated to modify the binding surface. For example, treatment with stearic acid (*i.e.*, octadecanoic acid) provides for a hydrophobic surface, as disclosed in U.S. 16/858,548 and PCT/US20/29949. For protein loading, treatment of the aragonite with phosphoric acid forms lamellar structures. Additional conjugation techniques for coupling reactive groups to the amino acid surface of aragonite are known in the art as disclosed, for example, in *Bioconjugate Techniques, Third Edition,* Greg T. Hermanson, Academic Press, 2013.

Oral/Mucosal Vaccine

[00259] Based at least in part on the above formulations, the present disclosure also provides methods and compositions for administering, monitoring, and assaying a vaccine. The contemplated methods include inducing immunity against a virus in a patient, administering a vaccine composition to the patient by administering a vaccine composition to the patient by delivery to the nasal mucosa, oral mucosa, and/or alimentary mucosa of the patient. Preferably, the vaccine targets SARS-like coronavirus (SARS-CoV-2). The oral vaccine compositions described herein can serve as a booster vaccination to any initial prime vaccination against SARS-CoV-2 S or N protein.

[00260] The oral vaccine compositions described herein can be used as a booster vaccine to any anti-SARS-CoV-2 vaccine directed against the SARS-CoV-2 spike (S) and/or nucleocapsid (N) proteins. This booster can work even in patients who were immunized with an anti-S or anti-N vaccine other than those described herein. In particular embodiments, the initial prime vaccine can be a lipid nanoparticle vaccine containing mRNA encoding the S protein, such as those vaccines currently being tested by Moderna and by Pfizer. In certain embodiments, the boost described

herein is administered at least 7 days after the initial prime vaccination, for example at least 8 days, at least 9 days, at least 10 days, at least 11 days, at least 12 days, at least 13 days, at least 14 days, at least 15 days, at least 16 days, at least 17 days, at least 18 days, at least 19 days, at least 20 days, at least 21 days, at least 28 days, at least 35 days, or at least 42 days. The boost as described herein can effectively improve both antibody production against SARS-CoV-2 and cell-mediate immunity against SARS-CoV-2.

[00261] Preferably, the vaccine administered for inducing immunity in the mucosal tissue of a patient is a vaccine against SARS-CoV-2. In exemplary embodiments, the vaccine a replication defective adenovirus construct, comprising an E1 gene region deletion and an E2b gene region deletion. In certain embodiments the adenovirus comprises a sequence (e.g. SEQ ID NO:12) encoding a SARS-CoV-2 spike fusion protein antigen with at least 80% (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%) primary sequence identity to SEQ ID NO:4. In certain embodiments the adenovirus comprises a sequence (e.g. SEQ ID NO:13) encoding a SARS-CoV-2 modified spike protein antigen with at least 80% (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%) primary sequence identity to SEQ ID NO:11. In certain embodiments, the adenovirus includes a sequence encoding a soluble ACE2 protein coupled to an immunoglobulin Fc portion, forming an ACE2-Fc hybrid construct that may also include a J-chain portion, as disclosed in U.S. 16/880,804 and U.S. 63/016,048, the entire contents of both of which are herein incorporated by reference. In other exemplary embodiments, the SARS-CoV-2 vaccine (e.g., an adenovirus construct) includes a mutant variant of a recombinant soluble ACE2 protein (e.g., SEQ ID NO: 10), wherein the mutant variant has at least one mutated amino acid residue (e.g., by substitution) that imparts an increased binding affinity of the ACE2 protein for the RBD protein domain of the SARS-CoV-2 spike protein as disclosed in U.S. 63/022,146, the entire content of which is herein incorporated by reference. In another exemplary embodiment, the SARS-CoV-2 vaccine (e.g., an adenovirus construct) includes a CoV2 nucleocapsid protein or a CoV2 spike protein fused to an endosomal targeting sequence (N- ETSD), as disclosed in U.S. 16/883,263 and U.S. 63/009,960, the entire contents of both of which are herein incorporated by reference. Additionally, or alternatively, the SARS-CoV-2 vaccine includes modified yeast cells (e.g., Saccharomyces cerevisiae) genetically engineered to express coronaviral spike proteins on the yeast cell surface thereby creating yeast presenting cells to stimulate B cells (e.g., humoral immunity) as disclosed in U.S. 63/010,010. The advantageous features of the compositions and methods described herein are further illustrated (but not limited) by the following examples.

Example 26: NHP Vaccination

[00262] Two groups of Rhesus macaques (5 per group) were immunized subcutaneously on day 0 with an adenoviral anti-SARS-CoV-2 vaccine as described above. Blood was drawn from each macaque before immunization. On day 14, one group of macaques (Group 1) received another subcutaneous booster injection of the same vaccine, while another group (Group 2) received an oral vaccine as described herein (E1-/E2b- Ad5 with SEQ ID NO:12 or SEQ ID NO:13). On day 28, both groups received an oral vaccine booster dose. Two macaques (Control) were vaccinated at the indicated time points with shams. Blood was drawn on days 14, 21, 28, 35, & 42.

[00263] Serum samples drawn at the indicated time points from these macaques was then assessed by ELISA for anti-spike protein IgG and IgM seroreactivity. Briefly, 96 well EIA/RIA plates (ThermoFisher, Cat#07-200-642) were coated with 50 µL/well of 1 µg/mL solution of purified recombinant SARS-CoV-2-derived Spike protein (S-Fusion. ImmunityBio, Inc.) suspended in coating buffer (0.05 M Carbonate-Bicarbonate, pH 9.6) and incubated overnight at 4°C. Individual 96 well plates were prepared for each immunoglobulins type (IgG or IgM) by washing three times each per well with 150 µL of TPBS solution (PBS + 0.05%Tween 20). 100 µL/well of blocking solution (2% non-fat milk in TPBS) was then added and incubated for 1 hour at room temperature (RT). Plasma and serum samples were heat-inactivated at 56°C for 1 hour before use. Serial dilutions of plasma, serum or antibody samples were prepared in 1% non-fat milk in TPBS. Plates were washed as described above and 50 µL/well of each serial dilution were added to the plate and incubated at RT for 1 hour. Plates were washed three times with 200 µL of TPBS. Dilutions (1:6000) of each goat anti-Human IgG (H+L) Cross-Adsorbed, HRP, Polyclonal; or Goat anti- Human IgM (Heavy chain) Cross-Adsorbed Secondary Antibody, HRP (ThermoFisher, Cat#62- 842-0 or A18841 respectively) were 1 prepared in 1% non-fat milk/TPBS and 50 µL/well of these secondary antibodies were added in separate reactions/plates per immunoglobulin type (IgG or IgM) and incubated for 1 hour at RT. Plates were washed three times with 200 µL of TPBS. One component (3,3',5,5'-tetramethylbenzidine (TMB) substrate, 50 µL/well, VWR, Cat#100359-156) was added to each well and incubated at RT for 10 minutes and then the reaction was stopped by addition of 50 µL/well of 1N Sulfuric acid (H2SO4). The optical density at 450 nm was measured with a Synergy 2 plate reader (BioTek Instruments, Inc). Data were analyzed using Prism 8 (GraphPad Software, LLC), and shown in **FIG.14** depicting ELISA results detecting IgG seroreactivity against SARS-CoV-2 spike in sera samples drawn from immunized macaques.

[00264] Example 27: NHP Challenge

[00265] On day 56, the macaques were challenged with respiratory exposure to the SARS-CoV-2 virus. Nasal swabs were collected daily from these macaques on days 56–63. Bronchoalveolar lavage (BAL) fluid was collected on days 57, 59, 61, & 63. The ability of serum to inhibit SARS-CoV-2 infectivity from the samples collected is shown in **FIG.15**. Panel A shows the ability of sera from vaccinated Group 1 macaques to inhibit SARS-CoV-2 infectivity *in vitro*. Panel B shows the ability of sera from vaccinated Group 2 macaques to inhibit SARS-CoV-2 infectivity *in vitro*. The dotted line indicates 20% inhibition. As can be seen, the sera from both the Group 1 and Group 2 macaques inhibited infectivity, with later collected sera inhibited more powerfully than early collected sera. Sera from control macaques had no inhibitory effect at any time point tested. Viral load over time in the nasopharynx is shown in **FIG.16**. Panel A shows viral load (qPCR) in nasal swabs from Group 1 macaques following SC+SC+oral vaccination, Panel B shows viral load (qPCR) in nasal swabs from Group 1 macaques following SC+oral+oral vaccination. Viral load over time in the lungs (BAL) is shown in FIG.17. **FIG.17**. Panel A shows viral load (qPCR) in BAL from Group 1 macaques following SC+SC+oral vaccination, Panel B shows viral load (qPCR) in BAL from Group 1 macaques following SC+oral+oral vaccination.

Example 28: Seroreactivity

[00266] Serum samples from various human volunteers who have received various experimental anti-SARS-CoV-2 vaccines were collected and assayed by ELISA as described above for IgG and IgM seroreactivity against SARS-CoV-2 S protein. The results are shown in **FIG.18** with ELISA results detecting IgG & IgM seroreactivity against SARS-CoV-2 spike in sera samples drawn from human patients immunized with various experimental anti-SARS-CoV-2 vaccines.

Example 29: Human Immunization

[00267] Human volunteers were divided into three cohorts. Cohort 1 (10 individuals) was immunized by subcutaneous injection with 5×10^{10} viral particles of a vaccine as described herein (E1-/E2b- Ad5 containing SEQ ID NO:12 or SEQ ID NO:13). Cohort 2 (10 individuals) was immunized by subcutaneous injection with 10^{11} viral particles of a vaccine as described herein. Cohort 3 (15 individuals) was immunized by subcutaneous injection with 10^{11} viral particles of a vaccine as described herein (or 5×10^{10} viral particles if safety concerns indicated a lower dose).

Blood was drawn from each volunteer on the same day as the initial prime vaccination was administered. Blood was drawn again on days 8, 15, & 22. A booster injection of the same vaccine was administered on day 22.

[00268] ELISpot tests were run on the blood collected on days 1 & 15 to assess cell-mediated immunity against SARS-CoV-2. 400,000 viable PBMCs from each blood draw per well (Cellometer K2 w/ AO/PI viability stain) were stimulated with empty medium, SARS-CoV-2 S, SARS-CoV-2 N, SARS-CoV-2 M, CD3/CD28/CD2, and CEFT. After 48 hrs of stimulation, supernatants were frozen (-80°C) for later testing. **FIG.19** shows the results of the ELISpot test from Th1 N-responsive patients 3, 6, & 11. **FIG.20** shows results from patient 4 (N-unresponsive) and patient 10 (weakly Th1 N-responsive). None of these patients showed a Th2 response to N.

Airway Protection

[00269] In still further experiments, the contemplated vaccine formulations and methods of use afforded protection of nasal and lung airways against SARS-CoV-2 challenge in a non-human primate. As is shown in more detail below, a dual-antigen COVID-19 vaccine incorporating genes for a modified SARS-CoV-2 spike (S-Fusion) protein and the viral nucleocapsid (N) protein with an Enhanced T-cell Stimulation Domain (N-ETSD) increases MHC class I/II responses. The adenovirus serotype 5 platform used, hAd5 [E1-, E2b-, E3-], previously demonstrated to be effective in the presence of Ad immunity, can be delivered in an oral formulation that overcomes cold-chain limitations. The hAd5 S-Fusion + N-ETSD vaccine was evaluated in rhesus macaques showing that a subcutaneous prime followed by oral boosts elicited both humoral and Th1 dominant T-cell responses to both S and N that protected the upper and lower respiratory tracts from high titer (1 x 10^6 TCID50) SARS-CoV-2 challenge. Notably, viral replication was inhibited within 24 hours of challenge in both lung and nasal passages, becoming undetectable within 7 days post-challenge. **FIG.25** depicts the hAd5 platform and the hAd5 S-Fusion + N-ETSD construct. (A) The human adenovirus serotype 5 vaccine platform with E1, E2b, and E3 regions deleted (*) is shown. The vaccine construct is inserted in the E1 regions (red arrow). (B) The dual-antigen vaccine comprises both S-Fusion and N-ETSD under control of cytomegalovirus (CMV) promoters and with C-terminal SV40 poly-A sequences delivered by the hAd5 [E1-, E2b-, E3-] platform.

[00270] The dual-antigen hAd5 S-Fusion + N-ETSD vaccine of **FIG.25** expresses a viral spike (S) protein (S-Fusion) fused to a signal sequence that, as predicted based on reports for similar

sequences, in the *in vitro* studies enhances cell-surface expression of the spike receptor binding domain (S RBD) as compared to S wildtype, the antigen used in the majority of other vaccines being developed. This vaccine also expresses the viral nucleocapsid (N) protein with an Enhanced T-cell Stimulation Domain (N-ETSD) that directs N to the endo/lysosomal subcellular compartment which is predicted to enhance MHC class II responses.

[00271] The SARS-CoV-2 vaccine antigens are delivered by an recombinant human adenovirus serotype 5 (hAd5) [E1-, E2b-, E3-] vector platform (**FIG.25**) can rapidly generate vaccines against multiple agents, allowing production of high numbers of doses in a minimal time frame. The hAd5 platform has unique deletions in the early 1 (E1), early 2 (E2b) and early 3 (E3) regions (hAd5 [E1-, E2b-, E3-]), which distinguishes it from other adenoviral vaccine platform technologies under development, and allows it to be effective in the presence of pre-existing adenovirus immunity. This platform produces vaccines against viral antigens such as Influenza, HIV-1 and Lassa fever and have shown induction of both antibodies and cell mediated immunity. In 2009, the vaccination of mice with the hAd5 [E1-, E2b-, E3-] vector expressing H1N1 hemagglutinin and neuraminidase genes elicited both cell-mediated immunity and humoral responses that protected the animals from lethal virus challenge.

[00272] The overwhelming majority of other SARS-CoV-2 vaccines in development target only the wildtype S antigen and are expected to elicit SARS-CoV-2 neutralizing antibody responses. The development of the vaccine prioritized the activation T cells to enhance the breath and duration of protective immune responses; the addition of N in particular was predicted to afford a greater opportunity for T cell responses. T cells may provide immune protection at least as important as the generation of antibodies. In a study of SARS-CoV-2 convalescent patients, virus-specific T cells were seen in most patients, including asymptomatic individuals, even those with undetectable antibody responses.

[00273] In preliminary studies of the hAd5 S-Fusion + N-ETSD vaccine in a murine model, the vaccine not only elicits T helper cell 1 (Th1)-dominant antibody responses to both S and N, it activates T-cells. MoDCs from SARS CoV-2 convalescent individuals were transduced with the dual-antigen vaccine, incubated the S-Fusion and N ETSD-expressing MoDCs with T cells from those same individuals. The vaccine antigens induce interferon-g (IFN-γ) secretion by both CD4+ and CD8+ T cells. This demonstrates that T cells from SARS-CoV-2 convalescent individuals 'recall' the S-Fusion and N-ETSD antigens presented by transduced MoDCs as if they were re-

exposed to the virus itself. This T-cell recall of vaccine antigens suggests that, conversely, hAd5 S-Fusion + N-ETSD vaccination will generate T cells that will recognize SARS-CoV-2 antigens upon viral infection and protect the vaccinated individual from disease.

[00274] The generation of T-cell responses may be a critical feature for a vaccine to be efficacious against the many variants whose emergence, at least in part, may be an escape response to antibodies generated by either first wave virus (28) or by antibody-based vaccines. As reported elsewhere, neutralization by 14 of 17 of the most potent mRNA vaccine-elicited monoclonal antibodies (mAbs) was either decreased or abolished variants E484K, N501Y or the K417N:E484K:N501Y combination. They also found these variants were selected for when recombinant vesicular stomatitis virus (rVSV)/SARS CoV-2 S was cultured in the presence of these mAbs, which is highly suggestive that the presence of these antibodies act as an evolutionary force driving the appearance of new variants. T cells are not vulnerable to such forces and, if effectively established by vaccination, may provide protection against existing viral strains and escape mutants.

[00275] In the next step in development of the hAd5 S-Fusion + N-ETSD vaccine, GMP (Good Manufacturing Practice)-grade liquid and oral forms of the vaccine were tested in non-human primates (NHP). A key objective of the NHP study design was to assess the efficacy of a subcutaneous (SC) prime followed by a thermally stable oral boost. An oral boost provides several advantages in SARS-CoV-2 vaccination, including a greater potential for generating mucosal immunity particularly in the gastrointestinal tract one of the major sites of infection. SARS-CoV-2 is a mucosal virus and is only rarely detected in blood, therefore vaccines that specifically target mucosal immunity are of interest. Compelling additional advantages of a thermally stable oral boost are that it would likely transform the global distribution of vaccines, especially in developing nations and potentially enable patients to self-administer the boost(s) at home. Because the hAd5 S-Fusion + N-ETSD construct induces both humoral and CMI responses to both antigens, it also has the potential to serve as a 'universal' heterologous booster vaccine to the multitude of SARS-CoV-2 vaccines under development.

[00276] A study determined the efficacy of the hAd5 S-Fusion + N-ETSD vaccine in rhesus macaques when delivered as either an SC prime with SC and oral boosts (SC-SC-Oral; n =5) or as an SC prime and two oral boosts (SC-Oral-Oral; n = 5) both using a regimen of prime on Day 0 and boosts on Days 14 and 28 to maximize T cell responses. The goals of the study were to assess

the immunogenicity of a dual-antigen hAd5 vaccine in both SC and oral formulations, and the potential of oral dose to serve as a boost following a single SC prime. Cell-mediated T-cell response and protection of nasal passages and lung from SARS-CoV-2 infection after challenge was assessed, as well as the rate of viral clearance.

[00277] Clinical signs, hematology and clinical chemistry: No clinical signs were noted during the twice daily observations for clinical signs of toxicity due to vaccination and no animals died during the two weeks after one subcutaneous immunization of 1×10^{11} vaccine particles (VP) or a week after an oral booster of 1×10^{10} IU of hAd5-S-Fusion+N ETSD. In addition, no gross pathological effects or adverse events were observed and there were no notable changes in body weight. Lastly, hematology and clinical chemistry revealed no abnormalities as a result of vaccination.

[00278] An SC prime with oral boosts elicits generation of neutralizing, anti-spike antibodies: As shown in **FIG.21**, all SC-Oral-Oral vaccinated NHP produced anti-S IgG that increased after both the Day 14 and Day 28 oral boosts (Panels A and B). Sera from 4 of 5 SC-Oral-Oral NHP, taken at baseline and every week starting at Day 14 and up to Day 42, demonstrated inhibition in the neutralization assay (Panel C) that assesses the inhibition of binding of S RBD to recombinant angiotensin-converting enzyme 2 (ACE2) and is reported to correlate with the ability of sera to neutralize the SARS-CoV-2 virus. Anti-S IgG production was similar for SC-SC-Oral (where the first boost was SC) hAd5 S Fusion + N-ETSD vaccinated NHP (Panels D and E) and sera from all five NHP in this group demonstrated inhibition in the surrogate assay for viral neutralization (Panel F).

[00279] SC prime, oral boost vaccination reduces viral load in nasal passages and lung after SARS CoV-2 challenge: RT-qPCR analysis of genomic RNA (gRNA) was performed on nasal swab and bronchoalveolar lavage (BAL) samples to determine the amount of virus present. SC-Oral-Oral vaccination of NHP reduced SARS-CoV-2 gRNA in the nasal swab samples as compared to placebo control NHP from Day 57, the first day after challenge (**FIG.22**, Panels A and B). Viral gRNA in this group continued to diminish to levels that were very low or below the level of detection (LOD) in all vaccinated animals by Day 63, 7 days after challenge. Placebo controls had moderate to high levels (range 2E+09 – 8.4E+03 gene copies/mL) of SARS-CoV-2 present in nasal swab samples for the duration of the study.

[00280] In the lungs (bronchoalveolar lavage, BAL) of SC-Oral-Oral NHP, gRNA also decreased rapidly, with the geometric mean showing a ~2 log decrease in vaccinated NHP compared to placebo NHP at Day 57, just one day after challenge (**FIG.22**, Panels C and D). In the group receiving an SC and oral boost (SC-SC-Oral), SARS-CoV-2 gRNA in nasal swab samples was also reduced similarly to that seen in SC-Oral-Oral vaccinated primates, with viral gRNA decreasing to levels that were very low or below the LOD in all vaccinated animals by Day 63 (**FIG.22**, Panels E and F). In the lungs of SC-SC-Oral NHP, gRNA also showed a ~2 log decrease on Day 57 (**FIG.22**, Panels G and H).

[00281] SC prime, oral boost vaccination immediately inhibited viral replication 188 in nasal passages and lung after SARS-CoV-2 challenge: The presence of replicating virus in nasal swab samples was determined by RT qPCR of subgenomic RNA (sgRNA). By Day 60, 4 days post-challenge, sgRNA was below the LOD for two SC-Oral-Oral primates and, starting on Day 61, below the LOD for all primates that received only oral boosts (**FIG.23**, Panels A and B). In the lungs of SC-Oral-Oral NHP, sgRNA also decreased as compared to placebo starting at Day 57 and was below the LOD in all by Day 63 (**FIG.23**, Panels C and D). Evidence of replicating viruses in nasal passages also decreased rapidly in SC-SC-Oral NHP and was below the LOD by Day 59 in two primates and in all primates by Day 63 (**FIG.23**, Panels E and F); and in the lungs of this group, sgRNA decreased by ~2 logs compared to placebo control on Day 57, one day after challenge, with sgRNA being below the LOD at Day 63 in 4 of 5 primates, and just above the LOD in the 5th (**FIG.23**, Panels G and H). Not only was there a rapid decrease of both viral load and replicating viruses in nasal passages and lung, it is notable that there was no growth of viruses following challenge. This implies the presence of pre-existing humoral and cellular immunity resulting in rapid clearance of the virus upon infection.

[00282] Immediate protection of NHP from SARS-Cov-2 challenge may be due to the presence of T cells responsive to both S and N, and rapid viral clearance to activation of memory B cells Peripheral blood mononuclear cell (PBMC)-derived T-cell responses to the antigens delivered by the hAd5 S-Fusion + N+ETSD vaccine, spike and nucleocapsid, were determined by ELISpot on Day 0 before prime vaccination, on Day 14 (before boost) and on Day 35, one week after the second Day 28 boost. T cells from SC-Oral-Oral vaccinated primates secreted interferon-gamma (IFN-γ) in response to both S and N peptides on Days 14 and 35 (**FIG.24**, Panel A). Interleukin-4 (IL-4) secretion was very low (**FIG.24**, Panel B), indicating the T-cell responses were T helper cell 1 (Th1) dominant, as reflected by the IFN-γ/IL-4 ratio (**FIG.24**, Panel C).

[00283] For sera collected in the post-challenge period, a microneutralization assay (see Methods) was used to assess SARS-CoV-2 neutralization capability as reflected by the 'MN50', that is, the serum dilution that correlates to a 50% reduction in viral infectivity as compared to a no-serum control. A rapid increase in neutralization capability of sera for NHP receiving only oral boosts was seen over the two weeks following challenge (**FIG.24**, Panel D) that mirrored decreases in nasal gRNA and sgRNA (**FIG.24**, Panels E and F, respectively) and lung gRNA and sgRNA (**FIG.24**, Panels G and H, respectively). Notably, sera from placebo group primates did not show an increase in neutralizing capability after challenge (**FIG.24**, Panel D and L), suggesting the existence of memory B cells in the vaccinated group and the absence of such cells in the unvaccinated placebo group. Further studies are needed to confirm this hypothesis.

[00284] For SC-SC-Oral vaccinated NHP, findings were very similar for reactive T cells during the pre-challenge vaccination period (**FIG.24**, Panels I-K) and for neutralization capability in the post-challenge period (**FIG.24**, Panel L), including the mirroring of decreasing gRNA and sgRNA in nasal passages and lung (**FIG.24**, Panels M-P).

[00285] The presence of cytotoxic T cells due to vaccination (**FIG.24**, Panels A-C and I-K) led to the almost immediate decrease in viral replication within the first 24 hours post-challenge (**FIG.24**, Panel F, H, N, and P), and the continued decreases over the following two weeks that mirrored increases in neutralization capability of sera from vaccinated, but not placebo, NHP reflect the contribution of anti-S-producing memory B cells (**FIG.24**, Panels D and L).

[00286] This study demonstrates that in the rhesus macaque NHP model, subcutaneous prime and oral boost dual-antigen hAd5 S-Fusion + N-ETSD vaccination protects both nasal and lung airways against SARS-CoV-2 challenge. The inhibition of viral replication in nasal passages as evidenced by decreased sgRNA on the first day after viral challenge was notable, as was continuous clearance of virus to levels below detection within 7 days of challenge in all (10/10) animals (**FIGS.22 and 23**); and, while the rhesus macaque is not a model for the assessment of transmission, these rapid reductions in nasal viral replication are encouraging and support the investigation of the ability of this vaccine to prevent transmission in future studies.

[00287] The ability of hAd5 S-Fusion + N-ETSD vaccination to elicit virus-neutralizing anti-S antibodies (**FIG.21**) and T cells responsive to both S and N (**FIG.23**), particularly when viewed with the rapid increase in the neutralization capability of sera post-challenge that is likely

indicative of the presence of memory B cells, suggests the vaccine establishes broad immunity against severe SARS-CoV-2 infection.

[00288] The potential of the hAd5 S-Fusion + N-ETSD SC prime, oral boost vaccine to generate cytotoxic T cells is a key feature, given the critical role of T cells play in protection from infection in COVID-19 convalescent patients where SARS-CoV-2 specific T cells were identified even in the absence of antibody responses. The apparent nearly immediate reduction of viral replication by the hAd5 S-Fusion + N-ETSD vaccination is in contrast to the reported findings for other adenovirus-vectored S-only vaccine NHP studies, wherein there was evidence of continued viral replication in some animals for at least a day after challenge. Even when challenged with the relatively high titer of 1×10^6 281 TCID50/mL as compared to titers used in some other NHP vaccine studies, vaccinated animals in the study appeared to be protected from the earliest time point assessed. This rapid protection and clearance was particularly evident in the lung, where both viral load and viral replication were ~1-2 logs lower than placebo in both vaccinated groups just one day after challenge.

[00289] The protection conferred by hAd5 S-Fusion + N-ETSD vaccination of NHPs by SC and oral boost administration particularly reveal the potential for this vaccine to be developed for worldwide distribution, especially in light of the escape variants resistant to antibodies and convalescent plasma, now rapidly spreading throughout the world. The oral hAd5 S-Fusion + N-ETSD formulation would not require ultra-cold refrigeration like many COVID vaccines currently in development. Dependence on the cold-chain for distribution to geographically remote or under developed areas causes shipping and storage challenges and will likely reduce the accessibility of the RNA-based COVID-19 vaccines.

[00290] The thermally stable oral hAd5 S-Fusion + N-ETSD vaccine, due its expression of S and N, also has the potential to act as a 'universal' boost to other previously administered vaccines that deliver only S antigens. This use would also be facilitated by cold-chain independence.

Saliva Testing

[00291] In still further contemplated aspects, methods and compositions are disclosed herein for administering, monitoring, and assaying a vaccine. The contemplated methods include inducing immunity against a virus in a patient, administering a vaccine composition to the patient by administering a vaccine composition to the patient by delivery to the nasal mucosa, oral mucosa,

and/or alimentary mucosa of the patient. Preferably, the vaccine targets SARS-like coronavirus (SARS-CoV-2).

[00292] Notably the disclosed methods also include obtaining a sample of saliva from the patient at a period of time after administering the vaccine. Typically, the sample of saliva is preserved in a stabilizing solution comprising glutaraldehyde, sodium benzoate, citric acid, propyl gallate, EDTA, zinc, actin, chitosan, parabens, sodium azide, or any combination thereof. More typically, the stabilizing solution comprises glutaraldehyde at 0.10 to 2.0% weight per volume (w/v), sodium benzoate at 0.10 to 1.0% w/v, and/or citric acid at 0.025 to 0.20% w/v. Additional embodiments include analyzing the sample of saliva for at least one selected from antibodies targeting the virus or a protein specific to the virus, wherein in the absence of antibodies in the sample saliva, the method further comprises administering a booster of the vaccine to the patient.

[00293] Additionally, the stabilizing solution further comprises aragonite particle beads having an average particle size of between 100 nm to 1 mm. The aragonite particle beads are capable of binding to immunoglobulin (Ig) proteins, anti-SARS-CoV-2 antibodies, or a SARS-CoV-2 viral protein. In exemplary embodiments, the aragonite particle beads are coupled to a recombinant ACE2 protein or a recombinant ACE2 alpha helix protein.

[00294] The contemplated subject matter also includes an aragonite composition formulated for binding an immunoglobulin (Ig) protein, an anti-SARS-CoV-2 antibody protein, or a SARS-CoV-2 viral protein. The aragonite composition includes a plurality of aragonite particle beads having an average particle size of between 100 nm to 1 mm, wherein the plurality of aragonite particle beads are functionalized with a moiety capable of binding to an immunoglobulin (Ig) protein, the anti-SARS-CoV-2 antibody protein and/or the SARS-CoV-2 viral protein.

[00295] In specific embodiments, the plurality of aragonite particle beads are functionalized with a moiety capable of binding to the anti-SARS-CoV-2 comprises a recombinant ACE2 protein. For example, the moiety capable of binding to the anti-SARS-CoV-2 may be selected from a recombinant ACE2 protein having at least 85% sequence identity to SEQ ID NO:9, a recombinant alpha-helix ACE2 protein of SEQ ID NO:10, or the recombinant alpha-helix ACE2 protein having at least one mutation selected from T27F, T27W, T27Y, D30E, H34E, H34F, H34K, H34M, H34W, H34Y, D38E, D38M, D38W, Q24L, D30L, H34A, and/ D355L.

[00296] The contemplated subject matter includes methods for administering a vaccine to a patient by more than one route of administration to induce both local and systemic immune responses to the vaccine. The contemplated subject matter also includes compositions and methods for assaying the presence or absence of the relevant antibodies (*e.g.*, anti-SARS-CoV-2 antibodies) in a patient sample (*e.g.*, saliva, nasal mucosa, alimentary mucosa, or serum). The antibody status in the patient's sample may be used to assess the need for an additional vaccine dose (*e.g.*, a booster dose/shot).

[00297] In addition to the coveted molecular epitopes presented in a vaccine, the route of administration of the vaccine as well as the regimen for administering additional (i.e., booster) doses of the vaccine, can also affect whether or not the patient's immune response is robust enough to establish protection.

[00298] For an emerging virus such as the SARS-like coronavirus (SARS-CoV-2), the duration of immunity (both humoral and cell-mediated) in a patient recovered from a SARS-CoV-2 infection is not yet completely known, and furthermore, a vaccine protocol has not yet been tested across a varied population. Considering the current SARS-CoV-2 pandemic and the high rate of transmission for the SARS-CoV-2 virus, there is a need for a robust vaccination protocol and effective testing for the virus or immunity to the virus (*e.g.*, presence of anti-SARS-CoV-2 antibodies).

[00299] The presently disclosed contemplated methods for inducing immunity in a patient include administering a vaccine by at least oral administration, and preferably by oral administration and by injection to the blood supply. Many vaccines are given via the intramuscular (IM) route to optimize immunogenicity with the direct delivery of the vaccine to the blood supply in the muscle to induce systemic immunity. The IM administration is typically preferred over subcutaneous (SC) injection which is more likely to have adverse reactions at the injection site than IM injections.

[00300] In addition to IM injection, induction of mucosal immunity has been reported to be essential to stop person-to-person transmission of pathogenic microorganisms and to limit their multiplication within the mucosal tissue. Furthermore, for protective immunity against mucosal pathogens, (*e.g.*, SARS coronaviruses) immune activation in mucosal tissues instead of the more common approach of tolerance to maintain mucosal homeostasis allows for enhanced mucosal immune responses and better local protection. For example, nasal vaccination (delivery of a

vaccine by nasal administration) induces both mucosal immunity as well as systemic immunity (see, *e.g.*, Fujkuyama et al., 2012, *Expert Rev Vaccines,* 11:367-379 and Birkhoff et al., 2009, *Indian J. Pharm. Sci.,* 71:729-731).

[00301] In order to induce both mucosal and systemic immunity in a patient, embodiments of the present disclosure include providing a vaccine to the patient by at least administration to the nasal mucosa, oral mucosa, and/or alimentary mucosa of the patient. In some embodiments, the routes of administration include administering the vaccine to the nasal mucosa, oral mucosa, and/or alimentary mucosa of the patient together with injection into the blood supply (*e.g.*, intramuscular (IM), intravenous (IV), or subcutaneous (SC)). As used herein, oral administration of a vaccine composition includes nasal injection, nasal inhalation, ingestion by mouth, and administration (*e.g.,* inhalation, ingestion, injection) to the alimentary mucosa. Preferably, the routes of administering the vaccine include oral administration selected from delivery to the alimentary mucosa, nasal injection, nasal inhalation, ingestion by mouth, or inhalation by mouth together with administration by intramuscular (IM) injection.

[00302] Notably, the vaccine administered for inducing immunity in the mucosal tissue of a patient is a SARS-CoV-2 vaccine. In exemplary embodiments, the SARS-CoV-2 vaccine (*e.g.,* an adenovirus construct) includes a soluble ACE2 protein coupled to an immunoglobulin Fc portion, forming an ACE2-Fc hybrid construct that may also include a J-chain portion, as disclosed in U.S. 16/880,804 and U.S. 63/016,048, the entire contents of both of which are herein incorporated by reference. In other exemplary embodiments, the SARS-CoV-2 vaccine (*e.g.,* an adenovirus construct) includes a mutant variant of a recombinant soluble ACE2 protein (*e.g.,* SEQ ID NO: 10), wherein the mutant variant has at least one mutated amino acid residue (e.g., by substitution) that imparts an increased binding affinity of the ACE2 protein for the RBD protein domain of the SARS-CoV-2 spike protein as disclosed in U.S. 63/022,146, the entire content of which is herein incorporated by reference. In another exemplary embodiment, the SARS-CoV-2 vaccine (*e.g.,* an adenovirus construct) includes a CoV2 nucleocapsid protein or a CoV2 spike protein fused to an endosomal targeting sequence (N-ETSD), as disclosed in U.S. 16/883,263 and U.S. 63/009,960, the entire contents of both of which are herein incorporated by reference. Additionally or alternatively, the SARS-CoV-2 vaccine includes modified yeast cells (*e.g., Saccharomyces cerevisiae*) genetically engineered to express coronaviral spike proteins on the yeast cell surface thereby creating yeast presenting cells to stimulate B cells (*e.g.,* humoral immunity) as disclosed in U.S. 63/010,010.

[00303] In some embodiments, more than one vaccine composition as disclosed herein may be administered to a patient to induce immunity to SARS-CoV-2. For example, a patient may be administered genetically modified yeast cells expressing corona viral spike proteins as a single type of vaccine, or the genetically modified yeast cells may be administered together or concurrently with one or more SARS-CoV-2 adenovirus constructs as disclosed herein.

[00304] In further embodiments, one can monitor or assess a patient's immune response either to a vaccine administered as disclosed herein (*e.g.*, by oral administration and injection into the blood supply), or to infection by the virus. In particular, disclosed herein are compositions and methods for assessing the continued presence of antibodies in a patient's respiratory and digestive mucosa following infection with SARS-CoV-2 or following inoculation against SARS-CoV-2 with administration of a SAR coronavirus vaccine.

[00305] For assaying a sample from a patient having received a vaccine against a pathogenic infection (*e.g.*, targeting SARS-CoV-2) and/or having been infected with a virus (*e.g.*, SARS-CoV-2), the presence of antibodies against the pathogen may be carried out using any one of many diagnostic tests. In some embodiments, the diagnostic test is a cell viability assay that allows for the detection of antibodies in the presence of antigen. Diagnostic tests using a cell viability assay for anti-SARS-CoV-2 antibody detection are disclosed in U.S. 62/053,691, the entire contents of which are herein incorporated by reference. The cellular diagnostic assay relies on the expression of the target receptor for a given pathogen (*e.g.*, ACE2 for SARS-CoV-2 infection) on the surface of an immune effector cell line (*e.g.*, killer T cells, natural killer cells, NK-92 cells and derivatives thereof, *etc.*) and the expression of the pathogen ligand (*e.g.*, Spike proteins for SARS-CoV-2 infection) on the surface of a surrogate cell line (*e.g.*, HEK293 cells or SUP-B15 cells).

[00306] Additional diagnostic tests using recombinant protein variants of the ACE2 protein (the human receptor targeted by SARS-CoV-2 spike protein) are disclosed in U.S. 16/880,804, the entire contents of which are herein incorporated by reference.

[00307] In order to more easily monitor a patient for the presence of anti-pathogen antibodies, assaying a saliva sample from the patient allows for expedited sample collection, increased patient participation, and may allow for the patient to obtain the sample themselves and either mail or transport the sample to the lab for testing. However, in order to assay saliva for the presence of neutralizing antibodies against SARS-CoV-2, it may be necessary to stabilize proteins in the saliva against degradation during transport and storage after sample collection prior to testing.

[00308] Upon collection of the saliva sample, the saliva is placed into a preservative solution to stabilize the components (*e.g.*, anti-SARS CoV-2 antibody or viral spike protein) therein. Preservatives for biological samples are disclosed, for example, in Cunningham *& al.* (2018) report ("Effective Long-term Preservation of Biological Evidence," U.S. Department of Justice grant # 2010-DN-BX-K193) and US 6,133,036. For example, a stabilizing preservative solution for a patient's saliva sample may include any one of glutaraldehyde, sodium benzoate, citric acid, propyl gallate, EDTA, zinc, actin, chitosan, parabens, sodium azide, and any combination thereof.

[00309] In specific embodiments, saliva samples may be mixed with stabilizing preservative solutions of glutaraldehyde to achieve a final glutaraldehyde concentration between 0.1%(w/v) and 2.0%(w/v), for example about 0.2%(w/v), about 0.3%(w/v), about 0.4%(w/v), about 0.5%(w/v), about 0.6%(w/v), about 0.7%(w/v), about 0.8%(w/v), about 1.0%(w/v), about 1.1%(w/v), about 1.2%(w/v), about 1.3%(w/v), about 1.4%(w/v), about 1.5%(w/v), about 1.6%(w/v), about 1.7%(w/v), about 1.8%(w/v), or about 1.9%(w/v).

[00310] In additionally or alternatively embodiments, saliva samples may be mixed with a stabilizing preservative solution of about 0.10% to about 1.00% sodium benzoate (weight/volume of sample) and/or about 0.025% to about 0.20% citric acid (weight/volume of sample). For example, the saliva sample may be mixed with 0.10%, 0.20%, 0.30%, 0.40%, 0.50%, 0.60%, 0.70%, 0.80%, 0.90%, or 1.00% w/v sodium benzoate. In additional embodiments, the saliva sample is mixed a stabilizing preservative solution of at least 0.5 mg/mL (for example, at least 0.6 mg/mL, at least 0.7 mg/mL, at least 0.8 mg/mL, at least 0.9 mg/mL, at least 1 mg/mL, at least 1.5 mg/mL, at least 2 mg/mL, at least 2.5 mg/mL, at least 3 mg/mL, at least 3.5 mg/mL, at least 4 mg/mL, at least 4.5 mg/mL, or even 5 mg/mL) of benzoic acid and/or at least 0.2 mg/mL (for example, at least 0.2 mg/mL, at least 0.25 mg/mL, at least 0.3 mg/mL, at least 0.35 mg/mL, at least 0.40 mg/mL, at least 0.50 mg/mL, at least 0.75 mg/mL, at least 1.0 mg/mL, at least 1.25 mg/mL, at least 1.5 mg/mL, at least 1.75 mg/mL, or even 2.0 mg/mL) of citric acid. As used herein, "benzoic acid" is interchangeable with benzoate salt (*e.g.*, sodium benzoate) and "citric acid" is interchangeable with citrate salt (*e.g.*, sodium citrate).

[00311] The saliva samples with preservatives as described above are stable for storage at temperatures between 15°C and 40°C for at least one hour (*e.g.*, at least 5 hours, at least 10 hours, at least 12 hours, at least 24 hours, at least 48 hours, or even 36 hours). Therefore, disclosed herein is a method of preserving a saliva sample for neutralizing antibody testing, the method including

mixing the saliva sample with the stabilizing solution made of one or more of glutaraldehyde, sodium benzoate, citric acid, propyl gallate, EDTA, zinc, actin, chitosan, parabens, and/or sodium azide and storing between 15°C and 25°C for at least one hour, and up to 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 36, or 48 hours. In some embodiments, the saliva sample is mixed with a glutaraldehyde concentration between 0.1% (w/v) and 2.0% (w/v), and the glutaraldehyde-saliva is stored between 15°C and 25°C. In certain embodiments, the glutaraldehyde-saliva may further comprise citric acid and/or benzoic acid at a concentration of as disclosed herein.

[00312] Aragonite: In some embodiments, any antibody proteins or any specific antibody protein may be captured from the saliva sample with oolitic aragonite particles. For example, the saliva preserving solution of glutaraldehyde, sodium benzoate and citric acid, propyl gallate, EDTA, zinc, actin, chitosan, parabens, sodium azide, and any combination thereof as disclosed herein, may also include oolitic aragonite (calcium carbonate, $CaCO_3$) particles. Use of aragonite particles for binding to proteins is disclosed, for example, in U.S. 16/858,548 and PCT/US20/29949, the entire contents of both of which are herein incorporated by reference. Accordingly, aragonite particles may be added to that have been modified to capture (*e.g.*, bind to) any antibodies present in the saliva sample or specifically capture an antibody against a specific antigen. For example, aragonite may be functionalized with moieties capable of binding to an immunoglobulin (Ig) protein. Preferably, the Ig protein is an immunoglobulin A (IgA), immunoglobulin G (IgG), or immunoglobulin E (IgE) protein. More preferably, the aragonite is functionalized to bind to an IgA protein. Most preferably, the aragonite particles are functionalized with moieties capable of binding to specific antibodies. For example, the aragonite particles may be coupled with a moiety specific to anti-SARS-CoV-2 antibodies. Preferably, the aragonite particle is coupled with a recombinant ACE2 protein as disclosed, for example, in U.S. 16/880,804, *supra.* In typical embodiments, the aragonite particle is coupled with a recombinant human ACE2 protein having at least 85%, at least 90%, or at least 95% sequence identity to SEQ ID NO: 9.

[00313] In additional or alternative embodiments, the aragonite particle is functionalized (*e.g.*, coupled to) a recombinant soluble ACE2 protein (*e.g.*, SEQ ID NO: 10). For more efficient capture or binding of an anti-SARS-CoV-2 antibody or the spike protein of SARS CoV-2, the recombinant soluble ACE2 may be mutated to form ACE2 variants having higher binding affinities for SARS-CoV-2 spike protein (*e.g.*, the RBD domain of the spike protein). These ACE2 variant mutants of

the recombinant soluble ACE2 protein include T27F, T27W, T27Y, D30E, H34E, H34F, H34K, H34M, H34W, H34Y, D38E, D38M, D38W, Q24L, D30L, H34A, and/or D355L.

[00314] As used herein, the term "functionalized" refers to coupling or binding of a moiety to the aragonite particle thereby imparting any function of the coupled moiety to the aragonite particle. For example, the aragonite particle may be functionalized with a protein moiety. Methods for preparing and using aragonite particle beads are disclosed in U.S. 16/858,548 and PCT/US20/29949. In some embodiments, the aragonite composition includes a plurality of aragonite particle beads. Preferably, the plurality of aragonite particle beads have an average particle size of between 100 nm to 1 mm.

[00315] In some embodiments a protein moiety is coupled directly to the natural, untreated surface of aragonite particles. Aragonite particles have approximately a 2-3% amino acid content, including aspartic acid and glutamic acid rendering the aragonite surface hydrophilic. Accordingly, in some embodiments, protein moieties may be directly coupled to the surface of the aragonite particles.

[00316] In alternative embodiments, the aragonite particle surface may be treated to modify the binding surface. For example, treatment with stearic acid (*i.e.*, octadecanoic acid) provides for a hydrophobic surface, as disclosed in U.S. 16/858,548 and PCT/US20/29949. For protein loading, treatment of the aragonite with phosphoric acid forms lamellar structures. Additional conjugation techniques for coupling reactive groups to the amino acid surface of aragonite are known in the art as disclosed, for example, in *Bioconjugate Techniques, Third Edition,* Greg T. Hermanson, Academic Press, 2013.

[00317] Patients who do not show sufficient titers of (*e.g.*, presence of) neutralizing antibody in their saliva may be sent oral dosages of the respective vaccine (*e.g.*, a SARS-CoV-2 vaccine as disclosed herein). The patients inhale or ingest these vaccine dosages, and then two weeks later send another saliva sample—prepared and stored in the same manner as above—to the test facility to confirm that the oral vaccine dose has restored their anti-SARS-CoV-2 antibody (*e.g.*, IgA) titers.

[00318] Accordingly, in additional embodiments, a kit for collecting a saliva sample from a patient includes a collection container with the saliva preservative solution as disclosed herein. For example, the kit includes a collection container with a solution of any of one or combination of

glutaraldehyde, sodium benzoate and/or citric acid, propyl gallate, EDTA, zinc, actin, chitosan, parabens, and sodium azide. The kit may also include adhesive packaging and/or mailing supplies in order to secure the collection container with the saliva sample for transport or mailing. In some embodiments, the kit may also include at least one dose of the vaccine for oral administration.

Immune stimulation

[00319] In addition to vaccination, or even as an alternative treatment for subjects likely to be or actually diagnosed with a corona virus infection, and especially SARS-CoV-2, immune stimulation with one or more immune stimulatory cytokines may prevent or alleviate lymphopenia that is frequently associated with COVID-19. Among other immune stimulatory cytokines, N-803 is particularly contemplated.

[00320] COVID-19 infection causes lymphopenia, specifically a suppression of NK and CD8 T cells, and severe cases and subsequent fatalities are associated with this significant decline in lymphocytes. Evidence also suggests that COVID-19 infection results in macrophage killing and reduction of natural killer (NK) cells and CD8+ T cells. An analysis of data from 1099 patients with laboratory-confirmed COVID-19 from 552 hospitals throughout China revealed that upon hospital admission, lymphopenia was found to be present in 83.2% of the patients. On average, patients with severe disease had more prominent laboratory abnormalities, such as lower lymphocyte counts, as compared to patients with non-severe disease. Likewise, another group of researchers showed that median lymphocyte counts were significantly lower for patients admitted to the ICU as compared to those who did not require ICU care.

[00321] Ona cellular level, COVID-19 appears to induce immune evasion by a reduction in NK, CD4+ and CD8+ T cells. The mechanism of lymphopenia remains unclear; however, the rapid decrease in both CD4 and CD8 T cells may be associated with an adverse outcome. Lymphopenia and increasing viral load in the first 10 days of SARS suggest immune evasion by the SARS coronavirus. The lack of an interferon (IFN)-gamma response in SARS-infected cells has been reported *in vitro*, using human primary myeloid-derived dendritic cells and the epithelial 293 cell line. Others proposed a mechanism of immune evasion by SARS-CoV in dendritic cells (DCs), based on their findings of low expression of antiviral cytokines (TFN-alpha, TFN-beta, TFN-gamma, and IL-12p40), moderate upregulation of pro-inflammatory cytokines (tumor necrosis factor alpha [TNF-α] and IL-6), and significant upregulation of pro-inflammatory cytokines (MIP-1 a, RANTES, IP- I 0, and MCP-1). In addition to the depletion of CD8+ T cells, lack of T-cell

reactivity through poor T-cell diversity may contribute to a poor immune response. This occurs particularly in the elderly population.

[00322] N-803 has demonstrated significant ability to induce increases in broad T-cell reactivity both in preclinical (mice) and clinical (breast, lung, and pancreatic cancers) settings. It is possible that by activating T-cell diversity, cross-reactivity to past coronavirus infections may offer immunity to COVTD-19 following N-803 enhancement of T-cell reactivity and overcome immune evasion. N-803 significantly promotes NK cell and CD8- T-cell proliferation and activation in the peripheral circulation and lymphoid organs in healthy mice and cynomolgus monkeys, as well as in a variety of murine and rodent tumor models including bladder cancer, lung cancer, melanoma, lymphoma, multiple myeloma, colon cancer, breast cancer. and glioblastoma. Additional preclinical evidence exists for N-803's antiviral effect, including in murine and nonhuman primate (NHP) models.

[00323] Clinical evidence from early phase trials has also supported these preclinical studies showing NK cell and CD8+ T-cell proliferation and activation. N-803 increases the cytotoxic potential of these immune cells as indicated by upregulation of the expression of activation markers including perforin and granzyme B. The phenotypic changes induced in NK and CD8+ T cells by N-803 resulted in enhanced anticancer activity (through antibody-dependent cellular cytotoxicity, direct cellular cytotoxicity, and enhanced tumor-specific cytotoxicity) and prolonged survival *in vivo*.

[00324] In healthy volunteers, NK cell and CD8+ T-cell activation has been demonstrated. In a study of healthy volunteers administered N-803 at doses of 10 μg/kg and 20 μg/kg SC, N-803-treated subjects demonstrated an over 20-fold increase in Ki-67 stained NK and CD8+T cells. Clinical efficacy of N-803 in stimulating lymphopenic response has been demonstrated in patients with acute myeloid leukemia (AML), breast, lung, and pancreatic cancers.

[00325] Therefore, as preclinical *in vitro* and *in vivo* studies along with clinical data have demonstrated that N-803 binds with a higher affinity to IL-15 receptor presenting cells, has enhanced lymphoid distribution, prolonged half-life, and causes proliferation and activation of effector NK cells and CD8+ memory T cells resulting in antitumor activity, N-803 may be used as a treatment option to counter COVID-19 related lymphopenia in a manner similar to N-803 stimulating both NK and CD8 T cells and rescue lymphopenia in normal healthy subjects as well as patients with cancer. Thus, N-803 may be used for reversal or alleviation of lymphopenia in

patients infected with COVID-19 and to so improve disease outcomes. For example, an infected subject may be treated by receiving a subcutaneous (SC) injection of N-803 on day 1, and on day 15 (if needed) in the abdomen. Most typically, the N-803 will be provided in liquid injectable form at a dosage of 10 mcg/kg.

[00326] In some embodiments, the numbers expressing quantities of ingredients, properties such as concentration, reaction conditions, and so forth, used to describe and claim certain embodiments of the invention are to be understood as being modified in some instances by the term "about." Accordingly, in some embodiments, the numerical parameters set forth in the written description and attached claims are approximations that can vary depending upon the desired properties sought to be obtained by a particular embodiment. The recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein.

[00327] As used herein, the term "administering" a pharmaceutical composition or drug refers to both direct and indirect administration of the pharmaceutical composition or drug, wherein direct administration of the pharmaceutical composition or drug is typically performed by a health care professional (*e.g.*, physician, nurse, etc.), and wherein indirect administration includes a step of providing or making available the pharmaceutical composition or drug to the health care professional for direct administration (*e.g.*, via injection, infusion, oral delivery, topical delivery, etc.). It should further be noted that the terms "prognosing" or "predicting" a condition, a susceptibility for development of a disease, or a response to an intended treatment is meant to cover the act of predicting or the prediction (but not treatment or diagnosis of) the condition, susceptibility and/or response, including the rate of progression, improvement, and/or duration of the condition in a subject.

[00328] All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (*e.g.*, "such as") provided with respect to certain embodiments herein is intended merely to better illuminate the technologies disclosed herein and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the claimed invention.

[00329] As used in the description herein and throughout the claims that follow, the meaning of "a," "an," and "the" includes plural reference unless the context clearly dictates otherwise. Also, as used in the description herein, the meaning of "in" includes "in" and "on" unless the context clearly dictates otherwise. As also used herein, and unless the context dictates otherwise, the term "coupled to" is intended to include both direct coupling (in which two elements that are coupled to each other contact each other) and indirect coupling (in which at least one additional element is located between the two elements). Therefore, the terms "coupled to" and "coupled with" are used synonymously.

[00330] It should be apparent to those skilled in the art that many more modifications besides those already described are possible without departing from the inventive concepts herein. The inventive subject matter, therefore, is not to be restricted except in the scope of the appended claims. Moreover, in interpreting both the specification and the claims, all terms should be interpreted in the broadest possible manner consistent with the context. In particular, the terms "comprises" and "comprising" should be interpreted as referring to elements, components, or steps in a non-exclusive manner, indicating that the referenced elements, components, or steps may be present, or utilized, or combined with other elements, components, or steps that are not expressly referenced. Where the specification or the claims refer to at least one of something selected from the group consisting of A, B, C .... and N, the text should be interpreted as requiring only one element from the group, not A plus N, or B plus N, etc.

CLAIMS

What is claimed is:

1. A recombinant nucleic acid, comprising:
    a. a first portion encoding a severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein (N) that is fused to an endosomal targeting sequence (N-ETSD), wherein the first portion is functionally coupled to one or more regulatory elements that enable N-ETSD expression; and
    b. a second portion encoding a SARS virus spike protein (S), wherein the second portion is functionally coupled to one or more regulatory elements that enable S expression.

2. The recombinant nucleic acid of claim 1, wherein the SARS virus is SARS-CoV-2.

3. The recombinant nucleic acid of claim 2, wherein the endosomal targeting sequence of the N-ETSD is encoded at a 5'-end of the first portion.

4. The recombinant nucleic acid of claim 2, wherein the endosomal targeting sequence of the N-ETSD is encoded at a 3'-end of the first portion.

5. The recombinant nucleic acid of claim 2, wherein the first and second portions are arranged in a bicistronic sequence.

6. The recombinant nucleic acid of claim 3, wherein the N-ETSD has an amino acid sequence that has at least 90% identity to amino acid sequence SEQ ID NO:1.

7. The recombinant nucleic acid of claim 6, wherein the N-ETSD has amino acid sequence SEQ ID NO:1.

8. The recombinant nucleic acid of claim 7, wherein the first portion has nucleotide sequence SEQ ID NO:2.

9. The recombinant nucleic acid of claim 6, wherein the S protein has an amino acid sequence that has at least 90% identity to amino acid sequence SEQ ID NO:3 or SEQ ID NO:4.

10. The recombinant nucleic acid of claim 9, wherein the S protein has amino acid sequence SEQ ID NO:3.

11. The recombinant nucleic acid of claim 9, wherein the S protein has amino acid sequence SEQ ID NO:4.

12. The recombinant nucleic acid of claim 9, wherein the second portion has nucleotide sequence SEQ ID NO:5.

13. The recombinant nucleic acid of claim 9, wherein the second portion has nucleotide sequence SEQ ID NO:6.

14. The recombinant nucleic acid of any one of the previous claims, further comprising a third portion encoding a co-stimulatory molecule or an immune stimulatory cytokine.

15. The recombinant nucleic acid of any one of the previous claims, wherein the recombinant nucleic acid is integrated into a viral or yeast expression vector.

16. The recombinant nucleic acid of claim 15, wherein the viral expression vector is an adenoviral expression vector having an E1 gene region deletion and an E2b gene region deletion.

17. The recombinant nucleic acid of claim 15, wherein the yeast expression vector is an expression vector for *Saccharomyces cerevisiae*.

18. The recombinant nucleic acid of any one of the previous claims, wherein the nucleic acid is a deoxyribonucleic acid (DNA).

19. A recombinant replication defective adenovirus, comprising an E1 gene deletion, an E2b gene deletion, and a recombinant nucleic acid that includes:
    a. a first portion encoding a SARS coronavirus N-ETSD, wherein the first portion is functionally coupled to one or more regulatory elements that enable N-ETSD expression; and
    b. a second portion encoding a SARS S protein, wherein the second portion is each functionally coupled to one or more regulatory elements that enable S expression.

20. The adenovirus of claim 19, wherein the SARS virus is SARS-CoV-2.

21. The adenovirus of claim 20, wherein the endosomal targeting sequence of the N-ETSD is encoded at a 5'-end of the first portion.

22. The adenovirus of claim 20, wherein the endosomal targeting sequence of the N-ETSD is encoded at a 3'-end of the first portion.

23. The adenovirus of claim 22, wherein the first and second portions are arranged in a bicistronic sequence.

24. The adenovirus of claim 22, wherein the N-ETSD has an amino acid sequence that has at least 90% identity to amino acid sequence SEQ ID NO:1.

25. The adenovirus of claim 24, wherein the N-ETSD has an amino acid sequence that has amino acid sequence SEQ ID NO:1.

26. The adenovirus of claim 24, wherein the first portion has nucleotide sequence SEQ ID NO:2.

27. The adenovirus of claim 24, wherein the S protein has an amino acid sequence that has at least 90% identity to amino acid sequence SEQ ID NO:3 or SEQ ID NO:4.

28. The adenovirus of claim 27, wherein the S protein has amino acid sequence SEQ ID NO:3.

29. The adenovirus of claim 27, wherein the S protein has amino acid sequence SEQ ID NO:4.

30. The adenovirus of claim 27, wherein the second portion has nucleotide sequence SEQ ID NO:5.

31. The adenovirus of claim 27, wherein the second portion has nucleotide sequence SEQ ID NO:6.

32. The adenovirus of any one of claims 19–31, further comprising a third portion encoding a co-stimulatory molecule or an immune stimulatory cytokine.

33. The adenovirus of any one of claims 19–32, wherein the recombinant adenovirus has an E3 gene region deletion.

34. A recombinant yeast, comprising a recombinant nucleic acid that includes:
    a. a first portion encoding a SARS coronavirus N-ETSD, wherein the first portion is functionally coupled to one or more regulatory elements that enable N-ETSD expression; and

b. a second portion encoding a SARS S protein, wherein the second portion is each functionally coupled to one or more regulatory elements that enable S expression.

35. The recombinant yeast of claim 34, wherein the SARS virus is SARS-CoV-2.

36. The recombinant yeast of claim 35, wherein the endosomal targeting sequence of the N-ETSD is encoded at a 5'-end of the nucleocapsid protein.

37. The recombinant yeast of claim 35, wherein the endosomal targeting sequence of the N-ETSD is encoded at a 3'-end of the nucleocapsid protein.

38. The recombinant yeast of claim 37, wherein the first and second portions are arranged in a bicistronic sequence.

39. The recombinant yeast of claim 37, wherein the N-ETSD has an amino acid sequence that has at least 90% identity to amino acid sequence SEQ ID NO:1.

40. The recombinant yeast of claim 49, wherein the N-ETSD has amino acid sequence SEQ ID NO:1.

41. The recombinant yeast of claim 40, wherein the first portion has nucleotide sequence SEQ ID NO:2.

42. The recombinant yeast of claim 39, wherein the S protein has an amino acid sequence that has at least 90% identity to amino acid sequence SEQ ID NO:3 or SEQ ID NO:4.

43. The recombinant yeast of claim 42, wherein the S protein has amino acid sequence SEQ ID NO:3.

44. The recombinant yeast of claim 42, wherein the S protein has amino acid sequence SEQ ID NO:4.

45. The recombinant yeast of claim 42, wherein the first portion has nucleotide sequence SEQ ID NO:5.

46. The recombinant yeast of claim 42, wherein the first portion has nucleotide sequence SEQ ID NO:6.

47. The recombinant yeast of any one of claims 35–46, further comprising a third portion encoding a co-stimulatory molecule or an immune stimulatory cytokine.

48. The recombinant yeast of any one of claims 35–47, wherein the yeast is *S. cerevisiae*.

49. The recombinant yeast of claim 48, wherein the yeast is a lysed yeast.

50. A vaccine composition comprising the recombinant nucleic acid of any one of claims 1–18.

51. The vaccine composition of claim 50, wherein the recombinant nucleic acid is encapsulated in a lipid nanoparticle.

52. A vaccine composition comprising aragonite particles admixed with the recombinant replication defective adenovirus of any one of claims 19–33, wherein the recombinant replication defective adenovirus is lyophilized.

53. The vaccine composition of claim 52, wherein the aragonite particles have an average particle size of between 100 nm and 1 mm.

54. A recombinant nucleic acid according to any one of claims 1–18, for use in generating a vaccine against a SARS virus.

55. A recombinant nucleic acid according to any one of claims 1–18, for use in inducing immunity against a SARS virus.

56. A recombinant replication defective adenovirus according to any one of claims 19–33, for use in inducing immunity against a SARS virus.

57. A recombinant yeast according to any one of claims 34–49, for use in inducing immunity against a SARS virus.

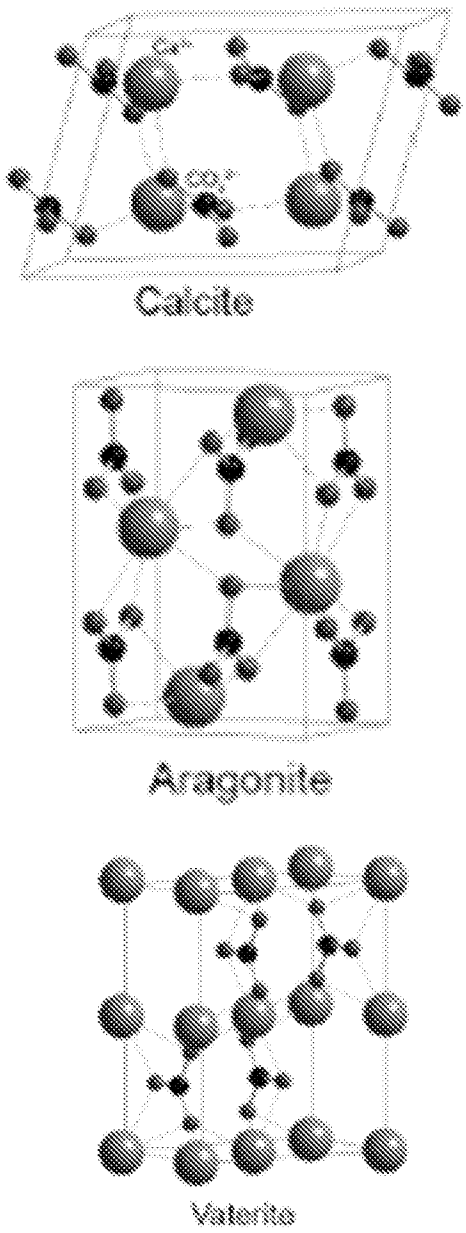


FIG.1

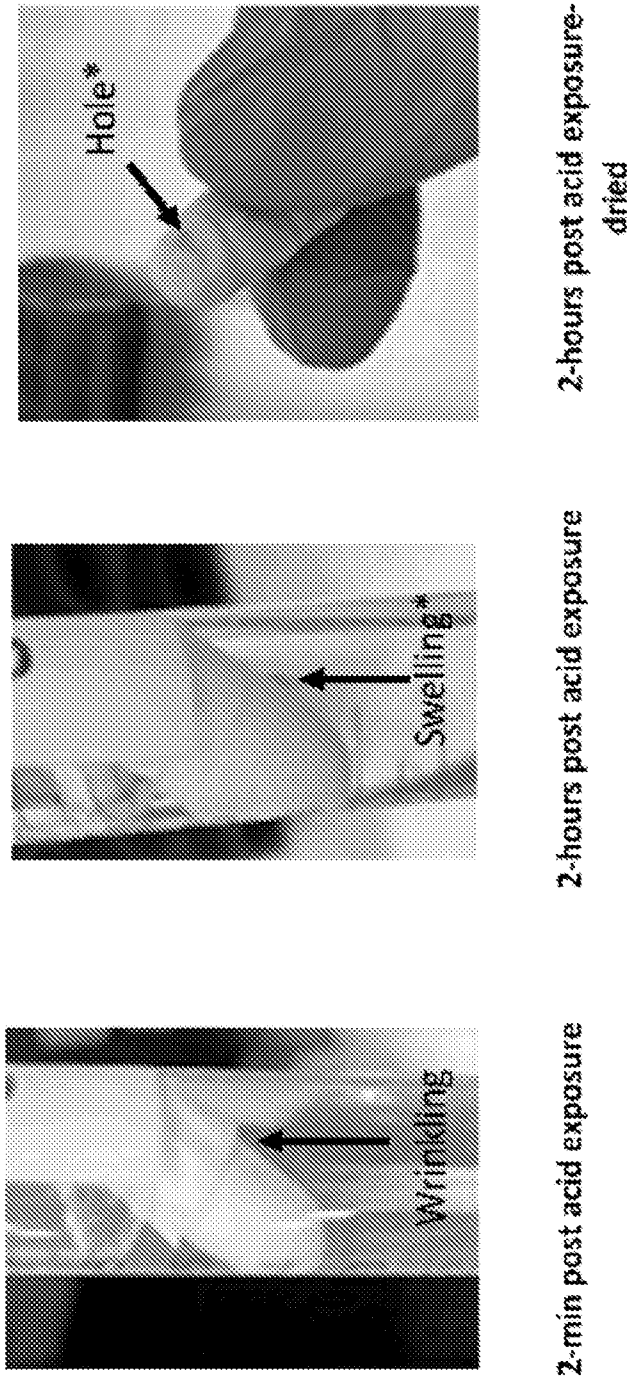

Hole*
2-hours post acid exposure-dried
Swelling*
2-hours post acid exposure
Wrinkling
2-min post acid exposure

FIG.2

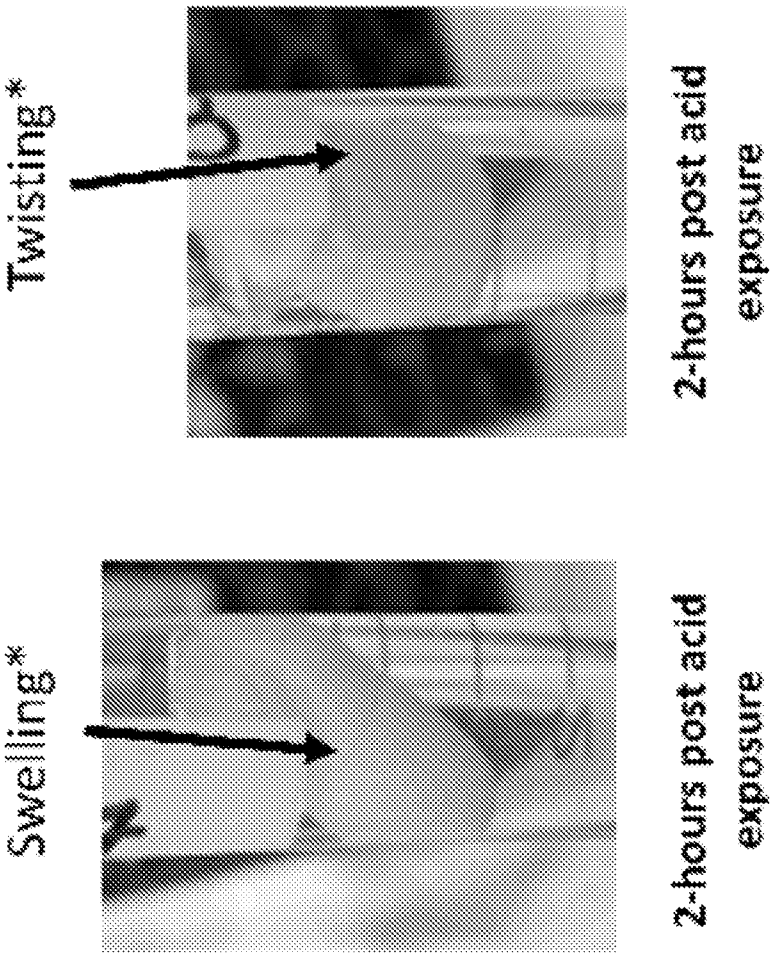

Twisting*
2-hours post acid exposure
Swelling*
2-hours post acid exposure

FIG.3

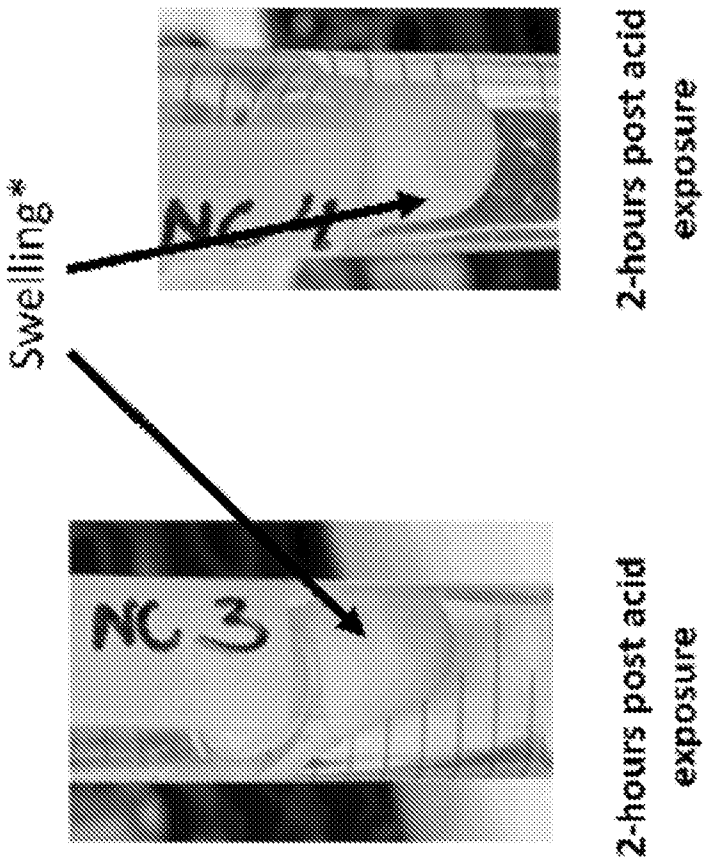

Swelling*
NC 4
NC 3
2-hours post acid exposure
2-hours post acid exposure

FIG.4

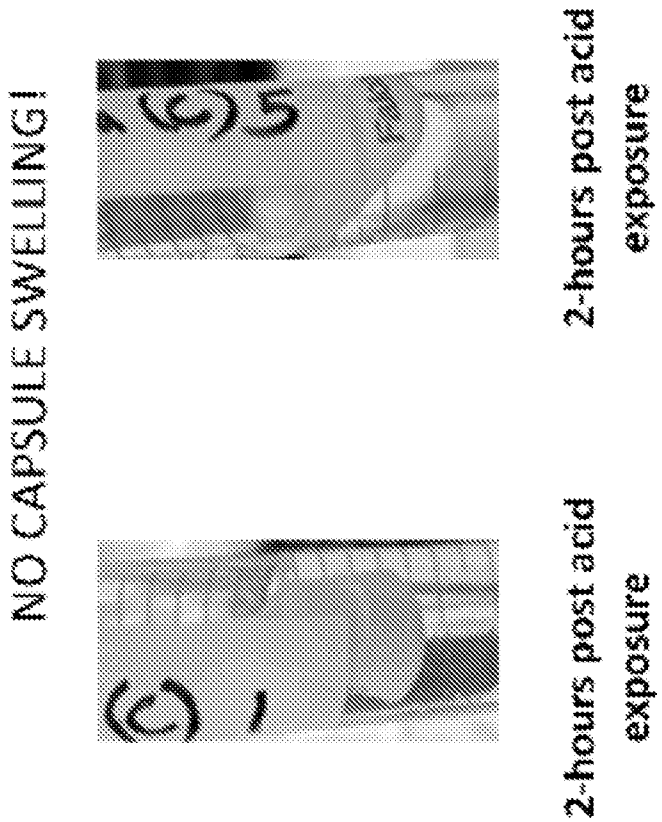

NO CAPSULE SWELLING!
2-hours post acid exposure
2-hours post acid exposure

FIG.5

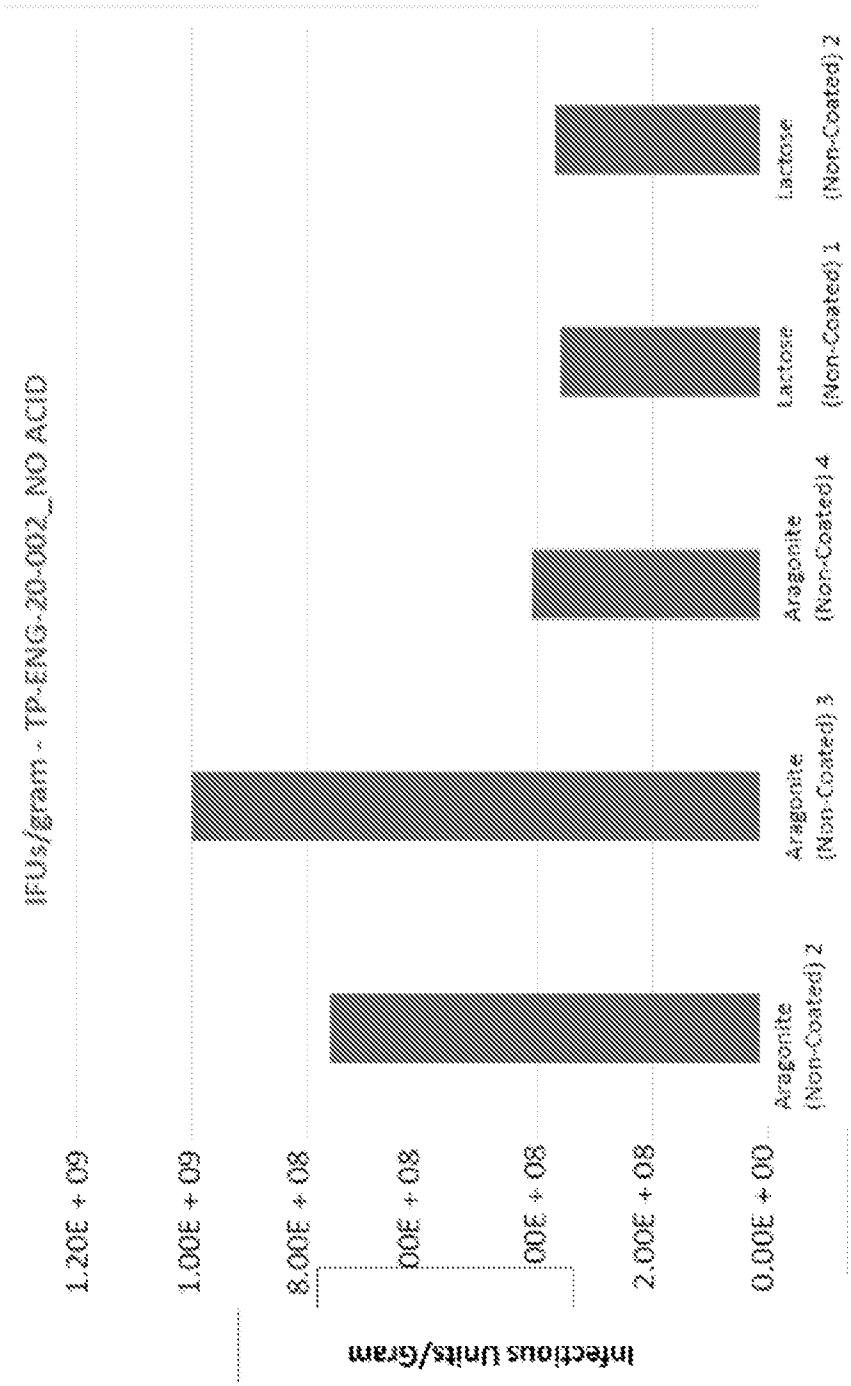

IFUs/gram - TP-ENG-20-002_NO ACID
1.20E + 09
1.00E + 09
8.00E + 08
6.00E + 08
4.00E + 08
2.00E + 08
0.00E + 00
Infectious Units/Gram
Aragonite (Non-Coated) 2
Aragonite (Non-Coated) 3
Aragonite (Non-Coated) 4
Lactose (Non-Coated) 1
Lactose (Non-Coated) 2
CAPSULE TYPE

FIG.6

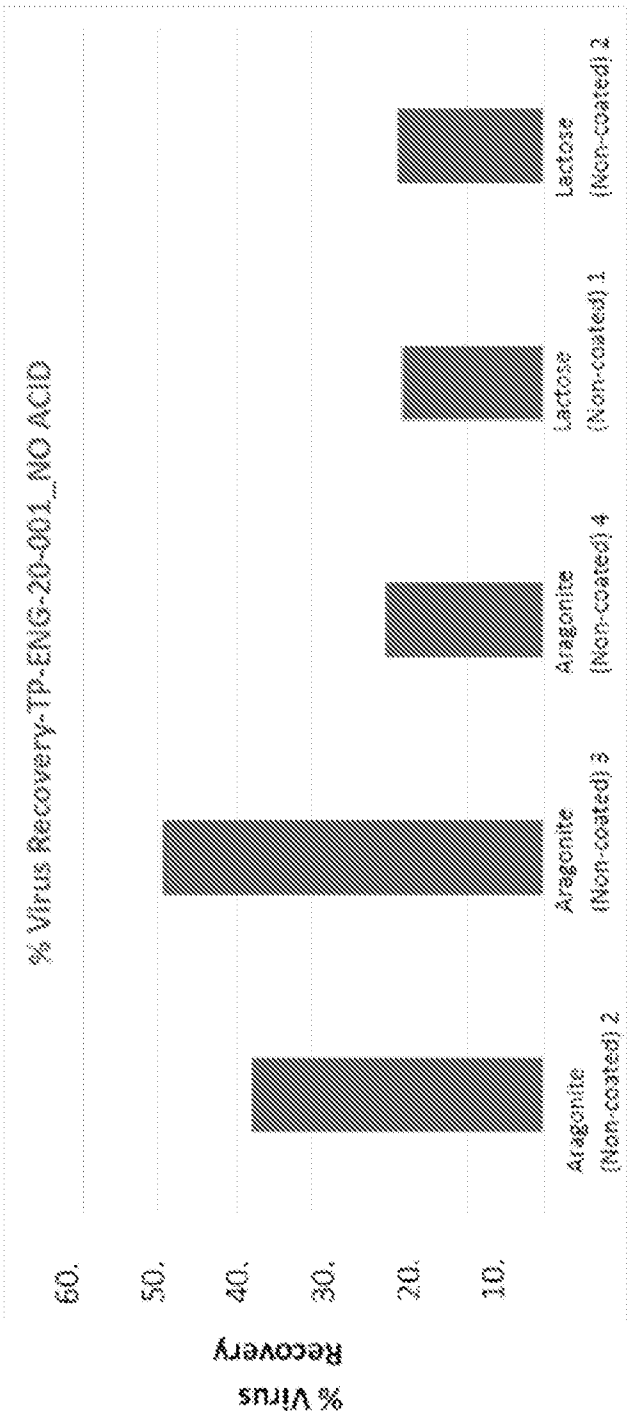

% Virus Recovery-TP-ENG-20-001_NO ACID
% Virus Recovery
60.
50.
40.
30.
20.
10.
Aragonite (Non-coated) 2
Aragonite (Non-coated) 3
Aragonite (Non-coated) 4
Lactose (Non-coated) 1
Lactose (Non-coated) 2
CAPSULE TYPE

FIG.7

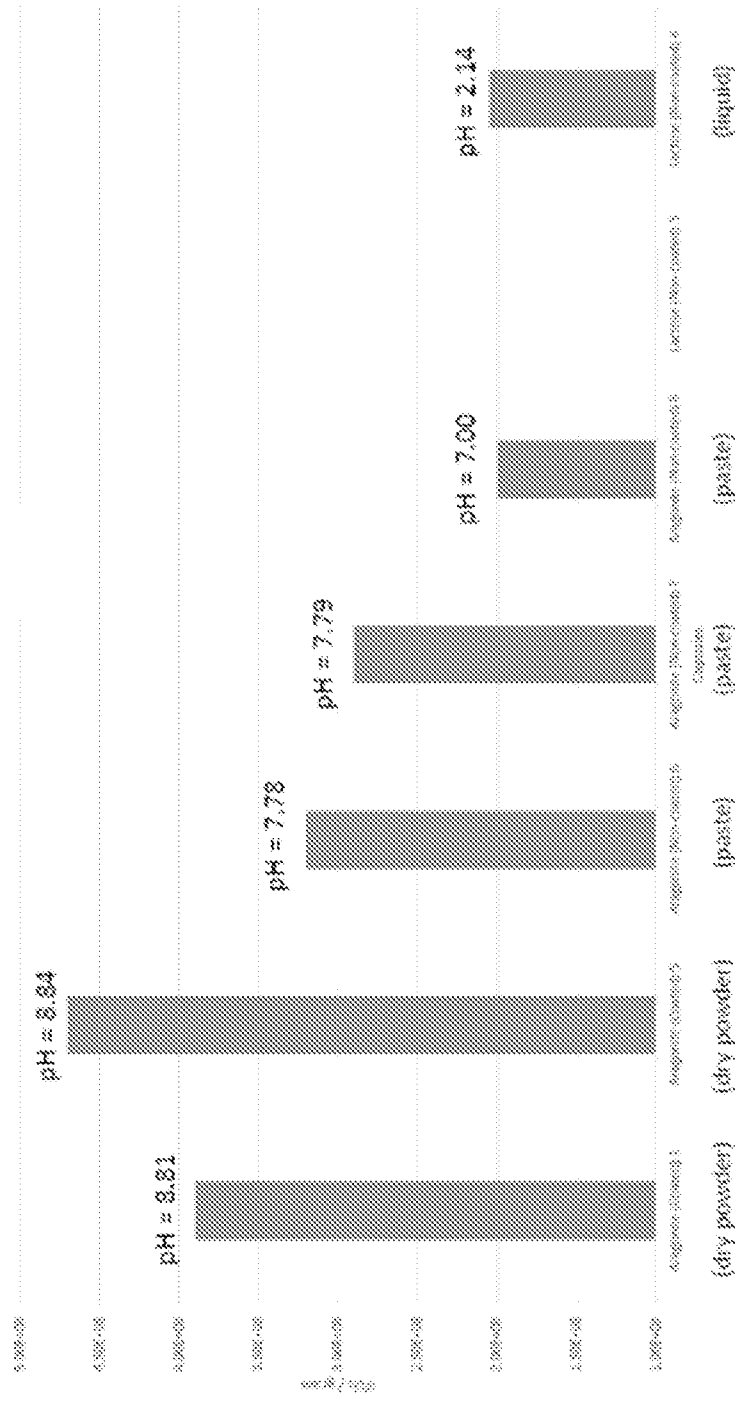

IFUs/gram - TP-ENG-20-002+ACID
pH = 8.84
pH = 8.81
pH = 7.78
pH = 7.79
pH = 7.00
pH = 2.14
(dry powder)
(dry powder)
(paste)
(paste)
(paste)
(liquid)

FIG.8

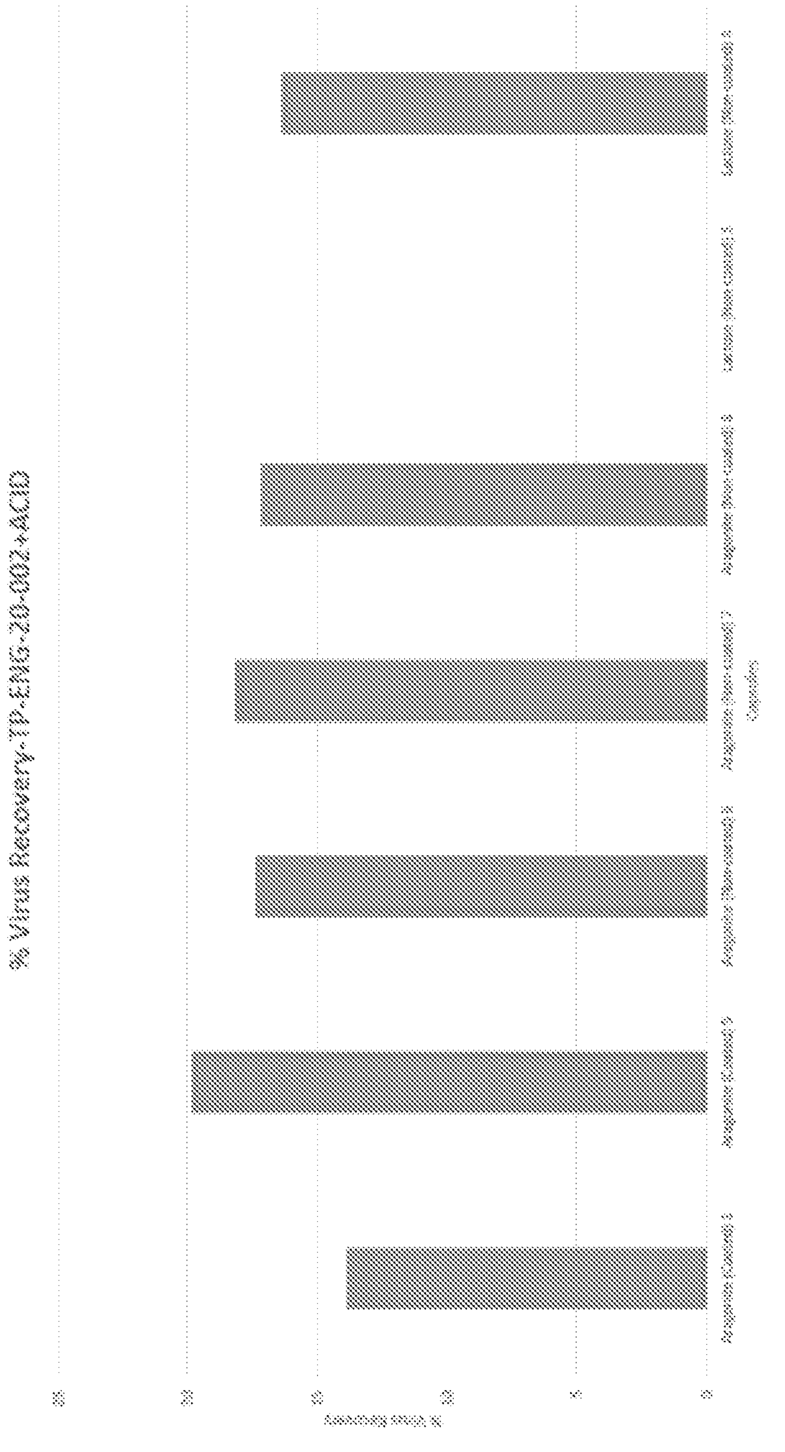


FIG.9

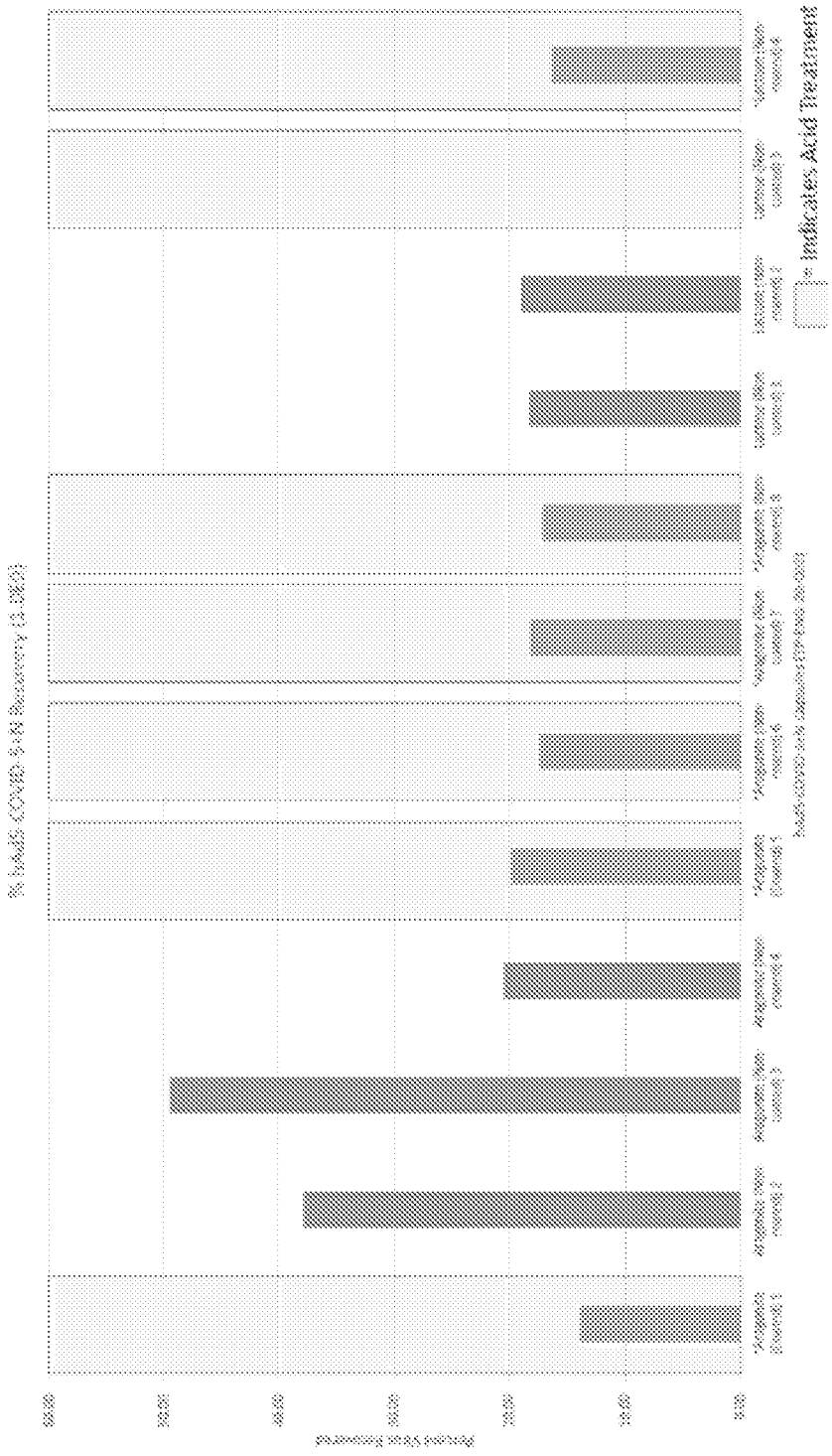


FIG.10

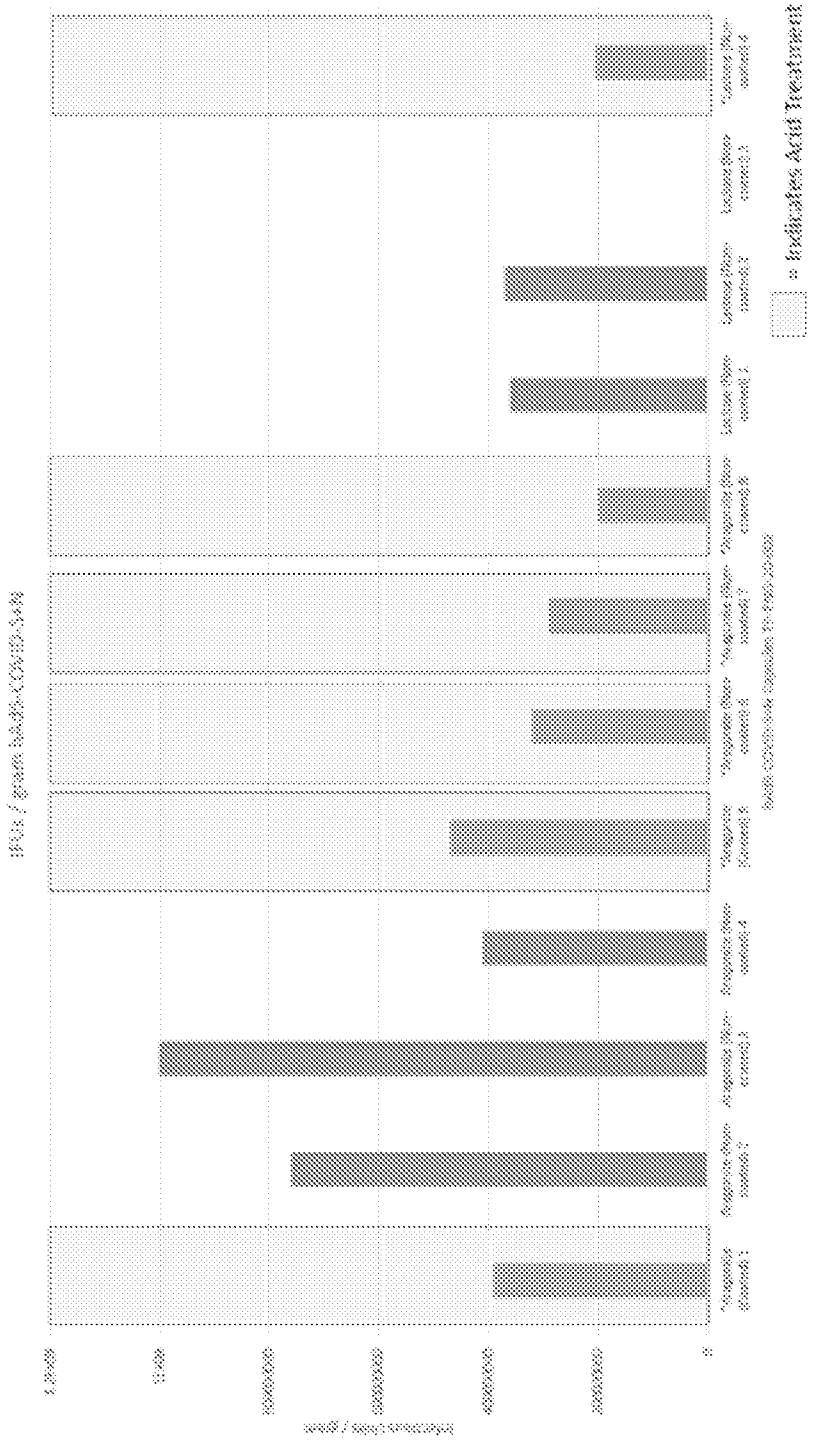


FIG.11

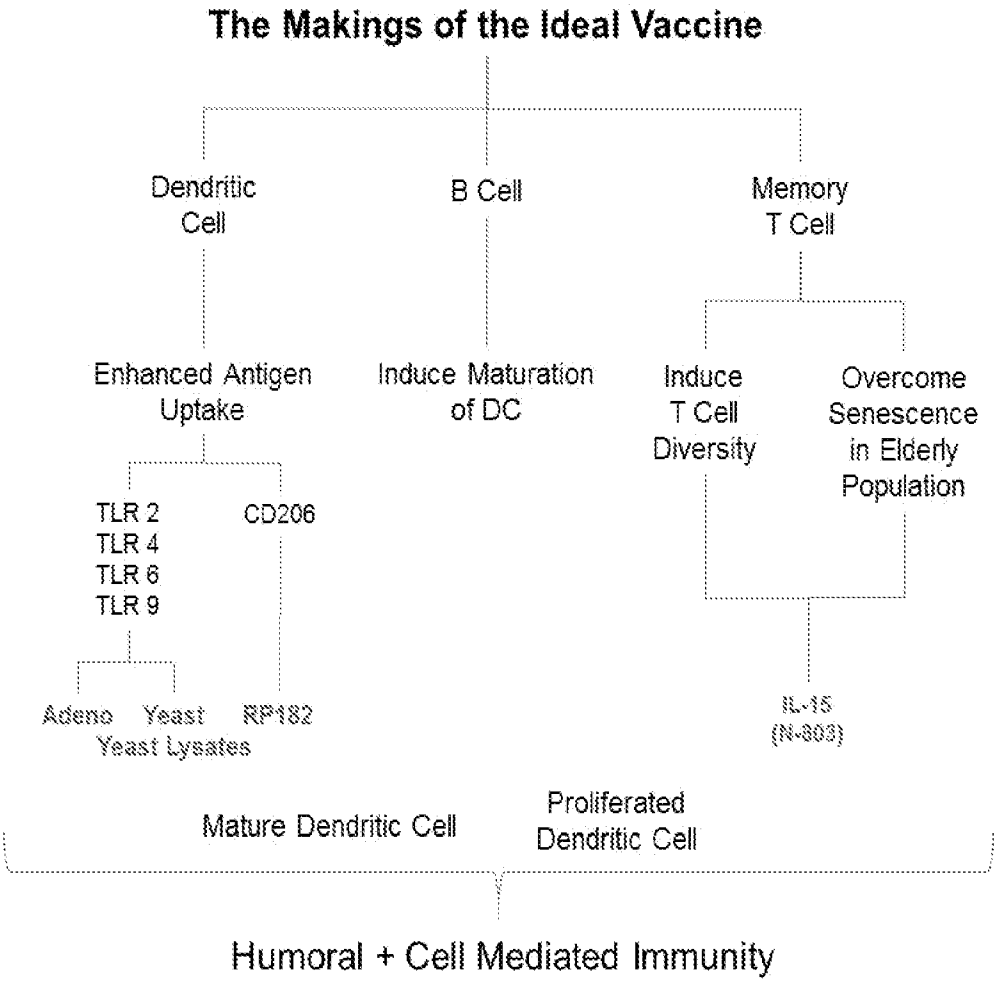

The Makings of the Ideal Vaccine
Dendritic Cell
B Cell
Memory T Cell
Enhanced Antigen Uptake
Induce Maturation of DC
Induce T Cell Diversity
Overcome Senescence in Elderly Population
TLR 2
TLR 4
TLR 6
TLR 9
CD206
Adeno
Yeast
Yeast Lysates
RP182
IL-15
(N-803)
Mature Dendritic Cell
Proliferated Dendritic Cell
Humoral + Cell Mediated Immunity

FIG.12

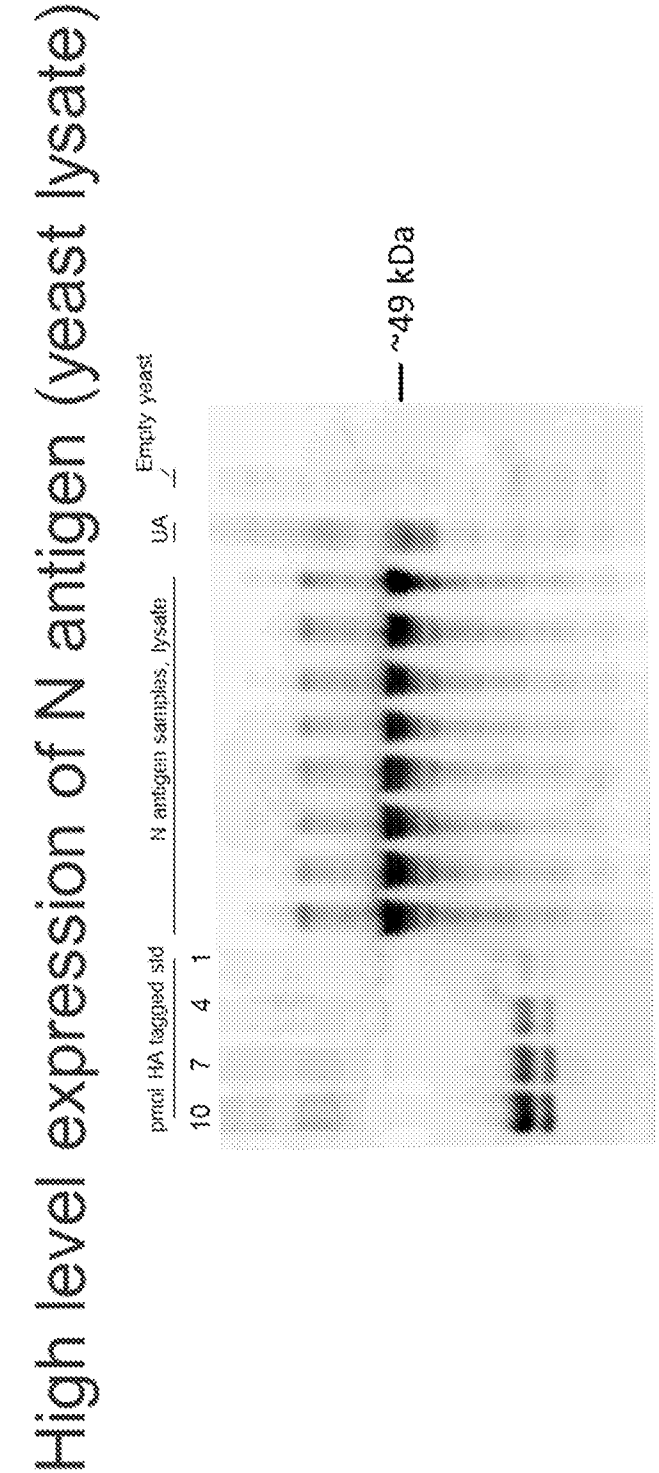

High level expression of N antigen (yeast lysate)
pmol HA tagged std
10 7 4 1
N antigen samples, lysate
UA
Empty yeast
~49 kDa
N is expressed at ~20-25% of total cellular protein
and is stable in lysates from heat inactivated yeast

FIG.13

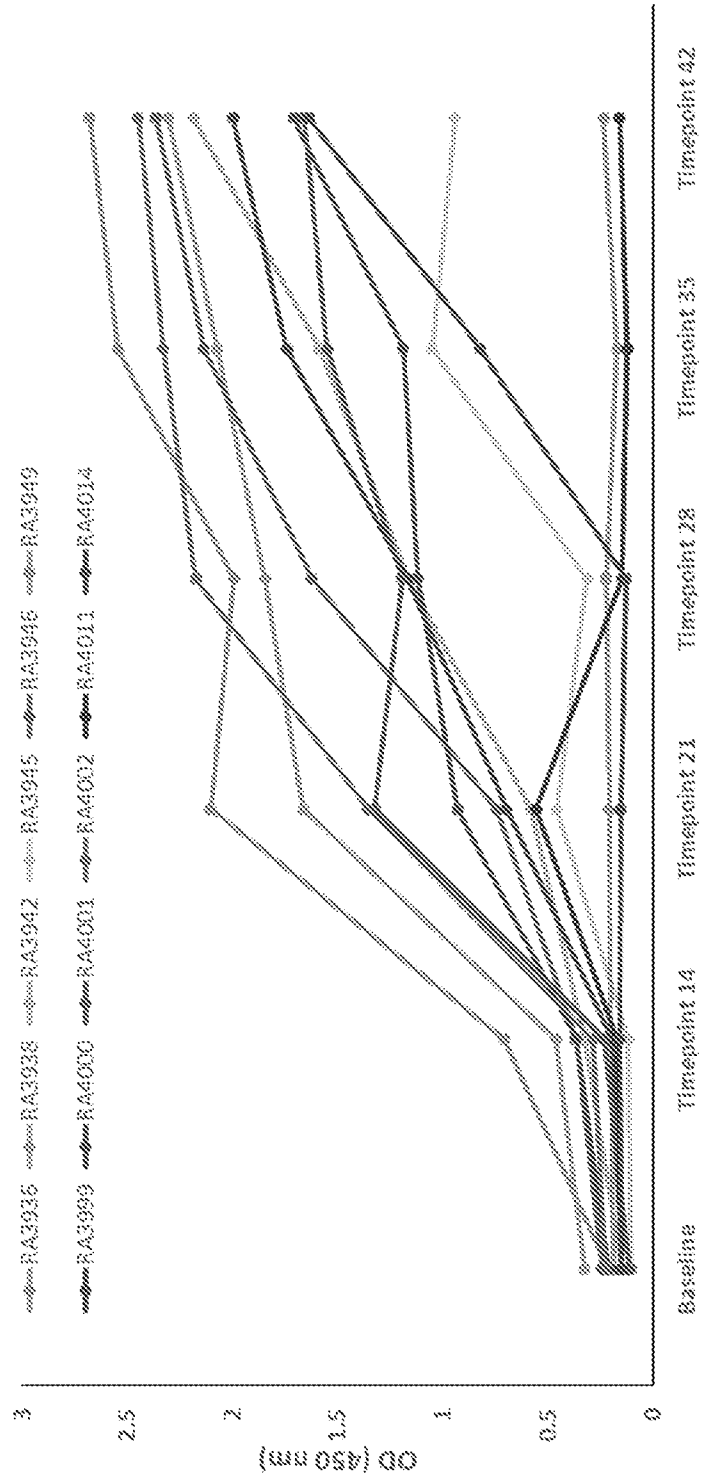

RA3936
RA3938
RA3942
RA3945
RA3946
RA3949
RA3939
RA4030
RA4031
RA4032
RA4011
RA4014
OD (450 nm)
3
2.5
2
1.5
1
0.5
0
Baseline
Timepoint 14
Timepoint 21
Timepoint 28
Timepoint 35
Timepoint 42

FIG.14

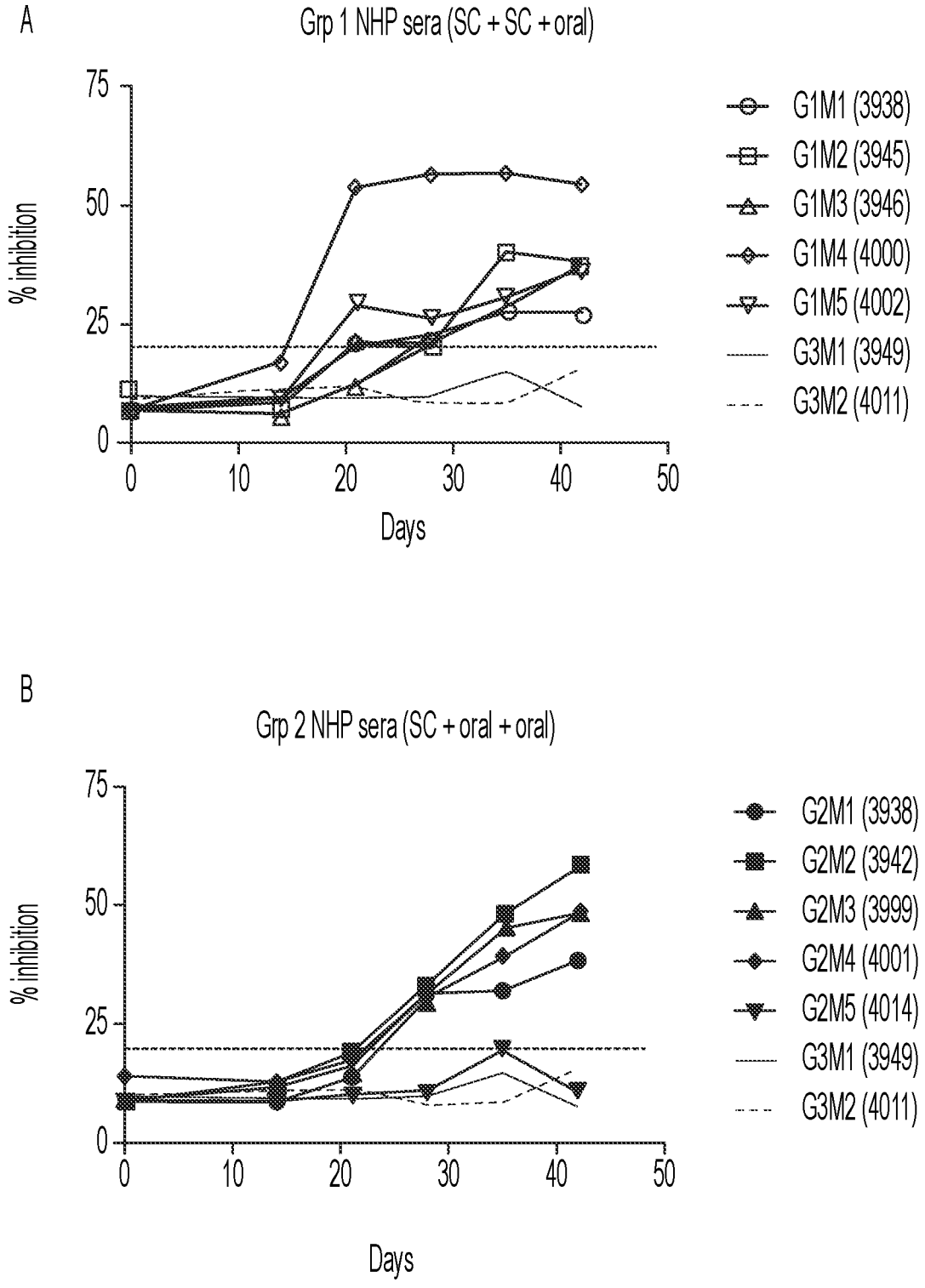

A
Grp 1 NHP sera (SC + SC + oral)
% inhibition
75
50
25
0
0
10
20
30
40
50
Days
G1M1 (3938)
G1M2 (3945)
G1M3 (3946)
G1M4 (4000)
G1M5 (4002)
G3M1 (3949)
G3M2 (4011)
B
Grp 2 NHP sera (SC + oral + oral)
% inhibition
75
50
25
0
0
10
20
30
40
50
Days
G2M1 (3938)
G2M2 (3942)
G2M3 (3999)
G2M4 (4001)
G2M5 (4014)
G3M1 (3949)
G3M2 (4011)

FIG. 15

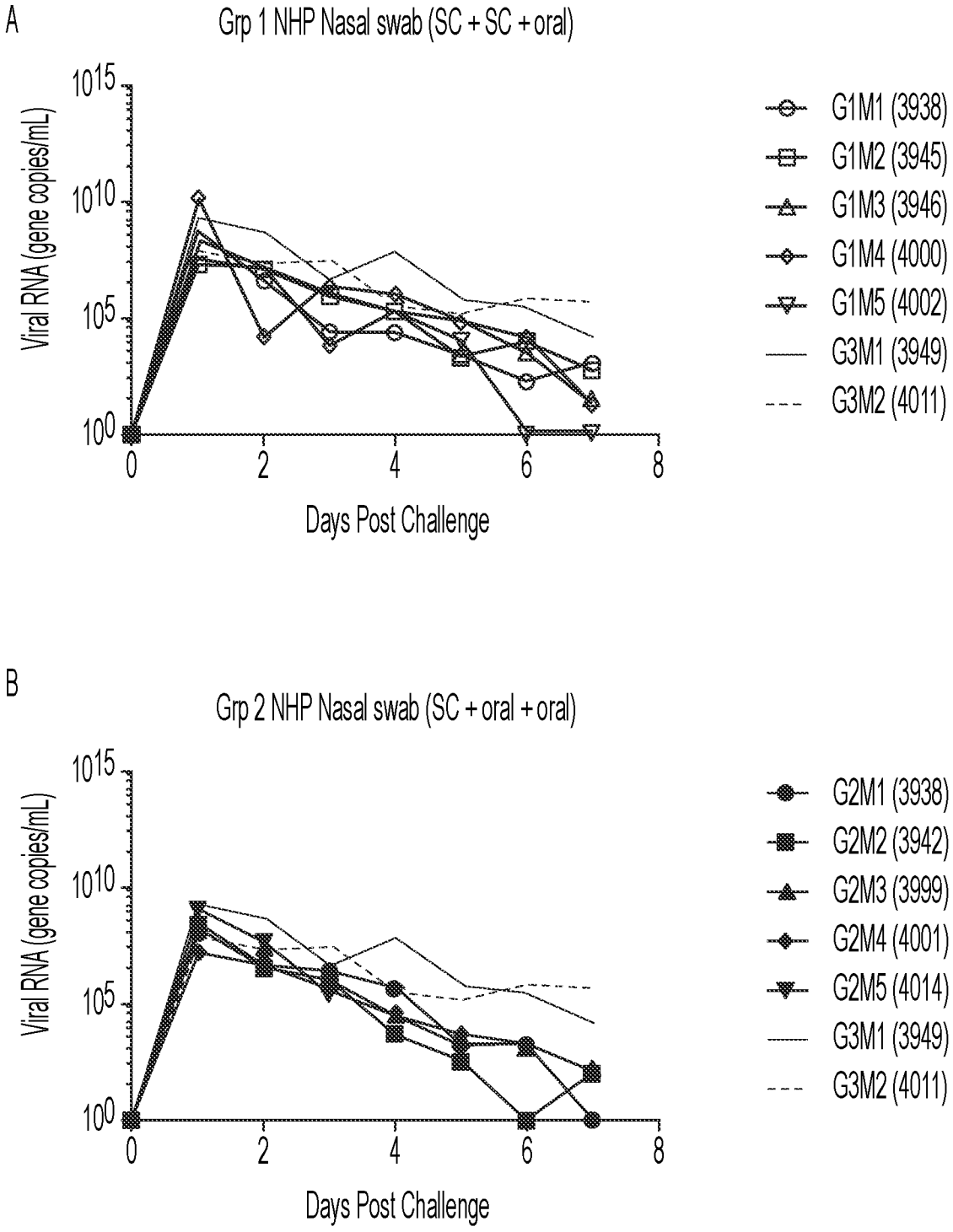
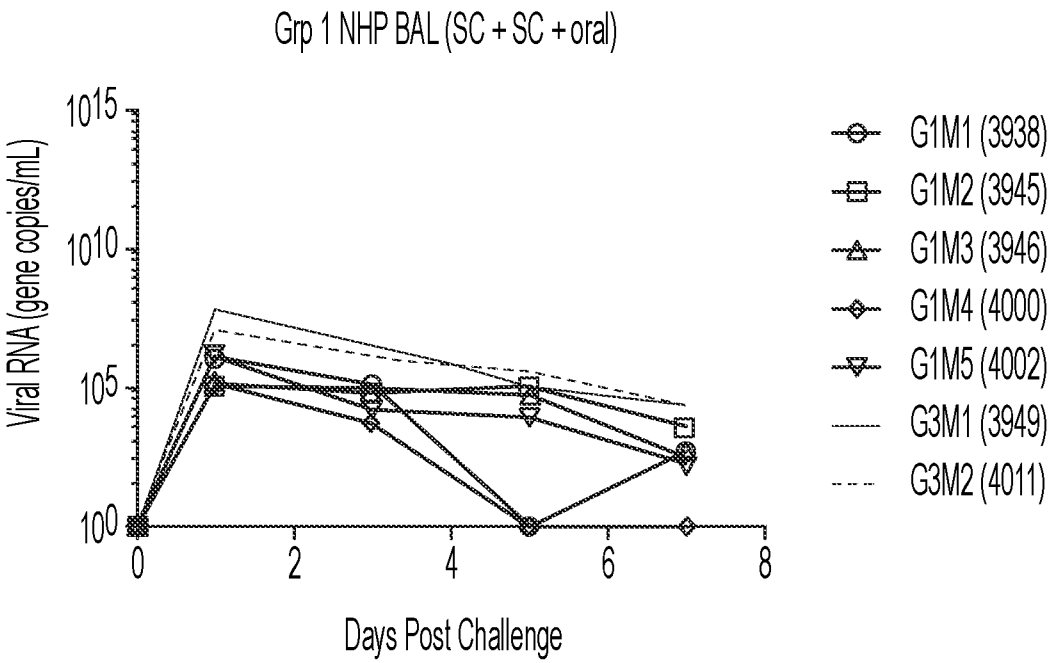

A
Grp 1 NHP Nasal swab (SC + SC + oral)
Viral RNA (gene copies/mL)
10^15
10^10
10^5
10^0
0
2
4
6
8
Days Post Challenge
G1M1 (3938)
G1M2 (3945)
G1M3 (3946)
G1M4 (4000)
G1M5 (4002)
G3M1 (3949)
G3M2 (4011)
B
Grp 2 NHP Nasal swab (SC + oral + oral)
Viral RNA (gene copies/mL)
10^15
10^10
10^5
10^0
0
2
4
6
8
Days Post Challenge
G2M1 (3938)
G2M2 (3942)
G2M3 (3999)
G2M4 (4001)
G2M5 (4014)
G3M1 (3949)
G3M2 (4011)

FIG. 16

A


Grp 1 NHP BAL (SC + SC + oral)
Viral RNA (gene copies/mL)
10^15
10^10
10^5
10^0
0
2
4
6
8
Days Post Challenge
G1M1 (3938)
G1M2 (3945)
G1M3 (3946)
G1M4 (4000)
G1M5 (4002)
G3M1 (3949)
G3M2 (4011)

B

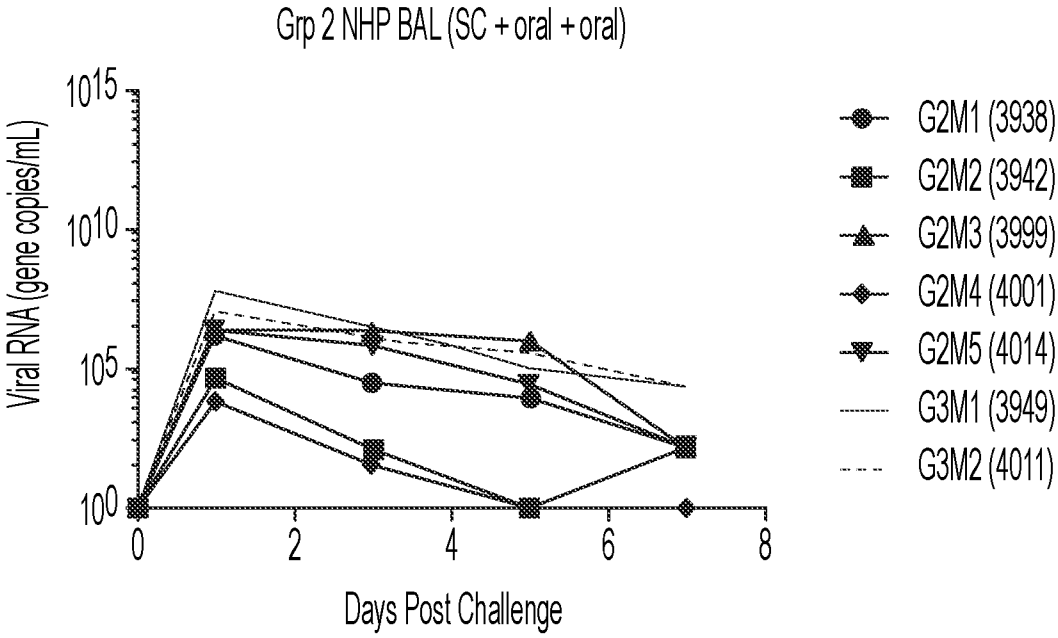

Grp 2 NHP BAL (SC + oral + oral)
Viral RNA (gene copies/mL)
10^15
10^10
10^5
10^0
0
2
4
6
8
Days Post Challenge
G2M1 (3938)
G2M2 (3942)
G2M3 (3999)
G2M4 (4001)
G2M5 (4014)
G3M1 (3949)
G3M2 (4011)

FIG. 17

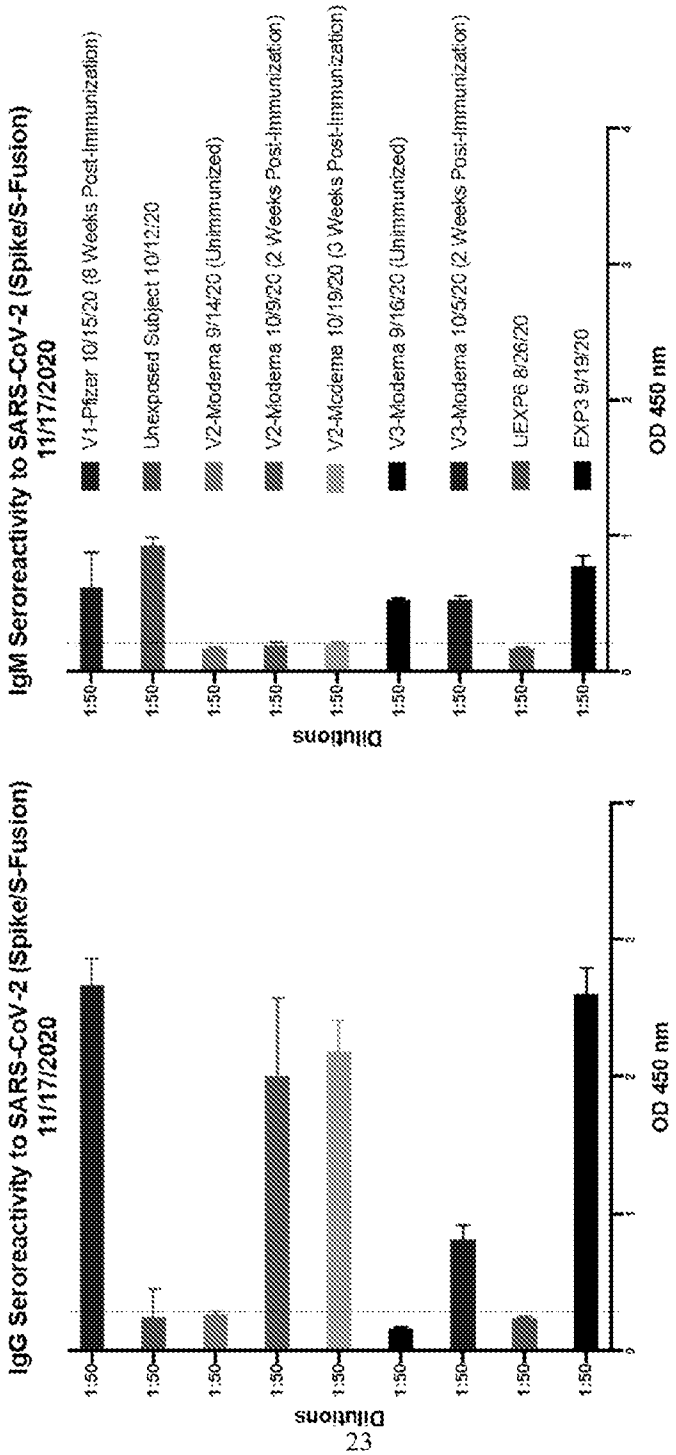

IgM Seroreactivity to SARS-CoV-2 (Spike/S-Fusion)
11/17/2020
V1-Pfizer 10/15/20 (8 Weeks Post-Immunization)
Unexposed Subject 10/12/20
V2-Moderna 9/14/20 (Unimmunized)
V2-Moderna 10/9/20 (2 Weeks Post-Immunization)
V2-Moderna 10/19/20 (3 Weeks Post-Immunization)
V3-Moderna 9/16/20 (Unimmunized)
V3-Moderna 10/5/20 (2 Weeks Post-Immunization)
UEXP6 8/26/20
EXP3 9/19/20
OD 450 nm
Dilutions
1:50
IgG Seroreactivity to SARS-CoV-2 (Spike/S-Fusion)
11/17/2020
OD 450 nm
Dilutions
1:50
23

FIG.18

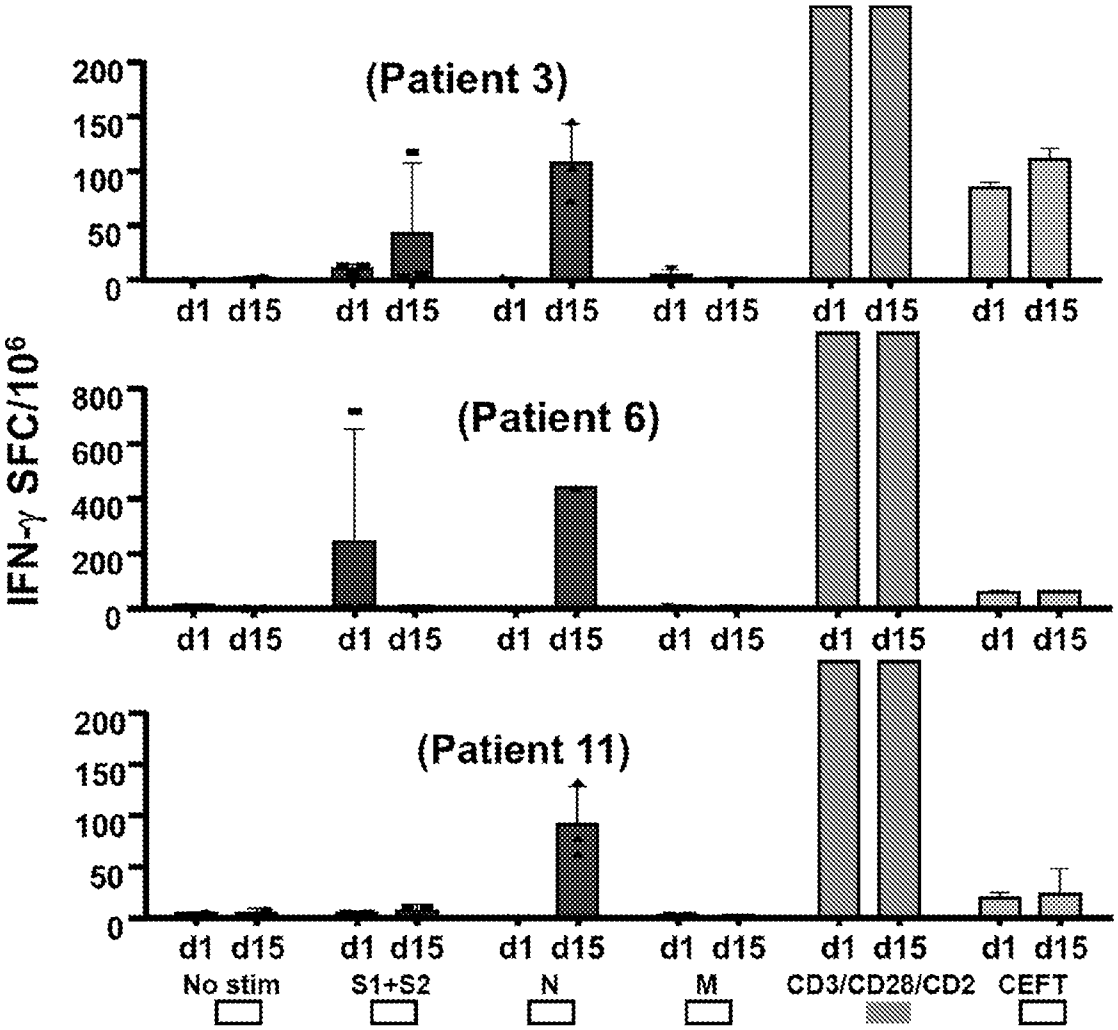

(Patient 3)
(Patient 6)
(Patient 11)
IFN-γ SFC/10^6
200
150
100
50
0
800
600
400
200
0
d1 d15
No stim
S1+S2
N
M
CD3/CD28/CD2
CEFT

FIG.19

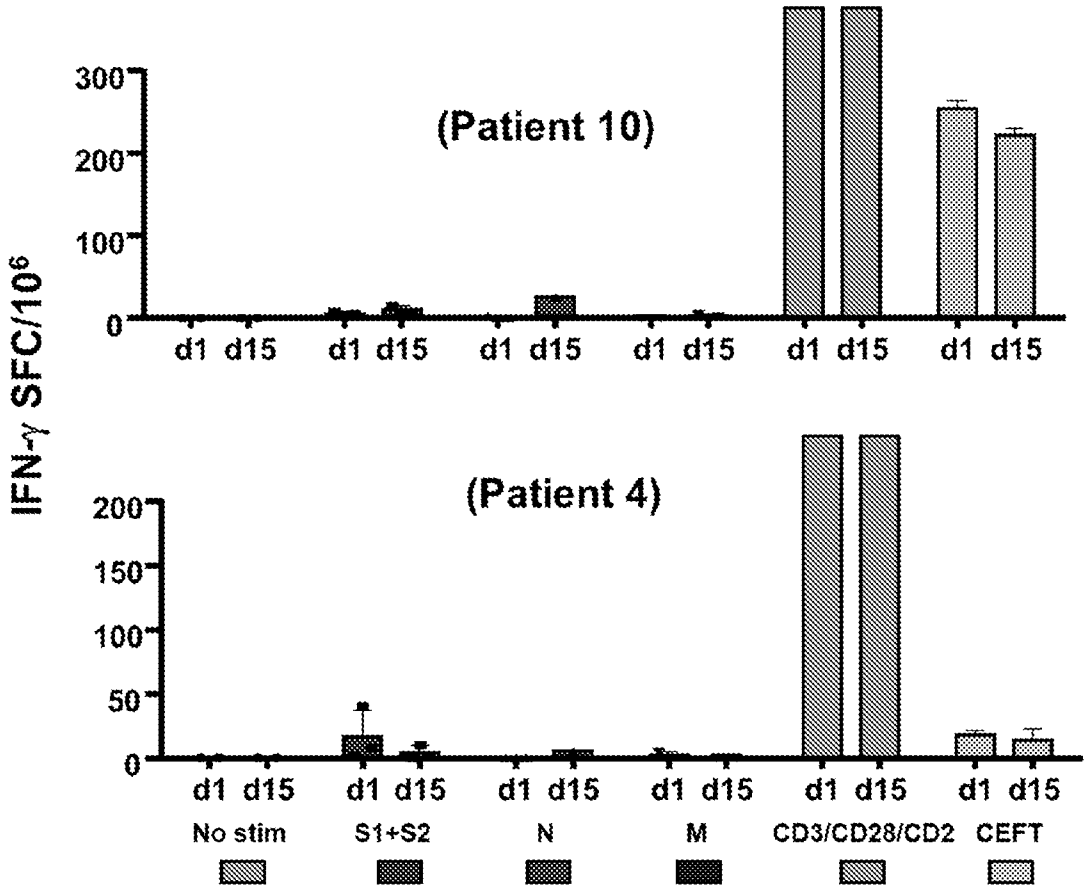

IFN-γ SFC/10^6
(Patient 10)
300
200
100
0
d1 d15 d1 d15 d1 d15 d1 d15 d1 d15 d1 d15
(Patient 4)
200
150
100
50
0
d1 d15 d1 d15 d1 d15 d1 d15 d1 d15 d1 d15
No stim
S1+S2
N
M
CD3/CD28/CD2
CEFT

FIG.20

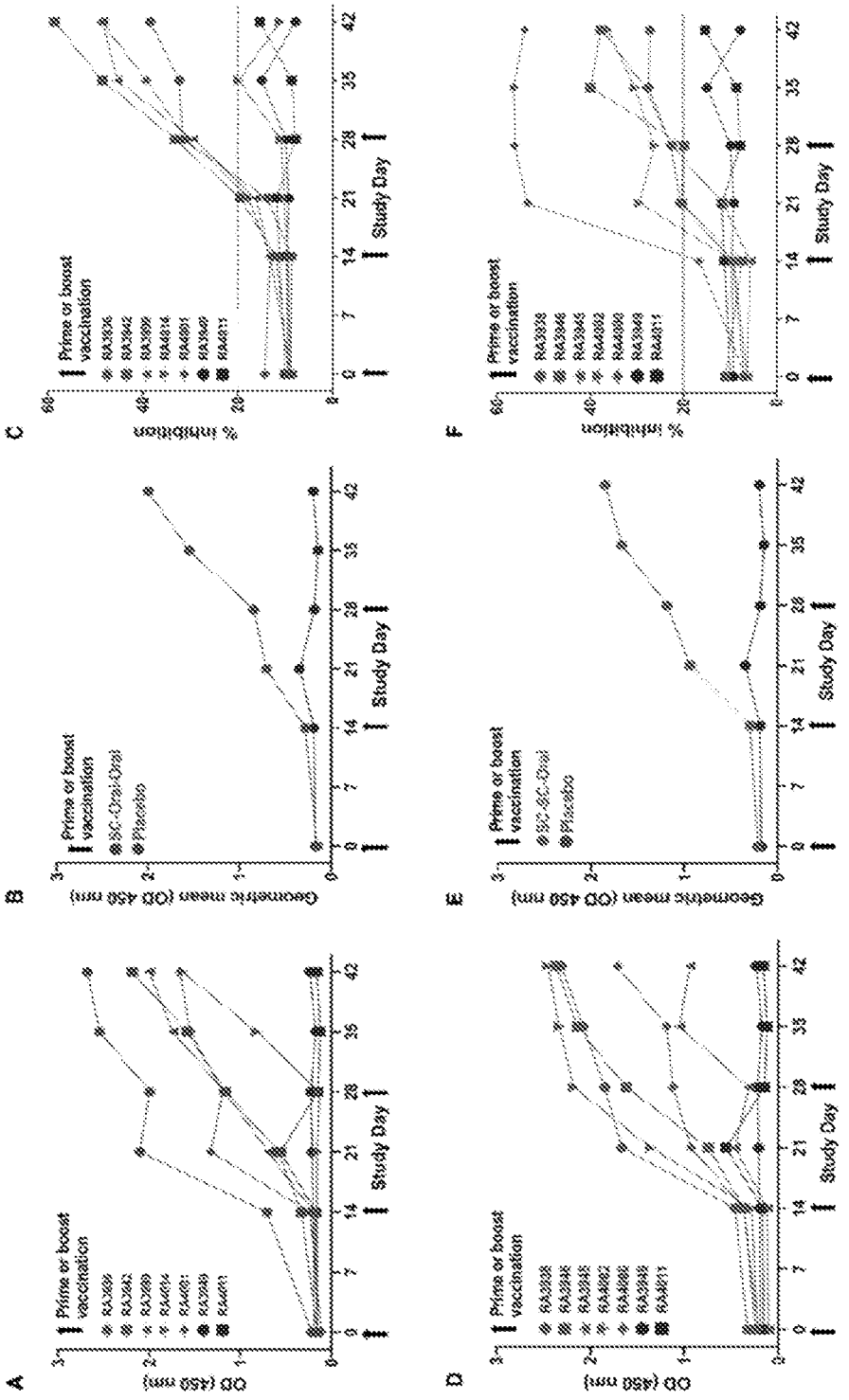

A
B
C
D
E
F
Prime or boost vaccination
Study Day
OD (450 nm)
Geometric mean (OD 450 nm)
% inhibition

FIG.21

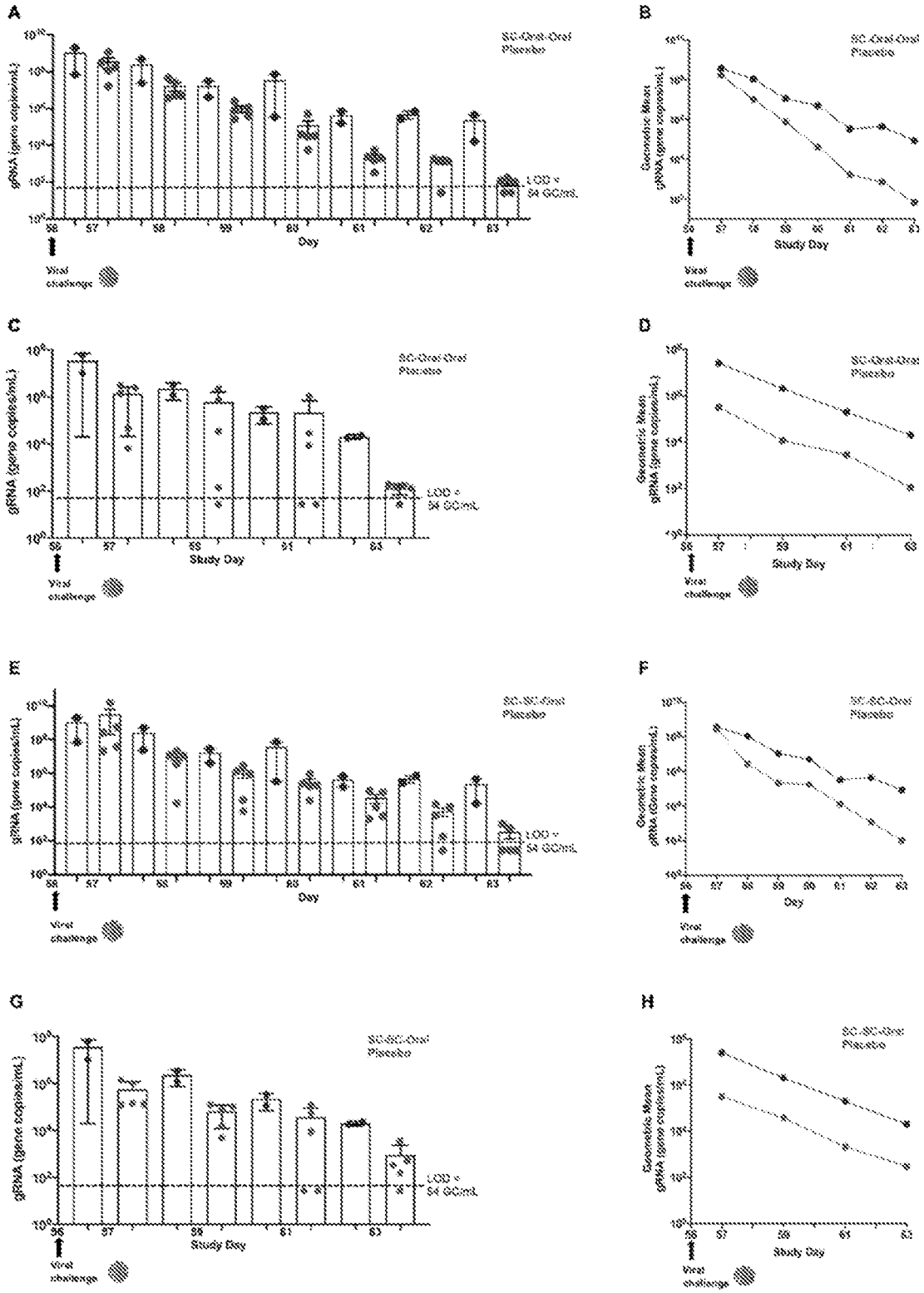


FIG.22

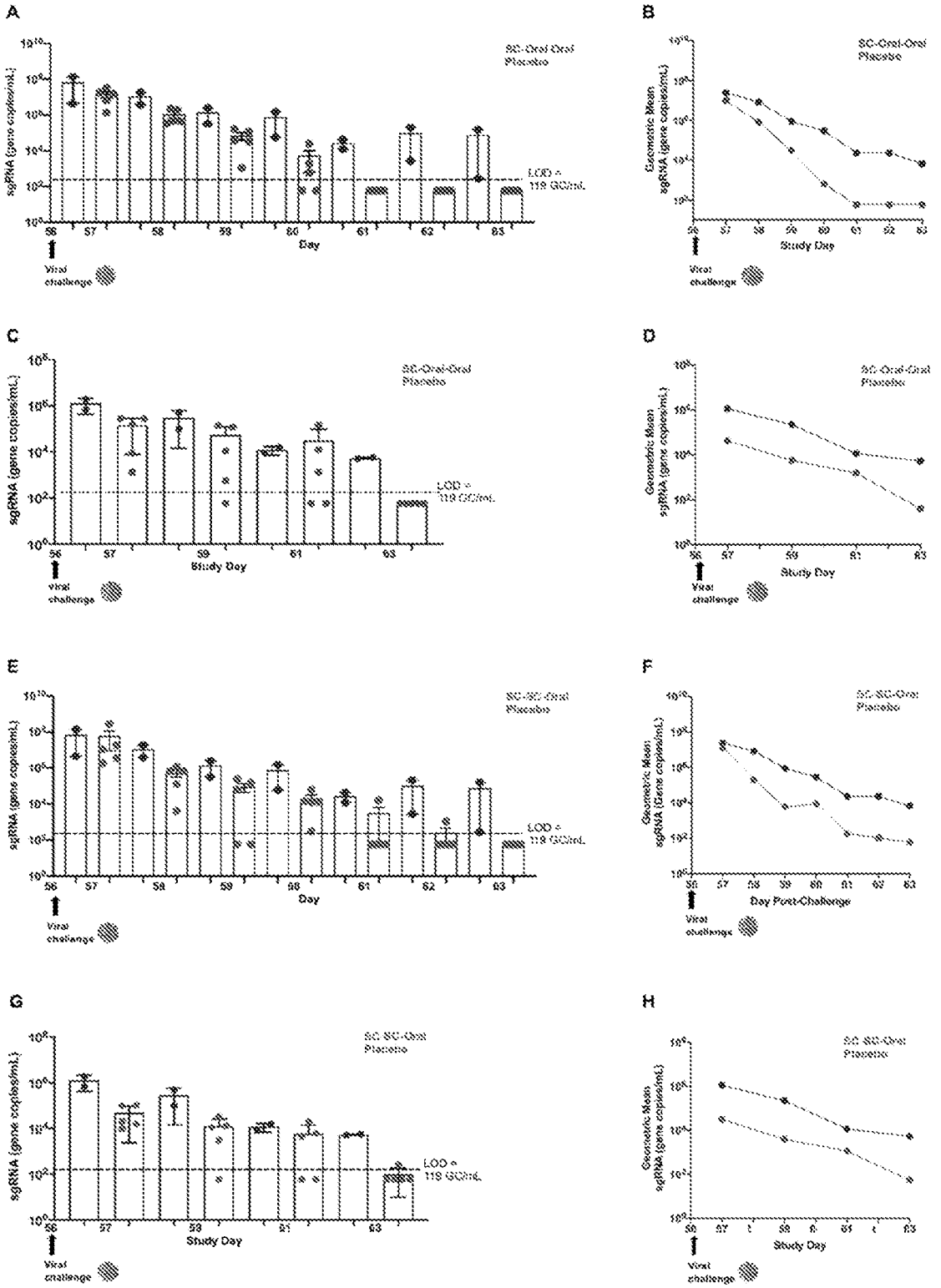


FIG.23

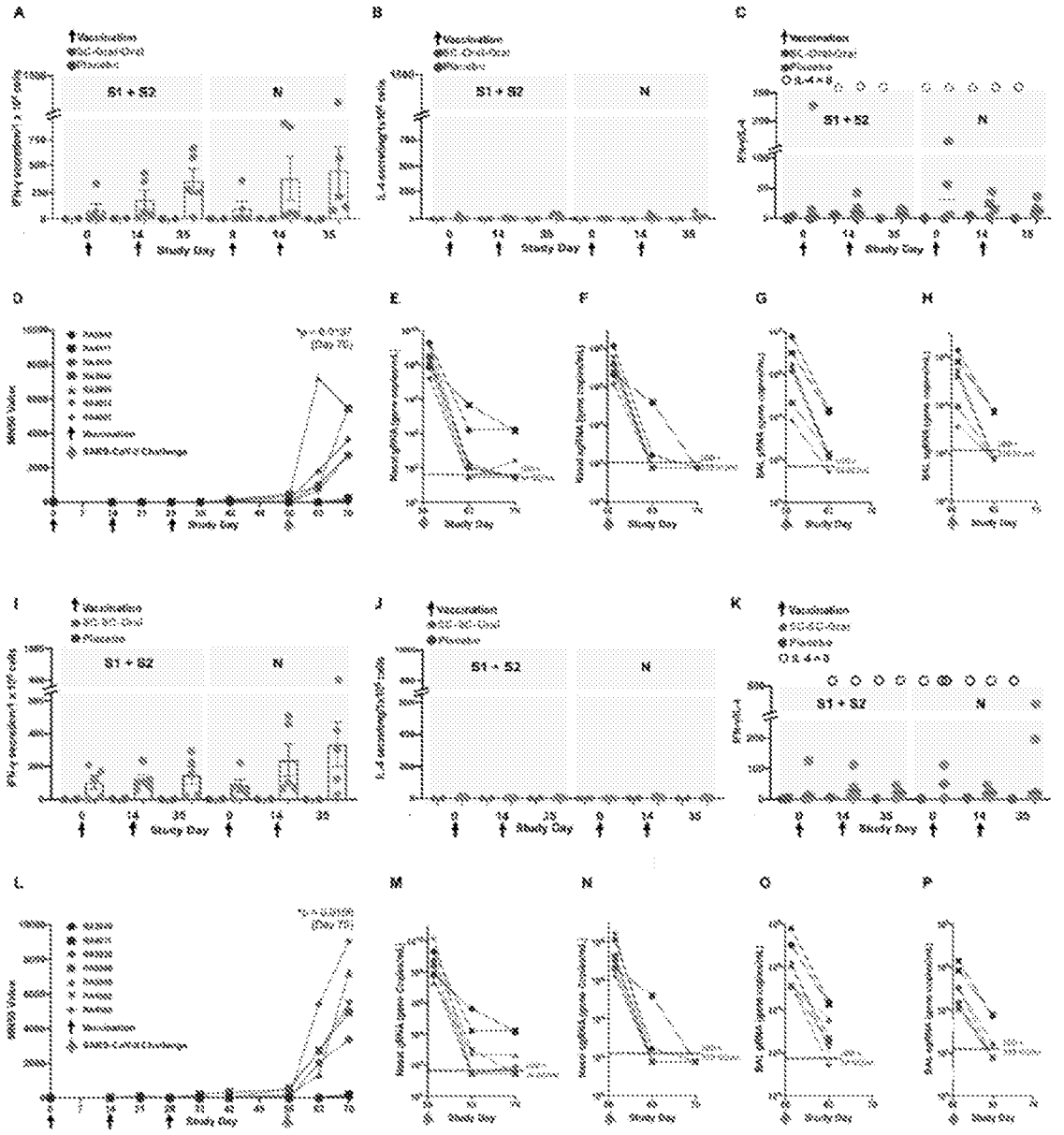


FIG.24

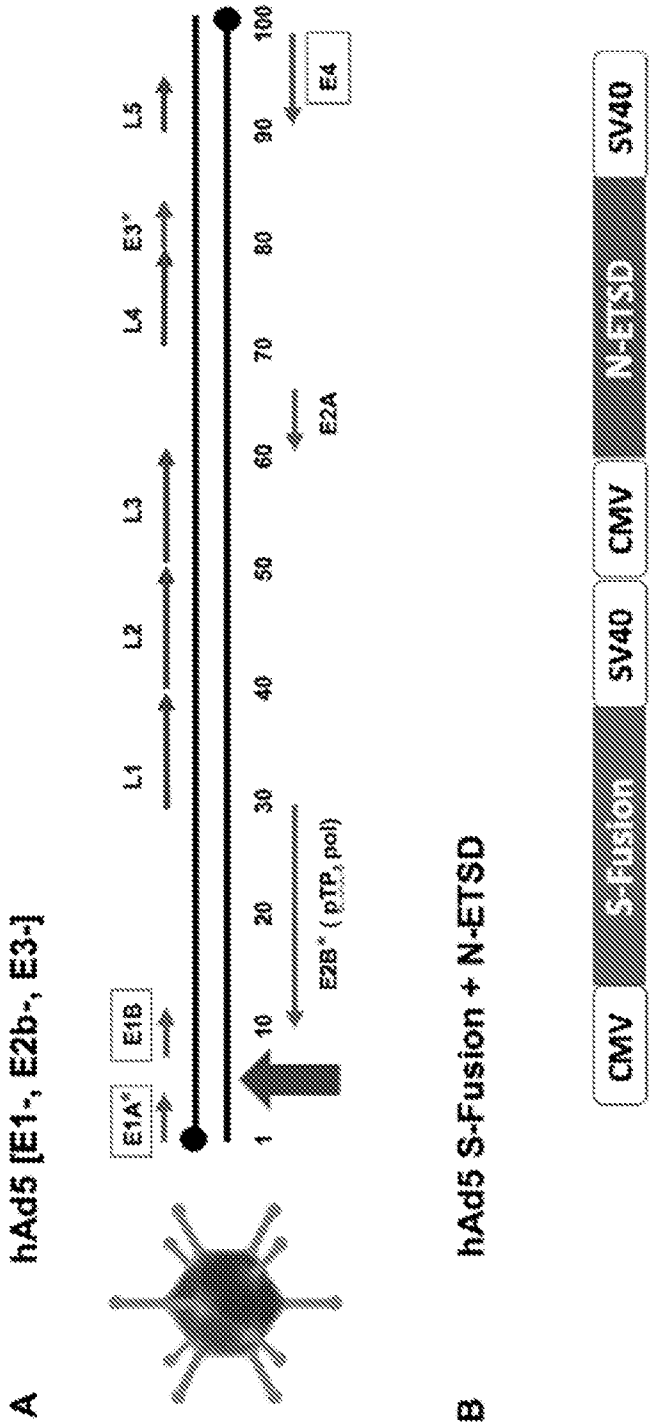

A
hAd5 [E1-, E2b-, E3-]
E1A*
E1B
L1
L2
L3
L4
E3*
L5
E4
E2A
E2B* (pTP, pol)
1
10
20
30
40
50
60
70
80
90
100
B
hAd5 S-Fusion + N-ETSD
CMV
S-Fusion
SV40
CMV
N-ETSD
SV40

FIG.25

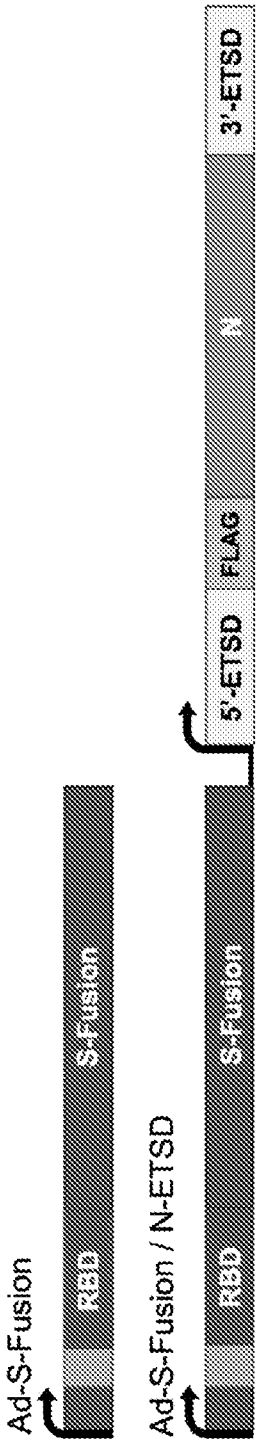

Ad-S-Fusion
RBD
S-Fusion
Ad-S-Fusion / N-ETSD
RBD
S-Fusion
5'-ETSD
FLAG
N
3'-ETSD

FIG.26

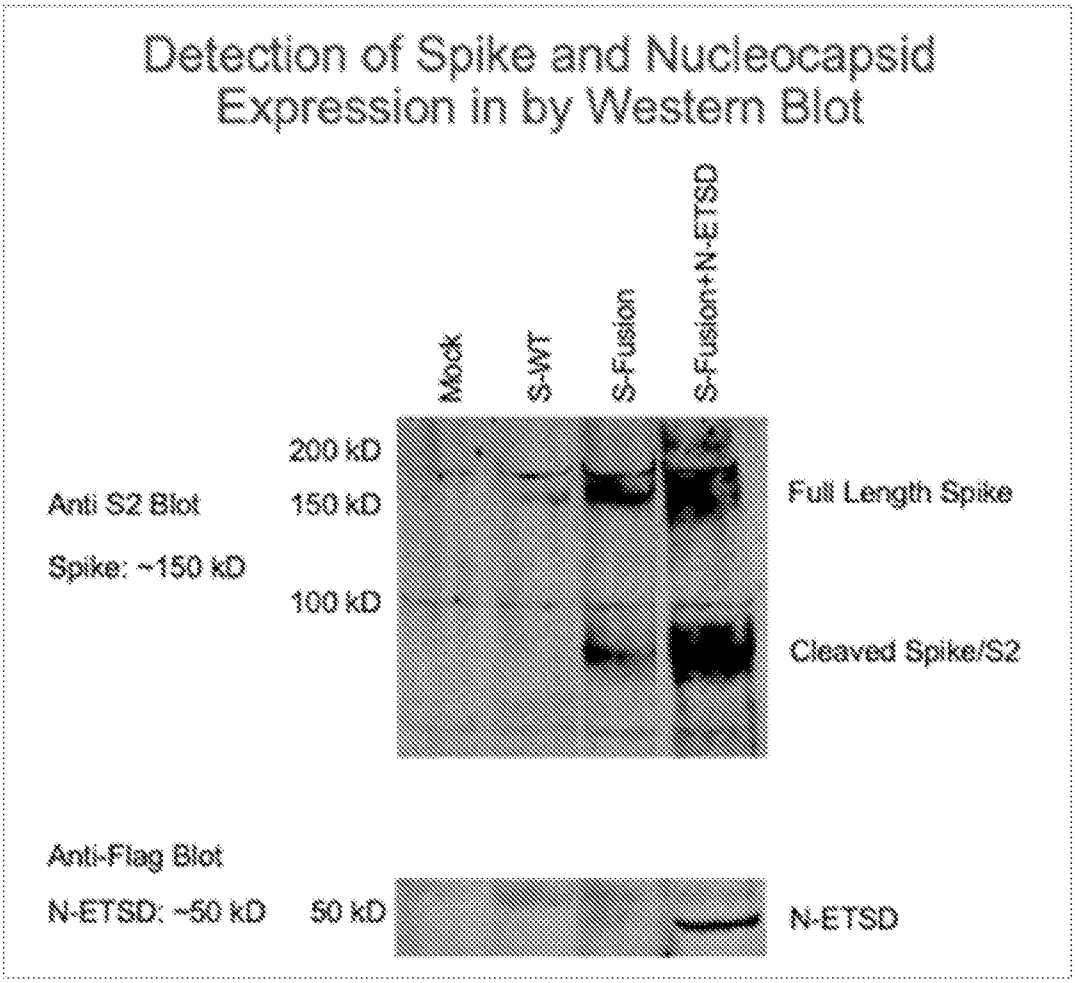

Detection of Spike and Nucleocapsid Expression in by Western Blot
Mock
S-WT
S-Fusion
S-Fusion+N-ETSD
200 kD
150 kD
100 kD
Anti S2 Blot
Spike: ~150 kD
Full Length Spike
Cleaved Spike/S2
Anti-Flag Blot
N-ETSD: ~50 kD
50 kD
N-ETSD

FIG.27

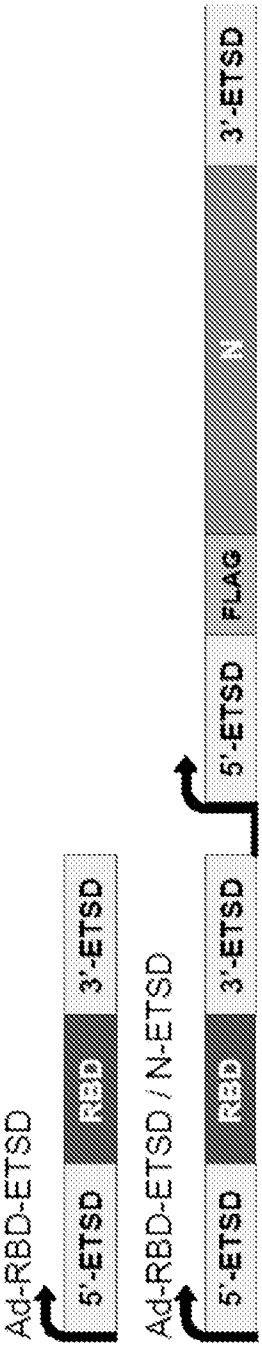

Ad-RBD-ETSD
5'-ETSD
RBD
3'-ETSD
Ad-RBD-ETSD / N-ETSD
5'-ETSD
RBD
3'-ETSD
5'-ETSD
FLAG
N
3'-ETSD

FIG.28

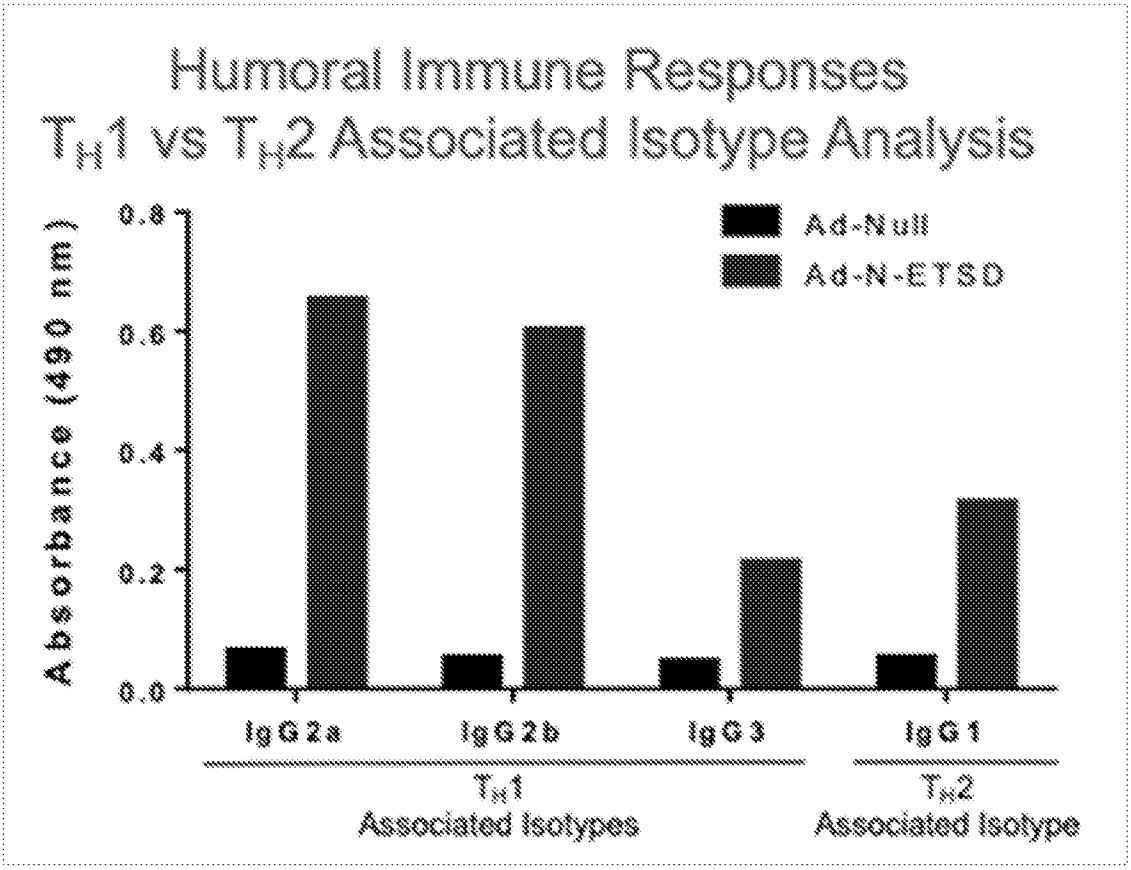

Humoral Immune Responses
TH1 vs TH2 Associated Isotype Analysis
Absorbance (490 nm)
0.0
0.2
0.4
0.6
0.8
Ad-Null
Ad-N-ETSD
IgG2a
IgG2b
IgG3
IgG1
TH1
Associated Isotypes
TH2
Associated Isotype

FIG.29

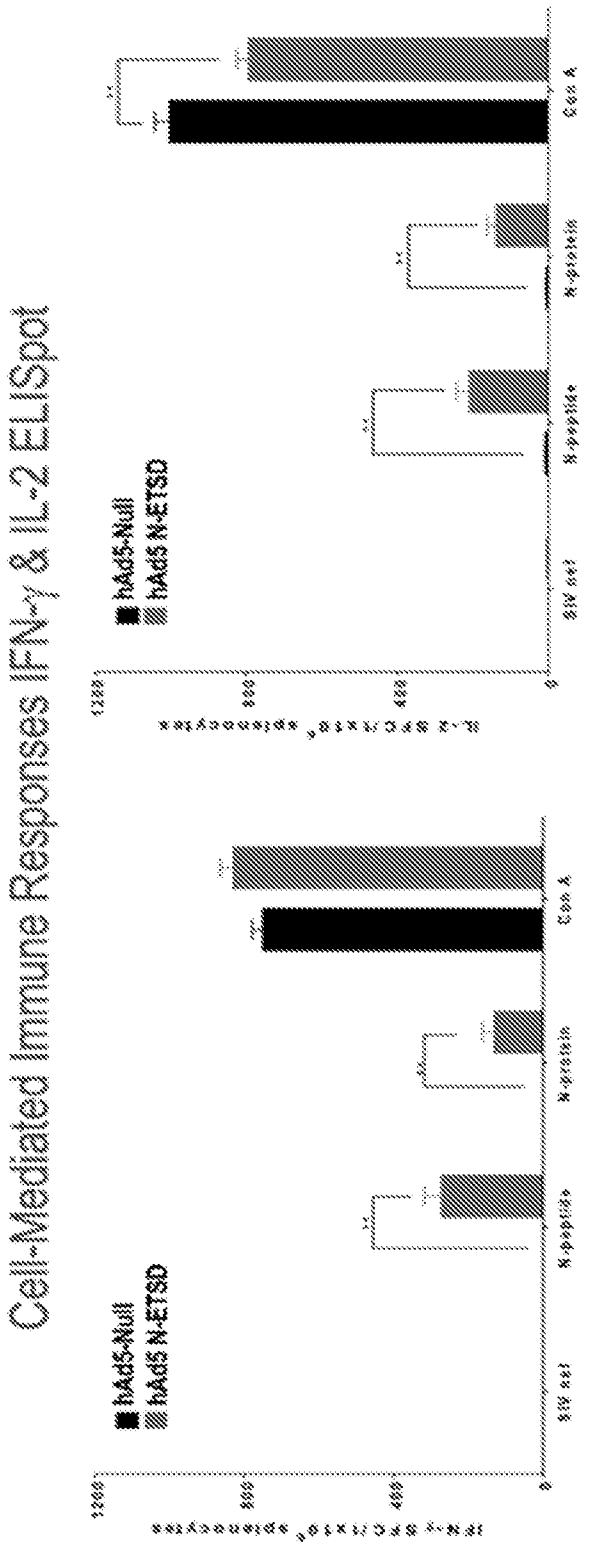

Cell-Mediated Immune Responses IFN-γ & IL-2 ELISpot
hAd5-Null
hAd5 N-ETSD
Con A
N-peptide
SW ctrl

FIG.30

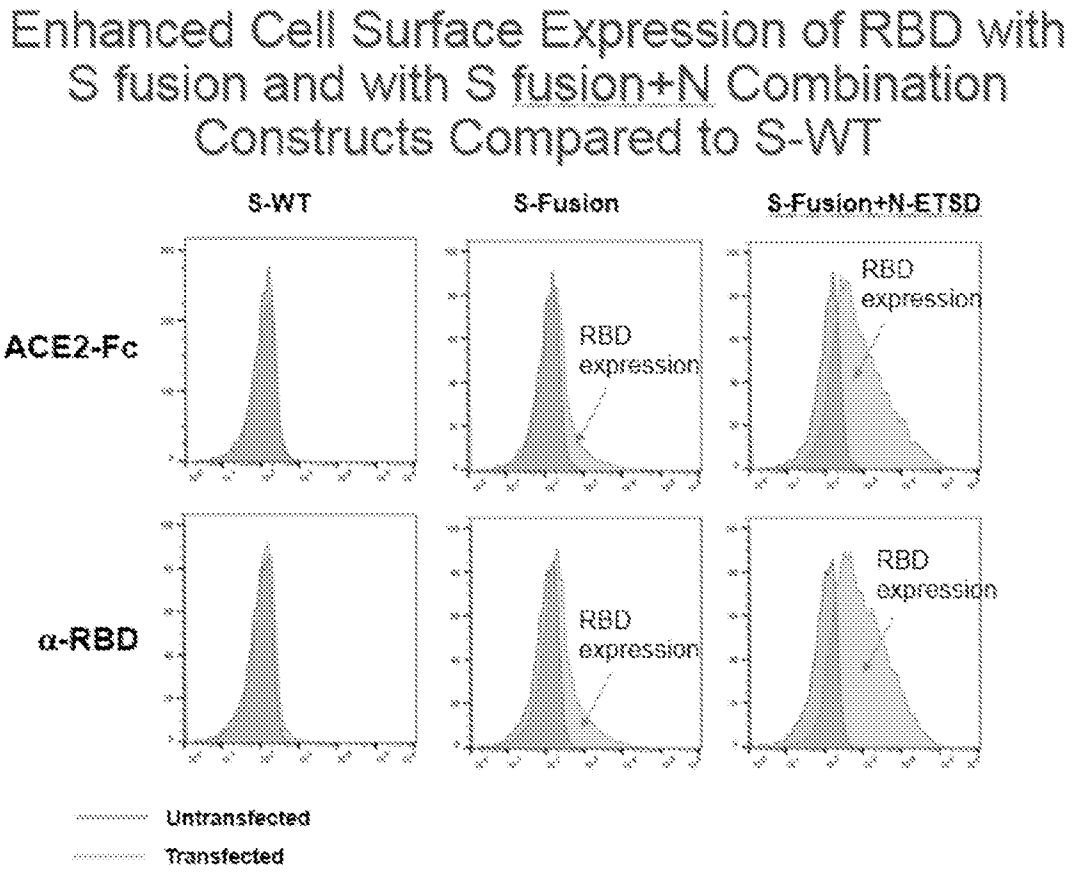

Enhanced Cell Surface Expression of RBD with S fusion and with S fusion+N Combination Constructs Compared to S-WT
S-WT
S-Fusion
S-Fusion+N-ETSD
ACE2-Fc
α-RBD
RBD expression
Untransfected
Transfected

FIG.31

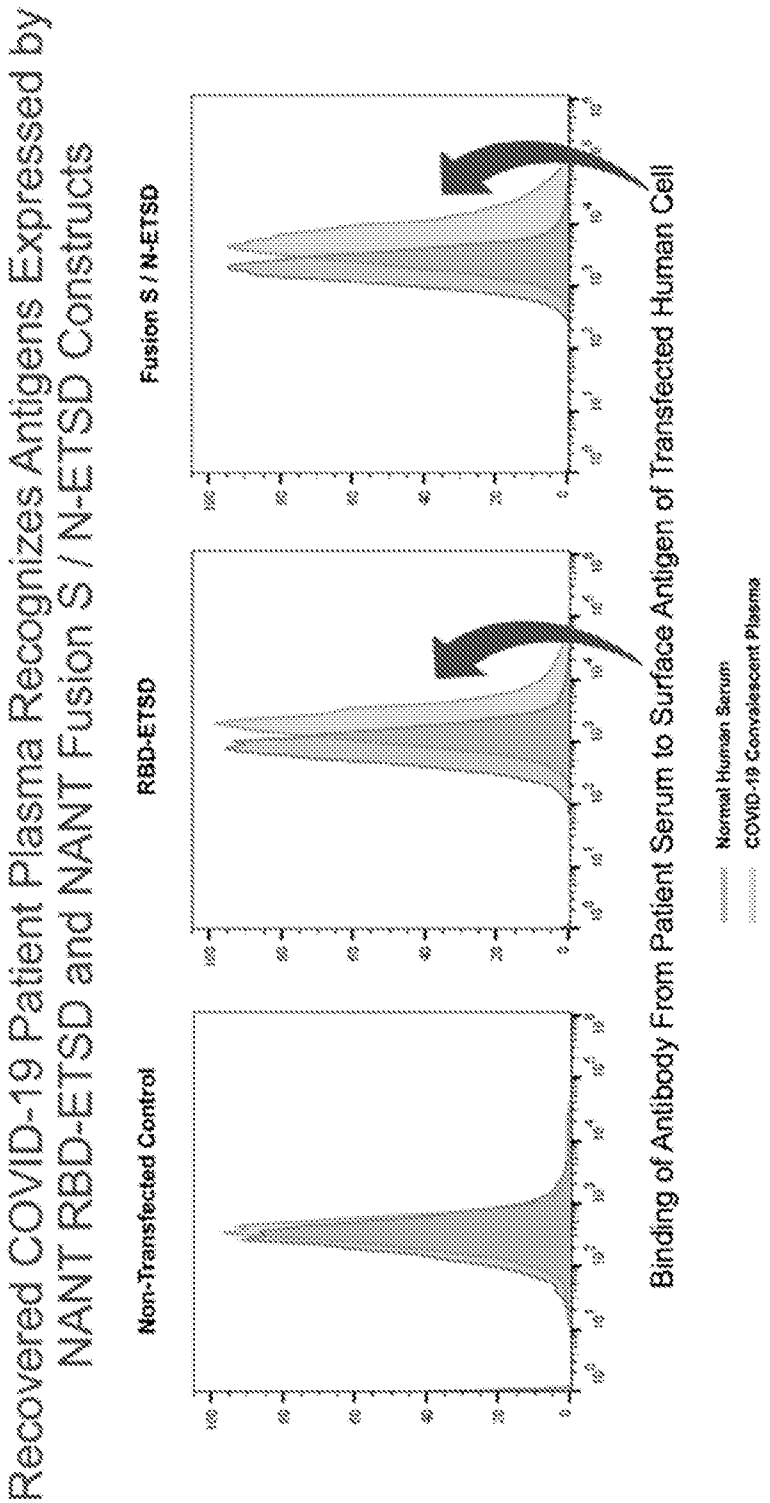

Recovered COVID-19 Patient Plasma Recognizes Antigens Expressed by NANT RBD-ETSD and NANT Fusion S / N-ETSD Constructs
Non-Transfected Control
RBD-ETSD
Fusion S / N-ETSD
Binding of Antibody From Patient Serum to Surface Antigen of Transfected Human Cell
Normal Human Serum
COVID-19 Convalescent Plasma

FIG.32

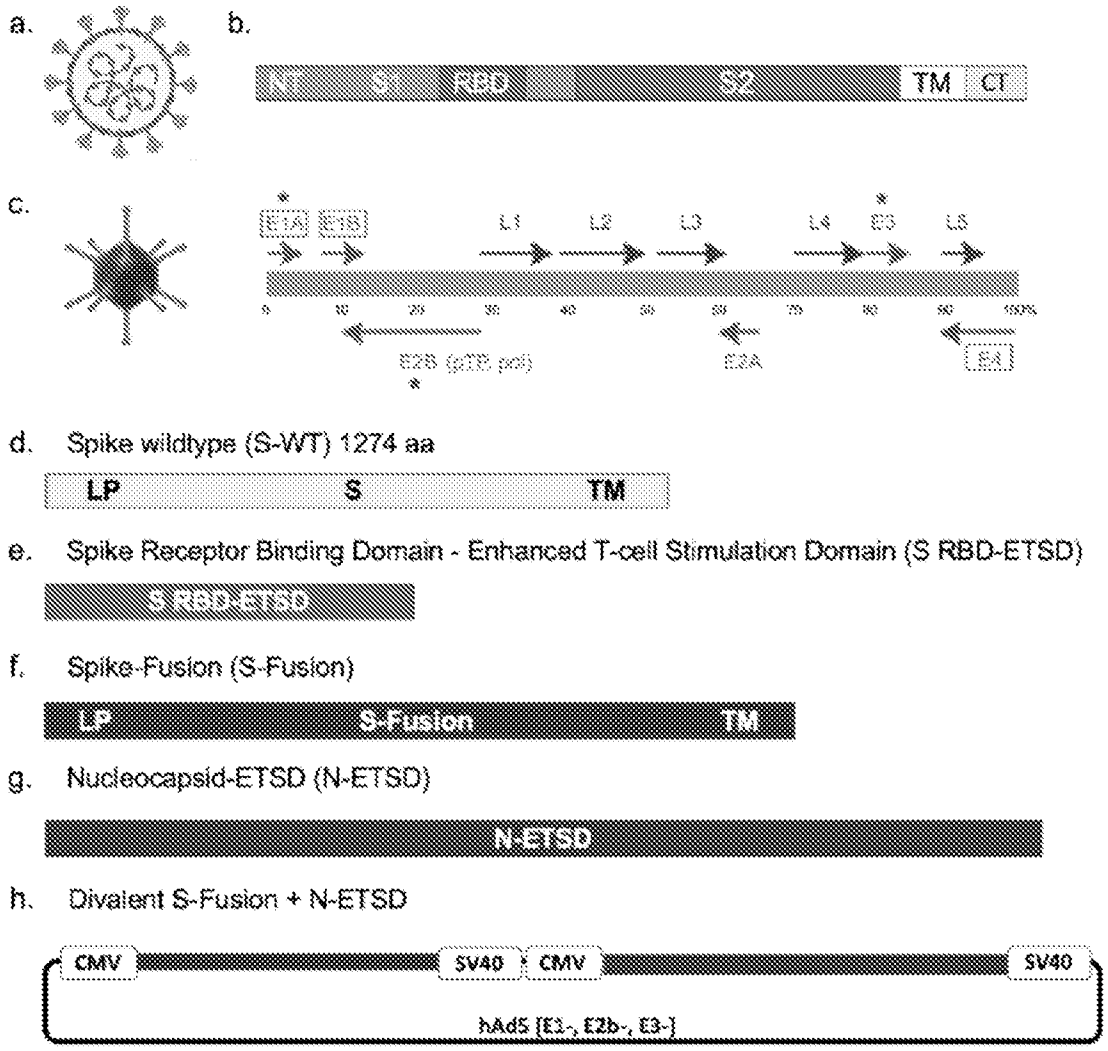

a.
b.
NT
S1
RBD
S2
TM
CT
c.
E1A
E1B
L1
L2
L3
L4
E3
L5
E2B
E2A
E4
d. Spike wildtype (S-WT) 1274 aa
LP
S
TM
e. Spike Receptor Binding Domain - Enhanced T-cell Stimulation Domain (S RBD-ETSD)
S RBD-ETSD
f. Spike-Fusion (S-Fusion)
LP
S-Fusion
TM
g. Nucleocapsid-ETSD (N-ETSD)
N-ETSD
h. Divalent S-Fusion + N-ETSD
CMV
SV40
CMV
SV40
hAd5 [E1-, E2b-, E3-]

FIG.33

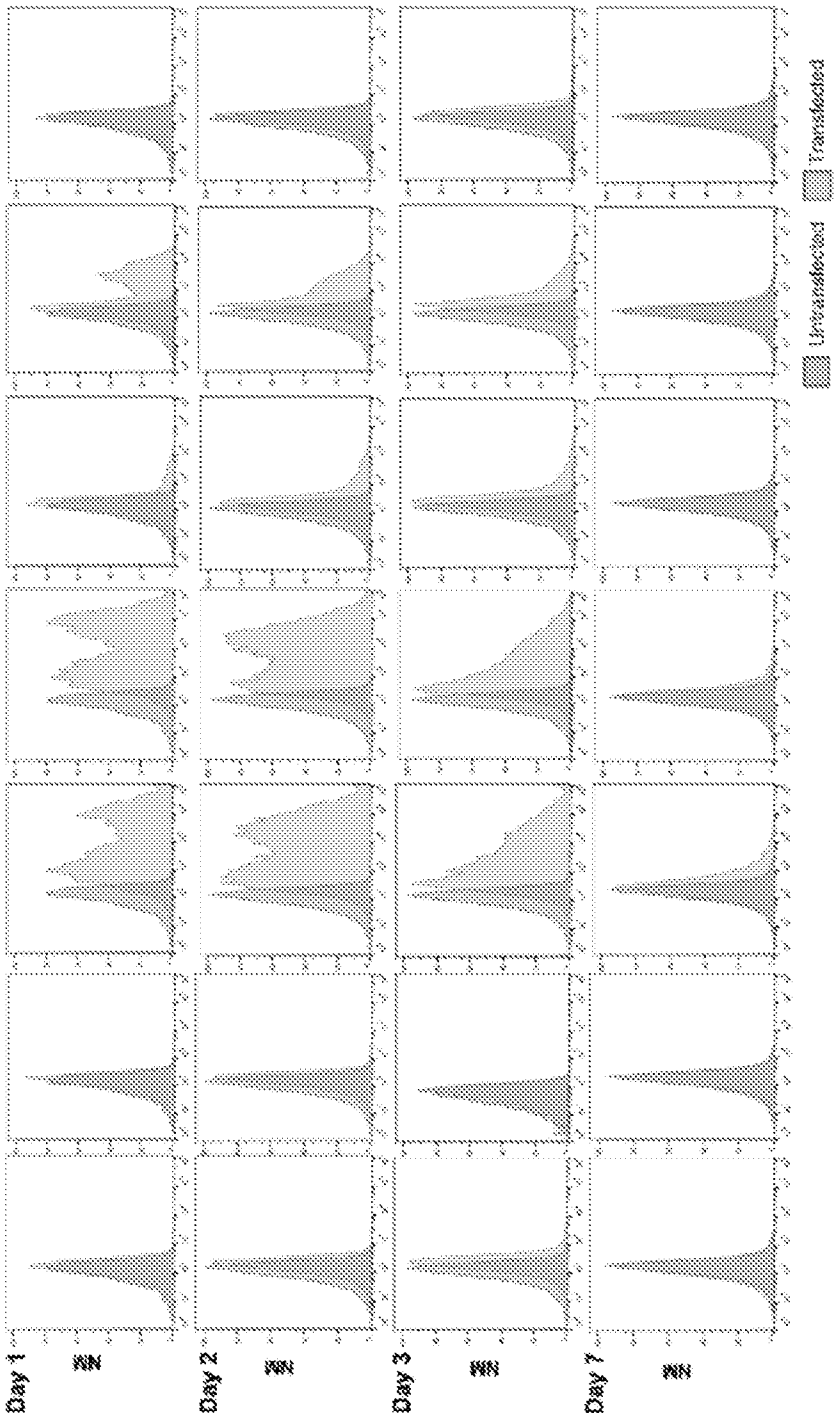

a. S-WT
b. S-WT + N-ETSD
c. S RBD-ETSD
d. S RBD-ETSD + N-ETSD
e. S-Fusion
f. S-Fusion + N-ETSD
g. N-ETSD
Day 1
Day 2
Day 3
Day 7
Untransfected
Transfected

FIG.34

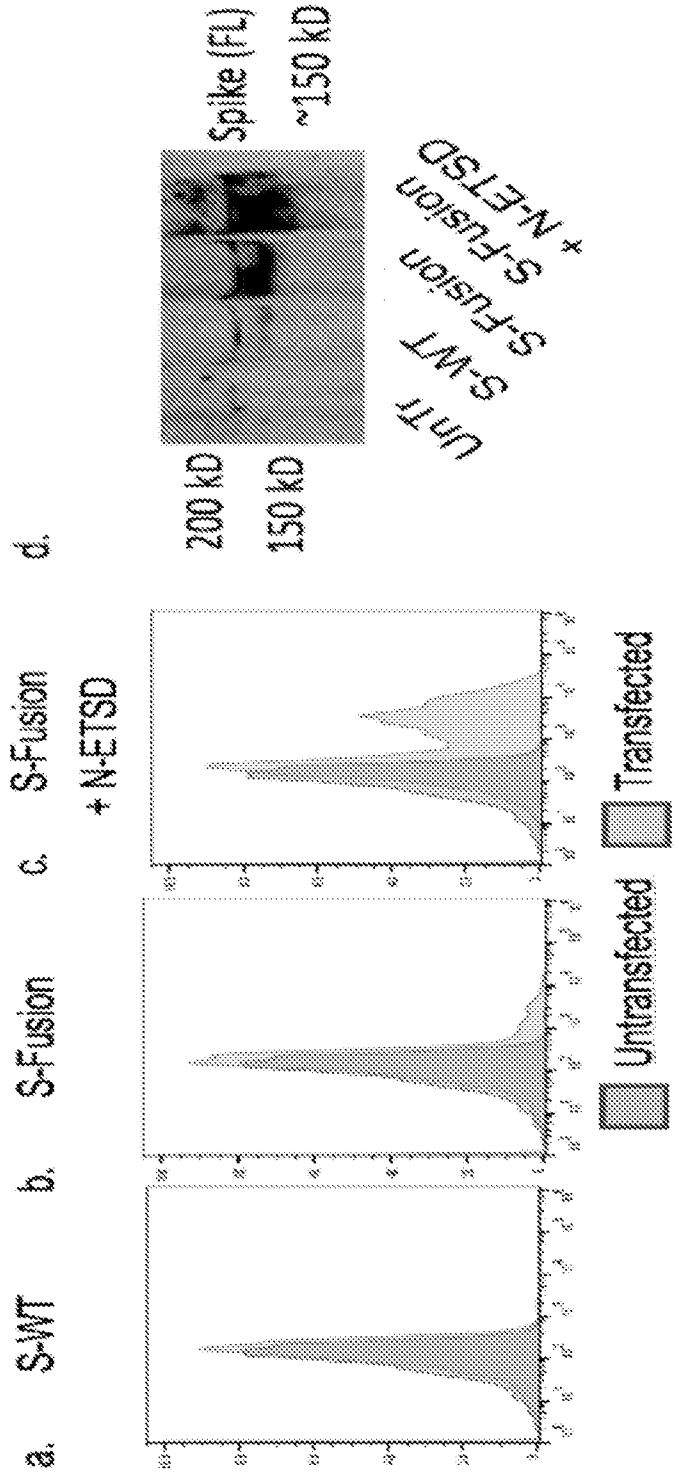

a. S-WT
b. S-Fusion
c. S-Fusion + N-ETSD
d.
Untransfected
Transfected
200 kD
150 kD
Spike (FL) ~150 kD
Untr
S-WT
S-Fusion
S-Fusion + N-ETSD

FIG.35

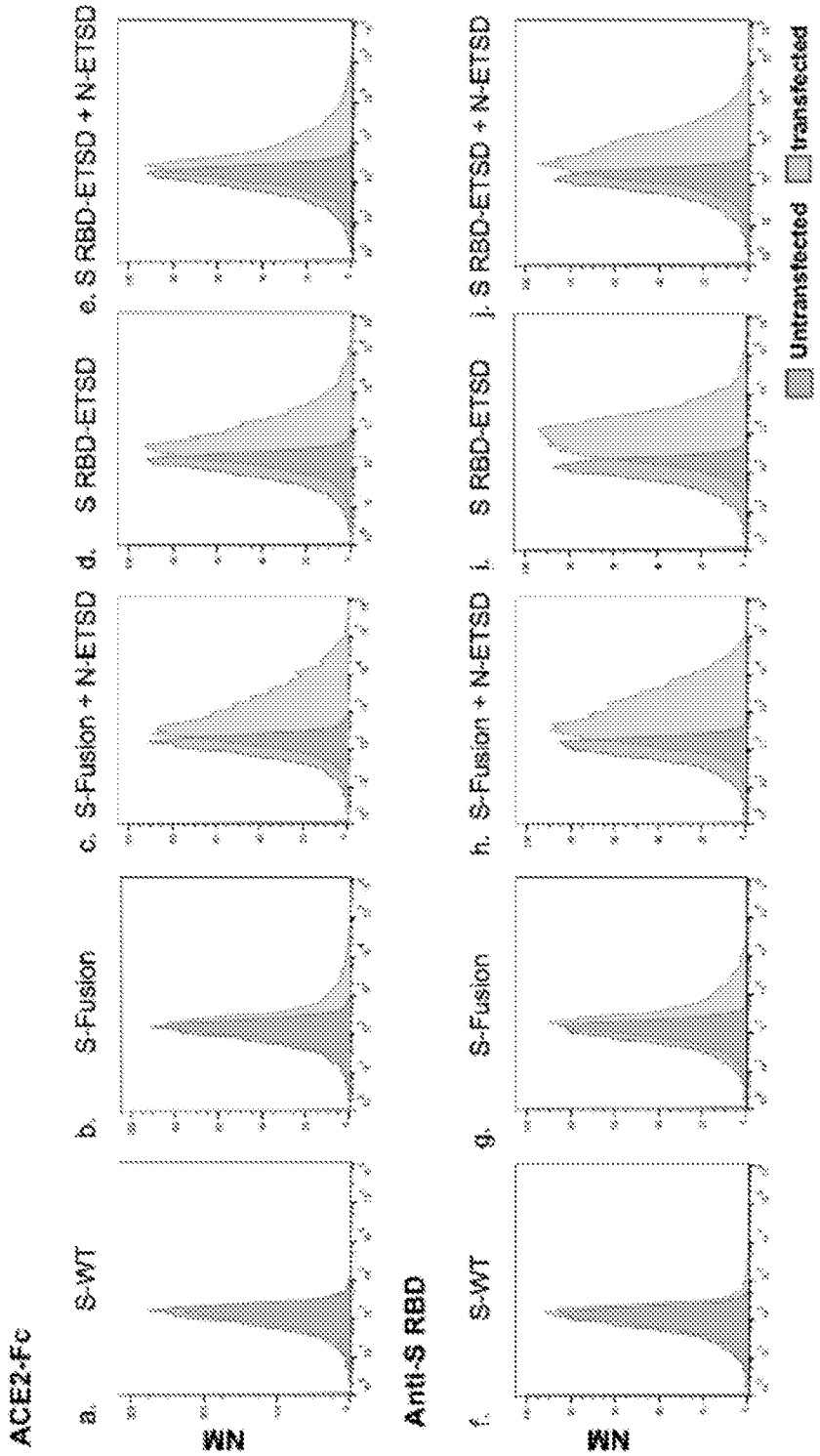

ACE2-Fc
a. S-WT
b. S-Fusion
c. S-Fusion + N-ETSD
d. S RBD-ETSD
e. S RBD-ETSD + N-ETSD
Anti-S RBD
f. S-WT
g. S-Fusion
h. S-Fusion + N-ETSD
i. S RBD-ETSD
j. S RBD-ETSD + N-ETSD
NM
Untransfected
transfected

FIG.36

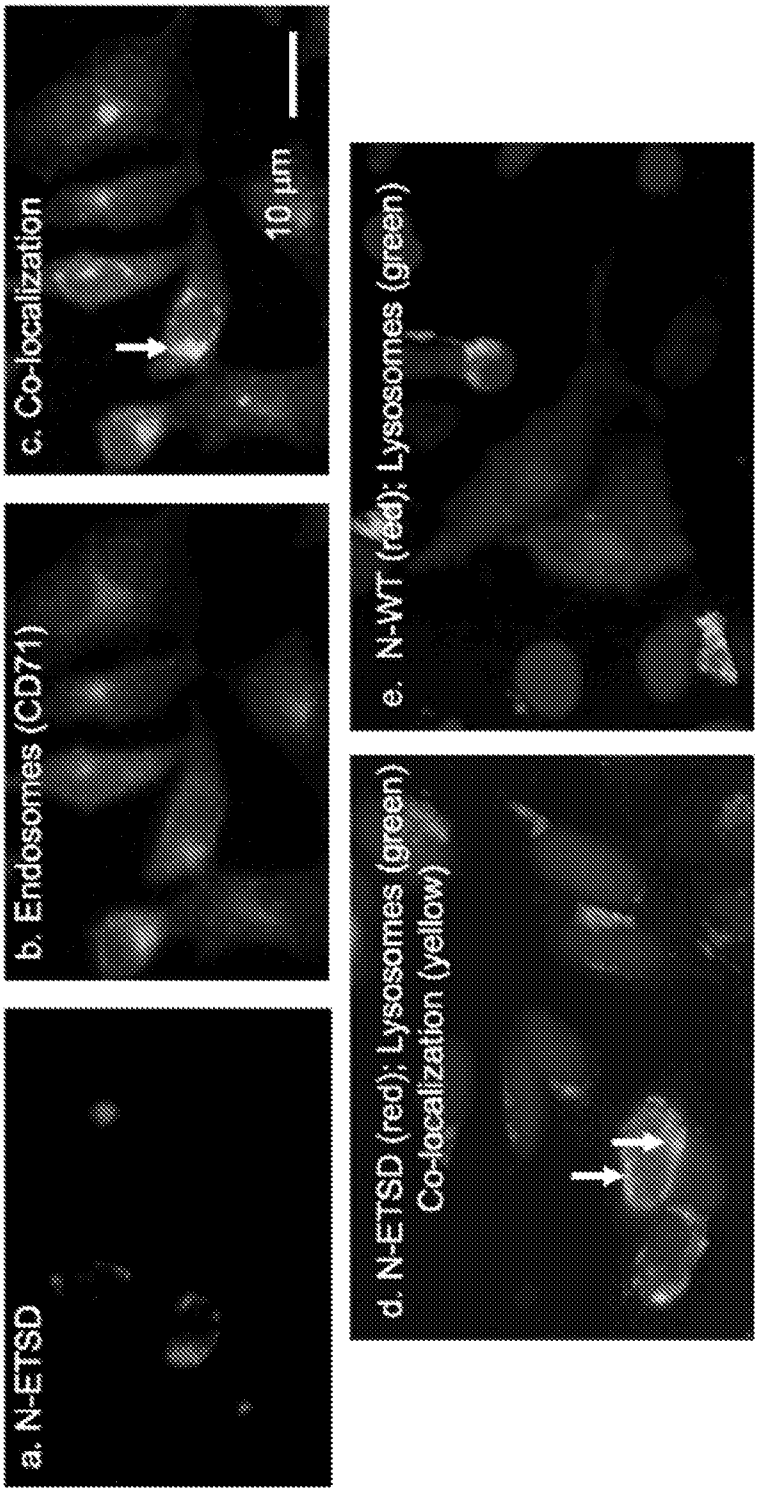

a. N-ETSD
b. Endosomes (CD71)
c. Co-localization
10 µm
d. N-ETSD (red); Lysosomes (green)
Co-localization (yellow)
e. N-WT (red); Lysosomes (green)

FIG.37

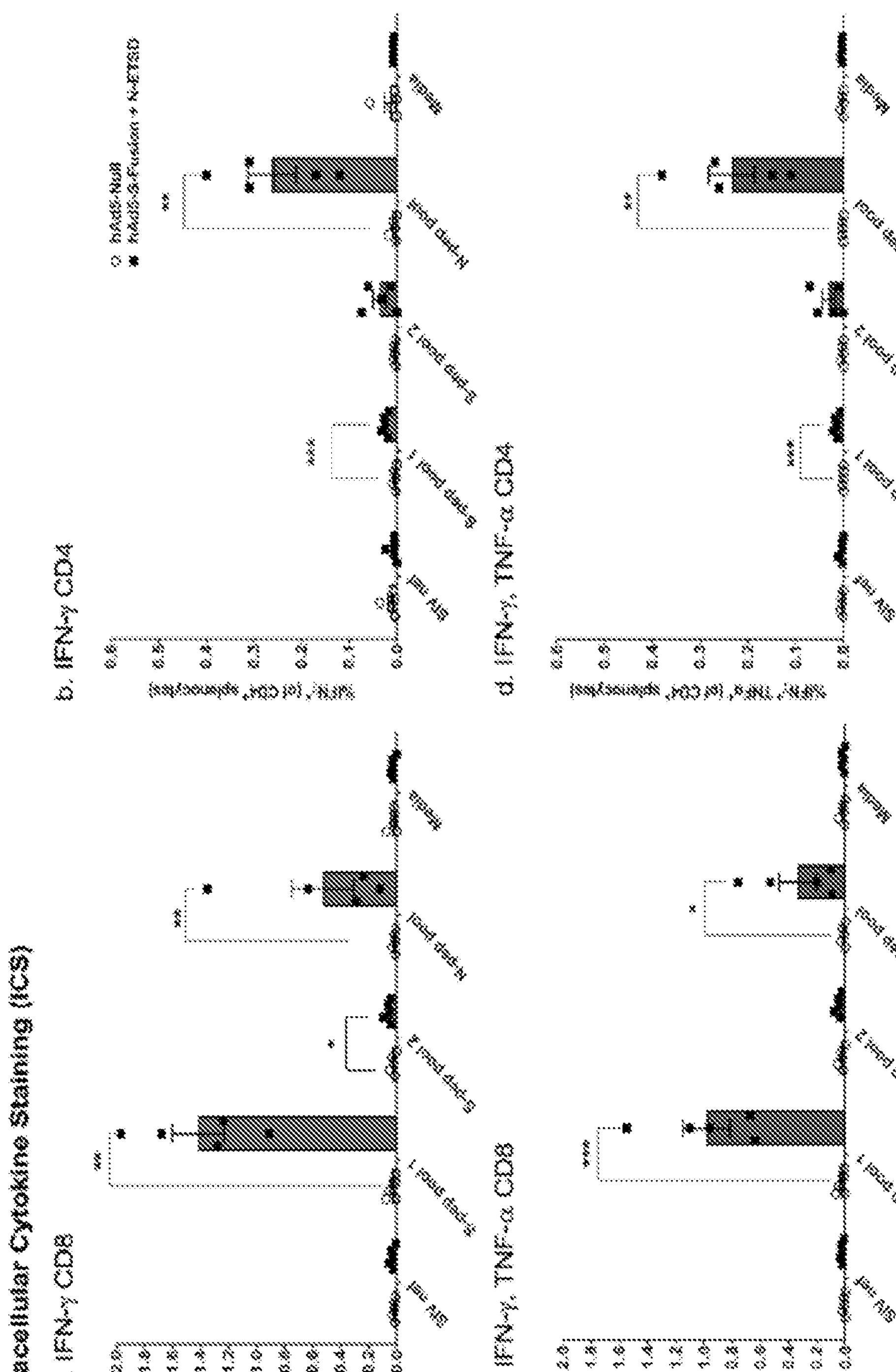

Intracellular Cytokine Staining (ICS)
a. IFN-γ CD8
b. IFN-γ CD4
c. IFN-γ, TNF-α CD8
d. IFN-γ, TNF-α CD4
hAd5-Null
hAd5-S-Fusion + N-ETSD
SIV nef
S-pep pool 1
S-pep pool 2
N-pep pool
Media

FIG.38

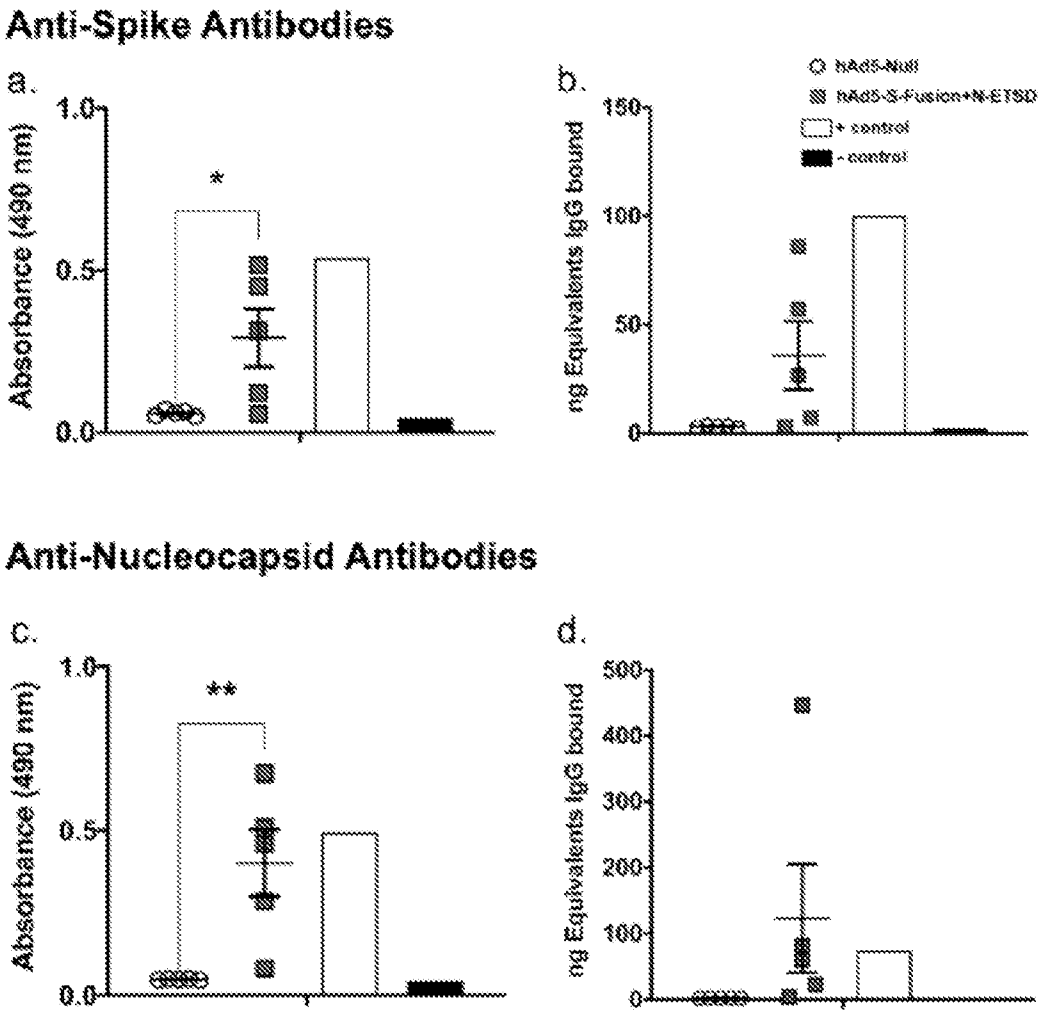

Anti-Spike Antibodies
a.
Absorbance (490 nm)
1.0
0.5
0.0
*
b.
ng Equivalents IgG bound
150
100
50
0
hAd5-Null
hAd5-S-Fusion+N-ETSD
+ control
- control
Anti-Nucleocapsid Antibodies
c.
Absorbance (490 nm)
1.0
0.5
0.0
**
d.
ng Equivalents IgG bound
500
400
300
200
100
0

FIG.39

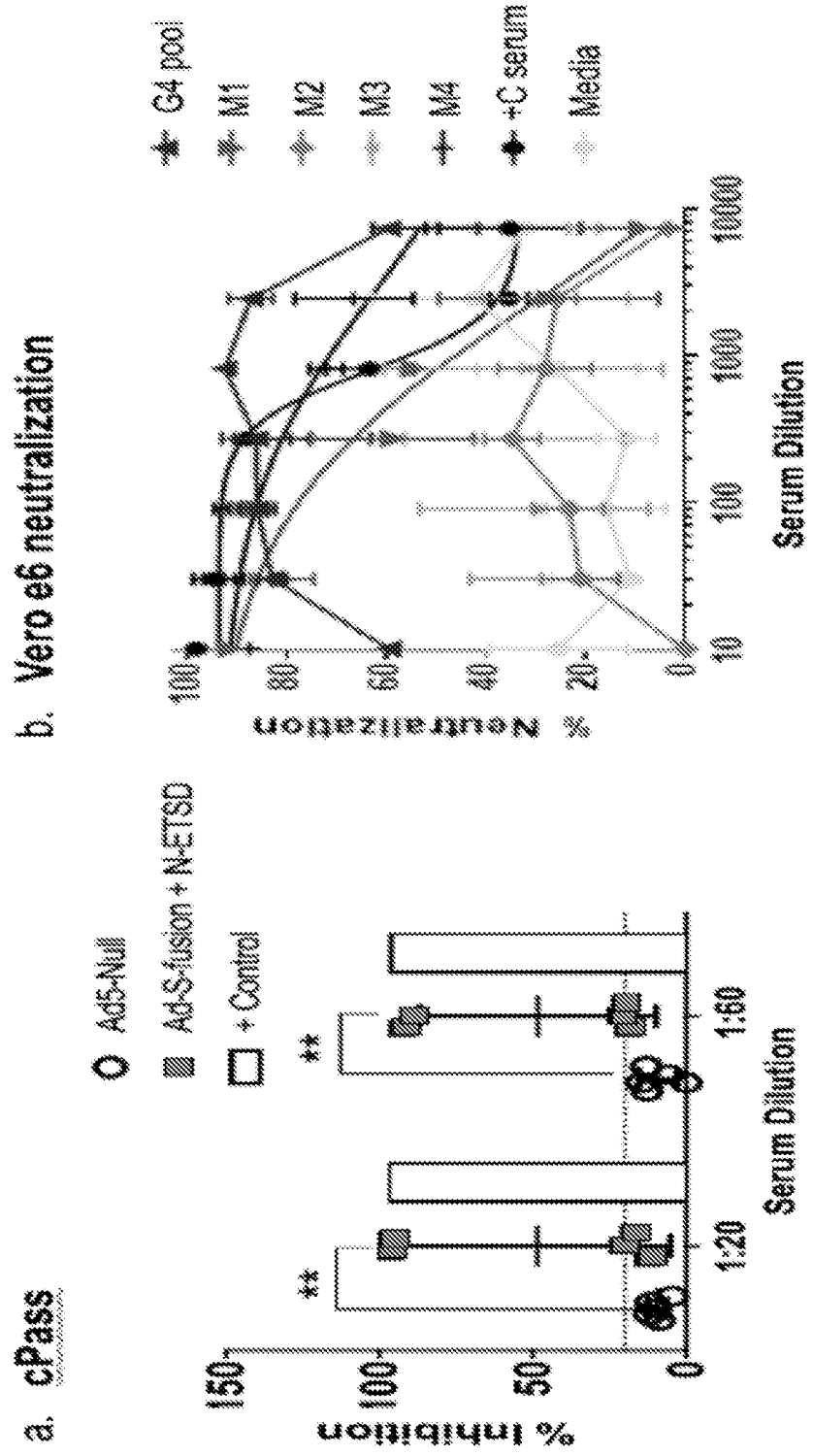

a. cPass
Ad5-Null
Ad5-Fusion + N-ETSD
+ Control
% Inhibition
150
100
50
0
**
**
1:20
1:60
Serum Dilution
b. Vero e6 neutralization
G4 pool
M1
M2
M3
M4
+C serum
Media
% Neutralization
100
80
60
40
20
0
10
100
1000
10000
Serum Dilution

FIG.40

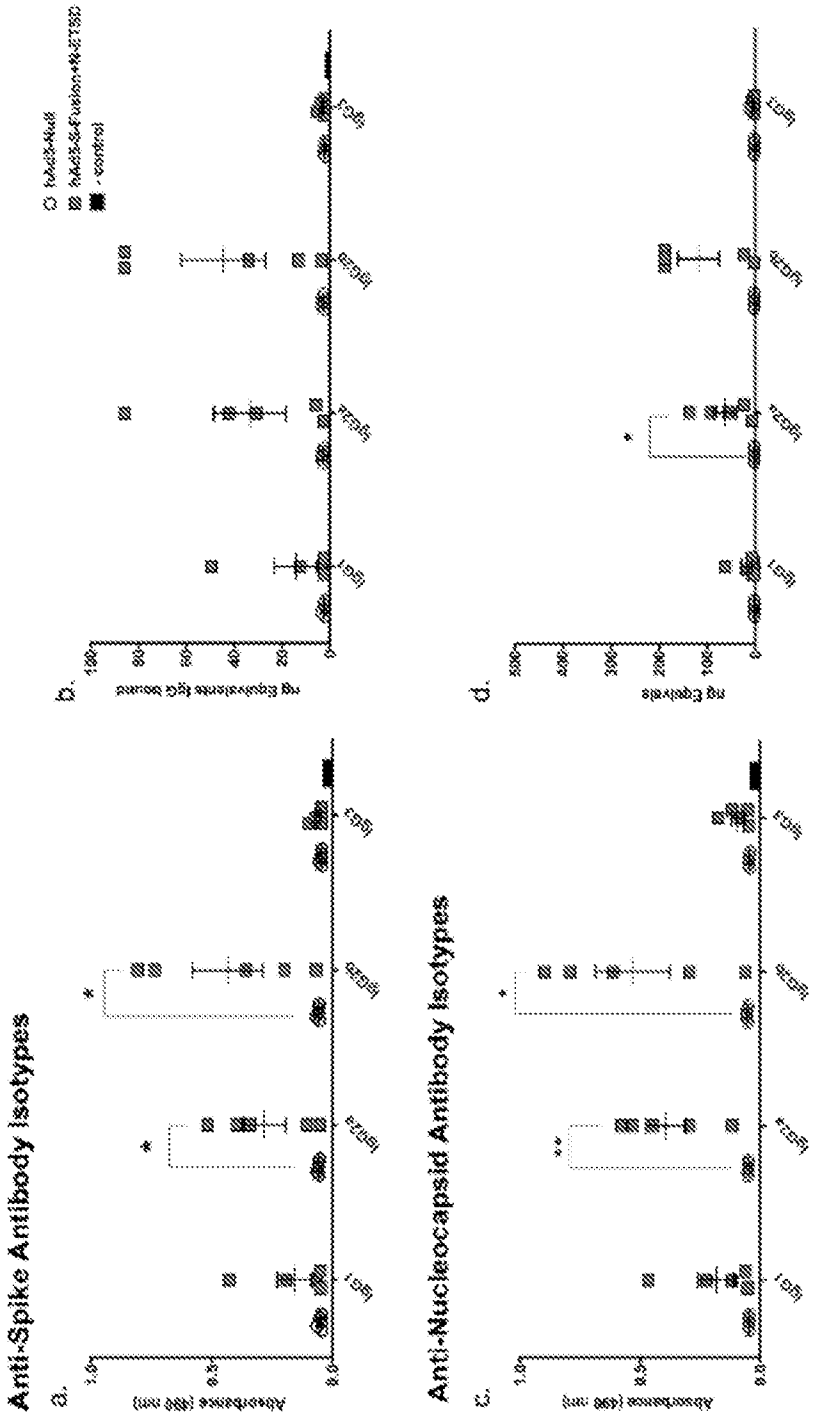

Anti-Spike Antibody Isotypes
a.
Absorbance (450 nm)
1.0
0.5
0.0
IgG1
IgG2a
IgG2b
IgG3
b.
ng Equivalents IgG Bound
100
80
60
40
20
0
c.
Anti-Nucleocapsid Antibody Isotypes
d.
ng Equivalents
500
400
300
200
100
0

FIG.41

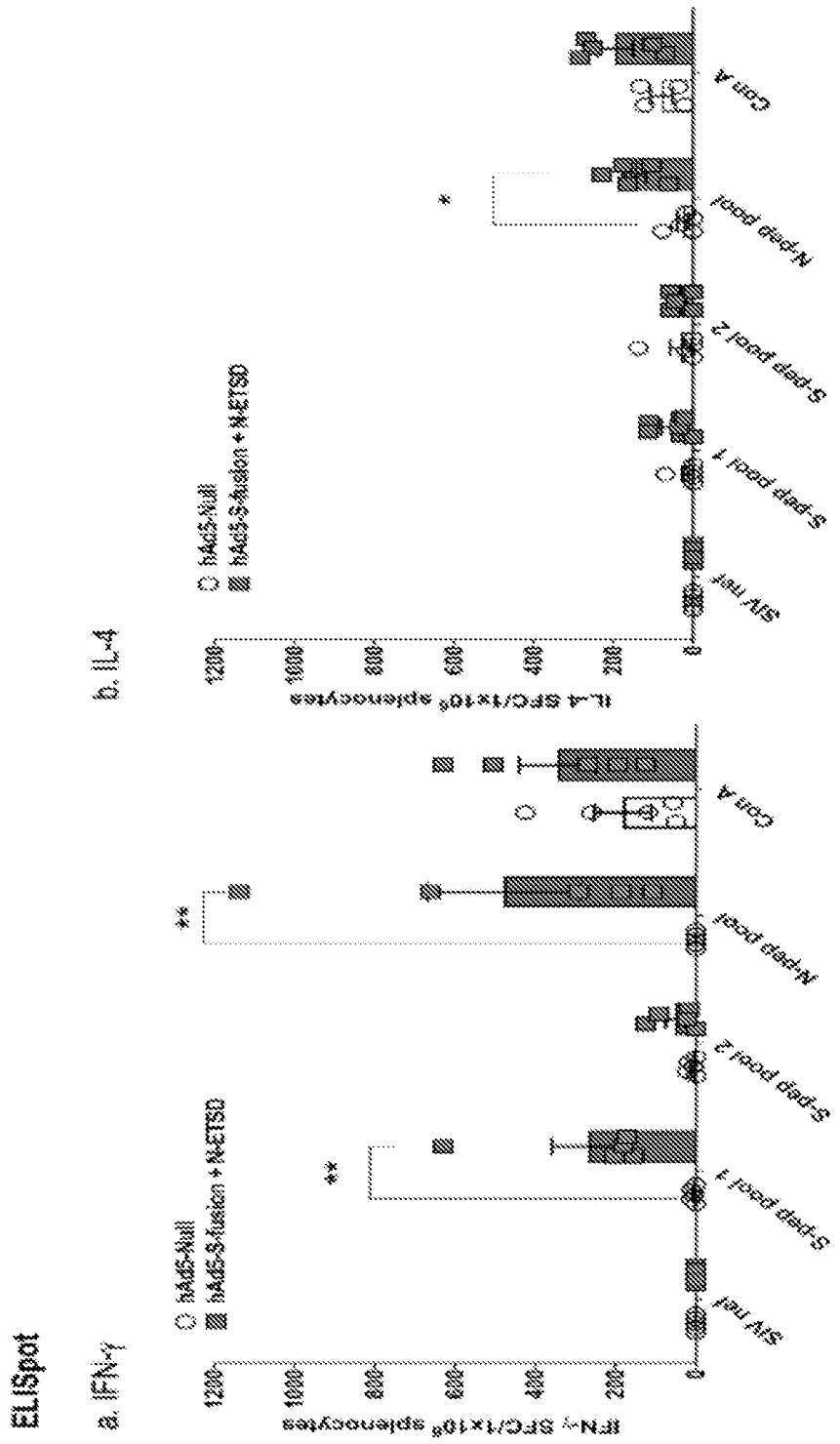

ELISpot
a. IFN-γ
hA45-Null
hA45-3-fusion + N-ETSD
IFN-γ SFC/1×10^6 splenocytes
1200
1000
800
600
400
200
0
**
**
Con A
N-pep pool
S-pep pool 2
S-pep pool 1
SIV nef
b. IL-4
hA45-Null
hA45-3-fusion + N-ETSD
IL-4 SFC/1×10^6 splenocytes
1200
1000
800
600
400
200
0
*
Con A
N-pep pool
S-pep pool 2
S-pep pool 1
SIV nef

FIG.42

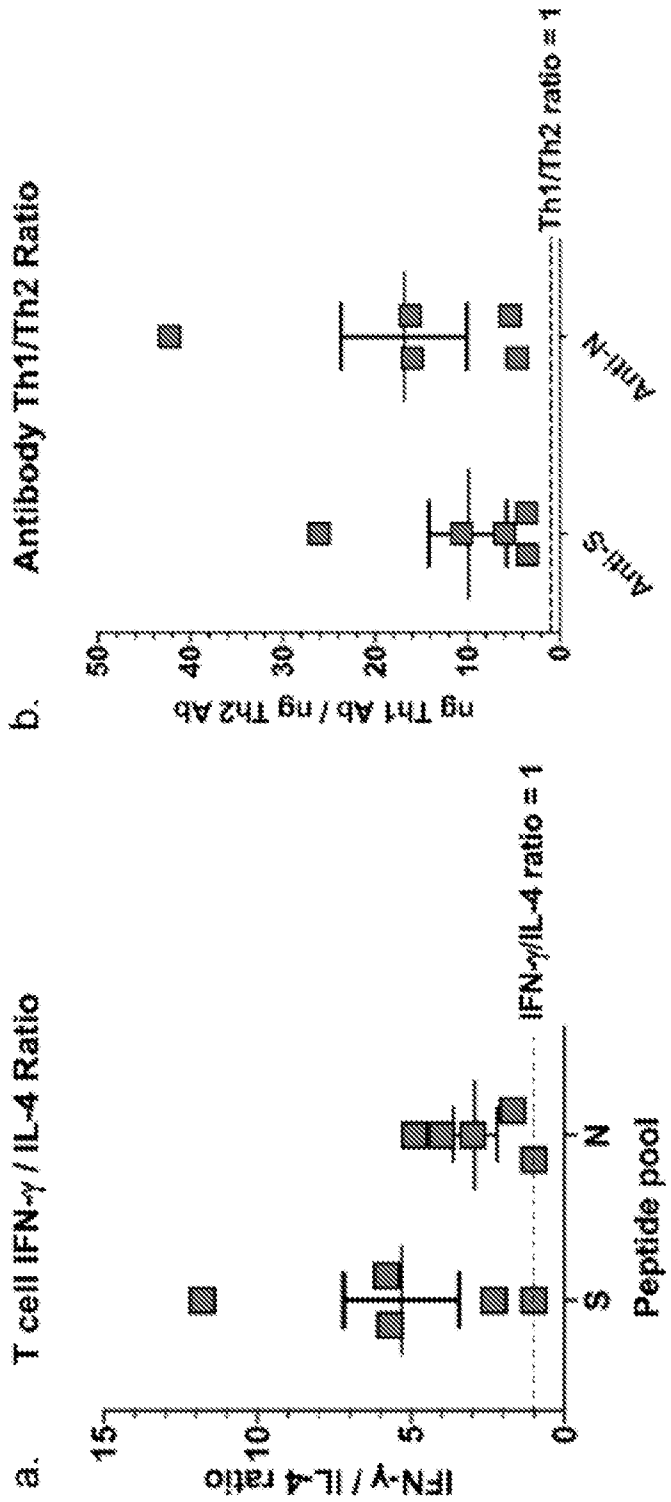

a.
T cell IFN-γ / IL-4 Ratio
IFN-γ / IL-4 ratio
15
10
5
0
IFN-γ/IL-4 ratio = 1
S
N
Peptide pool
b.
Antibody Th1/Th2 Ratio
ng Th1 Ab / ng Th2 Ab
50
40
30
20
10
0
Th1/Th2 ratio = 1
Anti-S
Anti-N

FIG.43

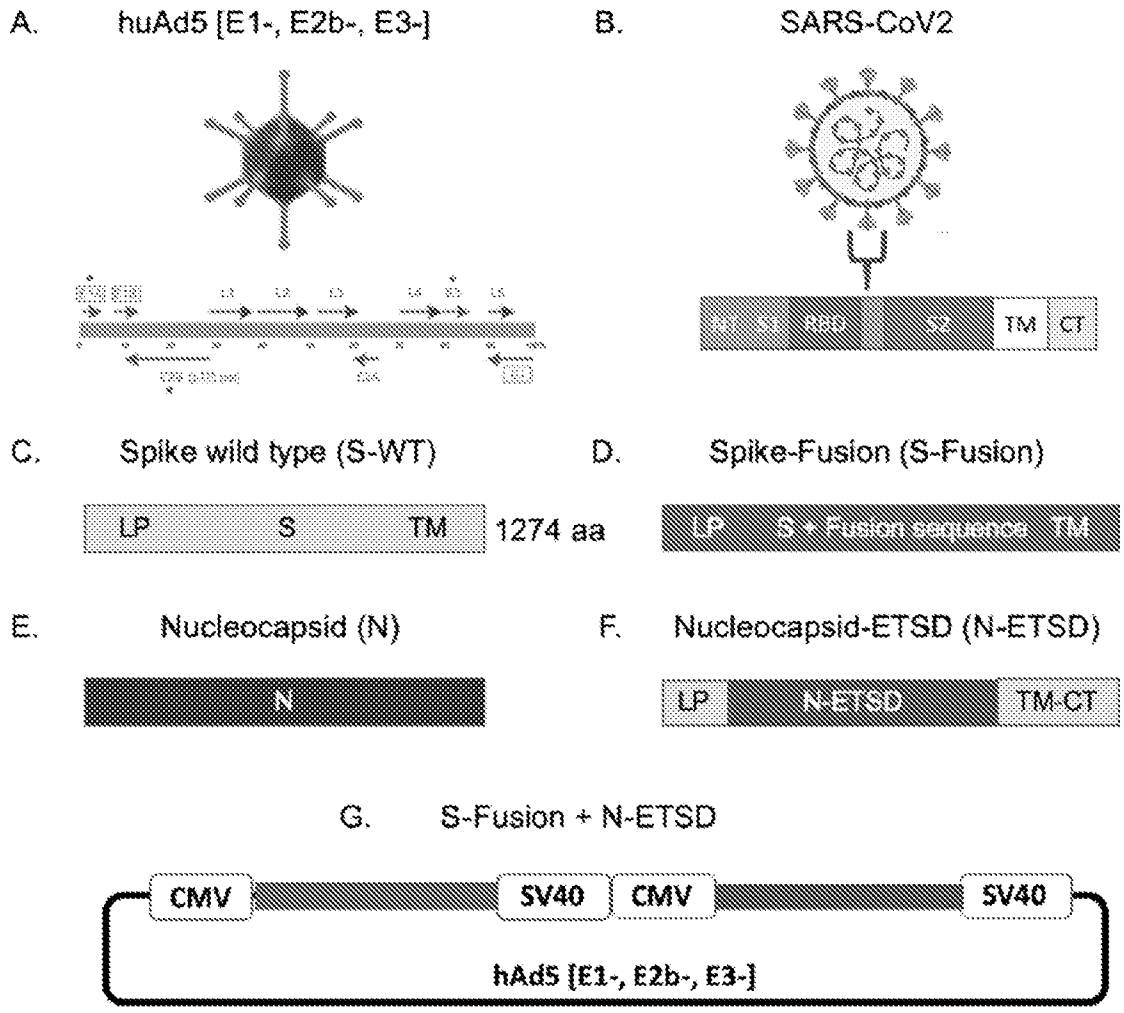

A. huAd5 [E1-, E2b-, E3-]
B. SARS-CoV2
S1
RBD
S2
TM
CT
C. Spike wild type (S-WT)
LP
S
TM
1274 aa
D. Spike-Fusion (S-Fusion)
LP
S + Fusion sequence
TM
E. Nucleocapsid (N)
N
F. Nucleocapsid-ETSD (N-ETSD)
LP
N-ETSD
TM-CT
G. S-Fusion + N-ETSD
CMV
SV40
CMV
SV40
hAd5 [E1-, E2b-, E3-]

FIG.44

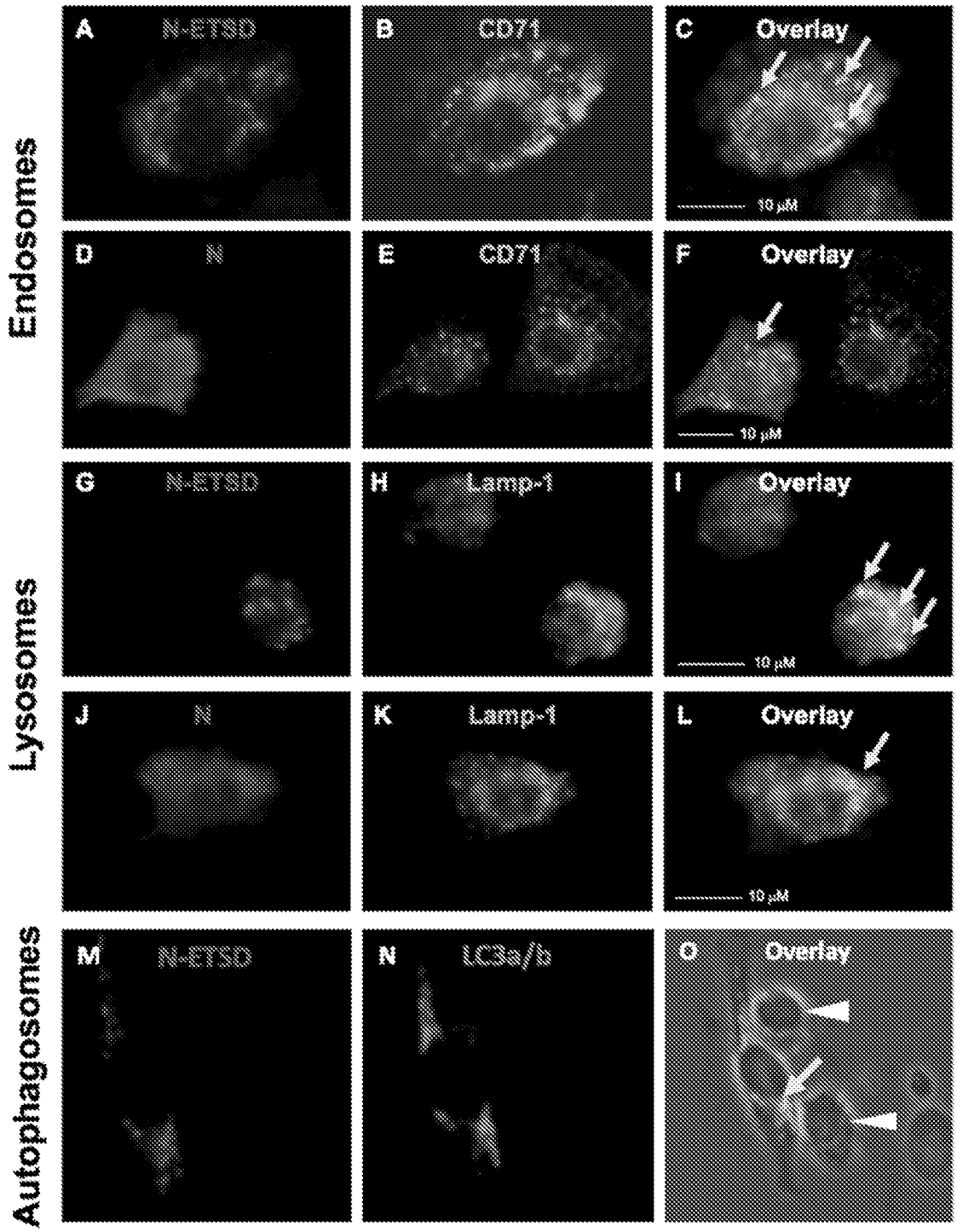

Endosomes
Lysosomes
Autophagosomes
A N-ETSD
B CD71
C Overlay
10 μM
D N
E CD71
F Overlay
10 μM
G N-ETSD
H Lamp-1
I Overlay
10 μM
J N
K Lamp-1
L Overlay
10 μM
M N-ETSD
N LC3a/b
O Overlay

FIG.45

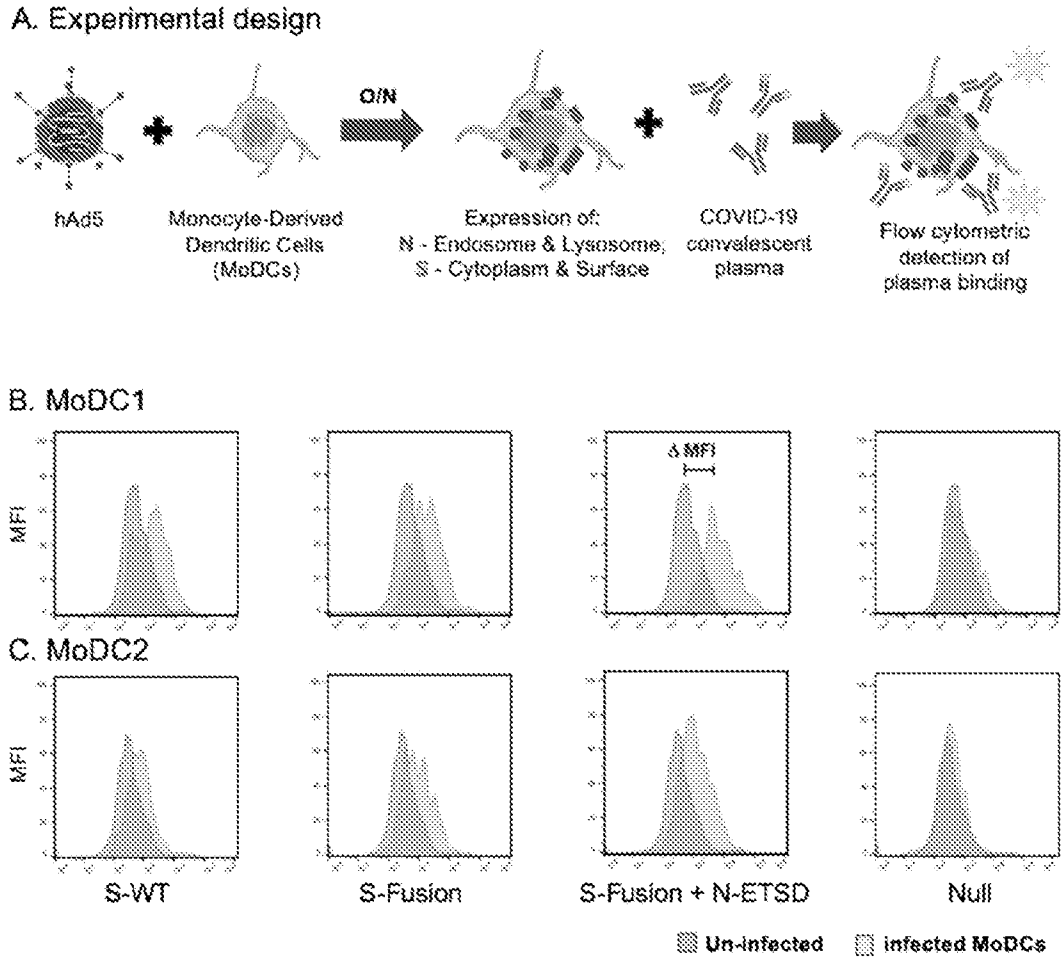

A. Experimental design
O/N
hAd5
Monocyte-Derived Dendritic Cells (MoDCs)
Expression of: N - Endosome & Lysosome; S - Cytoplasm & Surface
COVID-19 convalescent plasma
Flow cytometric detection of plasma binding
B. MoDC1
MFI
Δ MFI
C. MoDC2
MFI
S-WT
S-Fusion
S-Fusion + N-ETSD
Null
Un-infected
infected MoDCs
D. ΔMFI for COVID-19 plasma binding to hAd5 antigen-expressing MoDCs
ΔMFI
4000
3000
2000
1000
0
MoDC1
MoDC2
S-WT
S-Fusion
S-Fusion + N-ETSD
Null

FIG.46

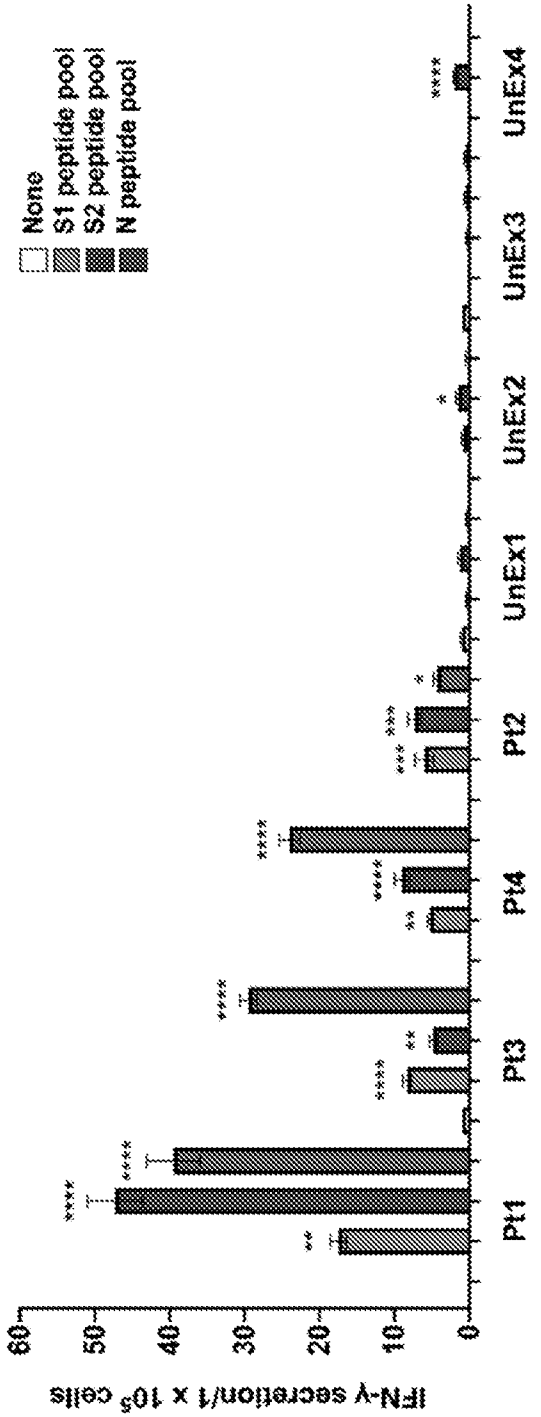

None
S1 peptide pool
S2 peptide pool
N peptide pool
IFN-γ secretion/1 x 10^5 cells
0
10
20
30
40
50
60
Pt1
Pt3
Pt4
Pt2
UnEx1
UnEx2
UnEx3
UnEx4

FIG.47

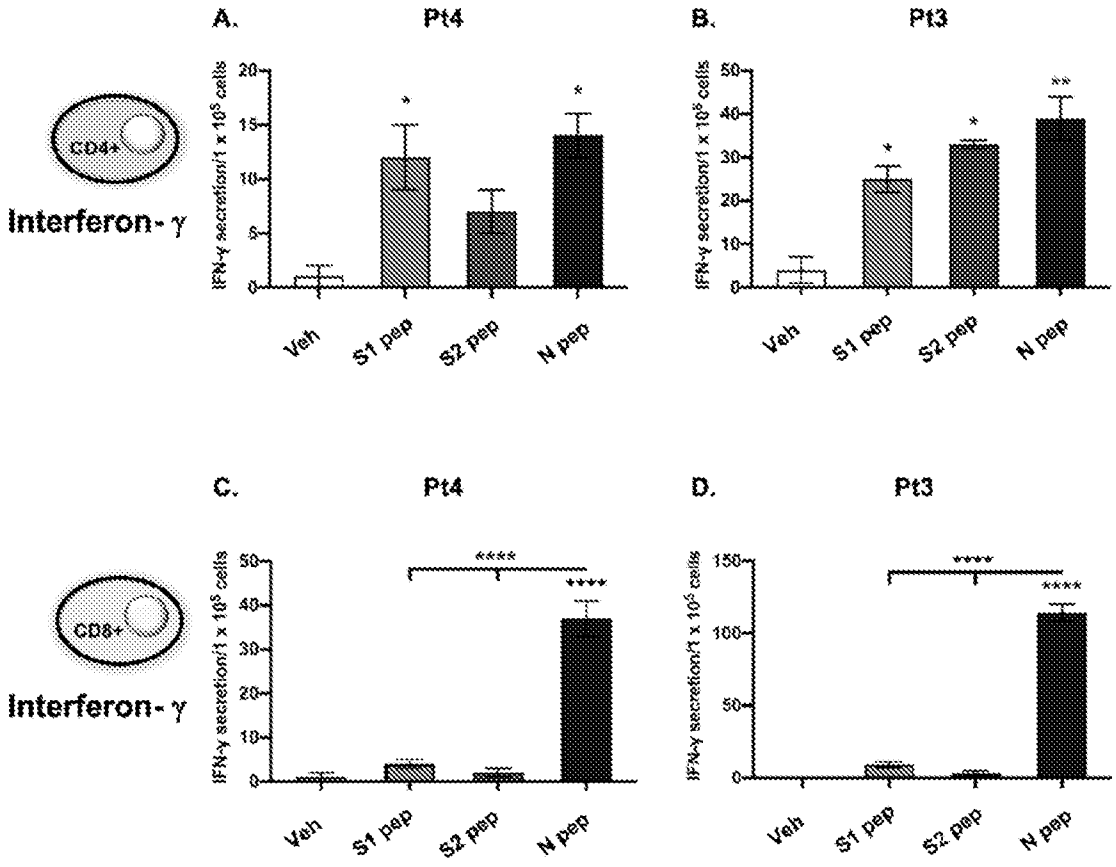

A.
Pt4
B.
Pt3
CD4+
Interferon-γ
IFN-γ secretion/1 x 10^5 cells
Veh
S1 pep
S2 pep
N pep
C.
Pt4
D.
Pt3
CD8+
Interferon-γ
IFN-γ secretion/1 x 10^5 cells
Veh
S1 pep
S2 pep
N pep

FIG.48

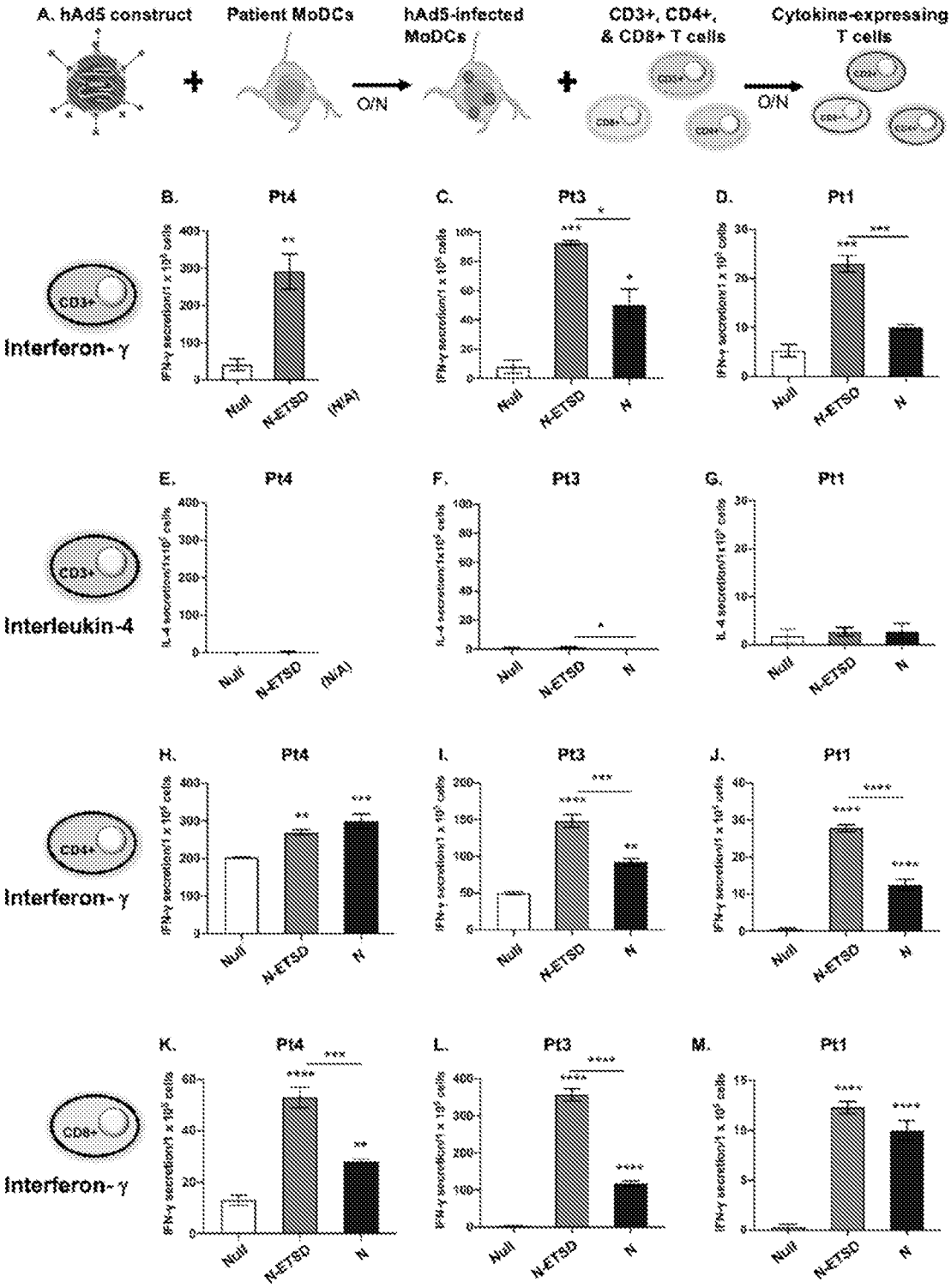

A. hAd5 construct
Patient MoDCs
hAd5-infected MoDCs
O/N
CD3+, CD4+, & CD8+ T cells
Cytokine-expressing T cells
O/N
B.
Pt4
C.
Pt3
D.
Pt1
CD3+
Interferon-γ
E.
Pt4
F.
Pt3
G.
Pt1
CD3+
Interleukin-4
H.
Pt4
I.
Pt3
J.
Pt1
CD4+
Interferon-γ
K.
Pt4
L.
Pt3
M.
Pt1
CD8+
Interferon-γ
Null
N-ETSD
N
(N/A)

FIG.49

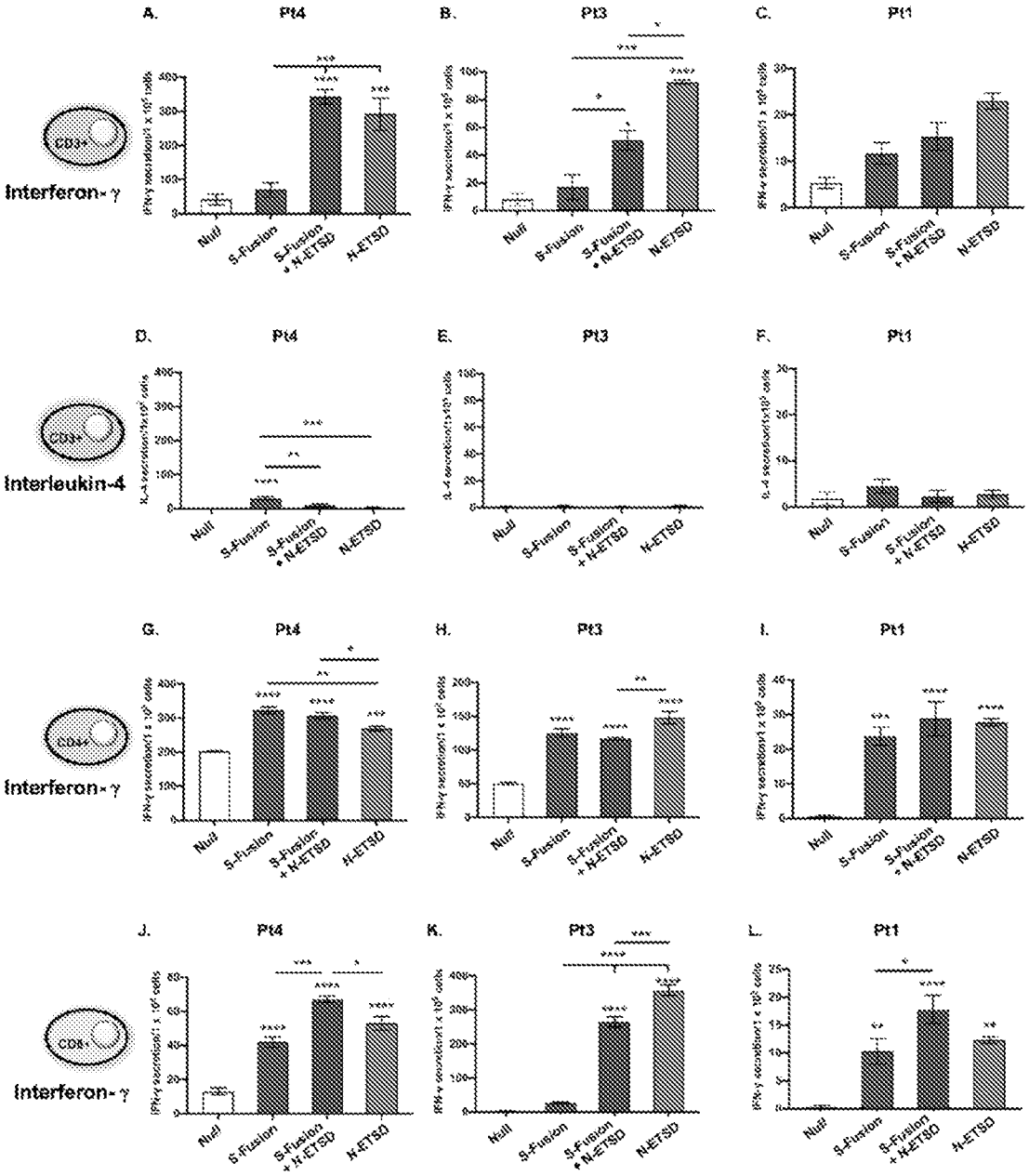

A.
Pt4
B.
Pt3
C.
Pt1
CD3+
Interferon-γ
D.
Pt4
E.
Pt3
F.
Pt1
CD4+
Interleukin-4
G.
Pt4
H.
Pt3
I.
Pt1
CD4+
Interferon-γ
J.
Pt4
K.
Pt3
L.
Pt1
CD8+
Interferon-γ
Null
S-Fusion
S-Fusion + N-ETSD
N-ETSD

FIG.50

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2021/021737

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. ☑ forming part of the international application as filed:
      ☑ in the form of an Annex C/ST.25 text file.
      ☐ on paper or in the form of an image file.
   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2021/021737

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☑ Claims Nos.: **16,17,40,41,49,51,53**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 16,17,40,41,49,51,53 are regarded to be unclear because they refer to claims which do not comply with PCT Rule 6.4(a).

3. ☑ Claims Nos.: **15,18,33,47-48,50,52,54-57**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2021/021737

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/62(2006.01)i; **C07K 14/005**(2006.01)i; **C12N 15/81**(2006.01)i; **C12N 7/00**(2006.01)i; **A61K 39/215**(2006.01)i; **A61P 31/14**(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/62(2006.01); A61K 39/215(2006.01); C07K 14/165(2006.01); C07K 16/10(2006.01); C12N 7/00(2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: COVID-19, recombinant nucleic acid, SARS, nucleocapsid protein, endosomal targeting sequence, spike protein, replication defective adenovirus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZHAO, J. et al., 'Identification and characterization of dominant helper T-cell epitopes in the nucleocapsid protein of severe acute respiratory syndrome coronavirus', Journal of virology, 2007, vol. 81, no. 11, pp. 6079-6088 abstract	1-14,19-32,34-39,42-46
A	GABITZSCH, E. S. et al., 'Anti-tumor immunotherapy despite immunity to adenovirus using a novel adenoviral vector Ad5 [E1-, E2b-]-CEA', Cancer immunology immunotherapy, 2010, vol. 59, pp. 1131-1135 the whole document	1-14,19-32,34-39,42-46
A	FAN, H. et al., 'The nucleocapsid protein of coronavirus infectious bronchitis virus: crystal structure of its N-terminal domain and multimerization properties', Structure, 2005, vol. 13, pp. 1859-1868 the whole document	1-14,19-32,34-39,42-46
A	EP 1508615 A1 (AMSTERDAM INSTITUTE OF VIRAL GENOMICS B.V.) 23 February 2005 (2005-02-23) the whole document	1-14,19-32,34-39,42-46

☑ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 June 2021	**25 June 2021**

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office **189 Cheongsa-ro, Seo-gu, Daejeon** **35208, Republic of Korea**	**Jung, Da Won**
Facsimile No. **+82-42-481-8578**	Telephone No. **+82-42-481-5373**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2021/021737

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2018-0244756 A1 (THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY, DEPT. OF HEALTH AND HUMAN SERVICES) 30 August 2018 (2018-08-30) the whole document	1-14,19-32,34-39,42-46
PX	SIELING, P. et al., 'Th1 Dominant Nucleocapsid and Spike Antigen-Specific CD4+ and CD8+ Memory T Cell Recall Induced by hAd5 S-Fusion+ N-ETSD Infection of Autologous Dendritic Cells from Patients Previously Infected with SARS-CoV-2', medRxiv, 2020[06 November 2020]<URL:https://www.medrxiv.org/content/10.1101/2020.11.04.20225417v1.full.pdf> abstract; page 9, figure 1	1-14,19-32,34-39,42-46

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US2021/021737

Patent document cited in search report	Publication date (day/month/year)	Patent family member(s)	Publication date (day/month/year)
EP 1508615 A1	23 February 2005	AT 500320 T	15 March 2011
		CA 2536235 A1	24 February 2005
		CA 2536235 C	21 November 2017
		CN 1867667 A	22 November 2006
		CN 1867667 B	04 June 2014
		CN 1867667 C	22 November 2006
		DK 1526175 T3	27 June 2011
		EP 1526175 A2	27 April 2005
		EP 1526175 A3	04 May 2005
		EP 1526175 B1	02 March 2011
		EP 1526175 B2	14 April 2021
		EP 1553169 A1	13 July 2005
		JP 2007-502612 A	15 February 2007
		US 2006-0024668 A1	02 February 2006
		US 2011-0065091 A1	17 March 2011
		US 2015-0050308 A1	19 February 2015
		US 7803918 B2	28 September 2010
		US 8835107 B2	16 September 2014
		US 9945856 B2	17 April 2018
		WO 2005-017133 A1	24 February 2005
US 2018-0244756 A1	30 August 2018	EP 3261665 A1	03 January 2018
		KR 10-2017-0140180 A	20 December 2017
		US 10301377 B2	28 May 2019
		US 10759846 B2	01 September 2020
		US 2019-0256579 A1	22 August 2019
		WO 2016-138160 A1	01 September 2016