Low-dose self-amplifying mRNA COVID-19 vaccine drives strong protective immunity in non-human primates against SARS-CoV-2 infection

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The coronavirus disease 2019 (COVID-19) pandemic continues to spread globally, highlighting the urgent need for safe and effective vaccines that could be rapidly mobilized to immunize large populations. We report the preclinical development of a self-amplifying mRNA (SAM) vaccine encoding a prefusion stabilized severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike glycoprotein and demonstrate strong cellular and humoral immune responses at low doses in mice and rhesus macaques. The homologous prime-boost vaccination regimen of SAM at 3, 10 and 30 μg induced potent neutralizing antibody (nAb) titers in rhesus macaques following two SAM vaccinations at all dose levels, with the 10 μg dose generating geometric mean titers (GMT) 48-fold greater than the GMT of a panel of SARS-CoV-2 convalescent human sera. Spike-specific T cell responses were observed with all tested vaccine regimens. SAM vaccination provided protective efficacy against SARS-CoV-2 challenge as both a homologous prime-boost and as a single boost following ChAd prime, demonstrating reduction of viral replication in both the upper and lower airways. The SAM vaccine is currently being evaluated in clinical trials as both a homologous prime-boost regimen at low doses and as a boost following heterologous prime.
The current severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has spurred the rapid development and approval of multiple vaccines; however, the virus continues to spread globally with devastating humanitarian and economic tolls. Novel vaccine platforms, such as self-amplifying mRNA (SAM), which could potentially be dose sparing due to the platform’s ability to replicate post vaccination, should be explored to address the ongoing or future pandemics.

Two main vaccine platforms have come to the fore in this pandemic, mRNA (mRNA-1273, Moderna; BNT162b2, Pfizer/BioNTech) and adenovirus based vaccines (ChAdOx1/AZD1222, AstraZeneca; Ad26.COV2.S, Janssen), both of which have demonstrated protection from hospitalization and death. Adenovirus (Ad) based vaccine vectors have previously been shown to be potent inducers of both humoral and cellular immunity, and the authorized adenovirus COVID vaccines demonstrated effectiveness in the range of 64.3–71%. mRNA-based vaccines are relatively new to the clinic, with the BNT162b2 and mRNA-1273 COVID vaccines being the first to receive emergency use authorization. Both have demonstrated potency in preclinical and clinical testing, with a standard vaccination regimen of two doses given 3–4 weeks apart. The authorized mRNA COVID vaccine doses are either 30 μg for the BNT162b2 vaccine or 100 μg for mRNA-1273, and both provide clinical protection in the range of 88–93%. Production of mRNA vaccines by a cell-free transcription reaction enables simple downstream purification and rapid vaccine release, providing for a cost-effective manufacturing process. Non-viral delivery systems, such as lipid nanoparticles (LNPs), allow repeated RNA administrations without inducing anti-vector immunity.

SAM vaccines offer the potential benefit of driving potent immune responses at low doses, as the mRNA replicates intracellularly, leading to high and durable antigen expression. We have developed and tested SAM based vaccines, initially in immuno-oncology applications, and have demonstrated their safety and potency in boosting neoantigen-specific T-cell responses primed by a chimpanzee adenovirus (ChAd68) vaccine in clinical trials (manuscript in press).

In this study, we evaluated a SAM-SARS-CoV-2 vaccine either as a homologous prime/boost vaccine regimen (SAM/SAM), or as a boost vaccine following a ChAd-SARS-CoV-2 prime (ChAd/SAM) in both mice and non-human primates (NHP). We demonstrate that SAM in both homologous and heterologous regimens can provide protection against SARS-CoV-2 replication post infection in NHP, comparable to currently authorized conventional mRNA vaccines and achieved at lower doses.

Results
SAM and ChAd vaccines encoding full-length SARS-CoV-2 spike were evaluated for immunogenicity in mice. Serum was collected at various timepoints post a single immunization and assessed for total anti-S1 IgG and pseudovirus neutralizing antibodies (nAb).

Due to the different kinetics of the humoral and cellular immune response, a separate cohort of mice was immunized and splenocytes collected at 2-weeks post-immunization to assess T-cell response by IFNγ ELISPot. While the ChAd-Spike(V1) vaccine induced detectable nAb titers and spike-specific T-cell response (Fig. 1B, C), similar to those observed with other adenoviral spike vectors, the immune response induced by the SAM-Spike(V1) vaccine was significantly higher (Fig. 1B, D). This had not been observed before with other antigens, prompting optimization of the spike sequence to improve expression. Highly variable expression was observed with various codon-optimized spike sequences (Fig. 1A) in the context of ChAd and increased expression in vitro correlated with higher nAb titers in mice (up to 63-fold) with a trend toward increased T-cell response (up to 1.7-fold) with the V2 sequence compared to the V1 sequence (Fig. 1B, C). A SAM-Spike(V2) vaccine resulted in similar immune response to that induced by the ChAd-Spike(V2) vaccine (Fig. 1B–D). Both ChAd & SAM Spike(V2) vaccines demonstrated increased durability of spike IgG titers compared to Spike(V1) (Supplementary Fig. 1). The Spike(V8) variant generated a similar immune response to Spike(V2) (Supplementary Fig. 1C, D).

To further improve vaccine potency, vectors expressing the prefusion-stabilized spike protein were generated. The Spike(V2) sequence was modified by mutation of the furin cleavage site (FurinΔ) and introduction of proline (P) substitutions to the S2 domain (2P: K986P, V987P) (Supplementary Fig. 2A), which have been shown to stabilize the spike protein in its prefusion form, leading to increased immunogenicity. The unmodified Spike(V2) is mostly in its uncleaved format with two smaller species at approximately 100 and 75 kDa. Incorporation of the furin mutation results in the loss of the 100 kDa band with the 75 kDa band still present and most likely is the TMPRSS2 cleaved product. The 2P and 6P substitutions appear to accentuate cleavage at the furin site as manifested by an intense band at 100 kDa but not at the TMPRSS2 site and the combination of FurinΔ and the 2P and 6P mutations maintains the spike protein in its uncleaved form (Supplementary Fig. 2B). The F2P variant demonstrated a further increase in nAb titers compared with V2 for both ChAd (2.9-fold) and SAM (2.1-fold) (Fig. 1E, Supplementary Fig. 2G). Vaccines with additional P-substitutions also increased nAb titers compared with V2 (Supplementary Fig. 2D, E) while T-cell responses remained potent but unchanged by the modifications (Supplementary Fig. 2C, F). Optimized Spike(V2)-F2P ChAd and SAM vaccines were evaluated in mice, as either a homologous (SAM/SAM) or heterologous (ChAd/SAM) regimen. Both regimens generated broad spike-specific T-cell responses to multiple epitopes spanning the spike antigen following prime immunization, which were increased following SAM boost (2.5 for heterologous and 5.2-fold for homologous) and were predominantly CD8+ and T111-biased (Fig. 2A; Supplementary Fig. 3 and 4). Spike-specific nAb titers were also increased by SAM boost (36-fold for heterologous and 22-fold for homologous) (Fig. 2B, Supplementary Fig. 3B). This demonstrates the increased immunogenicity of both heterologous and homologous prime/boost regimens utilizing ChAd and SAM vaccines compared to single dose regimens, similar to what has previously been observed in mice.

The robust immune response induced by SAM in mice, even at low doses (Supplementary Fig. 5), was explored further in NHP. Groups of 5 rhesus macaques received two immunizations of SAM-Spike(V2)-F2P (4-week interval) at either 3, 10, or 30 μg (Fig. 3A, Supplementary Table 1). Spike S1-specific IgG titers were observed in some NHP following a single SAM immunization in a dose-dependent manner and were increased to high levels in all NHP following a 2nd immunization at all doses (GMT 8880, 13,145, and 1223 at 30, 10, and 3 μg, respectively, Fig. 3B). Spike-specific T-cell responses were detected at all dose levels following two immunizations (Fig. 3C) and were significantly increased compared to control animals for all dose levels (adjusted p-value = 0.012, 0.024, and 0.024 for 30, 10, and 3 μg, respectively, by one-tailed Mann–Whitney test with Bonferroni correction for multiple comparisons). Strong IFNγ and minimal IL-4 responses were observed 1-week post boost in all vaccinated animals, indicating a T111-biased response (Fig. 3D). Strong nAb titers were detected following two SAM immunizations by both a pseudovirus neutralization (PNA) and live virus microneutralization assay (MNA), which strictly correlated (Fig. 3E, F; Supplementary Fig. 6). Similar nAb titers were detected in all ten NHP at both the 30 and 10 μg dose level (NT50
Lowering the dose of SAM to 3 μg resulted in overall lower, but detectable nAb titers in 5/5 NHP (GMT 245 2-weeks post boost by MNA) (Fig. 3F). Potent spike-specific nAb titers, higher than those previously reported to offer protection from SARS-CoV-2 infection in NHP14, were observed at all dose levels. While potent immune responses were detected with all dose levels, there was no increase in serum IFNα levels following SAM immunizations at doses ≤ 10 μg, which was clearly detectable at the 30 μg dose in this study, suggesting that innate immune pathways are not strongly activated at lower SAM doses (Supplementary Fig. 7).

One month following the 2nd SAM immunization (Fig. 3A), NHP were challenged with SARS-CoV-2. Consistent with previous studies of SARS-CoV-2 challenge in rhesus macaques, none of the vaccinated or control animals showed clinical signs of illness6,7,14–17. To assess vaccine efficacy in controlling SARS-CoV-2 replication post infection, viral load was assessed by PCR in bronchial alveolar lavage (BAL) fluid, oropharyngeal swabs, and nasal swabs post challenge, similar to other studies of SARS-CoV-2 vaccines in NHP7,9,15. In addition to total genomic RNA, subgenomic mRNA (sgRNA) was assessed to measure virus replication18. Three days post-challenge, 1/5 animals in the 30 μg and 3 μg groups and 0/5 animals in the 10 μg group had...
detectable sgRNA in BAL, as compared to 5/5 of the controls, demonstrating reduced viral replication at all dose levels (Fig. 4A). Comparison of peak viral titer for each animal between day 1 and day 10 post challenge demonstrated significant protection from viral replication at the 30 μg dose level compared to control animals (adjusted p-value = 0.021, Kruskal–Wallis followed by Dunn’s multiple comparison post-test, Supplementary Fig. 8). On day 3, none of the animals in the SAM 10 μg dose group and 2/5 in the SAM 30 and 3 μg groups had detectable sgRNA in nasal swabs, compared to 4/5 in the control group, demonstrating significantly decreased viral replication in nasal swabs at the 10 μg dose level (p = 0.026, peak viral load between day 2 and 14 post challenge compared to control, Kruskal–Wallis followed by Dunn’s multiple comparison post-test, Fig. 4C and Supplementary Fig. 8). Furthermore, total RNA levels were lower in BAL, oropharyngeal and nasal swabs in all SAM vaccinated animals compared to control animals, with improved viral clearance observed with the 30 and 10 μg of SAM compared with 3 μg SAM (Supplementary Fig. 9). This data demonstrates protective immunity in all SAM vaccinated animals, even at the 3 μg dose.

The potency of the SAM vaccine was further explored in a ChAd/SAM prime/boost regimen (5 × 10^{11} VP ChAd prime with 30 μg SAM boost, 6-week interval, Fig. 3A). Spike-specific IgG titers were observed following a single ChAd immunization, as reported for other adenovirus vaccines^{15,17} and were increased 2.5-fold following SAM boost to similar titers as those observed following two SAM immunizations (10 or 30 μg) (Fig. 3B). Higher spike-specific T_{H1} biased T-cell responses were detected after a single ChAd immunization compared to a single SAM immunization (at any dose), demonstrating the potency of the ChAd vaccine to rapidly drive high T-cell titers (Fig. 3C, D). T-cell responses were increased following the SAM boost to similar levels as those observed following two SAM immunizations. Spike-specific nAb titers were detected in 10/10 NHP following a single ChAd immunization (MNA NT50 GMT 663 4-weeks post prime in ChAd only treatment group) and were increased 2.3-fold following SAM boost (Fig. 3E, F). NHP were challenged with SARS-CoV-2 six weeks following a single ChAd immunization and at 4-weeks following the SAM boost (Fig. 3A).

On day 3 post challenge, only 1/5 animals in the ChAd only and 0/5 animals in the ChAd/SAM groups had detectable sgRNA in BAL, as compared to all control animals. On day 3, 1/5 NHP in the heterologous prime/boost group had detectable sgRNA in the nasal swab, compared to 4/5 in the ChAd only and control groups, demonstrating increased protective efficacy with heterologous prime/boost compared to a single ChAd immunization (adjusted p-values = 0.034 with ChAd/SAM and p < 0.001 with ChAd only, Kruskal–Wallis with Dunn’s multiple comparison post-test, peak viral load between day 2 and 10 post challenge compared to control, Fig. 4C and Supplementary Fig. 8). Collectively, these data demonstrate protection from SARS-CoV-2 replication post infection provided by the vaccines, with the 30 and 10 μg of SAM homologous prime/boost and the heterologous regimen providing complete protection from viral replication post infection and the 3 μg SAM homologous prime/boost offering slightly decreased immune response but clearly providing protection to animals challenged with live virus.

Discussion

Following extensive antigen sequence optimization, we demonstrate that a SAM vaccine encoding SARS-CoV-2 spike induces humoral and cellular immune responses in mice and NHP and provides protection from SARS-CoV-2 replication post-infection at low doses. A SAM-SARS-CoV-2 spike vaccine expressing full-length spike induced strong antigen-specific T-cell responses at low doses (Supplementary Fig. 5) and high nAb responses following a single immunization in mice (Fig. 1B, E). Unexpectedly, the ChAd vaccine expressing the first-generation spike antigen induced significantly weaker immune responses compared to SAM, prompting extensive sequence optimization, which resulted in selection of a spike sequence that strongly increased antibody and T-cell titers (Fig. 1). The difference in immunogenicity likely reflects differences in the biology of the two vaccine platforms, such as RNA splicing and routing to the cytosol required for ChAd but not SAM.

We evaluated the SAM vaccine in NHP to study immune correlates of protection. Spike-specific nAb titers were observed following two immunizations of SAM at low doses ranging from 3 to 30 μg. Potent nAb titers were generated at the 10 μg dose of
SAM, even after a single immunization, which increased following a 2nd immunization to levels similar to that observed with 10-fold higher doses (100 μg) of authorized SARS-CoV-2 mRNA vaccines6,7,16 in NHP, suggesting that SAM might present a dose sparing vaccine option. This may be due to the replication of SAM and increased mRNA half-life, which may lead to stronger and potentially more durable immune responses at lower doses compared to non-replicating mRNA vaccines. Another COVID-19 SAM vaccine, COVAC1, currently in clinical development, has been shown to induce overall weaker immune responses than the authorized mRNA vaccines, with only a 61% seroconversion rate at the 10 μg dose19, compared to 100% with either the Pfizer/BioNTech mRNA vaccine at doses ranging from 10–30 μg20 or the Moderna mRNA vaccine at 50 and 100 μg21. This decreased immunogenicity may have resulted from activation of innate immune pathways that can partly restrict SAM replication and antigen expression. Another SAM vaccine platform that uses an alternative non-prefusion stabilized spike sequence and a different LNP formulation, ARCT-021, induced low nAb titers below the GMT titer of convalescent sera, demonstrating decreased immunogenicity compared to the authorized mRNA vaccines22. Notably, our SAM vaccine differs from the COVAC1 and ARCT-021 SAM platforms in the LNP, the spike sequence and the SAM backbone. During the preclinical development of our SAM SARS-CoV-2 vaccine, we demonstrated that each of these components impact vaccine potency.

One difference between the authorized mRNA and the SAM vaccines in development is the use of modified nucleosides in the mRNA vaccines, which have been shown to reduce innate immunity post vaccination and may lead to increased vaccine-induced adaptive immune response. Induction of innate immunity and its impact on vaccine potency might differ between

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**Fig. 3 Immunogenicity in rhesus macaques.** A Schematic of SARS-CoV-2 vaccine evaluation and challenge in rhesus macaques. Orange circles represent ChAd immunization (5 × 10¹¹ VP), blue squares represent SAM vaccination at varying doses and black circle represents challenge with SARS-CoV-2 virus. N = 5/group. B Spike S1 IgG endpoint titers, geometric mean (annotated), and geometric SD. LOD = 50, samples below LOD set to ½ LOD. C Spike-specific T-cell responses assessed by overnight IFNγ ELISpot at specified timepoint post immunization. Mean (annotated) ± SEM. D IL-4 vs IFNγ ELISpot of PBMCs assessed 1-week post boost immunization. Individual animal values (n = 5/group). Diagonal lines represent unity line. Positive control stimulated wells were too numerous to count accurately. E Pseudovirus neutralization titers assessed in sera at specified timepoint post immunization. Mean (annotated) ± SEM. Positive control stimulated wells were too numerous to count accurately. F Live virus micro-neutralizing titer at specified timepoint post immunization and post SARS-CoV-2 challenge, geometric mean (annotated), and geometric SD. LOD = 20, samples below LOD set to ½ LOD.
mRNA and SAM vaccines, due to the replication of the SAM vaccine, which is an area of intense investigation. During the development of our SAM vaccine, various vaccine components were optimized to decrease activation of innate immune pathways. The NHP data suggest that our SAM SARS-CoV-2 vaccine does not induce strong systemic innate immunity at doses ≤10 μg (Supplementary Fig. 7).

Spike-specific T1 biased T-cell responses were observed in SAM immunized NHP at all doses, although with high heterogeneity across animals, potentially due to the small group size, lack of randomizing animals based on their MAMU haplotype, and/or variability in age and gender (Supplementary Table). Accumulating evidence has demonstrated the importance of T-cells in clearance of virus-infected cells and reduction of disease severity

T-cell responses are associated with improved survival in B-cell deficient COVID-19 patients with hematologic cancer, who mount weak humoral responses to the authorized COVID vaccines. Vaccine-induced T-cells may also contribute to broader protection against SARS-CoV-2 variants of concern that are sub-optimally neutralized by spike antibodies. Furthermore, T-cell memory may be more durable than B-cell memory and thus induction of both arms of the immune system may provide longer protection against future coronavirus variants.

We have demonstrated that homologous SAM prime/boost drives humoral and cellular immune responses and provides protection from SARS-CoV-2 replication post-infection at low doses in NHP. The SAM vaccine will be evaluated in a Coalition for Epidemic Preparedness Innovations (CEPI) sponsored clinical trial in South Africa. We further demonstrate that SAM provides a potent boost following a ChAd prime. The heterologous prime/boost concept has been evaluated clinically using Ad and mRNA vaccines, which led to comparable or increased antibody responses, compared to homologous boost.

In summary, SAM is a potent vaccine platform that drives robust T-cell and nAb responses at low doses, protects NHP from SARS-CoV-2 replication post-infection and is currently being tested in humans in both homologous and heterologous prime/boost regimens. It is adaptable, rapid to manufacture, and offers an additional vaccine platform in the fight against the ongoing SARS-CoV-2 pandemic and current and emerging infectious pathogens, as well as cancer.

**Methods**

**Codon optimization.** Spike nucleotide sequences based on the original Wuhan-Hu-1 strain (MN908947: https://www.ncbi.nlm.nih.gov/nuccore/MN908947.3) with the D614G mutation were designed for maximum expression in Homo sapiens using three different codon optimization tools. Spike-V1 was generated using the IDT (Coralville IA) optimization tool, V2-V7 were codon optimized using the COOL optimization algorithm (version 20160707a) and V8 was optimized using an SGI-DNA (La Jolla, CA) codon optimization tool (version 20190802). Each codon-optimized sequence was ordered as double-stranded DNA gBlocks (IDT) with at least a 30 bp overlap with sequences flanking the ChAd68 cloning site.
Vector generation. The ChAd68 nucleotide sequence was based on the wild-type sequence obtained by MiSeq (Illumina sequencing) of virus obtained from the ATCC (VR-594). The full-length sequence of the E1 (578–3404 bp) EL delete virus (2125–31,825 bp) was assembled into pUC19 from VR-594-derived and synthetic (SGI-DNA) fragments. An E4 deletion between E4ORF-2 was introduced by PCR. A CMV promoter/enhancer with an SV40 polyA was introduced into the E1 region and the spike gBlock sequences introduced by Gibson assembly (Codexis) and transfected into 2 mL of HEK293F cells. Error-free clones were identified by PCR and sequencing and plasmid DNA prepared at the Maxi-prep scale (Machery-Nagel). Furin and proline spike mutations, 2P or 6P, were introduced into the spike protein by overlapping PCR extension using primers to introduce the specific mutations and purified using Plasmid Maxi Kit (Machery-Nagel) and transfected into 2 mL of HEK293F cells (0.5 mL/mL) using TransIT-Lenti (Mirus Bio). The virus was amplified, harvested, and re-injected into 30 mL of HEK293F cells for 48–72 h. Cells and media were harvested and used to infect 400 mL of HEK293F cells. Cells were harvested after 48 h and lysed by a freeze/thaw step (−80 °C/37 °C) in 10 mM Tris pH 7.4. Following stimulation, cells were washed with PBS and stained with fixable viability dye (eBioscience, e506). Extracellular staining was performed in FACS buffer (PBS + 2% FBS + 2 mM EDTA). Cells were then washed, fixed, and permeabilized with the eBioscience Fixation/Permeabilization Solution Kit. Intracellular staining was then performed in permeabilization. Samples were collected on a Cytoflex LX (Beckman Coulter). Analysis of flow cytometry data was performed using FlowJo software (version 10.7.1).

Antibodies and dilutions. All antibodies used in this study were commercially available and are as follows with amounts/dilutions provided:

Flow cytometry. Rat Anti-Mouse IFNγ clone XMGI.21, Invitrogen, Cat #: 12-7311-82, PE (1 μL/×106 cells).

Rat-against-Mouse TNFα clone M6P-XT22, eBioscience, Cat#17-7312-82, APC (1.25 μL/×106 cells).

Rat-against-Mouse IL2 clone JES6-5H4, eBioscience, Cat#48-7021-82, eFluor450 (1.25 μL/×106 cells).

Rat-against-Mouse IL4 clone 11B11, BioLegend, Cat#504132, PE-Dazzle594 (1.25 μL/×106 cells).

Rat-against-Mouse IL10 clone JES-516E3, eBioscience, Cat#56-7101-82, AF700 (1.25 μL/×106 cells).

Rat-against-Mouse CD4 clone GK1.5, BioLegend, Cat#100451, BV605 (2.5 μL/×106 cells).

Rat-against-Mouse CD8 clone 53-6.7, BD, Cat#563332, BV786 (0.75 μL/×106 cells).

Live/dead stain, eBioscience Cat#65-0866-18, eFluor506 (1.000).

ELISPot. Anti-monkey IFNγ, clone 7B-6-1, biotin-conjugated (1.000), Mabtech, Kit catalog 3421LM-APW-10.

Mouse-against-IFNγ clone R-42-A2, biotin-conjugated (1.000), Mabtech, Kit catalog #3321-APW-10.

Anti-human IL4, clone IL-4-II, biotin-conjugated (1.000), Mabtech, Kit catalog #3410-APW-10.

IU Assay. Rabbit anti-Adenovirus antibody, Abcam Cat# Ab6982 (1.0000).

Goat-against-Rabbit HRP, Bethyl Labs, Cat#A120-101P (1.000).

Western blot. Mouse anti-Spike S2 monoclonal antibody clone 1A9, GeneTex, Cat#GTX632604 (1.000).

Rabbit-against-Mouse Actin polyclonal, Bethyl Labs, Cat#A300-485A (1.000).

Goat-against-Rabbit HRP, Bethyl Labs, Cat#A120-101P (1.000).

Commercial antibodies were validated by the vendor.

Commercial antibody information and validation info where applicable:

https://www.thermoscientific.com/antibody/product/IFN-gamma-Antibody-clone-XMG1-2-Monoclonal/12-7311-82

https://www.thermoscientific.com/antibody/product/TFN-alpha-Antibody-clone-M6P-XT22-Monoclonal/17-7312-82

https://www.thermoscientific.com/antibody/product/IL-2-Antibody-clone-JES6-5H4-Monoclonal/48-7021-82

https://www.biologend.com/en-nl/nl-products/pe-dazzle-594-anti-mouse-il-4-antibody-10715

https://www.thermoscientific.com/antibody/product/IL-10-Antibody-clone-JES-16E3-Monoclonal/56-7101-82

https://www.biologend.com/en-us/products/brightcolor-605-anti-mouse-cd4-antibody-10708/GroupID=BLG474

https://www.bdbiosciences.com/en-us/products/reagents/luminex-cytokine-reagents/research-reagents/single-color-antibodies-rbo/buv805-anti-mouse-cd8a.612898

https://www.thermoscientific.com/order/catalog/product/65-0866-14

https://www.genetex.com/order/catalog/product/65-0866-14

https://www.thermoscientific.com/antibody/product/Cytoskeletal-Actin-Antibody-Polyclonal/A300-485A

Eukaryotic cell lines. HEK293F (Thermo, #11625019).

HEK293-E3 (Expression Systems, LLC, #94-007F).

HEK293A (Thermo, #R0507).

Vero-E6 (ATCC, CRL-1586).

Vero-E6 (RIG, NR-596).

Cell lines were authenticated by original vendors. All cell lines tested negative for mycoplasma. No commonly misidentified lines were used.

Intracellular cytokine staining. Freshly isolated splenocytes were resuspended at a density of 5 × 10^6 cells/mL in complete RPMI and following an overnight rest at 4 °C, 1 × 10^6 cells per well were distributed into v-bottom 96-well plates. Cells were pelleted and resuspended in 100 μL of complete RPMI containing an overlapping peptide pool (each 15 amino acids in length, 11 amino acid overlap) spanning the SARS-CoV-2 spike antigen, at a final concentration of 0.5 μg/mL per peptide (GenScript). A second well with DMSO only was used as a negative control for each sample. After 1 h of incubation at 37 °C, Brefeldin A (Biologend) was added to a final concentration of 5 μg/mL and cells were incubated for an additional 4 h. Following stimulation, cells were washed with PBS and stained with fixable viability dye (eBioscience, e506). Extracellular staining was performed in FACS buffer (PBS + 2% FBS + 2 mM EDTA). Cells were then washed, fixed, and permeabilized with the eBioscience Fixation/Permeabilization Solution Kit. Intracellular staining was then performed in permeabilization. Samples were collected on a Cytoflex LX (Beckman Coulter). Analysis of flow cytometry data was performed using FlowJo software (version 10.7.1).
Non-human primate studies. Study was conducted in compliance with all relevant local, state, and federal regulations and was approved by the Battelle Institutional Animal Care and Use Committee (IACUC), Protocol #080082. Thirty Indian-origin male and female rhesus macaques (M. mulatta) >2.5 years old (Envigo) were housed at Battelle (Columbus, Ohio). NHPs were vaccinated with either ChAd-Spike(V2)-F2P (Group 1—5 × 10^5 PFU, study day 0; Group 2—5 × 10^5 PFU, study day 28); SAM-Spike(V2)-F2P (Group 1—30 μg, study day 42; Group 2—30 μg, study days 14 and 42; Group 5—10 μg, study days 14 and 42; Group 3—5 μg, study days 14 and 42), or PBS (Group 6, study days 0 and 42). All injections were bilateral intramuscular, 0.5 mL per leg (1 mL total) to the thigh. Note that vaccinations were staggered to enable challenge immunization on study day 70 for all NHPs. Each challenge includes divided immune groups and a challenge period of 1 week apart, to enable two staggered challenge groups. Animals were challenged with SARS-CoV-2 (strain USA-WA1/2020) via the intratracheal (0.5 mL) and intranasal (0.25 mL per nostril) routes with a target dose of ~1.6 × 10^6 PFU (undiluted). Serum was collected using serum separator tubes and whole blood collected in preparation tubes and peripheral blood mononuclear cells isolated and cryopreserved in CryoStorCS solution (Sigma-Aldrich).

ELISpot assays. IFNγ and IL-4 ELISpot assays were performed using pre-coated 96-well plates (Mabtech, Monkey IFNy ELISPOT PLUS, ALP; Mouse IFNy ELISPOT PLUS, ALP; or Human IL-4 ELISPOT PLUS, ALP) following manufacturer’s protocol. For NHP, frozen PBMCs were thawed at 37 °C and then rested overnight in RPMI 10% FBS. 1 × 10^5 PBMCs were plated per well in triplicate with a single overlapping peptide pool spanning N to C terminus (GenScript, 15 amino acid length, 11 amino acid overlap, 314 peptides total) at a final concentration of 1 μg/mL per peptide, and incubated overnight at 37 °C in RPMI 10% FBS. For mouse studies, freshly isolated splenocytes were stimulated overnight with either two (~120 peptides/each) or eight different overlapping peptide pools (36–40 peptides each) spanning the SARS-CoV-2 spike antigen, at a final concentration of 1 μg/mL per peptide (GenScript). Splenocytes were plated in duplicate in 1 × 10^5 cells per well and 2.5 × 10^4 cells per well (mixed with 7.5 × 10^4 naïve cells) for each stimulus. DMSO only was used as a negative control for each sample. For all mouse and NHP IFNy ELISPOT, positive control wells were stimulated with Phorbol Myristate Acetate (PMA, 15 ng/mL, Invivogen) and Ionomycin (500 ng/mL, Sigma) and included on each plate in triplicate. For NHP IL-4 ELISPOT, positive control stimulated wells controlled with Staphylococcal enterotoxin B (SEB, 1000 ng/mL, Sigma-Aldrich) were included on each plate in triplicate. Note that for all assays, positive control wells were too numerous to count accurately (well confluence >35%). For all ELISpot assays, triplicate wells with media only were also included on each plate. Plates were washed and then incubated with anti-monkey or anti-mouse IFNy mAb (biotin) (Mabtech) for 2 h, followed by an additional wash and incubation with Streptavidin-ALP (Mabtech) for 1 h. After final wash, plates were incubated for 10 min with BCIP/NBT (Mabtech) to develop the immunospots. Wells were imaged and spots enumerated using AID reader with Vspot v7 (Autoimmun Diagnostika). Samples with replicate well variability (variability = variance/median + 1) >10 and median > Lo f1 were excluded.55 Spot values were adjusted based on the well saturation according to the formula: AdjustedSpots = RawSpots + 2*(RawSpots’Saturation(100—Saturation))36 Each sample was background corrected by subtracting the average value of the negative control wells. Data were normalized to spot forming units (SFU) per 1 × 10^6 cells by multiplying the corrected spot number by 1 × 10^6/cell number plated. Data processing was performed using the R programming language and graphed using GraphPad Prism 9.

Pseudovirus neutralization assay. Mouse and human convalescent serum samples (courtesy of Helen Chu, University of Washington) were assessed by Nexelis (Laval, Quebec). NHP serum samples were assessed by Gritstone bio (Emeryville, CA) using the same pseudovirus, controls, reagents, and protocol. Pseudotyped virus particles were generated using a genetically modified Vesicular Stomatitis Virus from which the glycoprotein G was removed (VSVAg). The VSVAg virus was transduced in HEK293-E cells (Expression Systems, LLC) previously transfected with the spike glycoprotein of the SARS-CoV-2 coronavirus (Wuhan strain) for which the last 19 amino acids of the cytoplasmic tail were removed (VSVΔG). The VSVΔG virus was used in HEK293-E cells (Expression Systems, LLC) previously transfected with the spike glycoprotein of the SARS-CoV-2 coronavirus (Wuhan strain) for which the last 19 amino acids of the cytoplasmic tail were removed (VSVΔG). Beta coronavirus, at a final concentration of 1 μg/mL per peptide, and incubated overnight at 37 °C in RPMI 10% FBS. For mouse studies, freshly isolated splenocytes were stimulated overnight with either two (~120 peptides/each) or eight different overlapping peptide pools (36–40 peptides each) spanning the SARS-CoV-2 spike antigen, at a final concentration of 1 μg/mL per peptide (GenScript). Splenocytes were plated in duplicate in 1 × 10^5 cells per well and 2.5 × 10^4 cells per well (mixed with 7.5 × 10^4 naïve cells) for each stimulus. DMSO only was used as a negative control for each sample. For all mouse and NHP IFNy ELISPOT, positive control wells were stimulated with Phorbol Myristate Acetate (PMA, 15 ng/mL, Invivogen) and Ionomycin (500 ng/mL, Sigma-Aldrich) and included on each plate in triplicate. For NHP IL-4 ELISPOT, positive control stimulated wells controlled with Staphylococcal enterotoxin B (SEB, 1000 ng/mL, Sigma-Aldrich) were included on each plate in triplicate. Note that for all assays, positive control wells were too numerous to count accurately (well confluence >35%). For all ELISpot assays, triplicate wells with media only were also included on each plate. Plates were washed and then incubated with anti-monkey or anti-mouse IFNy mAb (biotin) (Mabtech) for 2 h, followed by an additional wash and incubation with Streptavidin-ALP (Mabtech) for 1 h. After final wash, plates were incubated for 10 min with BCIP/NBT (Mabtech) to develop the immunospots. Wells were imaged and spots enumerated using AID reader with Vspot v7 (Autoimmun Diagnostika). Samples with replicate well variability (variability = variance/median + 1) >10 and median > Lo f1 were excluded.55 Spot values were adjusted based on the well saturation according to the formula: AdjustedSpots = RawSpots + 2*(RawSpots’Saturation(100—Saturation))36 Each sample was background corrected by subtracting the average value of the negative control wells. Data were normalized to spot forming units (SFU) per 1 × 10^6 cells by multiplying the corrected spot number by 1 × 10^6/cell number plated. Data processing was performed using the R programming language and graphed using GraphPad Prism 9.

Nucleocapsid protein (N1) genomic analysis. Briefly, RNA was isolated using the IndiSpin QIAcube HT Pathogen Kit (Indical Bioscience, Germany) on the QIAcube HT robot. About 1 μg each of isolated RNA from RT-qPCR using the TaqMan Fast Virus 1-step Master Mix (Thermo Fisher Scientific) on a QuantStudio Flex 6 Real-Time PCR System (Applied Biosystems, Foster City, CA). The primers and probe were specific to the SARS-CoV-2 nucleocapsid gene, corresponding to the N1 sequences from the Centers for Disease Control and Prevention (CDC) 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel (https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html) except that the probe quencher was modified to Non-Fluorescent Quencher-Minor Groove Binder (NFQ-MGB) (Thermo Fisher Scientific). A standard curve comprised of synthetic RNA containing the target sequence from SARS-CoV-2 isolate WA1 sequence (GenBank Accession Number: MN985325.1) (Bio-Synthesis, Inc., Lewisville, TX) was included on each PCR plate for absolute quantitation of SARS-CoV-2 RNA copies in each sample. Thermocycling conditions were as follows: Stage 1—50 °C for 5 min for one cycle; Stage 2—95 °C for 20 s for one cycle; Stage 3—95 °C for 3 s and 60 °C for 30 s for 40 cycles. The data was analyzed using QuantStudio 6 software-generated values (total copies per well of each sample) and additional calculations to determine SARS-CoV-2 N1 copies per mL of fluid.

Viral RNA RT-qPCR
Envelope protein (E) subgenomic analysis. Following isolation and evaluation using the N1 genomic assay, the isolated RNA was then evaluated as described above using primers and probes specific to the SARS-CoV-2 E gene based on previously described sequences, and the reverse primer and probe sequences previously provided with this paper.

GAAAT-3′ subgenomic (Esg) RNA copies per mL of each sample) and additional calculations to determine SARS-CoV-2 E gene performed using the QuantStudio 6 software-generated values (total copies per well dose group and the control group as well as >80% power to detect a 3.3-fold change between any two vaccinated dose groups Assuming a standard deviation of two. Sample sizes of 5 per continuous parameter. For parameters that are not log-transformed, the sample sizes will provide >80% power to detect a 4.9 standard-unit change between each vaccinated group.

Student’s t-tests comparing any two of the vaccinated groups – Whitny tests. All statistical analysis performed using GraphPad Prism 9.

Statistics. For evaluating disease progression post-challenge in NHPs sample size was pre-determined using the power procedure in SAS (version 9.4). Sample sizes of 5 per group will provide >81% power to detect a 2.6-fold change between each vaccinated dose group and the control group as well as >80% power to detect a 3.3-fold change between any two vaccinated dose groups for any of the continuous parameters that are log-transformed for the analysis. This assumes a 40% coefficient of variation for the continuous parameter. For parameters that are not log-transformed, the sample sizes will provide >80% power to detect a 4.9 standard-unit change between each vaccinated dose group and the control group as well as >80% power to detect a 6.2 standard-unit change between any two vaccinated dose groups assuming a standard deviation of two. These power calculations performed for one-sided t-tests comparing vaccinated animals to controls and two-sided t-tests comparing any two of the vaccinated groups using a Bonferroni multiple comparison adjustment to control the overall Type I error level at no more than 5% for each set of tests.

P values reported for mouse ELLSpot comparisons determined by unpaired Student’s t-test. P values reported for mouse RNA comparisons determined by two-tailed Mann–Whitney tests.

Comparison of mRNA and PNA values assessed by nonlinear regression analysis (least-squares fit).

For NHP T-cell analysis, peak values for each animal over the duration of the study (prior to challenge) were compared for each of three SAM vaccinated groups compared to control group by nonparametric one-tailed Mann–Whitney tests. Reported p-values are adjusted using Bonferroni’s correction for multiple comparisons.

For NHP RT-qPCR analysis peak sgRNA value for each animal post challenge was compared for each vaccinated group to the control group by nonparametric Kruskal–Wallis ANOVA followed by Dunn’s multiple comparisons post-test.

Adjusted p-values are reported.

All statistical analysis performed using GraphPad Prism 9.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data generated in this study are provided in the Source data file. Source data are provided with this paper.

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Author contributions

C.D.S., L.G., S.J.H., and K.J. designed vaccine constructs. C.D.S., S.J.H., J.A.E., R.L.V., L.G., A.A., and M.F. produced the vaccines and performed in vitro evaluation. A.R.R., A.S., A.V.A., M.E., M.A.K., and G.B. designed and executed mouse studies and performed and analyzed serum IgG and ELISpot assays. A.R.R. and G.S. designed NHP study. G.B. performed and analyzed NHP ELISpot assays and cytokine MSD. M.A.K. performed and analyzed NHP PNA assays and M.E. performed and analyzed NHP IgG assays. G.S. oversaw performance and analysis of MNA and RT-PCR assays. A.R.R., S.J.H., C.D.S., and K.J. wrote the manuscript.

Competing interests

A.R.R., S.J.H., C.D.S., L.G., A.A., G.R.B., M.E., J.A.E., M.F., M.A.K., A.S., A.V.A., R.L.V., and K.J. are stockholders and either current or previous employees at Gritstone Bio, Inc. and may be listed as co-inventors on various pending patent applications related to the vaccine platform presented in this study. The remaining authors declare no competing interests.

Additional information

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