

T cell-mediated protection against lethal SARS-CoV-2 challenge by a synthetic long peptide vaccine targeting conserved CD4⁺ and CD8⁺ T cell epitopes

Esmé TI van der Gracht

ISA Pharmaceuticals

Iris N Pardieck

Leiden University Medical Center

Suzanne van Duikeren

Leiden University Medical Center

Peter van Rijn

ISA Pharmaceuticals

Dominique MB Veerkamp

Leiden University Medical Center

J. Frédérique de Graaf

Leiden University Medical Center

Kitty Kwappenberg

ISA Pharmaceuticals

Ingrid MC Kamerling

Centre for Human Drug Research

Annelieke C Kruithof

Centre for Human Drug Research

Johan L van der Plas

ISA Pharmaceuticals

Marissa E Linger

Leiden University Medical Center

Jonna R Bloeme-ter Horst

Leiden University Medical Center

Sebenzile K Myeni

Leiden University Medical Center

Willem-Jan Krebber

ISA Pharmaceuticals

Leon Hooftman

ISA Pharmaceuticals

Thomas JM Beenakker

ISA Pharmaceuticals

Ramon Arens

R.Arens@lumc.nl

Leiden University Medical Center

Cornelis JM Melief

ISA Pharmaceuticals

Anna-Sophia Wiekmeijer

ISA Pharmaceuticals

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Abstract

Background

The majority of approved SARS-CoV-2 prophylactic vaccines are based on mRNA or viral vector platforms and act through the induction of neutralizing IgG antibodies against conformational B cell epitopes of the viral Spike (S) protein, as well as the induction of virus-specific T cell responses.

Methods

In this study, we designed a synthetic long peptide (SLP) vaccine comprising multiple conserved peptides derived from the SARS-CoV-2 Spike (S), nucleocapsid (N), and membrane (M) proteins, containing predicted MHC class I and class II epitopes. Candidate peptides were selected using *in silico* HLA-binding and immunogenicity prediction algorithms combined with manufacturability filtering, and subsequently evaluated for immunogenicity by *ex vivo* stimulation of PBMCs from SARS-CoV-2 convalescent donors. A murine SLP analogue was generated and optimized for formulation, dose, and administration route, and tested *in vivo* in C57BL/6-based vaccination experiments, followed by prophylactic challenge studies in K18-hACE2 transgenic and human ACE2 knock-in mice. The potential impact of SARS-CoV-2 variants of concern on the targeted epitopes was evaluated *in silico*.

Results

We demonstrated strong reactivation of memory CD8⁺ T cell responses towards our selected coronavirus-SLP vaccine in PBMCs from SARS-CoV-2 convalescent individuals. In addition, we optimized the vaccination strategy and showed T cell-mediated protection against SARS-CoV-2 in the absence of neutralizing antibodies following prophylactic vaccination of K18-hACE2 transgenic mice and human ACE2 knock-in mice with a specifically designed mouse SLP vaccine adjuvanted with the Toll-Like Receptor (TLR)1/2 ligand Amplivant. *In silico* analysis indicated that the conserved regions targeted by the vaccine are largely preserved across five variants of concern, with only one or two peptides affected per lineage, suggesting that immunogenicity is expected to be retained across these variants.

Conclusion

Taken together, our data demonstrate that SLP vaccination targeting conserved SARS-CoV-2 epitopes induces robust T cell responses and mediates protection against SARS-CoV-2 challenge in preclinical models. These findings support the further evaluation of T cell-focused vaccination strategies, particularly for settings in which antibody-mediated immunity is limited.

Background

Prophylactic SARS-CoV-2 vaccination can be achieved using several platforms, including mRNA vaccines, DNA vaccines, recombinant viruses, and synthetic long peptides (SLP). Approved SARS-CoV-2 preventive vaccines, mostly based on mRNA and viral vectors, act through the induction of neutralizing

IgG antibodies against conformational B cell epitopes on the viral spike (S) protein, as well as through virus-specific T cell responses (1, 2). These vaccines are less protective against COVID-19 in patients with hereditary or acquired B cell deficiencies, or in patients receiving B cell-depleting anti-CD20 monoclonal antibodies, which are associated with impaired antibody formation (3). We hypothesize that focused T cell responses against T cell epitope-rich regions of SARS-CoV-2 proteins can be elicited by adjuvanted SLP vaccination. In the absence of protective neutralizing antibodies, these virus-specific T cell responses are expected to confer protection against SARS-CoV-2, even in individuals with defective B cell immunity (4).

Therefore, we developed an SLP vaccine against SARS-CoV-2, called Corona-SLP. This vaccine is intended both as a therapeutic modality and as a preventive measure for patients at increased risk of severe COVID-19 and COVID-19-related mortality, such as older individuals and patients with underlying conditions associated with impaired antibody-mediated protection (5, 6). Severe disease following SARS-CoV-2 infection appears to be characterized, among other features, by an impaired T cell immune response (5–8). This deficient T cell response may represent a relevant therapeutic vaccine target, provided that a profound decline in T cell numbers has not yet occurred.

SLP vaccines have been studied in combination with immune checkpoint inhibition and/or chemotherapy for the treatment of cancer, targeting viral antigens such as those expressed in human papilloma virus (HPV)-induced cancers (9–11) or mutation-derived neoantigens (12). A correlation between the magnitude of the SLP vaccine-induced T cell response and responses against HPV16 E6/E7-positive cancer has been observed in multiple preclinical and clinical studies (13–16). An advantage of SLP vaccines is that they contain multiple SLPs. By separating these into two drug products, antigenic competition is reduced, resulting in enhanced responses to individual SLPs when each drug product is administered in a different limb, thereby engaging distinct draining lymph nodes (17). Recently, a peptide vaccine comprising six HLA-DR-restricted SARS-CoV-2 peptides was developed for the treatment of patients with congenital or acquired B cell deficiency and tested in a Phase I/II trial (4). T cell responses against the Spike protein by this SLP vaccine were higher in B cell-deficient patients and immunocompetent COVID-19 convalescent individuals than those induced by mRNA-based vaccination (4), highlighting the potential of SLP vaccines to induce robust T cell immunity against SARS-CoV-2.

We have developed a vaccine comprising multiple conserved peptides harboring both MHC class I and MHC class II epitopes derived from the S, nucleocapsid (N), and membrane (M) proteins of SARS-CoV-2. We hypothesize that inclusion of both HLA class I and II-restricted epitopes will promote simultaneous presentation of multiple SARS-CoV-2-specific peptides, leading to enhanced SLP-specific CD4⁺ and CD8⁺ T cell responses, as CD4⁺ T cell help is essential for optimal CD8⁺ T cell effector function and memory formation (18).

Here, we report the reactivation of robust memory T cell responses by Corona-SLP *ex vivo* in PBMC from convalescent donors recovered from SARS-CoV-2 infection. In addition, we describe vaccination of mice

with a murine variant of Corona-SLP (mCorona-SLP), consisting of immunogenic human epitope-based SLPs combined with SLPs designed around validated C57BL/6 (H-2^b) murine epitopes. Adjuvant, administration route, vaccination interval, and SLP dose were evaluated to optimize T cell responses in mice, followed by protection studies against a lethal SARS-CoV-2 challenge in K18-hACE2 transgenic and human ACE2 knock-in mice. Finally, we assessed in silico the potential impact of major SARS-CoV-2 variants of concern on the conserved epitopes targeted by the vaccine.

Methods

Peptides and Amplivant

All reagents and solvents were purchased from Merck, VWR, Bachem, GL Biochem, Novabiochem, and Biosolve and used as received. Peptides were synthesized by solid-phase peptide synthesis on a Tetras peptide synthesizer (Advanced ChemTech) using Fmoc/tBu chemistry (19). Amplivant was synthesized using chirally pure FmocCys((R)-2,3-di(palmitoyloxy)-propyl)-OH following the same methodology. Peptides were purified by preparative HPLC/MS (Waters AutoPurification system) under acidic conditions (acetonitrile/water/TFA) and lyophilized overnight. Identity and purity were confirmed by UPLC-MS (Waters Acquity UPLC/TQD; C18 column, 220 nm detection). Amplivant was analyzed using a C4 column under the same system. Chromatograms were processed using MassLynx v4.1 with standard ApexTrack parameters. After MS confirmation, UV peak integration was used to determine purity. All peptides and Amplivant had $\geq 90\%$ purity, except unique mCorona-SLP peptides, which had $\geq 70\%$ purity per individual peptide. Peptide pools were prepared by dissolving peptides in 0.09% TFA in H₂O:ACN (1:1 v/v), mixing, and lyophilizing overnight. All Corona-SLP candidate peptides were produced by ISA Pharmaceuticals B.V. The five mCorona-SLP peptides designed for C57BL/6 mice were produced at the LUMC peptide facility.

PBMCs

Samples from COVID-19 patients were part of a clinical trial (NL8589) registered in the Dutch Trial Registry. All patients suffered from critical COVID-19 as categorized according to WHO guidelines (WHO/2019-nCoV/clinical/2020.4). A total of 15 individuals were included: 11 recovered COVID-19 patients and 4 healthy controls without known SARS-CoV-2 exposure. Donors were enrolled between 17 August and 16 September 2020. Symptom duration ranged from 3 to 45 days; most donors had fever and 6 had dyspnea. Other symptoms included headache, loss of smell and taste, cough, sore throat, hyperventilation, chest pain, gastrointestinal symptoms, shivering, rhinitis, myalgia, nausea, and fatigue. One donor required intensive care admission. No correlation was observed between disease duration or severity and immunogenicity of the candidate Corona-SLPs.

PBMCs were obtained and cryopreserved by the Centre for Human Drug Research using standard procedures. PBMCs from Sanquin were isolated from buffycoats at ISA Pharmaceuticals B.V. Biobanked

ICU PBMCs were used as previously described. Pre-May 2019 samples were randomly selected as SARS-CoV-2-naïve healthy controls.

In silico epitope prediction

An in-house proprietary immunological *in silico* prediction tool, named ISABELLA, was applied to identify immunogenic regions for the most frequent HLA types. ISABELLA is designed to perform HLA binding predictions and is an acronym for “ISA’s Best Epitope & Ligand Learning Algorithm”, referring to a proprietary Logistic Regression (LR) model that was trained on output produced by multiple common MHC binding affinity predictors (e.g. NetMHC-4.0), multiple proteasomal cleavage predictors (e.g. NetChop) and multiple TAP transport affinity predictors when applied to source protein segments containing confirmed epitopes as detailed by the Immune Epitope DataBase. For each prediction, the model outputs a value between 0 and 1. Higher values indicate a better chance that a peptide is immunogenic. During validation the model’s optimal cut-off for bulk-predicting immunogenic peptides was determined to be 0.05, translating to a positive predictive value of 0.30 and a sensitivity of 0.55. This model was used to design parts of Corona-SLP. More in-depth details on the model’s design and access to the model can be granted upon request to the authors. Identification of immunogenic regions for murine MHC (H-2) was performed with both NetMHC 4.0 and NetMHCpan 3.2.

In silico manufacturability prediction

An in-house proprietary *in silico* manufacturing prediction tool, named MONALISA, was applied to reduce the risk of failed SLP syntheses. MONALISA is an acronym for “Manufacturability Of Neo Antigens and Ligands by ISA”, referring to a proprietary Random Forest machine learning model that was trained on SLP features (e.g. length, hydrophobicity, number of cysteines, etc.) and results (percentage crude quality) from in-house SLP syntheses. The model outputs crude quality percentages between 0 and 100 for individual SLPs along with other SLP characterizations, which can be used as guidelines for initial filtering before starting a synthesis. More in-depth details on the model’s design and access to the model can be granted upon request to the authors.

Flow Cytometry

Cell surface staining and intracellular cytokine staining was performed as described (20). Fluorescently labelled monoclonal anti-human antibodies were used to detect CD8 (clone SK1, BD Biosciences), CD4 (SK3, BD Biosciences), CD137 (4B4-1, BD Biosciences), CD154 (Trap1, BD Biosciences), IFN- γ (B27, BD Biosciences), TNF (Mab11, Biolegend), IL-2 (MQ1-17H12, BD Biosciences). For mouse samples, fluorescently labelled monoclonal antibodies were used to detect CD3 (145-2C11, BD Biosciences), CD4 (RM4-5, BioLegend), CD8 (53 – 6.7, BioLegend), KLRG1 (2F1, ThermoFisher), CD44 (IM7, BioLegend), CD62L (MEL-14, BioLegend). 7-AAD (A1310, Invitrogen) cell viability dye was used to exclude dead cells. mSLP-specific CD8⁺ T cells were identified using H2-Kb tetramers for Spike_{263–270} (AAYYVGYL) and

Spike_{539–546} (VNFNFNGL). Acquisition was performed on a BD Fortessa flow cytometer (BD Biosciences).

ELISpot (human PBMC)

Cryopreserved PBMCs from healthy and SARS-CoV-2 convalescent donors were thawed and stimulated with individual SLPs (3 μ M) at 2×10^6 cells/well in 24-well plates, or with Candida antigen (0.175 μ g/mL) as a positive control. SARS-CoV-2 Peptivator mixes (Miltenyi Biotec) were used to assess SARS-CoV-2-specific memory responses. Cells stimulated with PHA (1 μ g/mL; Sigma-Aldrich) served as a positive control. After 48 h, 1×10^5 cells/well were transferred (in triplicate) to Multiscreen plates coated overnight with anti-IFN- γ antibody (mAb-1-D1K; Mabtech, 5 μ g/mL). Plates were blocked with IMDM containing 8% human serum (BioIVT) for ≥ 1 h at 37°C. After overnight incubation at 37°C, cells were removed and plates were washed with PBS/0.05% Tween-20. Detection was performed using biotinylated anti-IFN- γ antibody (mAb7-B6-1; 0.3 μ g/mL) for 2 h at room temperature, followed by streptavidin-ALP (1 h). Spots were developed with BCIP/NBT substrate and counted using an Astor ELISpot reader. Responses were considered positive if exceeding 90 SFU/ 10^6 cells, based on the HBV-derived negative control SLP.

ELISA (human serum)

Human serum samples were analyzed for the presence of SLP-specific IgG by ELISA. Nunc MaxiSorp plates were coated overnight at room temperature with 100 μ L SLP (1 μ g/mL in coating buffer). Plates were washed and blocked for 2 h at room temperature with PBS containing 5% BSA. After washing (PBS/0.05% Tween-20), serum samples were added at indicated dilutions in PBS/0.1% BSA/0.05% Tween-20 (100 μ L/well) and incubated for 2 h at room temperature. Plates were washed and incubated with biotinylated goat anti-human IgG, followed by streptavidin-poly-HRP (both diluted in HPE buffer) for 1 h at room temperature. Plates were developed with 100 μ L TMB per well, and the reaction was stopped with 100 μ L 1 M H₂SO₄. Absorbance was measured at 450 nm within 5 min using a SpectraMax M3.

Restimulation assay human PBMCs

Frozen PBMCs were thawed and cultured in a cell density of 1×10^6 cells in 100 μ L medium with 10% heat-inactivated FCS, L-glutamine and pen/strep. In brief, cells were stimulated with 21 peptides (Table S1 and S2) at day 0. At day 1, IL-15 and IL-2 were added. At day 6 and day 8, cells were harvested and stimulated with single peptides. One day later intracellular cytokine analysis was performed and samples were acquired on a flow cytometer. For the PBMCs from ICU patients, Peptivator mixes S, M, and N were prepared and used according to the PepTivator SARS-CoV-2 Prot_s protocol (Miltenyi). In addition, two peptide pools containing the human Corona-SLPs were used. Stimulation by the CMV pp65 peptide pool (Miltenyi) and PMA/ionomycin functioned as a positive control. 1% DMSO in normal culture medium functioned as a negative control. One hour after adding the peptides, brefeldin A (Golgiplug; BD

Pharming) was added in 5 µg/ml end concentration, 15 h later the stimulation was stopped by placing the plate at 4°C and washing for further FACS staining.

Multiplex bead-based cytokine analysis

For the multiplex cytokine assay, the Luminex technology with the Th1/Th2 Cytokine 11-Plex Human ProcartaPlex Panel (EPX110–10810-901, ThermoFisher) was used according to manufacturer's instructions. Levels of the following cytokines were measured: GM-CSF, TNF, IL-6, IL-2, IFN- γ , IL-4, IL-5, IL-13, IL-18, IL-12p70, and IL1 β . Plates were measured on a MAGPIX analyzer.

Mice and SLP vaccination

C57BL/6 (H-2^b) mice were purchased from Janvier Labs (Le Genest-Saint-Isle; France). K18-hACE2 transgenic mice expressing human ACE2 receptor (hACE2) under control of the cytokeratin 18 (K18) promoter (B6.Cg-Tg(K18-ACE2)2PrImn/J) and hACE2 knock-in mice (B6.129S2(Cg)-Ace2 <tm1(ACE2)Dwnt>/J) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and bred in-house. Both mouse strains were on an H-2^b background, matching the MHC haplotype of C57BL/6 mice. Mice were 7–8 weeks of age at the start of the experiments. Animals were housed under specific-pathogen free conditions in individually ventilated cages at the animal facility at Leiden University Medical Center (LUMC).

Mice were vaccinated subcutaneously, intramuscularly or intradermally with the indicated doses of in-house manufactured SLP and Amplivant dissolved in DMSO/Water for Injection (WFI). For Montanide-adjuvanted vaccines, Montanide ISA-51 was emulsified with DMSO/WFI dissolved SLP using a T-connector.

SARS-CoV-2 infection

SARS-CoV-2 infection was performed similar to a previous study (21). Clinical isolate SARS-CoV-2/human/NLD/Leiden-0008/2020 (here named SARS-CoV-2) was used for the SARS-CoV-2 infection of mice. Isolate Leiden-0008 was propagated and titrated in Vero-E6 cells (ATCC CRL-1586). K18-hACE2 transgenic and hACE-2 knock-in mice were anaesthetized with iso-flurane gas and intranasally infected. Mice received 1×10^4 plaque forming units (PFU) SARS-CoV-2 (K18 hACE2 transgenic mice) or 1×10^5 PFU (hACE2 knock-in mice) of the SARS-CoV-2 Leiden-08 isolate in a total volume of 50 µl DMEM. Mouse weight and clinical discomfort were monitored daily. Euthanasia criteria were weight loss of > 20 percent of body weight compared to the pre-study weight and/or clinical score. Clinical score consists of activity level, behavior, appearance of fur and posture and body weight. The quantification of SARS-CoV-2 viral RNA in lung homogenates was performed as previously described (22). All experiments with SARS-CoV-2 were performed in the Biosafety Level 3 Laboratories at the LUMC.

Ex vivo studies using murine splenocytes and serum

At different time points after vaccination, peripheral blood was collected from the tail vein. One week after the final vaccine administration, spleen and blood samples were collected. Blood samples were collected by heart puncture and transferred to an Eppendorf tube. After blood clot formation, the blood samples were centrifuged to obtain final serum of individual mice. Serum was stored at -20°C in cryovials.

Splenocytes were isolated by mincing spleens through a cell strainer. Subsequently, erythrocytes were lysed using lysis buffer, splenocytes were washed and plated for *in vitro* restimulation. For this, single peptides or peptide pools (1 µg per SLP in 5 µl) were added to the splenocytes, which were incubated overnight. The next day, intracellular cytokine staining was performed to study the induction of SLP-specific T cell responses as measured by flow cytometry.

ELISA murine serum

Serum samples were analyzed for SLP-specific IgG by ELISA. Nunc MaxiSorp plates were coated overnight at 4°C with 100 µL SLP (1 µg/mL in coating buffer). Plates were washed and blocked for 1 h at room temperature with blocking buffer (PBS, 1% BSA, 0.05% Tween-20). Serum samples at serial dilutions were added (100 µL/well) and incubated for 1 h at room temperature. After washing, goat anti-mouse IgG-HRP (1:4000, 100 µL/well) was added and incubated for 1 h at room temperature. Plates were washed and developed using 50 µL TMB per well, followed by incubation in the dark. The reaction was stopped with 50 µL 1 M H₂SO₄, and absorbance was measured at 450 nm (SpectraMax M3) within 5 min.

Pseudovirus neutralization assay

Generation of a rhabdoviral pseudotype expressing the Wuhan variant of the SARS-CoV-2 Spike protein was performed as described previously (23, 24). For pseudovirus neutralization, serum samples and controls were heat-inactivated for 30 min. at 56°C. Serum samples were two-fold serially diluted starting at 1:10 in cell culture medium (DMEM (Gibco), 5% FCS (Greiner), 2 mM L-glutamine (Gibco) and 100 IU/mL Penicillin/Streptomycin (Gibco)) and for 1 h at 37°C incubated with 500 FFU/well Spike protein-expressing viral particles. Next, the sample-virus mixture was transferred to VeroE6 cells at 100% confluence, that were seeded the day before. After 24 h incubation at 37°C, infected cells were visualized using an IncuCyte S3 (Sartorius). Fluorescent foci were analyzed using the IncuCyte GUI software (version 2020B) and the 50% pseudovirus neutralization titer (pVNT50) was calculated using a variable slope, four parameter regression analysis in Graphpad Prism (version 8). In the case of undetectable neutralization at an initial serum dilution of 1:10, the pVNT50 values were arbitrarily set to 20. Experiments were performed with internal standard controls, negative controls and virus-only controls to assess the nominal virus input for every single measurement.

Plaque assay

Viral titers were determined by plaque assay on VeroE6 cells. Homogenized lung samples were serially diluted (10^{-1} - 10^{-6}) in minimal essential medium (MEM, Gibco) supplemented with 2% FCS, penicillin,

streptomycin, and L-glutamine. After 1 h incubation at 37°C, the inoculum was replaced with a semi-solid overlay (DMEM containing 2.5% Avicell) and cells were incubated for 3 days at 37°C with CO₂. Next, cells were fixed with 3.7% PFA and stained with crystal violet. Plaques were counted per dilution to calculate PFU per gram of lung tissue.

Statistical analysis

To determine statistical significance, unpaired Student's t-test (two groups) or one-way ANOVA (three or more groups) and Sidak's post hoc test was performed to correct for multiple comparisons. Difference in survival was tested with the log-rank (Mantel-Cox) survival test. All P values were two sided, and $P < 0.05$ was considered statistically significant. GraphPad Prism 9 software (GraphPad Software, La Jolla, CA, USA) was used for statistical analyses.

Results

Synthetic long peptide design and selection

The design of the Corona-SLP vaccine is based on conserved amino acid sequences of the S, M and N proteins of SARS-CoV-2. These conserved regions contain several of the most dominant T cell epitopes identified in humans (25). An in-house proprietary immunological *in silico* prediction tool, named ISABELLA, was applied to identify strongly immunogenic regions for the most frequent HLA types. In addition, several SLPs were designed containing SARS-CoV derived T cell epitopes obtained by positive T cell or MHC binding assays, conserved for SARS-CoV and SARS-CoV-2 (26).

Next, an in-house proprietary manufacturability prediction tool, named MONALISA, was applied to select 45 SLPs with high expected manufacturability. After synthesis, 21 SLPs were further selected based on actual manufacturing feasibility. Of these 21 SLPs, SLP-19 was specifically designed by elongation at the C-terminus by one amino acid compared to SLP-18, and predicted to have 2.8-fold higher immunogenicity. In the final selection for the Corona-SLP SLP-19 replaced SLP-18. The population coverage (27) of the final Corona-SLP set is 99.915% for HLA-I and 100% for HLA-II epitopes, corresponding to an average of 15.72 HLA-I and 44.885 HLA-II hits in 90% of the European and North American populations (Additional file: Fig. S1 and Table S3). The Corona-SLP design and selection process is illustrated (Fig. 1), and SLP sequences are provided (Additional file: Tables S1 and S2).

Memory T cell responses in SARS-CoV-2 convalescent donors to Corona-SLP

The memory immune response to candidate Corona-SLPs was characterized to guide the final selection of the most immunogenic SLPs. SLP-specific T cell responses in blood from recovered COVID-19 patients and healthy donors were assessed *ex vivo* using peptide pools (Peptivator, Miltenyi Biotec) covering the S, N and M proteins, as well as the 21 candidate SLPs, to inform selection of the final Corona-SLP vaccine composition. PBMCs from 11 recovered COVID-19 patients and 4 healthy donors

were screened for T cell reactivity to candidate SLPs by IFN- γ ELISpot. All convalescent donors showed responses of > 100 Spot Forming Units (SFU)/ 1×10^6 cells to the different Peptivator mixes, except donor 1003 to Peptivator M, demonstrating the presence of SARS-CoV-2-specific memory T cells (Fig. 2A) and confirming prior SARS-CoV-2 infection. The same PBMC samples were subsequently screened for T cell reactivity to candidate SLPs by IFN- γ ELISpot. Positive responses in at least one convalescent donor were observed for 14 of the 21 candidate SLPs (Fig. 2B). SLPs eliciting positive responses in multiple convalescent donors included SLP-13, SLP-14, SLP-16, SLP-19, SLP-20 and SLP-21. These SLPs are derived from the N protein, except for SLP-21, which is derived from M. SLP-14 elicited positive responses in the highest number of convalescent donors (n = 6). SLP-19, which has a 2.8-fold higher predicted immunogenicity than the one-amino acid shorter SLP-18, indeed induced higher IFN- γ T cell responses as measured by ELISpot. Multiplex bead-based cytokine analysis was performed on culture supernatants to assess whether CD4⁺ T cell responses were skewed toward a Th1 or Th2 profile. High levels of IL-1 β and very low levels of IL-4 and IL-5 were produced (Additional file: Fig. S2), indicating induction of a Th1-biased rather than a Th2 response.

To determine the presence of linear B cell epitopes within the candidate SLPs, serum from the same donors was used to assess IgG antibody binding to Corona-SLP candidates using ELISA. No IgG antibodies were detected to SLP-1, SLP-2, SLP-4, SLP-6, SLP-8 and SLP-11. High responses (OD_{450 nm} > 0.5) were observed against SLP-3, SLP-16, SLP-20 and SLP-21 in convalescent donors (Additional file: Fig. S3). However, in healthy donors high responses were observed to SLP-3 and SLP-9. Overall, serum analysis showed that SLP-specific IgG antibody responses were detected for 15 of the 21 candidate SLPs but most responses were low or very low and were also detected in control donors, suggesting the presence of cross-reactive antibodies (Additional file: Fig. S3).

In addition, mononuclear cells (MNC) obtained by apheresis from 6 convalescent donors were screened for T cell reactivity to candidate Corona-SLPs by IFN- γ ELISpot (Fig. 2C). All donors showed responses to at least one SLP. SLP-12, SLP-14 and SLP-16 elicited IFN- γ responses in at least three donors tested (50% of tested donors) and were the most immunogenic. Four MNC samples were further tested in a short restimulation assay followed by intracellular cytokine analysis to delineate CD4⁺ and CD8⁺ T cell responses (Fig. 2D-F). CD8⁺ T cell responses to candidate SLPs were observed in three of four donors, with particularly high IFN- γ CD8⁺ T cell responses to SLP-20 (Donor 001) and SLP-5 and SLP-6 (Donor 002). Although CD4⁺ T cell responses were lower compared to CD8⁺ T cell responses, 16 out of 21 candidate SLPs induced positive CD4⁺ T cell responses. Overall, three of four tested donors showed both CD4⁺ and CD8⁺ memory T cell responses to 76% of the candidate SLPs upon short restimulation.

Furthermore, PBMC samples from ICU patients, during disease or after recovery, were tested for T cell responses to the candidate SLPs (in two peptide pools) and Peptivator mixes for S, N and M following one day of stimulation (Additional file: Fig. S4). IFN- γ and TNF-producing CD4⁺ and CD8⁺ T cell responses to the peptide pools were low, indicating that these patients could benefit from an SLP vaccine capable of inducing robust T cell responses against SARS-CoV-2.

In summary, 21 candidate Corona-SLPs containing conserved CD4⁺ and CD8⁺ epitopes were evaluated for their ability to induce *ex vivo* memory responses in SARS-CoV-2 convalescent donors. A final selection of the 11 most immunogenic SLPs covering the S, N and M SARS-CoV-2 antigens was made for the composition of the Corona-SLP vaccine (see Methods). Peptides were selected based on their capacity to elicit memory T cell responses, with manufacturability also taken into account.

Optimization of vaccine strategy using a murine Corona-SLP analogue

A murine vaccine variant (mCorona-SLP) was designed, consisting of 6 of the 21 candidate human Corona-SLPs, all of which contained predicted C57BL/6 (H-2^b) mouse MHC class I epitopes (Additional file: Table S1) and represented predicted epitopes from all three different SARS-CoV-2 proteins in this mouse strain. Three of these SLPs were also included in the final set of 11 Corona-SLPs for vaccination of humans (bold and italic in Additional file: S2), whereas the other three were present only in the initial candidate selection of 21 SLPs and not in the final human vaccine composition. (italic but not bold in Additional file: Table S1). In mCorona-SLP, these 6 SLPs were combined with 5 SLPs designed around validated murine C57BL/6 epitopes (mSLP1-5, (Additional file: Table S2)). Before investigating the protective capacity of this murine vaccine variant, the vaccination strategy was optimized. Because these vaccine parameters could not all be varied simultaneously, the route and adjuvant comparisons were first performed using the standard vaccination regimen established in our previous studies with Amplivant-conjugated SLP vaccines, i.e. three vaccinations at 2-week intervals with 50 µg per SLP and adjuvant (21). As the current study employed a vaccine consisting of multiple SLPs rather than a single SLP, vaccination interval, SLP dose, and Amplivant dose were subsequently investigated in separate experiments to determine their contribution to vaccine immunogenicity in this setting.

The impact of the vaccine administration route on the immunogenicity was assessed by vaccinating C57BL/6 mice intradermally, subcutaneously or intramuscularly with SLPs combined with Amplivant, a TLR1/2 ligand (Fig. 3A). Vaccination with the 11 SLPs in combination with Amplivant, intradermal administration resulted in high antigen-specific CD8⁺ T cell responses as determined by MHC class I tetramers, comprising 2–6% of the total CD8⁺ T cell population, as exemplified by Spike_{263–270} (AAY)-specific T cells induced by mSLP1 and Spike_{539–546} (VNF)-specific T cells induced by mSLP2. While AAY-specific CD8⁺ T cell responses increased after the first boost and remained high after the second booster, VNF-specific CD8⁺ T cell responses increased after each booster vaccination (Fig. 3B). One week after the third vaccination, splenocytes were obtained for *in vitro* restimulation with individual peptide or peptide pools. Restimulation of splenocytes from intradermally mCorona-SLP vaccinated mice with mSLP1 and mSLP2 resulted in strong IFN-γ-producing CD8⁺ T cells (Additional file: Fig. S5A). The remaining peptides within pool S induced also robust memory CD8⁺ T cell responses after intradermal vaccination. In contrast, mice vaccinated subcutaneously or intramuscularly did not show detectable responses to pool S. Memory CD4⁺ T cell responses were also detected following restimulation, albeit at lower levels (Additional file: Fig. S5B). Taken together, intradermal administration

of mCorona-SLP resulted in superior SARS-CoV-2-specific T cell responses compared with subcutaneous or intramuscular administration.

To investigate the impact of the adjuvant, intradermal vaccination with mCorona-SLP combined with Amplivant was compared with the HPV-16 vaccination strategy (ISA101), in which SLPs emulsified in Montanide are administered subcutaneously (sc) (13). Upon sc administration of mCorona-SLP with Montanide, VNF and AAY-specific CD8⁺ T cell responses in the blood circulation (Fig. 3C) were comparable to those induced by the same vaccine combined with Amplivant and administered intradermally. However, upon *in vitro* restimulation, no CD8⁺ T cell responses to pool S were detected (Fig. 3D), in contrast to vaccination with SLP plus Amplivant (Additional file: Fig. S5A). CD4⁺ T cell responses were also limited (Fig. 3E), although low responses were detected to SLP pool S and pool N. Vaccination with SLPs in the absence of adjuvant failed to induce detectable IFN- γ responses in CD4⁺ T cells and elicited only a minimal CD8⁺ T cell response against mSLP2 following ex vivo restimulation of splenocytes, underscoring the requirement for Amplivant-mediated adjuvant activity for efficient T cell priming (Additional file: Fig. S5C and S5D). Because the highest T cell responses, particularly to pool S, were observed following intradermal vaccination of mCorona-SLP combined with Amplivant, this strategy was selected for further optimization. Montanide was included as a comparator because it has been used in clinical studies with HPV16-SLP vaccines; however, due to its higher viscosity, it is less suitable for intradermal administration than Amplivant.

Next, the immune response induced by mCorona-SLP vaccination (comprising 11 SLPs) was compared with that induced by vaccination with a single SLP (mSLP2 of mCorona-SLP) to investigate potential antigenic competition (Fig. 3F). Vaccination with a single SLP resulted in higher VNF-specific CD8⁺ T cell responses in the circulation as determined by MHC class I tetramers (Fig. 3G). Nonetheless, mCorona-SLP elicited a broader response as represented by measurable AAY-specific CD8⁺ T cell populations (Fig. 3G). Moreover, restimulation of splenocytes followed by intracellular IFN- γ staining recapitulated the findings obtained with MHC class I tetramer analysis (Additional file: Fig. S6A). IFN- γ responses in CD4⁺ T cells from mCorona-SLP-immunized mice following restimulation with pool S peptides were clearly detectable, whereas no such response was observed after immunization with single SLP (Additional file: Fig. S6B). We conclude that although some evidence of antigenic competition is observed upon administration of multiple SLPs, as reflected in the circulating T-cell readout, inclusion of several SLPs increases the overall T cell response compared with vaccination using a single SLP. In addition, the effect of extending the interval between booster vaccinations (3 weeks compared to 2 weeks) was investigated (Fig. 3F). Vaccination at 2-week intervals was more immunogenic than vaccination at 3-week intervals, based on both antigen-specific CD8⁺ T cell frequencies in the circulation and T cell responses in splenocytes upon restimulation (Fig. 3G and Additional file: Fig. S6A).

A dose-finding study was subsequently performed for mCorona-SLP. C57BL/6 mice received three sequential vaccinations of mCorona-SLP (11 SLP, 50 μ g or 10 μ g per SLP) in combination with Amplivant (50 μ g), in a prime-boost-boost regimen with 2-week intervals. In particular the Spike_{539–546} (VNF)-

specific T cells, identified by MHC class I tetramers, were highest in the 50 µg SLP group (Fig. 4A). Also, upon restimulation of splenocytes, antigen-specific CD8⁺ T cell responses were highest in the 50 µg SLP group, whereas CD4⁺ T cell responses were comparable between the different dose groups (Fig. 4B). Similarly, reduction of the Amplivant dose resulted in decreased CD8⁺ T cell responses (Fig. 4C and Additional file: Fig. S7C and S7D). In addition, serum was collected 1 week after the third vaccination to assess induction of SLP-specific IgG antibodies. Vaccination with 50 µg per SLP induced IgG antibody responses to 7 of the 11 SLPs, whereas vaccination with 10 µg per SLP induced SLP-specific IgG responses to 5 of the 11 SLP (Additional file: Fig. S8A and S8B). However, vaccination with mCorona-SLP or single SLP did not induce antibodies with neutralizing capacity (Additional file: Fig. S8C).

Taken together, vaccination of C57BL/6 mice with mCorona-SLP induced increased CD8⁺ T cell responses and SLP-specific antibody responses when 50 µg SLP was administered in combination with Amplivant, compared with 10 µg per SLP. Similarly, vaccination with 50 µg Amplivant resulted in higher T cell responses than vaccination with 10 µg Amplivant. Moreover, 2-week intervals between sequential vaccinations yielded the highest overall immune responses. For induction of IFN- γ -producing CD4⁺ T cell responses, either 2 or 3-week intervals and a wider range of SLP doses can be used. Vaccination with a single SLP elicited CD8⁺ T cell responses comparable to those by the full 11-SLP formulation, indicating that antigenic competition does not substantially limit SLP-specific CD8⁺ T cell responses.

Overall, the Corona-SLP vaccination strategy was optimized with respect to adjuvant and administration route, SLP dose, Amplivant dose, and vaccination interval. This led to the current vaccination strategy of intradermal vaccination at 2-week intervals with 50 µg per SLP of the 11 Corona-SLPs, in combination with 50 µg Amplivant. Collectively, these optimization experiments showed that the vaccination parameters previously defined for single-SLP vaccination remained suitable in the context of a multi-SLP vaccine formulation.

Murine Corona-SLP vaccine provides protection against SARS-CoV-2 infection

We next investigated the *in vivo* protective capacity of the Corona-SLP vaccine in two different mouse models. K18-hACE2 transgenic mice express human angiotensin-converting enzyme 2 (hACE2), the receptor for SARS-CoV2, under the murine K18 promoter, resulting in high receptor expression in epithelial cells, including the lungs, but also in the central nervous system, which renders these mice highly susceptible to SARS-CoV-2 infection. In contrast, hACE2 knock-in mice, in which murine ACE2 is replaced with hACE2, show lower susceptibility to SARS-CoV-2 infection.

K18-hACE2 transgenic mice were prophylactically vaccinated with mCorona-SLP or a single SLP (mSLP2 from mCorona-SLP) adjuvanted with Amplivant, and protection against SARS-CoV-2 infection was assessed (Fig. 5A). On day 48, mice received 1×10^4 PFU (K18-hACE2 transgenic mice) or 1×10^5 PFU (hACE2 knock-in mice) of the SARS-CoV-2 Leiden-08 isolate intranasally. The weight of K18-hACE2 transgenic mice was monitored daily until day 62, and animals were observed daily for clinical signs, with

sacrifice performed if humane endpoints were reached. Upon vaccination of K18-hACE2 mice, VNF-specific CD8⁺ T cell responses induced by mSLP2, present in both single SLP and mCorona-SLP vaccines, were higher following single SLP vaccination compared to mCorona-SLP (Fig. 5B), and similar to the responses observed in C57BL/6 mice (Fig. 4). However, mCorona-SLP induced a broader response, as exemplified by clearly detectable AAY-specific CD8⁺ T cells. Sequential vaccination with mCorona-SLP in combination with Amplivant resulted in protection against SARS-CoV-2 infection in K18-hACE2 transgenic mice, as shown by minimal weight loss (Fig. 5C, Fig. 5D), lower clinical scores (Fig. 5E), and improved survival (Fig. 5F). In contrast, mice vaccinated with single SLP (mSLP2 also present in mCorona-SLP) in combination with Amplivant were not protected against SARS-CoV-2 infection.

In hACE2 knock-in mice, T cell responses following a similar vaccination schedule and challenge (Fig. 5G), were comparable to those observed in K18-hACE2 transgenic mice (Fig. 5H). Viral load in the lungs of hACE2 knock-in mice harvested four days after virus inoculation was significantly reduced in mice vaccinated with mCorona-SLP compared with unvaccinated controls, whereas vaccination with a single SLP induced only a modest, non-significant reduction (Fig. 5I). This decrease was accompanied by reduced viral infectivity in the lungs, as determined by plaque assay (Fig. 5J).

In conclusion, intradermal administration of mCorona-SLP three times at 2-week intervals in combination with Amplivant provided protection against SARS-CoV-2 infection in both K18-hACE2 transgenic mice and hACE-2 knock-in mice, as shown by their improved survival and reduced lung viral load, respectively.

Impact of SARS-CoV-2 variants on Corona-SLP epitopes

The Corona-SLP vaccine is directed against conserved regions of S, N and M proteins of SARS-CoV-2. However, emerging new SARS-CoV-2 variants may potentially impact Corona-SLP epitopes and thereby vaccine immunogenicity. Five variants of concern (VOCs) were assessed for their impact on Corona-SLP epitopes: B.1.1.7 (Alpha), B.1.351 (Beta), B.1.617.1 (Delta), P.1 (Gamma), and B.1.1.529 (Omicron; dominant worldwide). The potential impact of each variant on Corona-SLP epitopes was assessed by removing all epitopes and predicted binders from the wild-type dataset that are affected by one or more mutations in a given variant, and subsequently calculating the remaining number of epitopes and predicted binders. The assumption made here is that mutations in these peptide sequences contribute to escaping immune recognition while not introducing new epitopes, as mutations that reduce immune escape would be counter-selected before becoming fixed within a variant. The effect of each VOC on the final Corona-SLP selection is summarized (Additional file: Table S4). Only one SLP in the final set was affected by mutations in variants B.1.1.351 (SLP-2), P.1 (SLP-12) and B.1.1.529 (SLP-8). For B.1.1.529 (Omicron), this is depicted in Fig. 6, and for the other VOCs in Additional file: Fig. S9 (Alpha), S10 (Beta), S11 (Delta), and S12 (Gamma). For variants B.1.1.7 (Alpha) and B.1.617.1 (Delta), two SLPs were affected (SLP-8 and SLP-16, and SLP-5 and SLP-6, respectively). Although some affected SLPs showed a substantial reduction in putative epitopes, most SLPs in the final selection remained intact across all variants analyzed. Overall, the analyzed SARS-CoV-2 variants have a limited impact on the putative

targets of the Corona-SLP vaccine, as only one or two SLPs per lineage were affected, with minimal effect on overall epitope content. These findings indicate that the vaccine is expected to retain its immunogenicity across variants of concern.

Discussion

The Corona-SLP vaccine against SARS-CoV-2 was designed to target multiple conserved HLA class I and II epitopes in the S, N and M proteins of the virus. Multiple rounds of selection of individual SLPs involved prediction of processing, binding and stability of peptide binding to common HLA class I and II molecules, followed by *ex vivo* reactivation of memory T cells in PBMCs of convalescent donors, recently exposed to SARS-CoV-2. The selected Corona-SLPs elicited strong reactivation of memory CD8⁺ T cell responses in PBMCs from these individuals. Thus, *in silico*-predicted immunogenicity was validated using *ex vivo* assays in human PBMCs to guide selection of the most immunogenic SLPs. In addition to SARS-CoV-2 convalescent donors, candidate Corona-SLPs were tested using PBMCs from (recovered) ICU patients. Overall, PBMCs from ICU patients showed low IFN- γ and TNF-producing CD4⁺ and CD8⁺ T cell responses to the peptide pools both during disease and after recovery, indicating that these patients could benefit from an SLP vaccine capable of inducing T cell responses against SARS-CoV-2.

It is well understood that both humoral and cellular immunity plays a central role in the control of SARS-CoV-2 (1, 2). Neutralizing antibodies against conformational B cell epitopes of the Spike protein are a major component of effective protection (1). However, antigenic drift in B cell epitopes of SARS-CoV-2 can reduce antibody-mediated immunity and may require periodic vaccine updates. Moreover, antibody responses, but not cellular responses to SARS-CoV-2 vaccination are impaired by systemic B cell-depleting therapy in patients with B cell malignancies (28), providing opportunities to stimulate T cell responses to prevent (severe) COVID-19. Broad vaccination strategies aimed at inducing T cell responses may therefore be particularly protective in patients with hereditary or acquired B cell deficiencies. In patients lacking B cells due to treatment with rituximab or primary immunodeficiency, enhanced T cell reactivity and proliferative capacity have been observed after both SARS-CoV-2 infection and vaccination (29). Recently, Heitmann et al. showed that in patients with low or absent B cell numbers, a peptide vaccine, comprising six HLA-DR-restricted SARS-CoV-2 peptides induced more robust and durable T cell responses than standard mRNA preventive vaccines (4). Our results in mouse models demonstrate that such T cell responses directed against both MHC class I and II epitopes can provide protection in the absence of neutralizing antibodies against a lethal dose of SARS-CoV-2.

Therapeutic vaccination may also boost existing T cell responses in patients with reduced CD8⁺ T cell activation following SARS-CoV-2 infection (30). In addition, the Corona-SLP vaccine elicits T cell responses not only to S protein but also to N and M proteins, thereby inducing a broader response compared with Spike-targeting mRNA vaccines. Furthermore, in contrast to short peptide vaccines, the SLP approach is HLA agnostic, as it includes HLA class I and II epitopes for multiple alleles resulting in high population coverage. Consistent with this rationale, our *in silico* analysis indicated that the conserved S, N and M regions targeted by Corona-SLP are largely preserved across the major variants of

concern, with only one or two peptides affected per lineage. As this assessment is based on epitope prediction rather than experimental re-challenge with variant viruses, the breadth of cross-variant protection remains to be confirmed *in vivo*; nonetheless, focusing on conserved regions offers a plausible route to variant-resilient T cell immunity.

The vaccination strategy was optimized using a murine vaccine homologue (mCorona-SLP), consisting of a selection of human SLPs immunogenic in mice and SLPs designed for C57BL/6 mice. Of the immunogenicity studies performed here, intradermal vaccination in combination with Amplivant resulted in the highest and broadest CD4⁺ and CD8⁺ T cell response. Vaccination according to this strategy with mCorona-SLP provided protection against SARS-CoV-2 challenge, whereas vaccination with a single SLP did not. Although SLP-specific IgG was induced upon mCorona-SLP vaccination, these antibodies showed no virus neutralizing activity. Thus, protection against SARS-CoV-2 infection by prophylactic mCorona-SLP vaccination was largely mediated by vaccine-induced T cell responses. Nevertheless, the mCorona-SLP vaccine contains several peptides that also include B cell epitopes, and the presence of SLP-specific IgG in serum of vaccinated mice could have contributed to the protection. Non-neutralizing antibodies can exhibit protective capacity in viral infections (31) and act through distinct mechanisms (32) to further enhance immune responses, including T cell responses. In line with this, previous data from Arieta et al., suggests that a combination of T cell and antibody inducing vaccines enhances protection (33). Further mechanistic studies, could provide additional insight into the immune cell populations essential for protection. In this regard, previous data showed that a single SLP containing only one CD8 epitope adjuvanted with CpG was able to provide protection against SARS-CoV-2 without the help of antibodies (21), which may be related to higher vaccine-elicited responses.

Our proprietary adjuvant Amplivant is a synthetic TLR1/2 ligand modified to induce strong dendritic cell maturation and *in vivo* T cell priming (34, 35). Amplivant-conjugated SLPs induced robust HPV16-specific T cell immunity in patients previously treated for HPV16 positive (pre-)malignancies (10). Recently, mucosal TLR2-activating protein-based vaccination, in contrast to parenteral vaccination, induced potent pulmonary immunity and protection against SARS-CoV-2 in mice (36). TLR2 expression by respiratory epithelia and immune cells facilitated early innate responses to mucosal vaccination, whereas TLR2 on hematopoietic cells was essential for optimal lung-localized, antigen-specific tissue-resident memory T cell responses, as shown with TLR2-deficient chimeric mice. Whether mucosal SLP vaccination in combination with Amplivant could further enhance responses remains to be explored. To date, the approved mRNA vaccinations only led to limited induction of lung-resident memory T cell responses against SARS-CoV-2 (37).

Conclusions

Taken together, our findings demonstrate that the Corona-SLP vaccine elicits robust T cell responses and reactivates memory T cell responses *ex vivo* in PBMCs from convalescent donors recovered from SARS-CoV-2 infection. In addition, the Amplivant-adjuvanted mCorona-SLP vaccine is efficacious in two independent *in vivo* models of SARS-CoV-2 infection, while emerging VOCs had little impact on its

predicted immunogenicity. Therefore, this vaccine warrants further development to provide better protection against SARS-CoV-2-induced disease, particularly in individuals with spontaneous or therapy-induced B cell deficiencies, by inducing protective anti-viral T cell immunity.

Abbreviations

ACE2	Angiotensin-converting enzyme 2
COVID-19	Coronavirus disease 2019
E	Envelope protein
HLA	Human leukocyte antigen
HPV	Human papilloma virus
ICU	Intensive Care Unit
M	Membrane protein
mCorona-SLP	murine variant of Corona-SLP
MNC	Mononuclear Cell
N	Nucleocapsid protein
PBMC	Peripheral Blood Mononuclear Cell
PFU	Plaque Forming Unit
pVNT50	50% pseudovirus neutralization titer
RdRp	RNA-dependent RNA polymerase
S	Spike glycoprotein
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SFU	Spot Forming Unit
SLP	Synthetic Long Peptide
VOC	Variants of concern

Declarations

Acknowledgements

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Authors' contributions

Conceptualization: EG, IP, RA, CM, AW. Methodology: EG, IP, SD, PR, DV, FG, IK, AK, JP, ML, JH, SM, TB. Analysis and Visualization: EG, IP. Investigation: EG, IP, SD, DV, FG, KK, ML, JH, SM. Writing—Original draft: EG, IP, RA, CM, AW; Writing—Review & Editing: TB, RA, CM, AW. Supervision: RA, CM, AW. Funding Acquisition: RA, CM.

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Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files. Raw data are available from the corresponding authors upon reasonable request.

Ethics approval and consent to participate

Ethical approval for the samples from COVID-19 patients from trial (NL8589) was granted by the Medical Ethical Committee Leiden-Den Haag-Delft (NL74814.058.20). Ethical approval for the Animal experiments were granted by the local and national committees under the permit numbers AVD11600202417987 and AVD1160020186804, and performed according to the recommendations and guidelines set by the Leiden University Medical Centre (LUMC) and by the Dutch Act on Animal Experimentation and EU Directive 2010/63/EU.

Consent for publication

Not applicable.

Competing interests

EG, PR, WK, LH, TB, CM, AW were salaried employees of ISA Pharmaceuticals, a biotech company aiming at the development of therapeutic synthetic long peptide vaccines against cancer and chronic infection

including SARS-CoV-2, when the study was performed. CM, TB, PR, AW are inventors on patent application [US20230143215A1/ EP4138876A1]/Immunization against sars-cov-related diseases] linked to the technology described in this manuscript.

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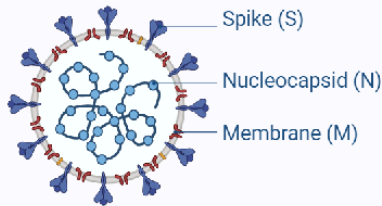
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Figures

Design

Target selection

Spike, Nucleocapsid and Membrane proteins of SARS-CoV-2



Conserved regions

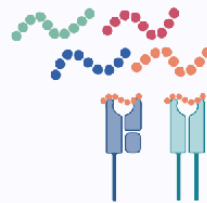


SLP Design

ISABELLA: HLA ligand prediction

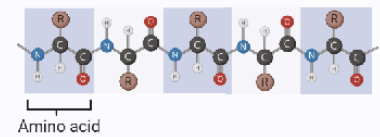
NetMHCpan *in silico* binding predictions

MHC class I and II presentation



SLP Synthesis

MONALISA: prediction of manufacturability

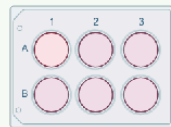
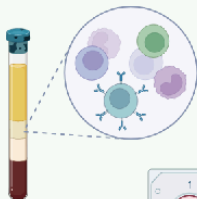


Biological activity

Ex vivo experiments

PBMCs convalescent donors

PBMCs ICU patients



In vivo experiments

Murine analogue of Corona-SLP comprising C57BL/6 epitopes

Adjuvant selection
Administration route
Dose-Findings

Protection against SARS-COV-2 infection



Number of SLPs

Design and Manufacture

45

Convalescents

21

ICU patients

21

Corona-SLP vaccine

11

Figure 1

Design and selection strategy of the Corona-SLP vaccine. Corona-SLP comprises multiple synthetic long peptides derived from the S, N, and M proteins of SARS-CoV-2. Previously reported T-cell epitopes and *in silico* prediction tools were used to identify immunogenic regions. Candidate peptides were subsequently evaluated using the MONALISA manufacturability prediction tool, resulting in the selection of 45 SLPs with favorable synthesis characteristics. Based on synthesis outcomes, 21 peptides were selected for biological evaluation using PBMCs from convalescent donors and ICU patients. In parallel, a

murine analogue of the vaccine was designed and evaluated in vivo to optimize the vaccination strategy and assess protection against SARS-CoV-2 infection. Created with BioRender.com.

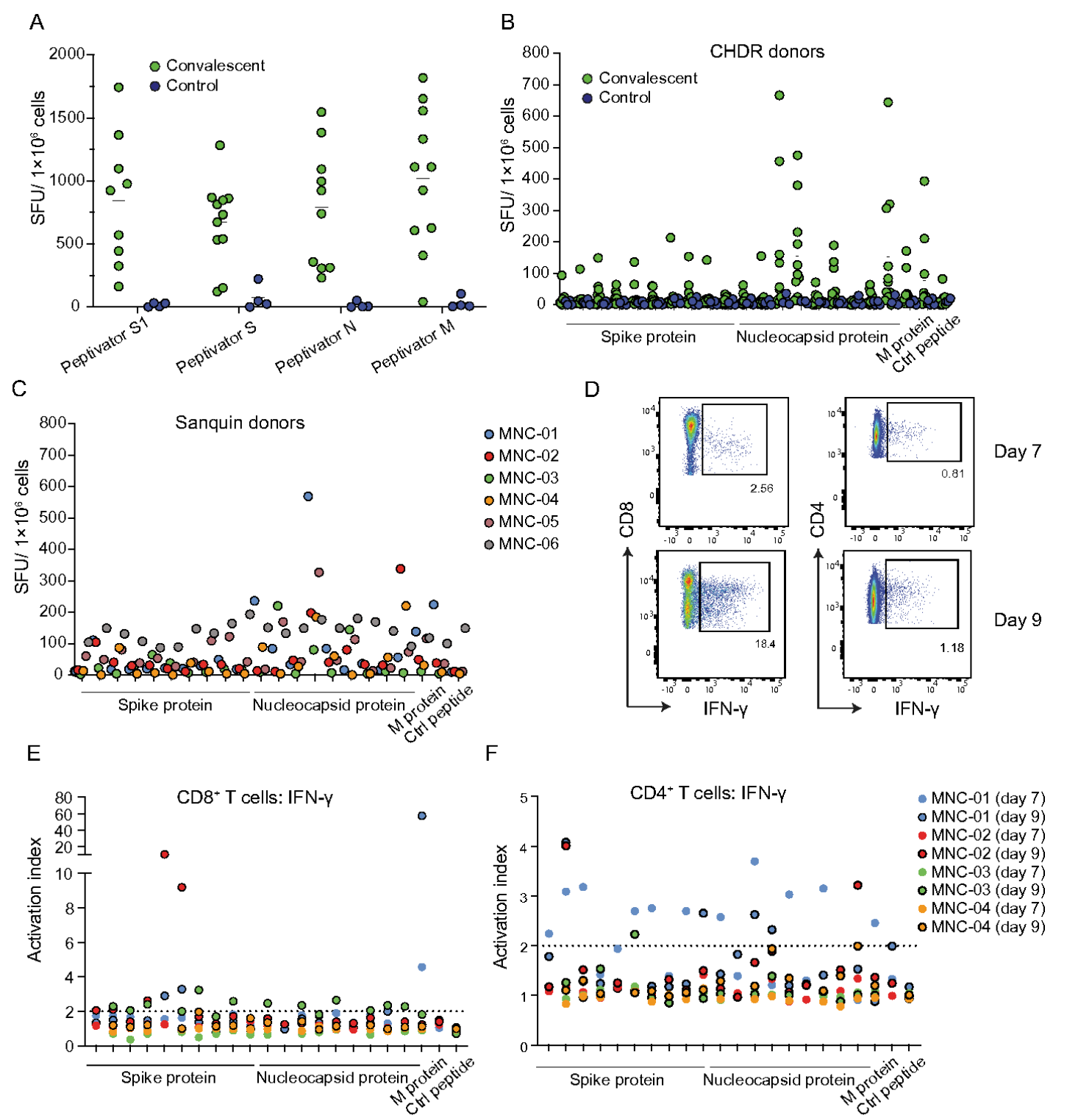


Figure 2

Corona-SLPs reactivate memory T-cell responses in PBMCs from convalescent donors. (A) IFN-γ ELISpot responses following restimulation of PBMCs from 11 convalescent donors and 4 healthy

controls with overlapping 15-mer peptide pools. (B) IFN- γ ELISpot responses following restimulation of PBMCs from 11 convalescent donors and 4 healthy controls with 21 candidate Corona-SLPs and a control SLP. (C) IFN- γ ELISpot responses following restimulation of mononuclear cells (MNCs) from 6 convalescent donors with 21 candidate Corona-SLPs and a control SLP. (D) Intracellular IFN- γ production following short-term restimulation of MNCs from 6 convalescent donors. Intracellular cytokine staining was performed on days 7 and 9 after stimulation, with analysis of total CD8⁺ and CD4⁺ T cells. (E, F) Activation index relative to the negative control for CD8⁺ T cells (E) and CD4⁺ T cells (F). A positive response was defined as a frequency of cytokine-producing T cells exceeding twofold the negative-control background, indicated by the dotted line.

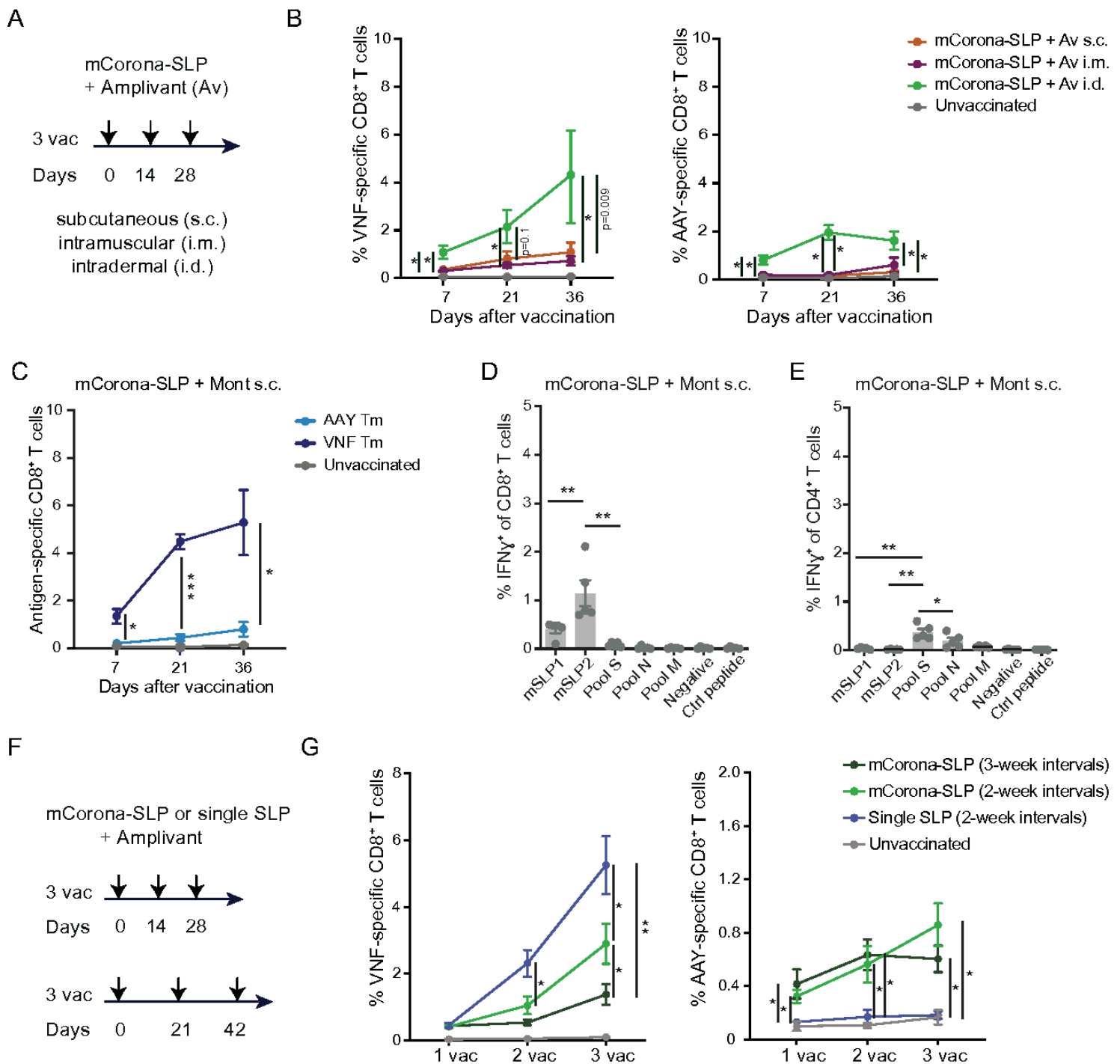


Figure 3

Intradermal administration of mCorona-SLP induces robust antigen-specific CD8⁺ T-cell responses. (A) Vaccination schedule. C57BL/6 mice received three sequential vaccinations in a prime–boost–boost regimen at 2-week intervals with mCorona-SLP (11 SLPs; 50 µg per SLP) combined with Amplivant (50 µg), administered i.d., s.c., or i.m. (B) AAY-specific CD8⁺ T-cell responses to mSLP1 and VNF-specific CD8⁺ T-cell responses to mSLP2 measured in peripheral blood 7 days after each vaccination. (C–E) C57BL/6 mice received three sequential vaccinations in a prime–boost–boost regimen at 2-week

intervals with mCorona-SLP (11 SLPs; 50 µg per SLP) emulsified in Montanide. (C) Antigen-specific CD8⁺ T-cell responses in peripheral blood measured 7 days after each vaccination. (D, E) Splenocytes harvested 7 days after the third vaccination were restimulated in vitro with mSLP1, mSLP2, or peptide pools derived from the S, N, and M proteins. IFN-γ responses of CD8⁺ T cells (D) and CD4⁺ T cells (E) are shown. (F) Vaccination schedule for the comparison of mCorona-SLP (11 SLPs) with a single SLP (mSLP2) and of 2- versus 3-week booster intervals; all groups received 50 µg per SLP combined with 50 µg Amplivant administered intradermally. (G) Antigen-specific CD8⁺ T-cell responses in peripheral blood measured 7 days after each vaccination with MHC class I tetramers. Dots represent individual mice. Data are presented as mean ± SEM. Statistical significance was assessed using one-way ANOVA with Šidák's multiple-comparison test (B, D, G) or Student's t-test (C). *P < 0.05; **P < 0.01.

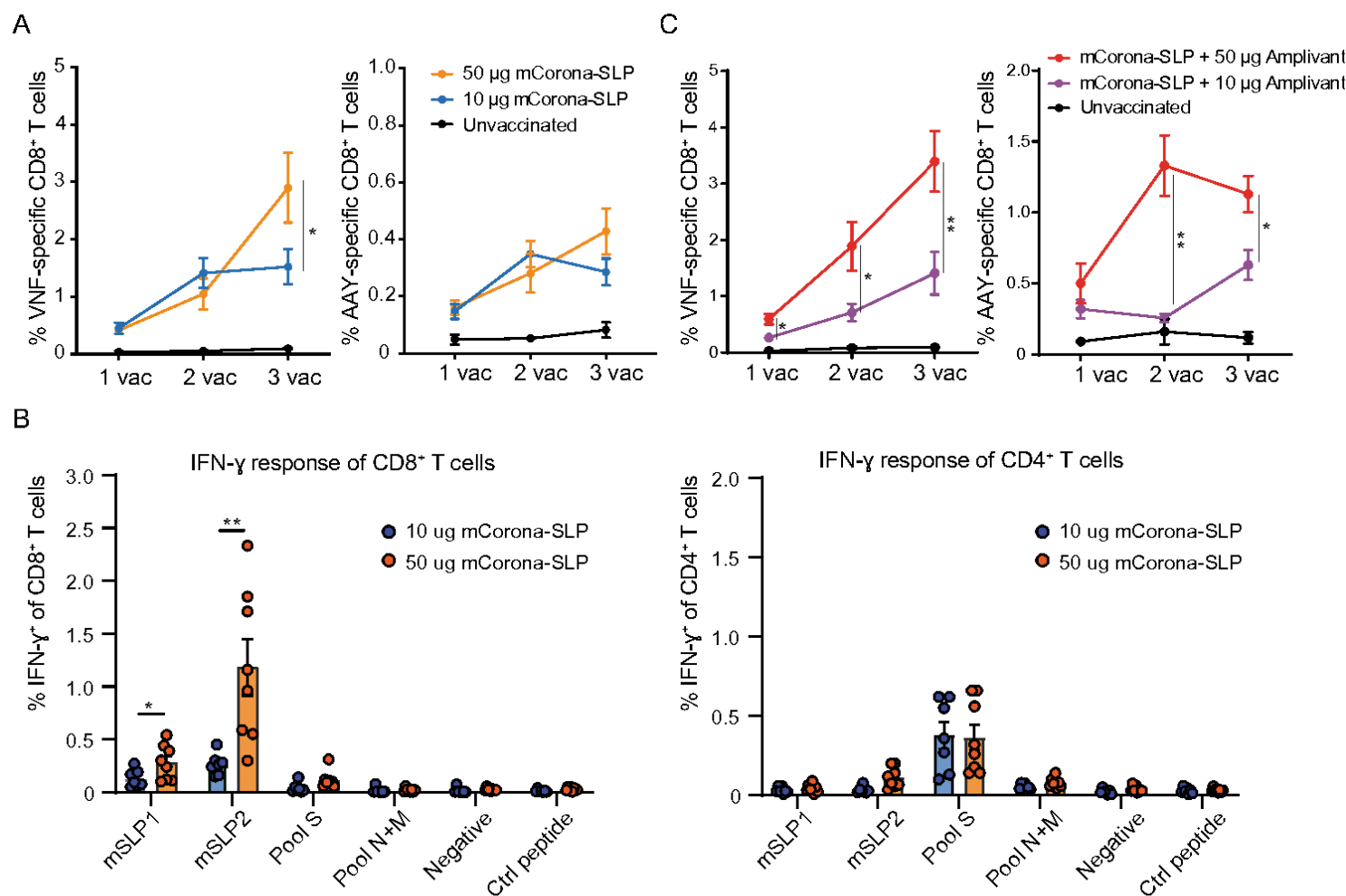


Figure 4

Optimization of mCorona-SLP vaccination parameters. (A) C57BL/6 mice received three sequential vaccinations in a prime–boost–boost regimen at 2-week intervals with mCorona-SLP (11 SLPs; 50 µg or 10 µg per SLP) combined with Amplivant (50 µg). Antigen-specific CD8⁺ T-cell responses measured with MHC class I tetramers are shown. (B) Splenocytes harvested 7 days after the third vaccination were

restimulated in vitro with mSLP1, mSLP2, or peptide pools derived from the S, N, and M proteins. IFN- γ responses of CD8 $^{+}$ T cells (left graph) and CD4 $^{+}$ T cells (right graph) are shown. (C) C57BL/6 mice received three sequential vaccinations at 2-week intervals with mCorona-SLP (11 SLPs; 50 μ g per SLP) combined with either 50 μ g or 10 μ g Amplivant. Antigen-specific CD8 $^{+}$ T-cell responses are shown. Dots represent individual mice. Data are presented as mean $\pm$ SEM. Statistical significance was assessed using Student's t-test. *P < 0.05; **P < 0.01.

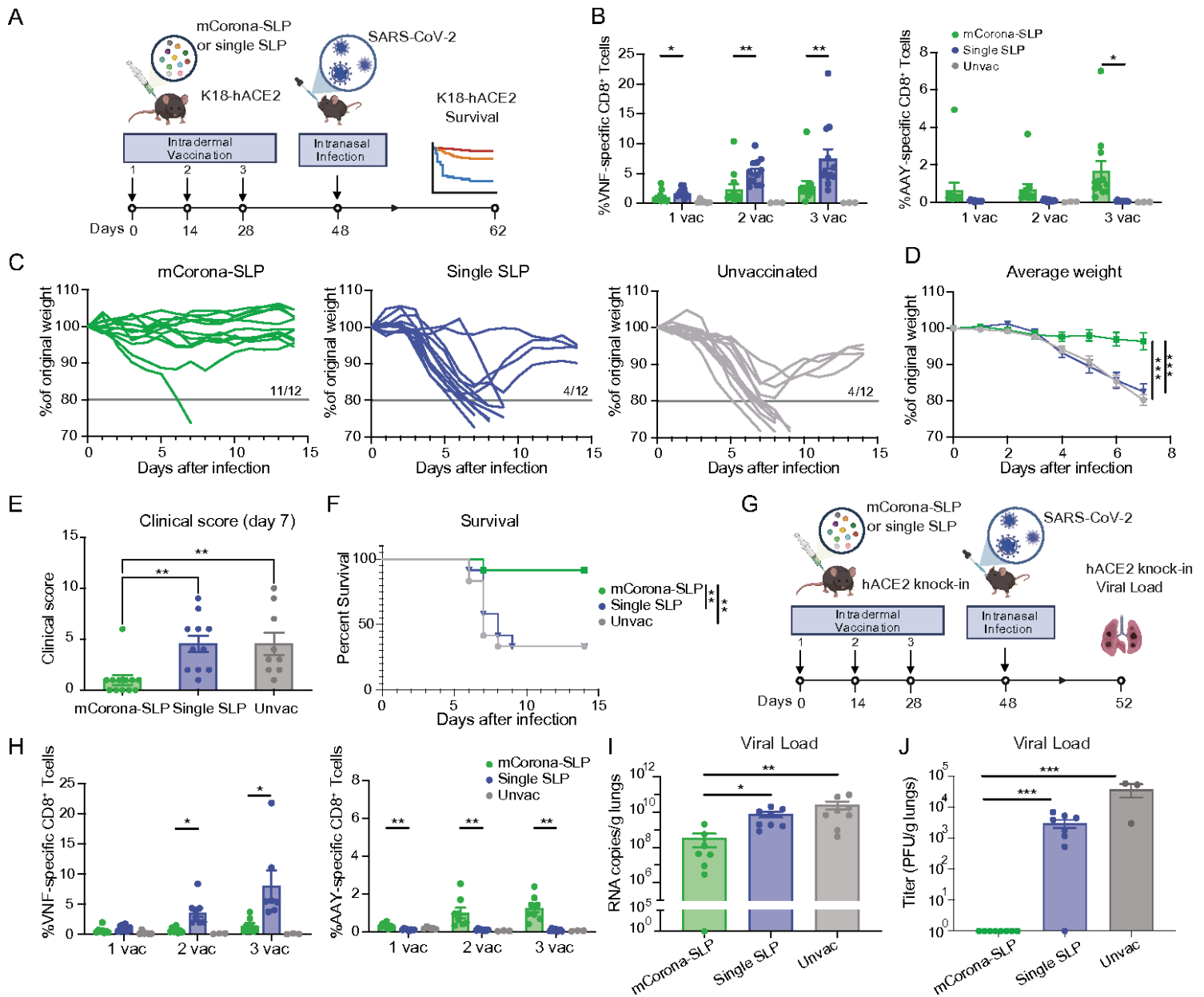


Figure 5

mCorona-SLP protects against SARS-CoV-2 challenge in mice. (A) K18-hACE2 mice (B-F) were vaccinated three times at 2-week intervals with mCorona-SLP or a single SLP (mSLP2). On day 48 after the first vaccination, mice were challenged intranasally with the SARS-CoV-2 Leiden-08 isolate. Created with BioRender.com. (B) AAY-specific CD8 $^{+}$ T-cell responses to mSLP1 and VNF-specific CD8 $^{+}$ T-cell

responses to mSLP2 measured 7 days after each vaccination. (C) Body weight of individual mice following SARS-CoV-2 challenge. (D) Mean body weight per group following challenge. (E) Clinical scores at day 7 after infection. Clinical scoring included activity, behavior, fur appearance, posture, and body weight. (F) Survival following SARS-CoV-2 challenge. (G) hACE2 knock-in mice (H-J) were vaccinated and challenged according to the same schedule described in (A). Created with BioRender.com. (H) Antigen-specific CD8⁺ T-cell responses measured 7 days after each vaccination. (I) Viral load in lung tissue determined by quantitative PCR. (J) Viral load in lung tissue determined by plaque assay. Symbols in (B), (E), and (H–J) represent individual mice. Data are presented as mean ± SEM. Statistical significance was assessed using one-way ANOVA with Šidák’s multiple-comparison test. *P < 0.05; **P < 0.01; ***P < 0.001.

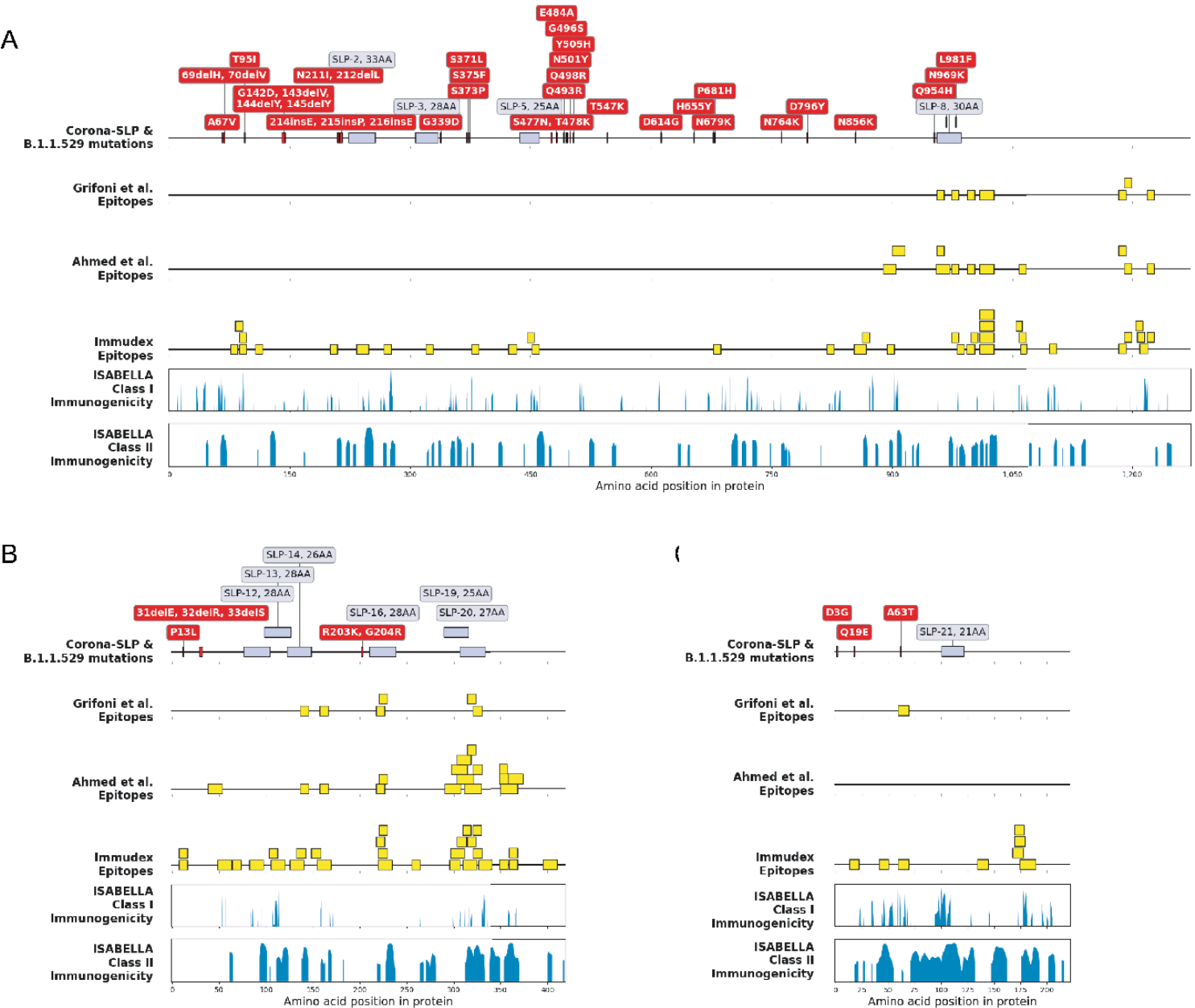


Figure 6

Alignment of mutations of Omicron VOC with Corona-SLPs to S, N and M proteins. (A–C) Overview of the S (A), N (B), and M (C) proteins showing the positions of Corona-SLPs (blue), B.1.1.529 (Omicron variant of concern) mutations (red), reported epitopes (yellow), and cumulative ISABELLA scores across the wild-type protein sequence (bottom panels). The alignment illustrates the overlap between these features and demonstrates that most Corona-SLPs remain unaffected by Omicron-associated mutations while retaining substantial predicted immunogenicity. Images were generated using the matplotlib and dna_features_viewer Python packages.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementarySLPSARSCoV2.docx](#)