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A class II MHC-targeted vaccine elicits immunity against SARS-CoV-2 and its variants

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The pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in over 100 million infections and millions of deaths. Effective vaccines remain the best hope of curtailing SARS-CoV-2 transmission, morbidity, and mortality. The vaccines in current use require cold storage and sophisticated manufacturing capacity, which complicates their distribution, especially in less developed countries. We report the development of a candidate SARS-CoV-2 vaccine that is purely protein based and directly targets antigen-presenting cells. It consists of the SARS-CoV-2 Spike receptor-binding domain (SpikeRBD) fused to an alpaca-derived nanobody that recognizes class II major histocompatibility complex antigens (VHHMHCII). This vaccine elicits robust humoral and cellular immunity against SARS-CoV-2 and its variants. Both young and aged mice immunized with two doses of VHHMHCII-SpikeRBD elicit high-titer binding and neutralizing antibodies. Immunization also induces strong cellular immunity, including a robust CD8 T cell response. VHHMHCII-SpikeRBD is stable for at least 7 d at room temperature and can be lyophilized without loss of efficacy.

**Significance**

Vaccines remain the best hope of curtailing SARS-CoV-2 transmission, morbidity, and mortality. Currently available vaccines require cold storage and sophisticated manufacturing capacity, complicating their distribution, especially in less developed countries. We report a protein-based SARS-CoV-2 vaccine that directly and specifically targets antigen-presenting cells. It consists of the SARS-CoV-2 Spike receptor-binding domain (SpikeRBD) fused to a nanobody that recognizes class II major histocompatibility complex antigens (VHHMHCII). Our vaccine elicits robust humoral (high-titer binding and neutralizing antibodies) and cellular immunity against SARS-CoV-2 and its variants in both young and aged mice. VHHMHCII-SpikeRBD is stable for at least 7 d at room temperature and can be lyophilized without loss of efficacy, desirable attributes for logistical reasons.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, has caused a global pandemic, infecting over 230 million people, and leading to millions of deaths (1). Rapid distribution of effective vaccines on a global scale is the most effective means of mitigating the political, social, and economic destabilization caused by the SARS-CoV-2 pandemic.

The SARS-CoV-2 spike (S) protein is a trimeric transmembrane protein that binds to the cell surface receptor angiotensin-converting enzyme 2 (ACE2) via its receptor-binding domain (RBD) and mediates fusion with host membranes (2). SARS-CoV-2 S is the primary target for neutralizing antibodies and elicits both CD4 and CD8 T cell responses during infection (3–7). Most vaccines in current use or in development target S, or fragments of S, as the primary antigen (8). Because several variants of concern have emerged, many of which contain mutations in S that partially resist neutralization by vaccine-elicted and COVID-19–elicted antibodies, vaccines that offer protection against new variants are necessary (9–11).

Leading vaccine candidates use an array of diverse vaccine platforms. These include inactivated virions, DNA-based vaccines, recombinant subunit preparations, lipid-encapsulated mRNA formulations, as well as live-attenuated, replication-incompetent viral vectored, and replication-competent viral vectored vaccines (8). None of them directly and specifically target antigen-presenting cells (APCs). We hypothesized that targeted delivery of antigen to professional class II MHC\textsuperscript{+} APCs would improve access to the processing and presentation pathways that generate CD4 and CD8 T cell responses, in addition to provoking a robust antibody response. Our earlier efforts to generate an anti-HPV16 CD8 T cell response relied on fusions of an anti-CD11b nanobody to the immunodominant epitope of the HPV16 E7 protein as a vaccine. Its success in eradicating established tumors inspired us to pursue a similar effort to deliver the RBD of the SARS-CoV-2 S protein as a fusion with a nanobody that targets APCs (12). Most vaccines in current use require specialized storage conditions.
(13–15). The development of vaccines with enhanced stability to allow storage at ambient temperature and rapidly adjustable to emerging variants of the virus therefore remains a priority. Moreover, vaccines that can be produced rapidly in a scalable manufacturing process would improve access.

Here we report the development of a recombinant protein vaccine that consists of the SARS-CoV-2 Spike RBD (SpikeRBD) fused to an alpaca-derived nanobody that targets class II major histocompatibility (MHC II) complex antigens (VHHMHCII-SpikeRBD). This vaccine delivers the antigen directly to class II MHC+ APCs. Immunization of both young and aged mice with two doses of VHHMHCII-SpikeRBD resulted in robust binding and neutralizing antibody responses against SARS-CoV-2 and emerging variants. Immunization also induced prominent CD8 T cell responses against conserved SpikeRBD-derived epitopes. VHHMHCII-SpikeRBD can be produced in high yield in mammalian cells and tolerates both storage at room temperature for at least 7 d and lyophilization without loss of efficacy.

Results

VHHMHCII-SpikeRBD Elicits High-Titer Anti-SpikeRBD and Neutralizing Antibodies in Mice. We have characterized a single-domain antibody fragment that binds class II MHC antigens (VHHMHCII) with nanomolar affinity. Immunization of mice with an influenza A virus (IAV) H2A antigen, conjugated to VHHMHCII-protected mice against a lethal IAV challenge (16). To apply this vaccine platform to SARS-CoV-2, we generated a recombinant protein that consists of a fusion between VHHMHCII and the SARS-CoV-2 RBD (Fig. 1A). VHHMHCII-SpikeRBD was expressed in Exp293 cells and purified by means of its C-terminal His6-tag followed by size-exclusion chromatography. The identity of the product was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) (Fig. 1B). A 200-mL culture of Exp293 cell supernatant yielded 20 mg of recombinant protein.

To examine the immunogenicity of VHHMHCII-SpikeRBD, we used a two-dose immunization regimen. Preimmune serum was collected at 17 d and C57BL/6j mice (H-2b haplotype) were primed (day –14) intraperitoneally (i.p.) with adjuvanted (poly dIdC and anti-CD40 monoclonal antibody) VHHMHCII,SpikeRBD (20 μg), with an equimolar amount of adjuvanted SpikeRBD (13.5 μg), or with adjuvant alone. Mice were boosted with each corresponding preparation 14 d postprime (day 0) (Fig. 1C and SI Appendix, Fig. S1A). Serum was collected from all animals 14 d postboost (day 14). IgG titters were determined by ELISA against recombinant SARS-CoV-2 SpikeRBD (Wuhan Hu-1 strain) (Fig. 1D and SI Appendix, Fig. S1B). Immunization with two doses of VHHMHCII-SpikeRBD elicited high levels of anti-SpikeRBD antibodies in all animals, reaching mean endpoint titers in excess of 1/23,600,000, an approximate 34,000-fold increase over mice immunized with two doses of SpikeRBD. Analysis of immunoglobulin subclasses showed evidence of class switching in mice immunized with VHHMHCII-SpikeRBD, judged by the levels of IgA, IgG1, and IgG2b detected at day 14 (Fig. 1E and SI Appendix, Fig. S1C). Mean endpoint titers were higher in mice immunized with VHHMHCII-SpikeRBD than in mice immunized with SpikeRBD, with mean endpoint titers reaching >1/7,300 (IgM; fourfold increase over SpikeRBD), 1/8,900 (IgA; 66-fold increase over SpikeRBD), 1/3,160,000 (IgG1; 6,870-fold increase over SpikeRBD), and >1/90,000 (IgG2b; 87-fold increase over SpikeRBD). Immunization with VHHMHCII-SpikeRBD induced high levels of IgA, which suggests improved mucosal protection in immunized animals. Immunization of mice with two doses of VHHMHCII-SpikeRBD thus induces a robust humoral immune response, significantly higher than that induced by the SpikeRBD alone.

Since VHHMHCII-SpikeRBD binds class II MHC antigens encoded by the I-A locus on target APCs, we immunized a different inbred mouse strain (BALB/c; H-2b haplotype) to confirm efficacy against different MHC haplotypes. Immunization with two doses of VHHMHCII-SpikeRBD led to comparable levels of total IgG in C57BL/6j (H-2b) and BALB/cJ (H-2d) mice (SI Appendix, Fig. S2A and B). Immunoglobulin class switching was evident in both mouse strains, and mean SpikeRBD-specific IgM, IgA, IgG1, and IgG2b titers were comparable (SI Appendix, Fig. S2C).

We next measured total and subclass immunoglobulin levels at days 7, 14, and 21 in mice immunized with one or two doses of adjuvanted VHHMHCII-SpikeRBD or control (SI Appendix, Fig. S3A). Mice immunized with two doses of VHHMHCII-SpikeRBD quickly achieved peak IgG titers on day 7, with levels persisting until at least day 21 (SI Appendix, Fig. S3A), while animals immunized with a single dose produced lower levels of total IgG and other subclass immunoglobulins (SI Appendix, Fig. S3B).

Because SARS-CoV-2 variants with mutations in S that enable partial immune escape have emerged, we next examined whether serum from mice immunized with VHHMHCII-SpikeRBD recognized recombinant SpikeRBD with the K417T, E484K, and N501Y mutations. These mutations are found individually in many S variants and in combination in the P.1 (Gamma or Brazil) variant and partially in the B.1.351 (Beta or South Africa) variant which has K417N instead of K417T (17). Immunization with two doses of adjuvanted wild-type SpikeRBD induced antibodies (day 14) with a low capacity to bind the triple-mutant SpikeRBD, while immunization with two doses of adjuvanted VHHMHCII-SpikeRBD elicited antibodies still capable of recognizing the mutant SpikeRBD to high titters (mean endpoint titer of 1/178,000) (Fig. 1F and SI Appendix, Fig. S1D). Two doses of VHHMHCII-SpikeRBD were required to maintain high titers of IgG against the mutant SpikeRBD (SI Appendix, Fig. S3C).

We next measured the levels of neutralizing antibodies induced by immunization with two doses of adjuvanted VHHMHCII-SpikeRBD by using replication-competent vesicular stomatitis viruses that express eGFP and variants of the SARS-CoV-2 spike (VSV-SARS-CoV-2) in place of their native glycoprotein (18, 19). Mice immunized with two doses of adjuvant failed to neutralize VSV-SARS-CoV-2 expressing the Wuhan Hu-1-D614G spike, as well as those carrying the spikes of B.1.1.7 (Alpha or United Kingdom), P.1, and B.1.351 variants (Fig. 1G) (20). Alternatively, immunization with two doses of adjuvanted VHHMHCII-SpikeRBD induced high-titer neutralizing antibodies against VSV-SARS-CoV-2 expressing the spikes of Wuhan Hu-1-D614G (mean effective concentration, 50% [EC50] titers of 1/2,426), B.1.1.7 (1/937), P.1 (1/250), and B.1.351 (1/488). Sera from mice immunized with two doses of VHHMHCII-SpikeRBD neutralized a clinical isolate of SARS-CoV-2 strain NL/2020 (mean EC50 titers of 1/499), as measured by a quantitative RT-PCR assay (SI Appendix, Fig. S4A). Neutralizing titers were comparable to that of a monoclonal neutralizing antibody, 4D11 (1/640 to 1/1280 dilution range, 0.05 μg/mL to 0.025 μg/mL) (Fig. 1H and SI Appendix, Fig. S4B) (21). While neutralizing titers were impacted by mutations in the RBD of circulating variants, mean EC50 titers remain high. Regardless, high neutralizing titers against the Wuhan Hu-1-D614G and B.1.351 after immunization with a matched RBD suggests that VHHMHCII-SpikeRBD carrying mutations found in the RBDs of circulating variants will significantly improve neutralizing titers against those variants. Other variants remain to be tested.

A Single Dose of VHHMHCII-SpikeRBD Elicits Strong Cellular Immunity. Because cellular immunity, particularly that exerted by T cells,
Fig. 1. Immunization with VHH\textsubscript{MHCII}-Spike\textsubscript{RBD} induces high-titer anti-Spike\textsubscript{RBD} and neutralizing antibodies in mice. (A) Schematic of VHH\textsubscript{MHCII}-Spike\textsubscript{RBD}. The structure shown is a representative example of a VHH. (B) Coomassie-stained SDS/PAGE gel of purified VHH\textsubscript{MHCII}, Spike\textsubscript{RBD}, and VHH\textsubscript{MHCII}-Spike\textsubscript{RBD}. (C) C57BL/6J mice were immunized i.p. with adjuvant only, adjuvanted Spike\textsubscript{RBD}, or adjuvanted VHH\textsubscript{MHCII}-Spike\textsubscript{RBD} on the indicated days. Serum samples were collected as indicated. (D and E) Total IgG (day −13 and day 14), or IgM, IgA, IgG1, and IgG2b (day 14) responses were evaluated from sera of immunized mice (n = 4 to 7 per group) by ELISA against recombinant Spike\textsubscript{RBD}. ELISA data were summarized as endpoint titers and presented as means ± SEM. (F) Humoral responses in sera of immunized mice were evaluated (n = 4 to 7 per group) by ELISA for anti-Spike\textsubscript{RBD} (K417T, E484K, N501Y mutations) IgG. ELISA data were summarized as endpoint titers and presented as means ± SEM. (G) Neutralization data for VSV, pseudotyped with the SARS-CoV-2 Spike glycoprotein Wuhan + D418G and other indicated variants. (H) Neutralization assay against clinical isolates of SARS-CoV-2/NL/2020 strain. All data are presented as means ± SEM; n.s., not significant; *P < 0.05, **P < 0.01, ***P < 0.001, unpaired t test with Holm–Sidak adjustment.
Humanized VHH\textsubscript{MHCIIC}–Spike\textsubscript{RBD} Elicits Both Humoral and Cellular Immunity in a Transgenic Mouse Model. Because the anti-MHC class II nanobody used for these experiments recognizes murine antigens independent of haplotype, we also generated a version of the vaccine that could be applied in a clinical setting. We used I-A\textsuperscript{d}–specific transgenic C57BL/6 mice, which lack wild-type murine MHC class II products and instead express transgenic hybrid MHC class II molecules composed of the peptide-binding portion of human HLA-DR4 and the membrane-proximal domains of mouse I-E (DR4-IE). We used a previously characterized nanobody (VHH\textsubscript{MHCIIC}) that recognizes nearly all allelic variants of human class II MHC molecules (HLA-DR specific, with the exception of HLA-DR-03*01) (VHH\textsubscript{MHCIIC}–Spike\textsubscript{RBD}) (28). Flow cytometry on splenocytes from HLA-DR4-IE–transgenic C57BL/6 IAb\textsuperscript{null} mice confirmed that VHH\textsubscript{MHCIIC} recognizes these hybrid DR4-IE molecules (Fig. 4D). We then generated a genetic fusion between VHH\textsubscript{MHCIIC} and either the Wuhan Hu-1 or B.1.1.7+E484K SARS-CoV-2 Spike\textsubscript{RBD}, as an illustration of rapid and straightforward vaccine adjustment in anticipation of emerging mutations in the RBD. Both constructs expressed well in mammalian cells. A 200-μL culture of Expi293 cell supernatant yielded 15 and 12.5 mg of VHH\textsubscript{MHCIIC}–Spike\textsubscript{RBD} (Wuhan Hu-1) and VHH\textsubscript{MHCIIC}–Spike\textsubscript{RBD} (B.1.1.7+E484K), respectively (Fig. 4B).

To examine the immunogenicity of this vaccine, we immunized HLA-DR4-IE–transgenic C57BL/6 IAb\textsuperscript{null} mice by i.p. administration with two doses of adjuvant only or 20 μg of adjuvanted VHH\textsubscript{MHCIIC}–Spike\textsubscript{RBD} (Wuhan Hu-1 RBD) (Fig. 4C and SI Appendix, Fig. S7A). Immunization with VHH\textsubscript{MHCIIC}–Spike\textsubscript{RBD} elicited high-titer anti-Spike\textsubscript{RBD} antibodies, reaching mean endpoint titers of 1/1,296,724 (total IgG), 1/17,853 (IgM), 1/208 (IgA), 1/349,669 (IgG1), and 1/8,707 (IgG2b) (Fig. 4D and SI Appendix, Fig. S7B and C). Total serum IgG retained the ability to bind mutant, recombinant Spike\textsubscript{RBD} with the K417T, E484K, and N501Y mutations found in the P1 variant at mean endpoint titers of 1/17,853 (Fig. 4E and SI Appendix, Fig. S7D). We also identified three peptides, 47, 48, and 49, that elicited strong IFN\gamma responses in splenocytes after immunization with a single dose (Fig. 4F). Not surprisingly, CD8 T cells are the dominant cell type implicated in IFN\gamma production upon peptide stimulation (Fig. 4G), as the HLA-DR4-IE–transgenic C57BL/6 IAb\textsuperscript{null} mice share the H-2b encoded class I MHC molecules.

**Discussion**

Curtailting the SARS-CoV-2 pandemic will require rapid and widespread distribution of effective vaccines. Here we report the development of VHH\textsubscript{MHCIIC}–Spike\textsubscript{RBD}, a purely...
Fig. 2. Immunization of mice with a single dose of VHHMHCII-SpikeRBD elicits strong cellular immunity. (A) C57BL/6J mice were immunized i.p. with one dose of adjuvanted VHHMHCII-SpikeRBD. Spleens were harvested 7 d postimmunization. (B) Amino acid sequence of the SARS-CoV-2 SpikeRBD. Residues circled in red denote residues in the SpikeRBD that are frequently mutated in circulating variants, including L452, S477, T478, E484, S494, and N501. Orange residues indicate a T cell stimulatory region of SpikeRBD, while magenta residues indicate a second T cell stimulatory peptide region of SpikeRBD. (C) The number of IFNγ-secreting cells in immunized mice was evaluated by ELISpot assays. Numbers on the x axis correspond to specific 15-mer peptides with 11-residue overlaps in the SpikeRBD; # indicates statistical comparison between SpikeRBD + adjuvant vs. VHHMHCII-SpikeRBD + adjuvant cohorts, whereas * indicates statistical comparison between adjuvant only vs. VHHMHCII-SpikeRBD + adjuvant cohorts; ** indicates statistical comparison between adjuvant only vs. VHHMHCII-SpikeRBD + adjuvant cohorts; *** indicates statistical comparison between adjuvant only vs. VHHMHCII-SpikeRBD + adjuvant cohorts, and adjuvant only vs. VHHMHCII-SpikeRBD + adjuvant cohorts, respectively. (D) IFNγ, IL-6, IL-2, and TNFα levels were measured 3 d after stimulating splenocytes with the indicated SpikeRBD peptide. (E and F) Flow cytometry analyses of splenocytes after incubation for 6 h in the presence of pooled peptides (42 and 47 to 50) and monensin. All data are presented as means ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, unpaired t test with Holm–Sidak adjustment. (E and F) Flow cytometry analyses of splenocytes after incubation for 6 h in the presence of pooled peptides (42 and 47 to 50) and monensin. All data are presented as means ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, unpaired t test with Holm–Sidak adjustment.
protein-based SARS-CoV-2 vaccine that specifically targets APCs. This preparation is easy to both produce and store. Immunization of mice with two doses of VHH\textsubscript{MHCI}-Spike\textsubscript{RBD} elicited high-titer binding and neutralizing antibodies against SARS-CoV-2 and several of its circulating variants, including B.1.1.7, P.1, and B.1.351. Strong immune responses were evoked in both young and aged mice, largely independent of the route of administration of the vaccine. A single dose was sufficient to induce cellular immunity to conserved regions of the RBD, as evident from cytokine production by CD8 T cells. The vaccine maintained its potency regardless of storage conditions, including ambient temperature and lyophilization. Humoral and cellular immune responses were both more consistent and potent in mice immunized with VHH\textsubscript{MHCI}-Spike\textsubscript{RBD} compared to immunization with the Spike\textsubscript{RBD}. A version of this vaccine suitable for clinical translation elicits robust immunity in a humanized mouse model. This approach would therefore complement ongoing active and passive immunization strategies.

Most currently used vaccines are difficult to manufacture and/or require specialized storage conditions. Vaccines with enhanced stability that tolerate lyophilization, such as the protein-based vaccine reported here or a different, nanoparticle-based vaccine, allow stockpiling at ambient temperature (29). This is an important attribute for distribution in countries where access to cold storage and/or effective transportation is a challenge. A unique feature of this vaccine approach is its ability to deliver antigen directly to MHC class II\textsuperscript{+} cells. Humoral and cellular immunity directed against the Spike\textsubscript{RBD} develops within a week of the first dose, unlike mRNA-based vaccines and nontargeting protein-based vaccines, which usually take longer (30).

In conclusion, we report a purely protein-based vaccine preparation that is unique, in that it directly targets professional APCs. The robust immunity afforded by this vaccine, combined with its ease of manufacture and stability, indicates the potential for a rapidly adjustable vaccine. Vaccination of mice with the VHH\textsubscript{MHCI}-Spike\textsubscript{RBD} adduct elicits CD4 and CD8 T cell as well as B cell responses, resulting in the formation of antibodies that neutralize not only recombinant VSV expressing the SARS-CoV-2 spike but also a SARS-CoV-2 isolate. Further studies in small animal models and nonhuman primates are needed to establish whether immunity elicited by VHH\textsubscript{MHCI}-Spike\textsubscript{RBD} protects against a SARS-CoV-2 challenge and to establish breadth of coverage. We suggest that this approach merits consideration for use in a clinical setting as a complement to ongoing active and passive immunization strategies.

Materials and Methods

Cells and Antibodies. Exp293F cells were maintained in humidified, shaking incubators at 37 °C, 8% CO\textsubscript{2} in Exp293 Expression Media (ThermoFisher Scientific). BSRT7/5, Vero-CCL81, Vero E6-TMPRSS2, and Vero-hACE2-TMPRSS2 cells were maintained in humidified incubators at 34 °C or 37 °C and 5% CO\textsubscript{2} in Dulbecco’s modified Eagle’s medium (DMEM) (Corning) supplemented with glucose, L-glutamine, sodium pyruvate, and 1% fetal bovine serum (FBS). Vero E6, Vero E6-TMPRSS2, and Vero-hACE2-TMPRSS2 were propagated and titrated on Vero-CCL81 cells at 34 °C. Vero E6 cell cultures (cRL-1586, ATCC) were grown in DMEM (Gibco) supplemented with 5% fetal calf serum (Sigma), 100 U/mL penicillin, and 100 μg/mL streptomycin. Vero E6 cells were harvested and filtered through a 0.45-μm filter. Virus supernatants were plaque purified on Vero-CCL81, Vero E6-TMPRSS2, or Vero-hACE2-TMPRSS2 cells in the presence of 25 μg/mL cytosine arabinoside (araC).

Plaques in agarose plugs were grown on Vero-CCL81, Vero E6-TMPRSS2, or Vero-hACE2-TMPRSS2 cells also in the presence of 25 μg/mL araC to generate P1 stocks. Working stocks were generated on Vero-CCL81 or Vero E6-TMPRSS2 at 34 °C. SARS-CoV-2 strain NL/2020 (EVA-E, Ref-SKU 010V-03903) was triturated to remove the C-terminal 63 nucleotides and were then cloned into an infectious molecular clone (complementary DNA [cDNA]) of VSV-eGFP in place of the native VSV G gene. To rescue the recombinant VSVs, BSR/T7 cells were infected with vaccinia virus encoding the bacteriophage T7 RNA polymerase (T7-T7) and subsequently transfected with T7-driven plasmids encoding VSV N, P, L, and G, as well as the infectious molecular cDNAs. Cell supernatants were harvested by centrifugation at 5,000 × g for 30 min and filtered through a 0.22-μm filter. Filtered virus was plaque purified on Vero-CCL81, Vero E6-TMPRSS2, or Vero-hACE2-TMPRSS2 cells in the presence of 25 μg/mL cytosine arabinoside (araC). Virus titer was measured by focus or plaque counts using 30% confluent monolayers of Vero-CCL81 cells and was recorded as focus-forming units (FFU)/mL. 100 μl of virus stock were added to each well in a 96-well plate and incubated with 2% methylcellulose for 1 h at 37 °C. 100 μl of medium were added and the wells were incubated for 3 days before the medium was discarded and 100 μl of 0.4% crystal violet was added. Plates were incubated for 30 min before washing with water.

Table 1. Peptide sequences for splenocyte stimulation experiments

| No. | Peptide sequence | No. | Peptide sequence | No. | Peptide sequence |
|-----|-----------------|-----|-----------------|-----|-----------------|
| 1   | RV320OPTESIFVPPQNTIT | 19  | CFTNVYADSF\textsubscript{460}VIRGD | 37  | PFERDIST\textsubscript{320}IEYQAGS |
| 2   | TESVRFPP\textsubscript{30}GNTNLCP | 20  | VYADF\textsubscript{40}VIRGDEQVRO | 38  | DIST\textsubscript{30}IEYQAGSTCP\textsubscript{40}NGVEG |
| 3   | VRFPP\textsubscript{30}GNTNLCPGPFGE330V | 21  | SF\textsubscript{40}VIRGDEQVRO\textsubscript{410}APG | 39  | IEYQAGSTCP\textsubscript{40}NGVEG |
| 4   | NITLCPGPFGE\textsubscript{320}VFNAT | 22  | RGDEQVRO\textsubscript{410}APGQGTKT | 40  | AGSTCP\textsubscript{40}NGVEGFCNY |
| 5   | LCPGPFGE\textsubscript{320}VFNATRFR | 23  | VRQ\textsubscript{410}APGQGKIAID240Y | 41  | PC\textsubscript{480}NGVEGFNCYF490PLQ |
| 6   | GE\textsubscript{320}VGNA\textsubscript{330}FASIV | 24  | APOGQGKIAID240YNYKLY | 42  | VEGFCNYF490PLQSYGF |
| 7   | NATRFAS\textsubscript{350}YAW \textsubscript{360}CVADYSVLY | 25  | TGKIAID240YNKYLPDFD | 43  | NCYF490PLQSYGFQGPFPT500N |
| 8   | FASV\textsubscript{350}YAW \textsubscript{360}CVADYSVLY | 26  | AD240YNYKLPDFDFT\textsubscript{430}GCV | 44  | PLQSYGFQGPFPT500NGVEG |
| 9   | YAWNKRRKSINN440L4CVADYSVLY | 27  | YKLPDFDFT\textsubscript{430}GCVIAW | 45  | YGQPFPT500NGVYQPR |
| 10  | RKRRKSINN440L4CVADYSVLY | 28  | DDFT\textsubscript{430}GCVIAWNSNN440L4 | 46  | PT\textsubscript{500}NGVYQYPVR510VVL |
| 11  | SN\textsubscript{320}NVADYSVLYNN\textsubscript{330}SAS | 29  | GC\textsubscript{410}VIAWNSNN440L4 | 47  | VGYQPFPT500VVL5FSEL |
| 12  | ADVSYLV\textsubscript{330}SN440L4SVS\textsubscript{340}YLYRLFRKSN | 30  | AWNSNN440L4DSKVGN | 48  | PYRVS10VVL5FSEL5HAA20P |
| 13  | Y\textsubscript{330}SN440L4SVS\textsubscript{340}YLYRLFRKSN | 31  | NN\textsubscript{440}DSKVGN | 49  | VVL5FSEL5HAA20PATV |
| 14  | SASFSTFKC\textsubscript{350}YLYRLFRKSN | 32  | SKVGN | 50  | FELLSA20PATVCVPGK |
| 15  | STFKC\textsubscript{350}YLYRLFRKSN | 33  | GNYN\textsubscript{450}YLYRLFRKSN | 51  | HASA20PATVCVPGKKS530TNL |
| 16  | CY\textsubscript{350}YLYRLFRKSN | 34  | YLYRLFRKSN | 52  | TVCGP5K530NVLNKK |
| 17  | SPTKLNDS\textsubscript{350}YLYRLFRKSN | 35  | LFRKSN | 53  | PPKS530NVLNKKNC5V541F |
| 18  | L\textsubscript{350}LNLD\textsubscript{350}CFTNYADSF\textsubscript{460}V | 36  | SN\textsubscript{460}LNLD | 54  | F |
Fig. 3. VHH<sub>MHC</sub>-Spike<sub>RBD</sub> elicits a strong humoral response, regardless of route of administration, storage temperature of the vaccine, lyophilization of the vaccine, and age. (A) C57BL/6J mice were immunized with two adjuvanted doses (days 0 and 14) of VHH<sub>MHC</sub>-Spike<sub>RBD</sub> under various conditions highlighted in B–D, and serum was collected 14 d postboost. (B) IgG, IgA, IgG1, and IgG2b levels were measured against recombinant Spike<sub>RBD</sub> following immunization with VHH<sub>MHC</sub>-Spike<sub>RBD</sub> by i.p., i.n., or i.m. administration. Control mice were immunized by the i.p. route with two doses of adjuvant alone. (C) IgG, IgA, IgG1, and IgG2b levels were measured against recombinant Spike<sub>RBD</sub> following immunization with two doses of adjuvant only, adjuvanted VHH<sub>MHC</sub>-Spike<sub>RBD</sub> incubated at either −20°C, 4°C, or 25°C for 1 wk, or with lyophilized and resuspended, adjuvanted VHH<sub>MHC</sub>-Spike<sub>RBD</sub>. (D) IgG, IgA, IgG1, and IgG2b levels were measured against recombinant Spike<sub>RBD</sub> following immunization of 8- to 12 wk-old mice with two doses of adjuvant only or adjuvanted VHH<sub>MHC</sub>-Spike<sub>RBD</sub>, or 72-wk-old mice with two doses of adjuvanted VHH<sub>MHC</sub>-Spike<sub>RBD</sub>. For B–D, n = 4 for all conditions, and curves are plotted as means of each condition. OD, optical density.
boiling for 5 min in sample buffer containing 1% (wt/vol) SDS and 1% (vol/vol) BME. Samples were analyzed on 10% or 15% SDS/PAGE. Gels were stained with Instant Blue (Abcam) and destained with ddH2O.

Mouse Models. All animals were housed in the animal facility of Boston Children’s Hospital (BCH) and were maintained according to protocols approved by the BCH Committee on Animal Care. C57BL/6J (CD45.2+ and BALB/c mice were either purchased from the Jackson Laboratory or bred in house. DR4-IE transgenic mice were purchased from Taconic. Only female mice aged 8 to 12 wk were used in this study unless indicated otherwise.

ELISA. Serum samples were collected on the indicated days and stored in BD Vacutainers; 96-well plates were coated with 2 μg/mL of either recombinant SpikeRBD or SpikeRBD (K417T, E484K, N501Y) proteins in phosphate-buffered saline (PBS). ELISA data were summarized as end point titers and presented as means ± SEM. (F) ELISpot assay measuring IFN-γ-secreting cells in splenocytes of DR4-IE transgenic C57BL/6J mice (n = 3 per condition) immunized with one dose of adjuvant only or adjuvanted VHHhMHCII–SpikeRBD. Splenocytes were harvested at day 7 post immunization. (G) Flow cytometry analyses of splenocytes after incubation for 6 h in the presence of pooled peptides (42 and 47 to 50) and monensin. All data are presented as means ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, unpaired t test with Holm–Sidak adjustment.

### Table 2. Commercial antibodies utilized in this study

| Target | Color | Clone | Manufacturer | Catalog number |
|--------|-------|-------|--------------|----------------|
| CD45   | BV605 | 30-F11| Biolegend    | 103139         |
| CD3    | PerCPCy5.5 | 17A2 | Biolegend    | 100218         |
| CD4    | PEcy7 | RM4-5 | Biolegend    | 100528         |
| CD8α   | FITC  | S3-6.7| Biolegend    | 100706         |
| TNFα   | APC   | MP6-XT22| eBioscience | 17-7321-82     |
| IL2    | BV421 | JES6-5H4| Biolegend   | 503826         |
| IFNγ   | PE    | XMG1.2| eBioscience | 12-7311-82     |
| Fc block (CD16/CD32) | Not Applicable | 93 | Biolegend | 101302 |
saline (PBS) overnight at 4 °C and incubated in blocking buffer (0.05% Tween 20 + 2% bovine serum albumin in PBS). Plates were incubated with diluted serum samples for 2 h at room temperature. Plates were then washed four times with PBS and incubated with goat anti-mouse IgG-HRP, anti-mouse IgM-HRP, anti-mouse IgG1-HRP, or IgG2b-HRP (SouthernBiotech) at 1:10,000 or with IgA-HRP at 1:2,000 in blocking buffer for 1 h. Plates were developed with 3,3′,5,5′-tetramethylbenzidine liquid substrate reagent (Sigma). The reaction was stopped with 1 N HCl, and absorbance was read at 450 nm.

Recombinant VSV Neutralization Assays. Serum samples were heat inactivated at 56 °C for 30 min. Neutralization assays were performed similarly to what has been described (19, 20). Briefly, threefold serial dilutions of sera, starting with a 1:20 dilution, were performed in 384-well plates and were incubated with 10^6 plaque-forming units (pfu) of VSV-SARS-CoV-2 expressing eGFP and the VSV-G gene. Wells were then emptied, and 1 × 10^5 spleenocytes from immunized mice were added to the plates. Cells were arrayed in the presence or absence of 15-mer peptides with 11-residue overlaps derived from the SARS-CoV-2 SpikeRBD (10 μg/mL) in 200 μL of complete medium and incubated overnight at 37 °C. Plates were then washed and incubated with a biotinylated IFNγ detection antibody (BD Biosciences, 51-1818X2) for 2 h at room temperature and incubated with streptavidin-horseradish peroxidase (BD Biosciences) for 1 h at room temperature. Plates were developed with 3-aminohexylcarbazole substrate (BD ELISPOT AEC Substrate kit (Invitrogen, 88-7324-22)) per manufacturer's protocol.

Flow Cytometry Analyses. Cells harvested from excised spleens were dispersed into RPMI 1640 ( Gibco) through a 40-μm cell strainer using the back of a 1-ml syringe plunger. Cell mixtures were subjected to hypotonic lysis (NH4Cl) to remove red blood cells, washed twice in flow cytometry buffer (2 mM (ethylenedinitrilo)tetraacetic acid and 1% FBS in PBS) and resuspended in flow cytometry buffer containing the corresponding fluorescent dye-conjugated antibodies. Staining steps were carried out at 1:100 dilutions in the presence of Fc block (Biolegend) for 30 min at 4 °C in the dark. Samples were washed twice with fluorescence-activated cell sorter (FACS) buffer before further analysis. All flow data were acquired on a FACS Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Lyoiphilization of Vaccine Preparations. VHHMHCII-SpikeRBD combined with adjuvant in PBS were flash frozen in a 2-ml Eppendorf tube. The tube was opened, placed in a 300-ml flask, and lyophilized overnight using a Freezone 4.5 Plus Freeze Dryer (Labconco). After overnight lyophilization, the resulting powder was left at room temperature for 1 wk before immersion.

Statistical Methods. All data represent at least two independent experiments. All statistical analyses were performed using Prism 6. Statistical methods used are indicated in the corresponding legend of each figure.

Data Availability. All data that support the findings in this publication are included in the article and SI Appendix.

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