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Improved Durability to SARS-CoV-2 Vaccine Immunity following Coimmunization with Molecular Adjuvant Adenosine Deaminase-1


Although severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccines have demonstrated strong immunogenicity and protection against severe disease, concerns about the duration and breadth of these responses remain. In this study, we show that codelivery of plasmid-encoded adenosine deaminase-1 (pADA) with SARS-CoV-2 spike glycoprotein DNA enhances immune memory and durability in vivo. Coimmunized mice displayed increased spike-specific IgG of higher affinity and neutralizing capacity as compared with plasmid-encoded spike-only–immunized animals. Importantly, pADA significantly improved the longevity of these enhanced responses in vivo. This coincided with durable increases in frequencies of plasmablasts, receptor-binding domain–specific memory B cells, and SARS-CoV-2–specific T follicular helper cells. Increased spike-specific T cell polyfunctionality was also observed. Notably, animals coimmunized with pADA had significantly reduced viral loads compared with their nonadjuvanted counterparts in a SARS-CoV-2 infection model. These data suggest that pADA enhances immune memory and durability and supports further translational studies.


S
ince the start of the pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), an estimated 3.8 billion people have been infected causing an estimated 15.1 million deaths globally (1). SARS-CoV-2 vaccines have demonstrated acute immunogenicity and protection. However, studies demonstrating decline in humoral responses postvaccination and postinfection, as well as the emergence of variants of concern against which current vaccines may be less effective (2), necessitate the continued study of vaccine modalities that promote robust, broad, and durable immunity. Recent studies evaluating the durability of SARS-CoV-2 mRNA vaccines, mRNA-1273 (K. Wu, A. Choi, M. Koch, L. Ma, A. Hill, N. Nunna, W. Huang, J. Oestreicher, T. Colpitts, H. Bennett, et al., manuscript posted on medRxiv, DOI: 2021.2005.2005.21256716) and Comirnaty (P. Naaber, L. Tserel, K. Kangro, E. Sepp, V. Jürjensson,

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E.K.H., E.N.G., and M.A.K. conceived the project. E.N.G. designed experiments; performed immunizations, harvests, and immunological assays; coordinated challenge experiments; and analyzed data. G.M.C. designed experiments; performed immunizations, harvests, and ELISA assays; and analyzed data. B.W. designed experiments and performed AAV6 transduction and containment challenge. M.R.B. performed immunizations, harvests, ELISPOT, and intracellular cytokine staining assays and analyzed data. J.C. performed harvests and immunological assays and analyzed data. N.J.T. designed experiments, performed harvests and immunological assays, and analyzed data. A.R.A. performed neutralization assays. B.T. performed harvests and immunological assays. S.Z., G.C., and I.M.C. performed surface plasmon resonance experiments and analysis. S.K.W. generated AAV6.2FF vectors. T.S. and S.R. designed experiments. E.A.G. performed area under the curve analysis. The manuscript was written by E.N.G., G.M.C., J.C., B.M.W., M.R.B., N.J.T., and G.C. with intellectual oversight from M.A.K., E.K.H., D.K., and D.B.W. The manuscript was submitted on behalf of all authors by G.M.C. and E.K.H.

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Abbreviations used in this article: AAV, adeno-associated virus; AAV6.2FF–hACE2, adeno-associated virus–6.2FF human angiotensin-converting enzyme-2; ADA-1, adenosine deaminase-1; AIM, activation-induced marker; AUC, area under the curve; Cat, catalog no.; GC, germinal center; hACE2, human angiotensin-converting enzyme-2; NIH, National Institutes of Health; PBS, phosphate-buffered saline; pADA, plasmid-encoded adenosine deaminase-1; pS, plasmid spike glycoprotein; RBD, receptor-binding domain; RLU, relative luciferase unit; Rmax, maximum binding capacity for each domain surface; RT, room temperature; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SPR, surface plasmon resonance; ssDNA, synthetic DNA; TCID50, tissue-culture infectious dose-50; TFH, T follicular helper cell.

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Increased frequencies of plasmablasts, receptor-binding domain enhanced in coimmunized mice. In this study, single pADA coimmunization SARS-CoV-2 vaccines and supports continued study of this novel adjuvant molecule for next generation vaccines. We and others have previously shown that ADA-1 is an enzyme with a role in the immune response and that ADA-1 is an adenosine deaminase-1 (ADA-1) has immune enhancement function. The enzymatic function of ADA-1 is to catalyze the irreversible deamination of adenosine into inosine, thus regulating intracellular and extracellular levels of adenosine. Among heritable SCIDs in humans, ~15% are caused by mutation in the ADA gene, and pegylated ADA is used therapeutically as a SCID treatment. We and others have previously shown that ADA-1 is an important immune modulator (4–6). During immune synapse formation between dendritic cells and T cells, ADA-1 can interact with receptors on immune cells both to modulate effective costimulatory signals and to promote T cell proliferation and differentiation (4, 5). We have recently demonstrated a novel property of ADA-1 wherein it improves the function and differentiation of the T follicular helper cell (Tfh) program as measured by the ability to provide B cell help in vitro (4). We extended these studies and further showed that coimmunization of plasmid-encoded adenosine deaminase-1 (pADA) in the context of an HIV DNA vaccine enhances germinal center (GC) formation, Tfh function, and Ab magnitude and quality in vivo (7).

A previous study demonstrated that two immunizations with synthetic DNA (synDNA) in the form of a vaccine plasmid encoding the full-length SARS-CoV-2 spike plasmid glycoprotein (pS) induced robust humoral and cellular immunity in mice and protected 100% of mice from SARS-CoV-2 replication in an adenovirus-human angiotensin-converting enzyme-2 (AAV6.2FF-hACE2)-transduction model of SARS-CoV-2 infection (8). In this report, we demonstrate that co-delivery of pADA with pS would improve spike-specific humoral and cellular responses durability and quality after only one immunization. Compared with pS only, mice immunized with pS and pADA mice exhibited increased concentrations of spike-specific IgG, which bound with increased affinity and exhibited increased neutralization capability. Increased frequencies of plasmablasts, receptor-binding domain (RBD)-specific memory B cells, and Tfh17 were also observed in coimmunized mice. Spike-specific T cell polyfunctionality was also enhanced in coimmunized mice. In this study, single pADA coimmunization protected 100% of challenged mice from viral replication in the lung as measured by tissue-culture infectious dose-50 (TCID$_{50}$) compared with only 40% of mice receiving a single immunization of pS alone. Importantly, at >400 d after final immunization, viral loads were completely reduced in pADA-coimmunized animals compared with those in the absence of pADA. These data suggest that pADA enhances the magnitude, quality, and duration of Ag-specific responses and supports continued study of this novel adjuvant molecule for next generation SARS-CoV-2 vaccines.

Materials and Methods

Plasmids and immunizations

Codon-optimized DNA plasmids encoding full-length SARS-CoV-2 pS were produced commercially and cloned into the pVax expression vector with an IgG leader sequence to facilitate in vivo secretion. Similarly, a synDNA construct encoding murine ADA-1 was generated in the pVax vector with an IgG leader sequence and commercially produced (GenScript, Piscataway, NJ) as previously described (7). In vitro studies revealed that expression of the spike and ADA proteins after transfection of cell lines with the vaccine constructs as previously described (7, 9). Female BALB/c mice aged 6–8 wk were immunized in the left tibialis anterior muscle with 30–50 µl of the formulated vaccines. Vaccines included 10 µg of pS alone or 10 µg of pS coformulated with 10 µg of pADA. Control mice remained unvaccinated or were immunized with 20 µg of empty plasmid vector (pVax) to ensure an equal amount of DNA was used across conditions for the experiments. Mice and female C57BL/6 mice at age 6–8 wk were also immunized in the left tibialis anterior muscle with 30 µl of the formulated vaccines. Immediately after vaccine injection, in vivo electroporation was performed using the CELLECTRA device (Inovio Pharmaceuticals, Bluebell, PA). Animals were housed in a temperature-controlled, light-cycled, specific-pathogen-free facility at Drexel University College of Medicine or Wistar Institute.

Ethical statement

In vivo electroporation of DNA vaccines in mice was conducted in accordance with the guidelines set forth by the NIH and performed under protocols approved by the Institutional Animal Care and Use Committee and Environmental Biosafety Committee–approved protocols at Drexel University College of Medicine and Wistar Institute.

Mouse sacrifice, sample collection, and tissue harvest

At the time points shown in the in vivo study design, mice were either bleed via cheek bleed or sacrificed. At sacrifice, blood, spleens, and popliteal and inguinal lymph nodes were collected. Blood collected via cheek bleed or cardiac puncture was collected into minicollect serum gel tubes (Grenier-Bio) and centrifuged at 13,000 rpm for 10 min at 4°C to separate serum. Spleens and lymph nodes were processed into single-cell suspensions. Cells within each suspension were washed and resuspended in RPMI media with 100 µl supplemented with 1% penicillin/streptomycin and 10% FBS. Cell concentrations and viabilities were determined using a Countess Automated Cell Counter (Invitrogen, Life Technologies).

ELISA assays

ELISA was used to determine RBD- and S1-specific IgG present in mouse serum. Mouse blood samples were collected via cheek bleed or cardiac puncture (intraperitoneal or intravenous). Anti-RBD immortalized mouse monoclonal (mAb) IgG1b was used for sandwich ELISA by sandwiching an immobilized RBD coupled to the sensor chip, which bound with a mouse IgG2a (2C11) as the control for binding to RBD (Purified Wuhan recombinant Spike RBD [Sino Biological, Beijing, China]; RBD-His 40592-VNAH, FC2). RBD directly coupled to the sensor was validated using ACE2 (Invitrogen, Life Technologies). The anti-RBD titration standard was an IgG1 mouse (in house, Drexel University). RBD directly coupled to the sensor was validated using ACE2 (10108-H08H) and VHH-72-huFc, the latter a generous gift from Integral Molecular (Philadelphia, PA). The anti-RBD assay was performed on a Biacore 3000 biosensor (Global Cytiva Lifesciences) at 25°C using PBS-P (10 mM phosphate, 150 mM NaCl [pH 7.4], 0.05% P-20) as the running buffer. A CM5 sensor chip (Cytiva, Marlborough, MA) was docked and derivatized with known quantities of mouse IgG (Thomas Scientific). The anti-RBD binding was calculated by interpolating the optical densities on calibration curves that were generated with known quantities of mouse IgG (Thomas Scientific).

Surface plasmon resonance assays

Surface plasmon resonance (SPR) experiments were performed on a Biacore core biosensor (Global Cytiva Lifesciences) at 25°C using PBS-P (10 mM phosphate, 150 mM NaCl [pH 7.4], 0.05% P-20) as the running buffer. A CM5 sensor chip (Cytiva, Marlborough, MA) was docked and derivatized by amine coupling with RBD using freshly prepared 100 mM Nhydroxysuccinimide and 400 mM 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (Invitrogen) mixed 1:1. Flow cell 1 was activated, blocked, and remained as the control of binding to RBD (Purified Wuhan recombinant Spike RBD [Sino Biological, Beijing, China]; RBD-His 40592-VNAH, FC2). RBD directly coupled to the sensor was validated using ACE2 (10108-H08H) and VHH-72-huFc, the latter a generous gift from Integral Molecular (Philadelphia, PA). The anti-RBD titration standard was an RBD-specific neutralizing mouse mAb (40592-MM57). Sera from postimmunization bleedings were diluted in sample buffer with 20 mg/ml CM-Dextran, saline or NSB reducer (Cytiva) to minimize nonspecific binding. The final serum sample dilutions were 1/15 and 1/20 (1.3-fold apart) in naive serum. Naïve sera mixed with CM-Dextran in equal proportion were used as negative controls, while mAb standards between 1.2 and 250 nM were also spiked in naive serum mixed with CM-Dextran. A surface density of 50 response units/µl RBD was experimentally found to offer partial mass transport limited binding of serum-spiked Ab standards injected over control and RBD flow cells at 5 µl/min. Under mass transport limited conditions, the Ab binding rates are dependent on the concentration of RBD-specific Ab binding. The anti-RBD mAb standard was used to calculate Ab concentrations.
of individual mouse sera. Duplicates of the mAb standards, controls, and serum samples were injected over all surfaces for 1 min (association phase) at flow 5 μl/min and washed with running buffer for 5 min (dissociation phase). The remaining bound Abs were removed with two 40-s pulses of 15 mM HCl (pH 2.0). Polyclonal Ab (pAb) initial rates of binding were translated into concentrations using Phenom, a software developed to systematize multiple data analysis. Anti-RBD mAb standards against each domain at four concentrations spanning 1.2–250 nM were subsequently injected in a kinetic format at flow 50 μl/min for 1 min to measure the kinetic profiles to each coupled domain.

To calculate the kinetics of pAb binding to RBD, we injected two individual sera dilutions in duplicate over all surfaces at flow 50 μl/min for 1 min, and bound pAbs were washed with running buffer for 5 min followed by surface regeneration. All binding profiles (sensorgrams) were double referenced to minimize the impact of instrument and solvent noise. Raw data analysis for the calculation of binding rates and kinetics was blinded to all personnel. Raw and binding kinetics (dR/dt = kₐ A × Rₘ₀ − kᵣ R) were generated for the E gene of SARS-CoV-2, as per the diagnostic protocol instructions. Quantitative RT-PCR detection of SARS-CoV-2 was performed on a QuantStudio 5 instrument (Applied Biosystems) according to the manufacturer’s instructions. Neutralization titers (infectious dose 50) were calculated using GraphPad Prism 8 and defined as the reciprocal serum dilution at which SLU were reduced by 50% compared with SLU in virus control wells after subtraction of background SLUs in cell control wells.

Generation of AA V6.2FF-hACE2

AAV6.2FF-hACE2 viruses were generated as previously described (8). Adeno-associated virus (AAV) vector titers were determined by quantitative PCR (qPCR) analysis as described elsewhere (10).

Pseudovirus neutralization assay

SARS-CoV-2 pseudoviruses were produced using HEK293T cells transfected with 1:1 ratio of IgG–SARS-CoV-2 S plasmid (Genscript) and pNL4-3. Luc-R-E plasmid (NIBS AIDS reagent) using Gene jammer (Agilent) as transfection reagent. Forty-eight hours posttransfection supernatant was collected, enriched with FBS to 12% final volume, and stored at −80°C. SARS-CoV-2 pseudovirus neutralization assay was set up using D10 media (DMEM supplemented with 10% FBS and 1x penicillin/streptomycin) in a 96-well format with huCHOAce2 cells (Cat: VCeL-Wyb019; Creative Bios). For neutralization assay, 10,000 CHO-ACE2 cells were plated in 96-well plates and rested at 17°C with 5% CO₂ for 24 h. FCS was added and sera from vaccinated and control groups were heat inactivated and serially diluted as desired. Sera were incubated with a fixed amount of SARS-CoV-2 pseudovirus for 90 min at RT, after which the mix was added to huCHO Ace2 cells and allowed to incubate in a standard incubator (37°C humidity, 5% CO₂ for 2 h). After 72 h, cells were lysed using BritePlu plus luminescence reporter gene assay system (Cat: 6067669; Perkin Elmer), and luciferase activity was measured using the BioTek plate reader. Neutralization titers (infectious dose 50) were calculated using GraphPad Prism 8 and defined as the reciprocal serum dilution at which SLU were reduced by 50% compared with SLU in virus control wells after subtraction of background SLUs in cell control wells.

Detection of SARS-CoV-2 in tissues

For measurement of viral titers in the lungs of infected mice, TCID₅₀ assays were performed. After necropsy, tissue samples were frozen at −80°C. Tissue samples were thawed and placed in MEM, supplemented with 1x tr-glutamine and 1% FBS, and homogenized with 5-mm stainless steel beads in a Bead Ruptor Elite Tissue Homogenizer (Omni). Homogenates were clarified by centrifugation at 1500 × g for 10 min. Ten-fold serial dilutions of tissue homogenates were made in MEM. Dilutions were added to 90–100% confluent Vero cells in triplicate wells, and cytopathic effect was read at 5 days postinfection. TCID₅₀ values per gram of tissue were calculated using the Reed and Muench method.

For detection of viral RNA, tissues collected were stored in RNA later. RNA was extracted using an RNasy mini kit plus QIAamp (Qiagen), according to the manufacturer’s instructions. RT-qPCR for SARS-CoV-2 was performed on a QuantStudio 5 instrument (Applied Biosystems) using a TaqPath 1-step RT-qPCR Master Mix (Applied Biosystems) and primers specific for the E gene of SARS-CoV-2, as per the diagnostic protocol.
ENHANCED QUALITY OF ANTI–SARS-CoV-2 Abs WITH ADA-1

We measured the ability of sera from immunized mice to neutralize SARS-CoV-2 pseudotyped viruses in vitro. Sera from pADA-coimmunized animals displayed increased neutralization capacity compared with pS-only–immunized animals out to day 127 postimmunization (Fig. 1F). Specifically, ADA-1 improved Ab neutralization abilities at days 35, 56, and 127 in coimmunized mice when compared with those elicited in the absence of ADA-1. Abs levels elicited at day 56 postvaccination showed a significant difference (p = 0.0257). We further evaluated the affinity of vaccine-induced polyclonal mouse sera for SARS-CoV-2 spike Ags. Specific binding of serum pAbs to RBD from pS-only– and pADA-coimmunized mice was measured using a rapid SPR screening assay for initial rates of binding. The follow-up SPR kinetic analysis of IgG binding allowed us to measure quantitative aspects of Ab binding, such as the equilibrium association constants (affinity, Kₐ, or Kᵢ), Kᵢ values measured the amount of Ab–Ag complex formed at equilibrium, independent of concentration. We examined the impact of ADA-1 on the affinity of anti-RBD Ab (Fig. 1G, 1H) at multiple days postvaccination. We showed that ADA-1 significantly improved the affinity of anti-RBD Abs at day 35 (p = 0.0238) and day 56 (p = 0.0079), and, importantly, a durable affinity at day 127 (p = 0.0286) was observed. Taken together, these data suggest that pADA coimmunization has a functional and qualitative impact on vaccine-induced humoral responses, supporting strong and durable virus neutralization and Ag affinity.

ADA-1 induces durable Ag-specific Tfh responses

Because the magnitude and quality of Ab response depends on persistent Tfh function, we examined whether pADA can elicit a durable Tfh function. For this, we used TCR-dependent activation-induced marker (AIM) assays to identify and quantify SARS-CoV-2–specific GC Tfh cells in vaccinated mice as a marker of robust GC responses (Fig. 2) as previously reported (11, 12). Six- to eight-week-old C57BL/6 mice (n = 8, four male and four female) were immunized with pS alone or in combination with pADA and were sacrificed at days 14 and 74 postimmunization to measure early and late Tfh responses (Fig. 2A). We stimulated splenocytes from mice vaccinated with pS alone or coimmunized with pADA with five peptide pools encompassing the entire pS. Con A was used a positive control, while DMSO was used as the negative control. Gating strategies to determine SARS-CoV-2–spike-specific AIM⁺ Tfh (OX40⁺ 41BB⁺) and RBD⁺ Memory B cells are shown in Fig. 2B and 2C. SARS-CoV-2 spike-specific Tfh (Fig. 2D) as measured by the AIM assay were significantly increased in both groups of mice (pS: p = 0.044, pADA: p = 0.044) at day 14 postimmunization when compared with immunization with empty vector pVax. Importantly, at day 74 postimmunization, the frequency of SARS-CoV-2 spike-specific Tfh in the pS vaccination group significantly contracts (p = 0.0104, average frequency = 0.91%), while the frequency of SARS-CoV-2 spike-specific Tfh in the pADA-coimmunized mice remains at similar frequency as day 14 (average frequency at day 14 = 4.9%, average frequency at day 74 = 4.0%), suggesting a sustained GC response with robust Ab production. Frequency of total Tfh was not changed at early (day 14) and late (day 74) postvaccination in both groups of mice (Fig. 2E) when compared with empty vector immunization likely due to the kinetics of the peak of total Tfh response, which could have occurred at earlier time points.

ADA-1 induces durable Ag-specific memory B cell responses

We further determined whether durable Ab response observed in the presence of ADA-1 is because of a durable Ag-specific B cell

Results

pADA coimmunization promotes robust and durable SARS-CoV-2–specific humoral immunity in vivo

To evaluate immune durability and memory after vaccination, 6- to 8-week-old female BALB/c (Fig. 1) or 6- to 8-week-old male and female C57BL/6 mice (Supplemental Fig. 2) were immunized once with a DNA vaccine construct expressing the full-length SARS-CoV-2 pS alone or in combination with a DNA plasmid expressing murine ADA-1. Control mice remained unvaccinated (Fig. 1) or received an empty plasmid vector (pVax) (Supplemental Fig. 2). All mice received a total of 20 μg of plasmid DNA to control for any nonspecific DNA-induced innate responses. Mice were bled longitudinally postimmunization, and serum was used to evaluate Ab levels, function, affinity, and durability. Fig. 1B and 1C depicts anti-S1 and anti-RBD Ab levels, respectively, as determined by ELISA. Coimmunization with pADA significantly improved the magnitude of Ab levels at multiple time points postvaccination and elicited a robust durable Ab response. In two independent experiments, ADA-1–coimmunized mice showed peak anti-RBD Ab responses at between days 21 (Supplemental Fig. 2B) and 56 (Fig. 1C) postimmunization. Specifically, ADA-1 improved the anti-S1 Ab response at day 35 postvaccination, but importantly, ADA-1 significantly (p = 0.0079) elicited a durable response up to 127 d when compared with those mice that did not receive ADA-1 (Fig. 1B). Similarly, coimmunization with ADA-1 elicited a significant increase in RBD-specific IgG in coimmunized mice at days 35 (p = 0.0016) and 56 (p = 0.0002) compared with PS-only–immunized mice. A trend of increased RBD IgG was exhibited in coimmunized mice at day 127 (Fig. 1C). To account for the impact of ADA-1 on multiple time points postvaccination, we measured AUC in mice immunized in the presence and absence of ADA-1. We showed that ADA-1 elicited a significantly higher response in S1 IgG (p = 0.016) (Fig. 1D) and RBD IgG (p = 0.0079) (Fig. 1E) when compared with Ab elicited in the absence of ADA-1. This confirms the ability of ADA-1 to elicit a strong Ab response over multiple time points. Collectively, these data suggest that pADA-adjuantated responses are robust and durable in vivo.

ADA-1 enhances Ab neutralization and affinity

Because the magnitude and quality of Ab response depends on persistent Tfh function, we examined whether pADA can elicit a durable Tfh function. For this, we used TCR-dependent activation-induced marker (AIM) assays to identify and quantify SARS-CoV-2–specific GC Tfh in vaccinated mice as a marker of robust GC responses (Fig. 2) as previously reported (11, 12). Six- to eight-week-old C57BL/6 mice (n = 8, four male and four female) were immunized with pS alone or in combination with pADA and were sacrificed at days 14 and 74 postimmunization to measure early and late Tfh responses (Fig. 2A). We stimulated splenocytes from mice vaccinated with pS alone or coimmunized with pADA with five peptide pools encompassing the entire pS. Con A was used a positive control, while DMSO was used as the negative control. Gating strategies to determine SARS-CoV-2–spike-specific AIM⁺ Tfh (OX40⁺ 41BB⁺) and RBD⁺ Memory B cells are shown in Fig. 2B and 2C. SARS-CoV-2 spike-specific Tfh (Fig. 2D) as measured by the AIM assay were significantly increased in both groups of mice (pS: p = 0.044, pADA: p = 0.044) at day 14 postimmunization when compared with immunization with empty vector pVax. Importantly, at day 74 postimmunization, the frequency of SARS-CoV-2 spike-specific Tfh in the pS vaccination group significantly contracts (p = 0.0104, average frequency = 0.91%), while the frequency of SARS-CoV-2 spike-specific Tfh in the pADA-coimmunized mice remains at similar frequency as day 14 (average frequency at day 14 = 4.9%, average frequency at day 74 = 4.0%), suggesting a sustained GC response with robust Ab production. Frequency of total Tfh was not changed at early (day 14) and late (day 74) postvaccination in both groups of mice (Fig. 2E) when compared with empty vector immunization likely due to the kinetics of the peak of total Tfh response, which could have occurred at earlier time points.

ADA-1 enhances durable Ag-specific memory B cell responses

We further determined whether durable Ab response observed in the presence of ADA-1 is because of a durable Ag-specific B cell
response. Therefore, we measured the frequency of SARS-CoV-2 RBD-specific memory B cells (Fig. 2F) and plasmablasts (Fig. 2G) at days 14 and 74 postimmunization. Ag-specific memory B cells were monitored by fluorescently labeled multimerized probes specific to RBD, and plasmablasts were gated as CD19<sup>+</sup>CD138<sup>+</sup>. Analysis of mice that were either immunized with pS or coimmunized with pADA revealed that frequencies of SARS-CoV-2 RBD-specific memory B cells were significantly higher in both groups of mice at day 14 postimmunization when compared with empty vector (pS: <i>p</i> = 0.0182, pADA: <i>p</i> = 0.0121). Importantly, mice that were coimmunized with pADA showed a significant (<i>p</i> < 0.001) durable response at day 74 postimmunization when compared with pS alone, suggesting the ability of pADA coimmunization to induce SARS-CoV-2–specific memory responses that last at least 2 mo.
FIGURE 2. pADA coimmunization supports sustained T<sub>FH</sub> and B cell responses. (A) Mice were immunized once with pS alone or coimmunized with pS and pADA (+pADA) and sacrificed at either day 14 or 74 postimmunization. (B) Gating schemes are depicted for AIM<sup>+</sup> memory T<sub>FH</sub> and (C) RBD<sup>+</sup> memory B cells. (D and E) The frequencies of total T<sub>FH</sub> (D) and SARS-CoV-2–specific T<sub>FH</sub> (E) were measured. SARS-CoV-2–specific T<sub>FH</sub> were quantified as percentage of AIM<sup>+</sup> (OX40<sup>+</sup>41BB<sup>+</sup>) cells after stimulation of splenocytes with peptide pools encompassing spike only (pS). Background from DMSO-treated splenocytes was subtracted from peptide-treated splenocytes, and the data are shown with geometric mean and geometric SD. (F and G) The frequencies of total plasmablasts (% of CD19<sup>+</sup> B cells) (G) and SARS-CoV-2 RBD-specific memory B cells (% of CD19<sup>+</sup> CD38<sup>+</sup> B cells) (G) were measured. *p < 0.05, as measured by two-tailed unpaired t test. Data are representative of one independent experiment with n = 8 mice per group.
postvaccination (Fig. 2F). pADA-coimmunized mice also exhibited increased frequencies of CD138⁺ plasmablasts at day 74 postimmunization compared with mice immunized with pS alone despite this increase not reaching statistical significance (Fig. 2G). Taken together, these data demonstrate that pS and pADA coimmunization induces more robust and durable Ag-specific memory B cell responses compared with immunization with Ag (pS) alone.

**ADA-1 enhances T cell monofunctionality and polyfunctionality**

The T cell response to a single immunization with pS alone or pADA coimmunization was determined via splenocyte stimulation with peptides encompassing SARS-CoV-2 pS followed by flow cytometry analysis. The frequencies of both monofunctional and polyfunctional CD8⁺ (Fig. 3A, 3B) and CD4⁺ (Fig. 3C, 3D) T cells were quantified using the gating strategy shown in Supplemental Fig. 1. In pADA-coimmunized mice, we observed a near-significant increase (p = 0.0688) in the frequency of TNF-α⁺ monofunctional CD8⁺ T cells (Fig. 3A) and a significant increase in the frequency of TNF-α⁻ (p = 0.0025) and IL-2⁻ (p = 0.0112) monofunctional CD4⁺ T cells (Fig. 3C) at 14 d postimmunization when compared with pS alone. At this time point, we also observed a significant boost in triple-positive (IFN-γ⁻/TNF-α⁻/IL-2⁻) (p = 0.0463) cells, as well as in IFN-γ⁻/IL2⁻ double-positive (p = 0.0127) CD8⁺ T cells in pADA coimmunized mice. An apparent increase in IFN-γ⁻/TNF⁻ double-positive CD8⁺ T cells was observed despite not being significant (Fig. 3A). Similarly, we found a significant increase in IFN-γ⁻/TNF⁻/IL-2⁻ triple-positive (p = 0.0002) and TNF⁻/IL-2⁻ double-positive (p = 0.0044) CD4⁺ T cells in pADA-coimmunized mice when compared with pS alone (Fig. 3C).

To understand the durability of the T cell response, we also measured CD4⁺ and CD8⁺ T cell responses at 74 d postimmunization. TNF-α⁺ monofunctional CD8⁺ (Fig. 3B) and CD4⁺ (Fig. 3D) cells and IL-2⁻⁺ monofunctional CD8⁺ T cells (Fig. 3B) persisted at significantly increased frequencies in pADA-coimmunized mice (p = 0.0079, p = 0.0238, and p = 0.0079, respectively) when compared with pS alone. In terms of polyfunctionality, we found that IFN-γ⁻/IL-2⁻ double-positive CD8⁺ T cells persisted (Fig. 3B). An increase in IFN-γ⁻/TNF-α⁻ and TNF-α⁻/IL-2⁻ double-positive CD4⁺ T cells was observed in pADA-coimmunized mice; however, this increase was not significant (Fig. 3D). Similarly, when BALB/c mice were immunized once with a suboptimal dose of pS (5 μg) alone or coimmunized with 5 or 10 μg of pADA, we observed a pADA-induced enhancement of cellular immunity at both acute (Supplemental Fig. 3A, 3B) and memory time points (Supplemental AB CD)
Fig. 3D), suggesting that the ability of pADA to enhance cellular responses is not mouse strain specific. These data suggest that pADA coimmunization supports vaccine-induced cellular immunity.

**pADA coimmunization enhances protection in an AAV6.2FF-mediated human angiotensin-converting enzyme-2 transduction model of SARS-CoV-2 challenge**

We previously reported that transduction of the mouse respiratory tract with modified adenovirus-6 (AAV6.2FF) results in robust transgene expression in the lung (13). Recently, we extended this work to model SARS-CoV-2 infection by expressing human angiotensin-converting enzyme-2 (hACE2) and demonstrated that this model supports replication of wild-type SARS-CoV-2 in the lungs of mice and provides an easily accessible experimental system with which to evaluate the efficacy of anti-SARS-CoV-2 vaccines and therapeutics (8). Using this model, we evaluated the effect of pADA coimmunization on the efficacy of our SARS-CoV-2 synDNA Ags. BALB/c mice were immunized at weeks 0 and 4 or week 4 only with 10 µg of SARS-CoV-2 pS alone or coimmunized with 10 µg of pS and 10 µg of pADA (Fig. 4A). In this instance, we observed trends toward enhanced serum pseudovirus neutralization capacity at day 14 after final immunization (Fig. 4B). After the final immunization, mice were transduced with AAV6.2FF-hACE2 and challenged 2 wk later with 10⁵ TCID₅₀ of SARS-CoV-2 (VIDO-01). Animals were sacrificed at 4 days postinfection to quantitate viral loads. Although a single immunization with pS alone protected 40% of animals from viral replication as measured by TCID₅₀ assay, a single pS and pADA coimmunization protected 100% of animals from viral replication (Fig. 4C). All immunized animals had decreased viral loads as measured by RT-PCR at the time of sacrifice (Fig. 4D). Importantly, we were able to demonstrate an inverse correlation between serum pseudovirus neutralization capacity prechallenge with postchallenge TCID₅₀ (Fig. 4E). Similar correlations were observed between prechallenge serum assays and viral RNA loads at sacrifice (Fig. 4F). These data indicate that pS coimmunization with pADA not only enhances the magnitude of humoral and cellular responses but has significant impact on challenge outcome in this model of wild-type SARS-CoV-2 infection.

To evaluate the longevity of pADA-induced enhanced protection in this model, we rested animals for >1 y before AAV6.2FF-hACE2 transduction and SARS-CoV-2 challenge (Fig. 4G). At this memory time point, animals receiving a single immunization with either pS alone or pS and pADA had similar amounts of detectable virus in their lungs. Animals receiving two pADA coimmunizations were completely protected from viral replication as measured by TCID₅₀ assay compared with 60% of animals receiving two pS-only immunizations (Fig. 4H). All immunized animals had decreased viral loads as measured by RT-PCR at the time of sacrifice (Fig. 4I). These data suggest that pADA coimmunization enhances synDNA vaccine-induced immune memory and supports the continued study of ADA as an adjuvant to enhance both the magnitude and the duration of Ag-specific immunity.

**Discussion**

It is becoming increasingly evident that most vaccines targeting SARS-CoV-2 do not induce durable Ab responses leading to a loss of protection against infection (Ref. 2, K. Wu et al., manuscript posted on medRxiv, DOI: 2021.2005.2005.212556716, and P. Naaber et al., manuscript posted on medRxiv, DOI: 2021.2004.2019.21255714). Thus, novel adjuvants that can improve COVID-19 vaccine durability and quality would represent important targets for development.

The most effective vaccines induce durable immunity through GC reactions resulting in long-lived plasma cells and memory B cells. GCs are dynamic sites within secondary lymphoid organs, where Tfh cells provide physical and cytokine-mediated stimulus to B cells resulting in somatic hypermutation and class switching of B cell receptors and differentiation to durable memory B cells and long-lived plasma cells (14). Thus, molecules such as ADA-1 that target Tfh cells would improve humoral immunity and likely induce long-lived humoral and cellular responses.

We have previously shown that ADA is a critical molecule of Tfh profile. Coclutire of Tfh with autologous B cells in the presence of ADA inhibitors blunted Ab secretion (4). We further reported that codelivery of pADA with an HIV-1 envelope DNA vaccine enhanced the formation of HIV-specific Abs (7). This enhancement was associated with increased frequencies of GC Tfh and resulted in the development of autologous HIV-1 virus neutralization (7).

In this study, we showed that ADA elicited long-term survival of SARS-CoV-2–specific Tfh and RBD-specific memory B cells for >4 mo after vaccination. In the absence of ADA, most of these Ag-specific Tfh and memory B cells waned significantly. The enhanced differentiation and persistence of SARS-CoV-2–specific Tfh driven by ADA supports the enhanced anti-RBD and anti-S1 Ab quantities, affinity, and neutralization we observed in ADA-immunized mice. ADA promotion of Tfh differentiation may be driven by the ability of ADA to promote dendritic cell maturation and production of IL-6, a cytokine known to play a crucial role in regulating Tfh differentiation and effector functions (7, 15). IL-6 has also been shown to induce the differentiation of IL-21–producing CD8⁺ T cells that provide B cell help and promote Ab production (15). IL-21 production from both CD8⁺ T (15) and CD4⁺ T cells (16) has been shown to enhance the cytolytic activity of CD8⁺ T cells (16), which is in line with the enhanced and durable cellular immunity observed in ADA-immunized mice.

We hypothesize that ADA promotes survival of Tfh and memory B cells in vivo by enhancing survival signals provided by B cell–T cell interactions such as CD40–CD40L binding (17). Prolonged survival of Tfh and memory B cells provides ample time for these cells to interact in GCs. This could lead to the early and persistent accumulation of GC Tfh cells, providing an extended time of interaction between Tfh and B cells in GC follicles, a process that favors enhanced somatic mutations and conversion of low-affinity B cell clones to high-affinity B cell clones. Our results of ADA-induced prolonged survival of SARS-CoV-2–specific Tfh cells and RBD-specific memory B cells in addition to the enhancement of overall anti-spike Ab levels, affinity, and neutralization strongly support this notion.

We interrogated the function of pADA-induced vaccine responses in an AAV6.2FF human ACE2 transduction model of SARS-CoV-2 infection in mice (8). In this model, we generated long-term expression of human ACE2 in the respiratory tract of wild-type mice, making them susceptible to SARS-CoV-2 infection. Transient models of human ACE2 expression in the mouse lung, such as the model used in this study, can provide rapid, quantifiable insights into vaccine-mediated protection in mice. In this model, we observed that a single pADA coimmunization enhances survival and protection with 100% of coimmunized animals protected from viral replication as measured by TCID₅₀ compared with only 40% of pS-only–immunized animals being protected. These data confirm our previous report that a single pS immunization protects only 50% of animals from replication in this model (8). Importantly, when animals were rested for more than a year and challenged in the model system, pADA coimmunization significantly impacted challenge outcomes. Although two immunizations were needed to observe statistically significant decreases in replication-competent virus in the lung postchallenge, only 60% of the animals receiving two immunizations with pS alone displayed significantly decreased viral
levels, while 100% of pADA-coimmunized animals had viral levels under the limit of detection. When viral RNA was measured by RT-PCR only, animals receiving pADA coimmunization (both one and two doses) had viral RNA loads that were decreased compared with naive animals. These data suggest that pADA has a significant impact on SARS-CoV-2 challenge outcome. We strongly believe that protection against SARS-CoV-2 infection in the presence of ADA is due to a combination of humoral and cellular effector functions that are improved in the presence of ADA.

Adenosine-deaminase replacement therapy using PEGylated bovine ADA in patients with SCID is well tolerated with only minor short-term side effects observed, even in pediatric patient populations (18). These studies demonstrate a novel use of a molecule with a proven record of clinical safety in the context of the safe, well-tolerated, and
immunogenic synDNA platform. The use of ADA-1 to adjuvant SARS-CoV-2 synDNA vaccine-induced responses represents a novel repurposing of an extant therapy with the potential to be fast-tracked for clinical application.

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